Oxidative stress plays important roles in a wide range of diseases such as cancer, inflammatory disease, neurodegenerative disorders, etc. Tyrosine nitration in a protein is a chemically stable oxidative modification, and a marker of oxidative injuries. Mass spectrometry (MS) is a key technique to identify nitrotyrosine-containing proteins and nitrotyrosine sites in endogenous and synthetic nitroproteins and nitropeptides. However, in vivo nitrotyrosine-containing proteins occur with extreme low-abundance to severely challenge the use of MS to identify in vivo nitroproteins and nitrotyrosine sites. A preferential enrichment of nitroproteins and/or nitropeptides is necessary before MS analysis. Current enrichment methods include immuno-affinity techniques, chemical derivation of the nitro group plus target isolations, followed with tandem mass spectrometry analysis. This article reviews the MS techniques and pertinent before-MS enrichment techniques for the identification of nitrotyrosine-containing proteins. This article reviews future trends in the field of nitroproteomics, including quantitative nitroproteomics, systems biological networks of nitroproteins, and structural biology study of tyrosine nitration to completely clarify the biological functions of tyrosine nitration.

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I. INTRODUCTION

Oxidative/nitrative stress-mediated tyrosine nitration is involved in a wide range of physiological and pathological processes (Zhan & Desiderio, 2004, 2009a), exists in normal physiology, and is enhanced in pathologies (Scaloni, 2006; Zhan & Desiderio, 2009a). Tyrosine nitration is not only a marker of oxidative injuries, but also alters the functions of proteins that participate in different pathophysiological processes such as cancer, inflammation disease, and neurodegenerative diseases (Zhan & Desiderio, 2004, 2006, 2009a). The nitrotyrosine residue has unique physical and chemical properties (Yee et al., 2003; Ghesquiere et al., 2006; Zhan & Desiderio, 2006) such as pKa value (the pKa value of the phenolic hydroxyl group of nitrotyrosine (pKa = ~7.1) is significantly lower than that of tyrosine (pKa = ~10)), spectrophotometric properties, bulk, electron-density factors (the electron-density of the phenolic ring of nitrotyrosine is lower than that of tyrosine), and reducible to aminotyrosine. Protein nitration has extensive biological consequences such as modification of enzymatic activities, sensitivity to proteolytic degradation, impact on protein tyrosyl-phosphorylation, immunogenicity, and implication in disease (Abello et al., 2009). Identification of nitrotyrosine-containing proteins and accurate location of each nitrotyrosine site are key steps to understand functions and roles of tyrosine nitration (Zhan & Desiderio, 2009a). However, identification of a nitrotyrosine-containing protein is very challenging because its extreme in vivo low abundance (Haddad et al., 1994; Shigenaga et al., 1997) and various MS behaviors (Petersson et al., 2001; Sarver et al., 2001; Zhan & Desiderio, 2006, 2009b; Zhan, Wang, & Desiderio, 2013). MS, coupled with different chemical derivation (Zhan & Desiderio, 2009b) and enrichment techniques (Zhan & Desiderio, 2006, 2009a; Zhang et al., 2007), is necessary to identify a nitrotyrosine-containing protein (Zhan & Desiderio, 2009a). For mass spectrometry (MS) analyses, a characteristic photodecomposition pattern of a nitro group is present in UV-laser-based MALDI MS analysis of a
nitrotyrosine-containing protein (Petersson et al., 2001; Sarver et al., 2001; Zhan & Desiderio, 2009b), but not for electrospray ionization (ESI)-MS (Kim et al., 2011; Lee et al., 2007, 2009b; Yeo et al., 2008; Zhan & Desiderio, 2009b). That photodecomposition pattern of a nitro group decreases signal intensities of a nitropeptide and complicates interpretation of a mass spectrum. However, that characteristic photodecomposition pattern can confirm the existence of a nitro group in an analyzed peptide (Zhan & Desiderio, 2009b).

Throughout this review, we clearly distinguish between endogenous in vivo nitroproteins/nitropeptides and synthetic in vitro nitroproteins/nitropeptides. Although many more publications relate to synthetic nitroproteins/nitropeptides, it is analytically much more challenging to analyze endogenous nitroproteins/nitropeptides because tyrosine nitration is an extremely low-abundance (1 in $\sim 10^6$ tyrosines) oxidative-stress process. The goals of nitroprotein analysis include the identity of the nitroprotein and each site of modification. A protein usually contains several tyrosine residues, and in a population of to-be-nitroated proteins, a tyrosine site might not be stoichiometrically nitrated.

Because of the sensitivity requirement of MS analysis and the in vivo low abundance of nitrotyrosine sites, a chemical derivation and targeted enrichment prior to MS analysis is needed (Dekker et al., 2012; Freeney & Schoneich, 2013; Zhan, Wang, & Desiderio, 2013), including (i) nitrotyrosine antibody-based immunoaffinity enrichment of nitroproteins (Zhan & Desiderio, 2006; Sultana, Reed, & Butterfield, 2009) and of nitropeptides (Gusanu, Petre, & Przybylski, 2011), (ii) conversion of a nitro group to an amino group coupled with derivatization of the amino group (Tsumoto, Taguchi, & Kohda, 2010). Briefly, protection of alpha and epsilon-amino groups in a protein or peptide with $^{13}$C$_0$/13C$_4$- or D$_0$/D$_6$-acetic anhydride, reduction of nitrotyrosine to aminotyrosine with sodium dithionite (also known as sodium hydrosulfite), and derivatization of aminotyrosine with 1-(6-methyl)[D$_0$/D$_3$]nicotinoyloxy) succinimide; (iii) conversion of a nitro group to an amino group coupled with target enrichment (Abello et al., 2010). Briefly, all amines are first acetylated, followed by conversion of nitrotyrosine to aminotyrosine, and biotinylation of aminotyrosine; (iv) reduction of the nitro group in a nitropeptide to an amino group and dansylated with dansyl chloride, followed with MS$^6$ analysis (Amoresano et al., 2007, 2008); (v) the use of “light”- and “heavy”-labeled acetyl groups to block N-terminal and lysine residues of tryptic nitropeptides, followed with reduction of nitrotyrosine to aminotyrosine with sodium dithionite and derivatization of light- and heavy-labeled aminotyrosine peptides with either tandem mass tags (TMT) or isobaric tags for relative and absolute quantification (iTRAQ), respectively (Robinson & Evans, 2012); (vi) a new quantitative identification strategy used iTRAQ reagents to selectively label nitrotyrosine residues (not primary amines) coupled to MS analysis (Chiappetta et al., 2009); (vii) use of selective chemoprecipitation and subsequent release of tagged species (conversion of nitro group to a small 4-formylbenzylamido tag) for analysis of nitropeptides with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Prokai-Tatrai, Guo, & Prokai, 2011); and (viii) use of combined fractional diagonal chromatography (COFRADIC) (Ghesquiere et al., 2009; Larsen et al., 2011) peptide sorting is based on a hydrophilic shift after reduction of the nitro group to its amino counterpart, followed with ESI-MS (Ghesquiere et al., 2009) and MALDI-MS (Larsen et al., 2011) identification of a nitropeptide. Except for the proteomics method based on anti-nitrotyrosine antibodies and gel-based separation, multidimensional chromatography, precursor-ion scanning, and/or chemical derivatization have also emerged to identify and quantify nitroprotein and nitrotyrosine sites (Zhang, Yang, & Poschl, 2011; Freeney & Schoneich, 2013).

In-depth analyses of nitrotyrosine-containing proteins is needed to clarify biological functions and roles of tyrosine nitration, and several aspects are worth discussing here: (i) quantitative proteomics to quantify a nitrotyrosine-containing protein in a pathological condition and the degree of nitration (Robinson & Evans, 2012), (ii) use of bioinformatics to locate nitrotyrosine sites within important protein domains and motifs (Zhan & Desiderio, 2006, 2011), (iii) use of systems biology methods to clarify important protein system networks that are involved in nitroproteins (Zhan & Desiderio, 2010a; Zhan, Wang, & Desiderio, 2013), (iv) effects of local primary structure on tyrosine nitration (Seeley & Stevens, 2012), structural biology to reveal the three-dimensional crystal structure of nitrotyrosine-containing proteins to address influences of tyrosine nitration on protein functions towards development of a drug against tyrosine nitration (Palamalai & Miyagi, 2010; Zhan, Wang, & Desiderio, 2013), and (v) development of body-fluid nitroproteomics and nitropeptidomics for discovery of body-fluid biomarkers for prediction, diagnosis, and prognosis of a disease (Zhan & Desiderio, 2010b; Zhan, Wang, & Desiderio, 2013).

II. CHARACTERISTICS OF MALDI-MS AND ESI-MS OF NITROTYROSINE-CONTAINING PEPTIDES

The MS characteristics of a nitrotyrosine-containing peptide (nitropeptide) differ significantly between matrix-assisted laser desorption ionization (MALDI)-and electrospray ionization (ESI)-MS (Petersson et al., 2001; Sarver et al., 2001; Zhan & Desiderio, 2009b). The high-energy laser in MALDI UV-laser MS (laser light at 337 nm) induces photochemical decompositions of the nitro group ($-\text{NO}_2$) to decrease the precursor-ion intensity of a nitropeptide and to complicate an MS spectrum (Petersson et al., 2001; Sarver et al., 2001; Lee et al., 2007; Zhan & Desiderio, 2009b). However, the photochemical decomposition pattern ([M+H]$^+$, [M+H−16]$^+$, [M+H−30]$^+$, and [M+H−32]$^+$) can clarify the decomposition pattern of a nitrotyrosine-containing peptide/protein (Petersson et al., 2001; Sarver et al., 2001; Zhan & Desiderio, 2009b). The scheme in Figure 1 shows generation of dityrosine and nitrotyrosine, and likely products of nitrotyrosine photochemical decomposition (Turko & Murad, 2005). ESI does not produce those decompositions (Petersson et al., 2001; Sarver et al., 2001; Lee et al., 2007, 2009b; Yeo et al., 2008; Zhan & Desiderio, 2009b; Kim et al., 2011). However, under ESI conditions, the characteristic immonium ion (m/z 181.06) that derives from a nitrotyrosine residue in an MS/MS spectrum can indicate the presence of a nitrotyrosine residue; meanwhile, the precursor-ion scanning for the immonium ion (m/z 181.06) for nitrotyrosine will accurately identify a nitrotyrosine-containing peptide/protein (Petersson et al., 2001).

The synthetic nitropeptide AAGFY(−NO$_2$)AR ([M+H]$^+$ = 800.4) was studied with MALDI-MS (Sarver et al., 2001). A
photochemical decomposition pattern ([M+H]⁺, [M+H – 16]⁺, [M+H – 14]⁺, [M+H – 32]⁺, and [M+H – 30]⁺) that corresponded to m/z 800.4, 784.4, 786.4, 768.4, and 770.4, respectively, was found in the MS spectrum (Fig. 2). The [M+H]⁺ ion (m/z 800.4) was the nitrotyrosine (Tyr-NO₂)-containing peptide; [M+H – 16]⁺ (m/z 784.4) was the nitrosotyrosine (Tyr-NO) containing peptide after loss of an oxygen atom from the nitro group; [M+H – 14]⁺ (m/z 786.4) was the hydroxylaminotyrosine (Tyr-NHOH)-containing peptide after reduction of the nitroso (Tyr-NO) group; [M+H – 30]⁺ (m/z 770.4) was the triplet nitrene (Tyr-N) group. Moreover, the –30/32 Da photodecomposition products (Tyr-NH₂ and Tyr-N) were considerably lower in abundance than the protonated molecule ion of (Tyr-NO₂), and the –14/16 Da photodecomposition products (Tyr-NHOH and Tyr-NO). The MALDI UV-induced photodecomposition pattern was also confirmed with nitropeptides from bovine serum albumin that was nitrated with tetrynitromethane (TNM) (Sarver et al., 2001), TNM-treated angiotensin II ([M+H]⁺, m/z 1092.5) (Petersson et al., 2001), and the synthetic peptides leucine enkephalin (LE1: Y-G-G-F-L, molecular weight (MW) = 555.1818 Da), nitro-Tyr-leucine enkephalin [LE2: (3-NO₂)Y-G-G-F-L, MW = 600.0909 Da] and d₅-Phe-nitro-Tyr-leucine enkephalin [LE3: (3-NO₂)Y-G-G-(d₅)F-L, MW = 605.1818 Da] (Zhan & Desiderio, 2009b). Moreover, the base-peak intensity of the [M+H]⁺ ion of leucine enkephalin (LE1, NL = 1.01E5) was much higher than that of nitro-Tyr leucine enkephalin (LE2, NL = 3.25E4) and d₅-Phe-nitro-Tyr leucine enkephalin (LE3, NL = 9.09E4) to demonstrate that photochemical decomposition decreased ion intensity and complicated the MS spectrum (Fig. 3) (Zhan & Desiderio, 2009b). In contrast, infrared-MALDI-Fourier transform ion cyclotron resonance mass spectrometry (IR-MALDI-FT-ICR-MS), which is a highly efficient method to determine protein nitration, did not fragment protonated molecule ions of nitrotyrosine peptides (Petre et al., 2005). The precise reason why MALDI with laser light at 337 nm induces photodecompositions of a nitro group in a nitropeptide—but not with infrared light—and the structures of these decomposition products remain unknown (Sarver et al., 2001); however, it is probable that the higher energy content at 337 nm induces loss of one or two oxygen atoms of the nitro group in a nitropeptide (Sarver et al., 2001).

The photodecomposition pattern of a nitro group was not found in an ESI-MS spectrum. TNM-nitrated angiotensin II [DRVY(–NO₂)IHPF; MW = 1090.76 Da] was analyzed with ESI-MS (Petersson et al., 2001). A mononitrated angiotensin II ion ([M + 2H]²⁺ m/z 546.38, [NO₂-Tyr]-angiotensin II) and dinitrinated angiotensin II ion ([M + 2H]²⁺ m/z 594.85, [NO₂₂-Tyr]-angiotensin II) were found in the ESI-MS spectrum, but no decomposition pattern was found (Fig. 4). The doubly charged precursor ions for mononitrated angiotensin II at m/z 546.38 and for dinitrinated angiotensin II at m/z 594.85 were further analyzed with MS/MS (Fig. 5); characteristic immonium ions at m/z 181.06 for mononitrated tyrosine and at m/z 226.0 for dinitrinated tyrosine were found in the ESI-MS/MS spectrum (Fig. 5). The characteristic immonium ion-based precursor-ion scan spectra accurately identify a nitropeptide in complicated sample (Fig. 6). The ESI-MS behavior of a nitropeptide and the precursor-ion scans for an immonium ion at m/z 181.06 were further confirmed with ESI-MS analysis of TNM-nitrated bovine serum albumin (Petersson et al., 2001).

In the MALDI-MS/MS analysis of synthetic peptides LE1 (Tyr-Gly-Gly-Phe-Leu; Y-G-F-L; 555.1818 Da), LE2 ((3-NO₂)Tyr-Gly-Gly-Phe-Leu; (3-NO₂)Y-G-F-L; 600.0909 Da), and LE3 ((3-NO₂)Tyr-Gly-Gly-(d₅)Phe-Leu; (3-NO₂)Y-G-G-(d₅)F-L; 605.1818 Da), b- and a-ions were the most-intense...
fragment ions compared to y-ions (Fig. 7) (Zhan & Desiderio, 2009b); those data were corroborated with MALDI-MS/MS analysis of nitrated angiotensin II (Petersson et al., 2001). Compared to the unmodified peptide (LE1), more collision energy optimized fragmentation of the nitropeptide (Fig. 8A) but increased the intensity of the a4-ion and decreased the intensity of the b4-ion (a-ion = loss of CO from a b-ion) (Fig. 8B). Furthermore, optimized laser fluence maximized fragmentation of the nitropeptide. Although MS3 analysis confirmed the MS2-derived amino acid sequence, MS3 analysis requires a higher amount of peptides relative to MS2 (Zhan & Desiderio, 2009b). Thus, MS3 analysis might not be suitable for routine analysis of endogenous low-abundance nitroproteins. Only when a target is determined can MS3 be used for confirmation. To detect a nitropeptide, the amount of peptide must reach the sensitivity of a mass spectrometer; for synthetic nitropeptides, the sensitivity of vMALDI-LTQ was 1 fmol for MS detection, and 10 fmol for MS2 detection (Zhan & Desiderio, 2009b).

The MS/MS (described above) of a nitrotyrosine-containing peptide was based on collision-induced dissociation (CID). However, the fragmentation behaviors of nitrotyrosine-containing peptides were different among CID-, electron-capture dissociation (ECD)-, electron-transfer dissociation (ETD)-, and metastable atom-activated dissociation (MAD)-MS (Jones et al., 2010; Cook & Jackson, 2011). Those studies found that the presence of nitration did not affect the CID behavior of the peptides. For doubly charged peptides, production of ECD sequence fragments was severely inhibited with nitration; ECD of triply charged nitrotyrosine-containing peptides produced some singly charged sequence fragments. ECD of nitropeptides was characterized with multiple losses of small neutral species, including hydroxyl radicals, water, and ammonia. The origin of neutral losses was investigated with activated ion (AI) ECD. Loss of ammonia appears to be the result of non-covalent interactions between a nitro group and protonated lysine side-chains (Jones & Cooper, 2010; Jones et al., 2010). Further studies found that high kinetic energy helium MAD produced extensive backbone fragmentation with significant retention of post-translation modifications (PTMs). Although the high electron affinity of a nitrotyrosine moiety quenched radical chemistry and fragmentation in ECD and ETD, MAD does produce numerous backbone cleavages in the vicinity of the nitration. Compared to CID, MAD produced more fragment ions, and

FIGURE 2. Photodecomposition pattern of the synthetic nitropeptide AAFGY(–NO2)AR in the MALDI-TOF spectrum in the (A) linear mode and (B) reflectron mode. The structure of 3-nitrotyrosine and the proposed photodecomposition products are shown next to various ions. Several small ions (asterisk) might represent metastable peaks (see text for details). A slight increase in the abundance of the ion at m/z 771.4 over what would be expected for the 13C isotope peak for the amino-tyrosine products at m/z 770.4 in the linear and reflectron spectra suggests that a small amount of a catechol product might have formed as well. Reproduced from Sarver et al. (2001), with permission from Elsevier Science, Inc., copyright 2001.
differentiated I/L residues in nitrated peptides. MAD induced radical-ion chemistry even in the presence of strong radical traps, and, therefore, offers unique advantages to ECD, ETD, and CID to determine nitrotyrosine-containing peptides (Cook & Jackson, 2011). Moreover, different types of CID-MS/MS have different abilities to identify nitrotyrosine-containing proteins (Li et al., 2011). For the same samples, a QSTAR Elite (QTOF) with CID was used to identify 119 3NT peptides and 23 multiply nitrated 3NT peptides, whereas a dual-pressure ion-trap mass spectrometer (LTQ Velos) with CID was used to identify 197 3NT peptides and 36 multiply nitrated 3NT peptides (Fig. 9) (Li et al., 2011). Therefore, the choice of an appropriate mass spectrometer is essential to analyze nitrotyrosine-containing peptides/proteins.

**FIGURE 3.** MALDI MS spectra of LE1 (A), LE2 (B), and LE3 (C). nY = nitro-Tyr. F(d5) = Phe residue with five 2H(D) atoms. Reproduced from Zhan and Desiderio (2009b), with permission from Elsevier Science, copyright 2009; Reproduced from Zhan, Wang, and Desiderio (2013), with permission from Hindawi Publishing Corporation. Copyright 2013 remains with authors due to the open-access article under the Creative Commons Attribution License.
**FIGURE 4.** ESI-MS spectrum of nitrated angiotensin II to show mono- and dinitrated angiotensin II. Reproduced from Petersson et al. (2001), with permission from Wiley-VCH, copyright 2001.

**FIGURE 5.** The MS² spectra of nitrated angiotensin II peptides. The doubly charged ions were selected as precursor ions for mononitrated angiotensin II at m/z 546.30 (A) and for the dinitrated angiotensin II at m/z 568.80 (B). Reproduced from Petersson et al. (2001), with permission from Wiley-VCH, copyright 2001.
proteins. Some synthetic peptides such as AAFGY(–NO₂)AR in vitro nitropeptide, it is crucial to develop methods to analyze sites, and the limitation of sensitivity of MS to detect a nitro (–NO₂) group during MS analysis, a nitrotyrosine-containing peptides, low-abundance of因为 of the particular MS characteristics of nitrotyrosine-containing proteins, low-abundance of in vivo nitrotyrosine sites, and the limitation of sensitivity of MS to detect a nitropeptide, it is crucial to develop methods to analyze in vitro nitrotyrosine-containing proteins due to the easily availability of in vitro synthetic or nitrated nitrotyrosine-containing peptides/proteins. Some synthetic peptides such as AAFGY(–NO₂)AR (Sarver et al., 2001), leucine enkephalin, nitro-Tyr-leucine enkephalin, and d₅-Phe-nitro-Tyr-leucine enkephalin (Zhan & Desiderio, 2009b) were used to study MS characteristics of a nitropeptide. Angiotensin II, bovine serum albumin (BSA), and ovalbumin (OVA) are the commonly used standard peptides and proteins that are in vitro nitrated with liquid TNM (Sokolovsky, Riordan, & Vallee, 1966; Petersson et al., 2001; Sarver et al., 2001; Ghesquiere et al., 2006). To simulate in vitro proteome situations, some proteomes such as human plasma were nitrated in vitro with tetranitromethane followed with nitroproteomics analysis (Prokai-Tatrai, Guo, & Prokai, 2011). Those prepared standard nitropeptides, nitroproteins, and nitroproteome samples in vitro were studied with several well-established nitroproteomic methods based on anti-3-nitrotyrosine antibody and gel-based separations (Petersson et al., 2001; Freeney & Schoneich, 2013). Methods that involved multidimensional chromatography, diagonal chromatography (Ghesquiere et al., 2009; Larsen et al., 2011), precursor-ion scanning (Petersson et al., 2001), and/or chemical derivation can identify and quantify protein nitration sites (Freeney & Schoneich, 2013).

Several chemical derivation methods have been developed to analyze nitrotyrosine-containing peptide/protein prior to MS (Dekker et al., 2012). All the chemical derivation methods of nitrotyrosine that have been developed employ reduction of nitrotyrosine to aminotyrosine, and derivatization of the generated amino group with specific reagents. (i) Conversion of a nitrotyrosine residue to an aminotyrosine residue via reduction readily discerns aminotyrosine peptides in a background of non-nitrated peptides, and aminotyrosine peptides were more stable in a single MS mode and led to easy-to-interpret peptide mass maps (Ghesquiere et al., 2006). (ii) The use of dansyl chloride to label nitration sites followed with MS/MS plus a precursor-ion scan (Amoresano et al., 2007, 2008). (iii) For MALDI-MS analysis of a nitropeptide, the optimum matrix was not 2,5-dihydroxybenzoic acid but sinapinic acid (Sheeley, Rubakhin, & Sweedler, 2005). (iv) A method was developed that specifically enriches nitropeptides to unambiguously identify nitrotyrosine peptides and nitration sites with LC-MS/MS, and includes conversion of nitrotyrosine to N-thioacetyl-aminotyrosine, followed with high-efficiency enrichment of sulfydryl-containing peptides with thiopropyl sepharose beads (Zhang et al., 2007). Derivation includes (a) acetylation with acetic anhydride to block all primary amines, (b) reduction of nitrotyrosine to aminotyrosine, (c) derivatization of aminotyrosine with N-succinimidyl S-acetyltioacetate, and (d) deprotection of S-acetyl on S-acetyltioacetate to form free sulfhydryl groups (Zhang et al., 2007). This method was used to study in vitro nitrated human histone H1.2, BSA, and mouse brain tissue samples (Zhang et al., 2007). (v) iTRAQ is an effective quantitative proteomics method, but is limited to primary amines. A new strategy was developed that was based on use of iTRAQ reagents coupled to MS analysis to selective label nitrotyrosine residues (Chiappetta et al., 2009) to simultaneously localize and quantify nitration sites in model proteins and biological systems (Chiappetta et al., 2009). (vi) A strategy that combined precursor isotopic labeling and isobaric tagging (cPILOT) increased the multiplexing capability to quantify a nitrotyrosine protein to 12 or 16 samples with TMT or iTRAQ, respectively. That method used light- and heavy-labeled acetyl groups to block N-termini and lysine residues of tryptic peptides. Nitrotyrosine was reduced to aminotyrosine with sodium dithionite, followed with derivatization of light- and heavy-labeled aminotyrosine peptides with either TMT or iTRAQ multiplex reagents (Robinson & Evans, 2012). This method demonstrated proof-of-principle in the analysis of

III. METHOD DEVELOPMENT FOR ANALYSES OF in vitro SYNTHETIC NITROTYROSINE-CONTAINING PROTEINS

Because of the particular MS characteristics of nitrotyrosine-containing peptides, low-abundance of in vivo nitrotyrosine sites, and the limitation of sensitivity of MS to detect a nitropeptide, it is crucial to develop methods to analyze in vitro nitrotyrosine-containing proteins due to the easily availability of in vitro synthetic or nitrated nitrotyrosine-containing peptides/proteins. Some synthetic peptides such as AAFGY(–NO₂)AR (Sarver et al., 2001), leucine enkephalin, nitro-Tyr-leucine enkephalin, and d₅-Phe-nitro-Tyr-leucine enkephalin (Zhan & Desiderio, 2009b) were used to study MS characteristics of a nitropeptide. Angiotensin II, bovine serum albumin (BSA), and ovalbumin (OVA) are the commonly used standard peptides and proteins that are in vitro nitrated with liquid TNM (Sokolovsky, Riordan, & Vallee, 1966; Petersson et al., 2001; Sarver et al., 2001; Ghesquiere et al., 2006; Zhang, Yang, & Poschl, 2011), gaseous nitrogen dioxide and ozone (NO₂ + O₃) (Zhang, Yang, & Poschl, 2011), or peroxynitrite (Fujigaki et al., 2006). Moreover, because the amino (–NH₂) group is more stable than the nitro (–NO₂) group during MS analysis, a nitrotyrosine-containing protein/peptide was reduced to an aminotyrosine-containing protein/peptide with reducing agent (Na₂S₂O₄) prior to MS analysis (Sokolovsky, Riordan, & Vallee, 1967; Saver et al., 2001; Ghesquiere et al., 2006). To simulate in vitro proteome situations, some proteomes such as human plasma were nitrated in vitro with tetranitromethane followed with nitroproteomics analysis (Prokai-Tatrai, Guo, & Prokai, 2011). Those prepared standard nitropeptides, nitroproteins, and nitroproteome samples in vitro were studied with several well-established nitroproteomic methods based on anti-3-nitrotyrosine antibody and gel-based separations (Petersson et al., 2001; Freeney & Schoneich, 2013). Methods that involved multidimensional chromatography, diagonal chromatography (Ghesquiere et al., 2009; Larsen et al., 2011), precursor-ion scanning (Petersson et al., 2001), and/or chemical derivation can identify and quantify protein nitration sites (Freeney & Schoneich, 2013).

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FIGURE 6. Precursor-ion scan spectra of nitrated angiotensin II for formation of the immonium ion at m/z 181.06 for mononitrated tyrosine (A) and at m/z 256.0 for dinitrated tyrosine (B). Reproduced from Peterson et al. (2001), with permission from Wiley-VCH, copyright 2001.

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in vitro nitrated BSA and mouse splenic proteins (Robinson & Evans, 2012). (vii) Improved chemical-labeling methods were designed to enrich nitrotyrosine-containing peptides independent of sequence context. In this procedure (Fig. 10), all amines were blocked with acetylation, followed by conversion of nitrotyrosine to aminotyrosine and biotinylation of aminotyrosine (Abello et al., 2010). Moreover, the entire reaction was carried out in a single buffer without any sample cleanup or pH changes to, thereby, reduce sample loss. Free biotin was removed with a strong-cation exchanger, labeled peptides were enriched with an immobilized avidin column, and enriched peptides were analyzed with LC-MS/MS (Abello et al., 2010). This method has been approved for in vitro nitrated samples (Abello et al., 2009, 2010). (viii) Because of the photodecomposition of a nitro group with a MALDI UV-laser, a strategy was developed that includes

**FIGURE 7.** MS² spectra of LE1 (A), LE2 (B), and LE3 (C). \(\text{nY} = \text{nitrato-Tyr}, \text{F(15)} = \text{Phe residue with five } ^2\text{H} \text{ (D)} \) atoms. Reproduced from Zhan and Desiderio (2009b), with permission from Elsevier Science, copyright 2009; Reproduced from Zhan, Wang, and Desiderio (2013), with permission from Hindawi Publishing Corporation. Copyright 2013 remains with authors due to the open-access article under the Creative Commons Attribution License.
(a) acetylation of N-terminal amines and epsilon-amines of lysine residues with acetic anhydride, (b) reduction of nitrotyrosine to aminotyrosine with sodium hydrosulfite, and (c) derivatization of aminotyrosine with 1-(6-methyl[\textsuperscript{13}C\textsubscript{0}/\textsubscript{13}C\textsubscript{3}]nicotinoyloxy) succinimide, followed with MALDI-TOF MS analysis (Tsumoto, Taguchi, & Kohda, 2010). (ix) The combined fractional diagonal chromatography (COFRADIC) approach (Ghesquiere et al., 2009; Larsen et al., 2011) was developed. Briefly, the basics of COFRADIC is reduction of nitrotyrosine to aminotyrosine with sodium dithionite; peptides are sorted with reverse-phase chromatography based on a hydrophilic shift from nitrotyrosine-containing peptide (more hydrophilic) to aminotyrosine-containing peptide (more hydrophobic) followed with ESI-MS (Ghesquiere et al., 2009) and MALDI-MS (Larsen et al., 2011) identification. COFRADIC has successfully been used to characterize tyrosine nitration in a TNM-nitrated BSA, peroxynitrite-nitrated proteome of human Jurkat cells (Ghesquiere et al., 2009; Larsen et al., 2011).

The interpretation of MS and MS/MS data of nitrotyrosine-containing (and especially endogenous) peptides is very challenging. To avoid any risk of linking MS/MS spectra to an incorrect amino acid sequence, the combination of reduction of nitrotyrosine to aminotyrosine and use of the Peptizer algorithm to inspect MS/MS quality-related assumptions (Ghesquiere et al., 2011) has been developed. The optimal approach to determine the amino acid sequence of a nitropeptide is a manual approach (Zhan & Desiderio, 2006).

**FIGURE 8.** Effect of collision energy on fragmentation of nitropeptides. A: Relationship between collision energy and product-ion intensity \((n = 3)\). B: Relationship between collision energy and product-ion \(b_4\) and \(a_4\) intensities \((n = 3)\). Reproduced from Zhan and Desiderio (2009b), with permission from Elsevier Science, copyright 2009; Reproduced from Zhan, Wang, and Desiderio (2013), with permission from Hindawi Publishing Corporation. Copyright 2013 remains with authors due to the open-access article under the Creative Commons Attribution License.
Tyrosine nitration is a low-abundance (1 in \( \sim 10^6 \) tyrosines) oxidative stress-related protein modification in an \textit{in vivo} proteome (Haddad et al., 1994; Shigenaga et al., 1997). MS is the key technique to characterize nitroproteins/nitropeptides and to accurately locate each nitrotyrosine site within a nitroprotein (Zhan & Desiderio, 2004, 2006, 2007). However, MS is limited in its sensitivity to the high-femtomole/low-picomole level (Zhan & Desiderio, 2009a). Therefore, isolation and preferential enrichment of nitroproteins/nitropeptides are essential prior to MS analysis (Zhan & Desiderio, 2007; Yeo et al., 2008, Lee et al., 2009b; Kim et al., 2011).

Several enrichment protocols have been developed to isolate and preferentially enrich \textit{in vivo} nitroproteins/nitropeptides from a biological proteome. (a) Two-dimensional gel electrophoresis (2DGE)-based nitrotyrosine Western blotting analysis (Fig. 11) (Aulak et al., 2001; Miyagi et al., 2002; Zhan & Desiderio, 2004, 2007; Butt & Lo, 2008; Zhan, Wang, & Desiderio, 2013). (b) Nitrotyrosine-affinity-column (NTAC) (Fig. 12) to enrich nitrotyrosine-containing proteins (Zhan & Desiderio, 2006; Justilien et al., 2007; Zhan, Wang, & Desiderio, 2013) and to enrich nitrotyrosine-containing peptides (Petre et al., 2012). (c) After acetylation of all primary amines in a nitrotyrosine-containing peptide, a nitrotyrosine residue is easily converted into aminotyrosine followed with enrichment with biotinylation of an aminotyrosine (Fig. 10) (Ghesquiere et al., 2006; Abello et al., 2010; Dekker et al., 2012). (d) After acetylation of all primary amines and reduction of nitrotyrosine to aminotyrosine, derivatization of aminotyrosine into a free sulphydryl group followed by enrichment of sulphydryl-containing peptides with thiopropyl sepharose beads (Zhang et al., 2007). (e) Dansyl chloride labeling of nitration sites in combination with a precursor-ion scan and MS3 analysis (Amoresano et al., 2007, 2008). (f) A new tagging reagent, (3R, 4S)-1-(4-(aminomethyl)phenylsulfonyl)pyrrolidine-3,4-diol (APPD) was used for selective fluorogenic derivatization of nitrotyrosine residues in peptides (after reduction to aminotyrosine) and boronate affinity enrichment (Dremina et al., 2011). (g) Use of COFRADIC to sort peptides according to the hydrophilic shift after reduction of a nitro group to an amino group, followed with ESI or MALDI-MS (Ghesquiere et al., 2009). (h) Quantitative identification of nitrotyrosine-containing proteins/peptides was also developed with TMT or iTRAQ (Chiappetta et al., 2009; Robinson & Evans, 2012); the “light”- and “heavy”-labeled acetyl groups were used to block \( N \)-termini and lysine residues of tryptic peptides, followed by reduction of nitrotyrosine to aminotyrosine and derivatization of light- and heavy-labeled aminotyrosine-containing peptides with either TMT or iTRAQ multiplex reagents (Chiappetta et al., 2009; Robinson & Evans, 2012). That method can relatively enrich and quantitatively identify nitrotyrosine-containing proteins/peptides.

Protocols (a), (b), and (g) have been used to identify endogenous nitrotyrosine-sites (Zhan & Desiderio, 2004, 2006, 2007; Justilien et al., 2007; Ghesquiere et al., 2009). Protocols (c), (d), (e), (f), and (g) have succeeded mainly with an \textit{in vitro} model peptide or protein, and with an \textit{in vitro} nitrotyrosine proteome (Ghesquiere et al., 2006, 2009; Amoresano et al., 2007; Zhang et al., 2007; Larsen et al., 2011), and provide promise for \textit{in vivo} studies of nitrotyrosine-containing proteins. Protocols (a)–(g) mainly focus on identification of nitropeptides, nitroproteins, and nitrotyrosine sites. However, to discover disease-related nitroproteins, except for identification of nitrotyrosine sites and nitroproteins, quantitative identification of nitrotyrosine-containing proteins is needed. Protocol (h) holds promise for that goal because it can relatively enrich nitrotyrosine peptides, identify nitrotyrosine sites, and quantify nitrotyrosine-containing proteins; it also has enhanced sample-multiplexing capabilities (Chiappetta et al., 2009; Robinson & Evans, 2012).

V. IDENTIFICATION OF IN VIVO NITROTYROSINE-CONTAINING PROTEINS IN DIFFERENT PATHOLOGICAL CONDITIONS

Oxidative/nitrative stress-mediated modifications in a protein play important roles in a wide range of cellular, physiological, and pathological processes (Dalle-Donne et al., 2005; Dalle-Donne, Scalon, & Butterfield, 2006). Protein tyrosine nitration is an important redox-related modification that alters activity of a protein. Endogenous nitrotyrosine-containing proteins and nitrotyrosine-sites have been identified in differential pathophysiological conditions, including several main categories (Table 1). (1) Tyrosine nitration is clearly involved in tumorigenesis. Nine nitroproteins, three nitroprotein-interacted proteins, and ten nitrotyrosine sites were identified from a pituitary adenoma (Zhan & Desiderio, 2006), and eight nitroproteins were identified from human pituitary control tissue (Zhan & Desiderio, 2004, 2007) (Table 2). Nitrated protein was also identified in human gliomas (Fiore et al., 2006) and rat glioma cell lines (Nakagawa et al., 2007). (2) Tyrosine nitration is...
involved in aging and aging-related diseases. Nitrotyrosine-containing proteins were discovered in aging rat skeletal muscle (Kanski, Hong, & Schoneich, 2005b; Sharov et al., 2006), rat heart (Kanski et al., 2005a), and mouse liver (Marshall et al., 2013). (3) Tyrosine nitration is involved in inflammation-related diseases. Nitrotyrosine-containing proteins were identified in a septic patient's rectus abdominis muscle (Lanone et al., 2002), bronchial epithelial cells and bronchoalveolar lavage with asthma (Ghosh et al., 2006), Chagas' disease (Dhiman et al., 2008), experimental sepsis (Chatterjee et al., 2009), and serum sample of a C57BL6/J mouse model with septic shock (Ghesquiere et al., 2009). (4) Tyrosine nitration is involved in neurodegenerative diseases. Nitrotyrosine-containing proteins were identified in mouse brain (Sacksteder et al., 2006; Bigelow & Qian, 2008; Zhang et al., 2010), spinal cords of a mouse model of familial amyotrophic lateral sclerosis (Casoni et al., 2005), Parkinson's disease (Danielson et al., 2009), and HT22 hippocampal cells (Yoon et al., 2010). (5) Tyrosine nitration is involved in the neurovisual system. Nitroproteins were identified in SOD2-knockdown mouse eye cup (Justilien et al., 2007), photoreceptor rod outer segments (Palamalai et al., 2006), and human Bruch's membrane (Murdagh et al., 2010). (6) Tyrosine nitration is also involved in the cardiovascular system and related diseases. For example, nitroproteins have been identified in ischemia reperfusion injury (Liu et al., 2009; Chen et al., 2008; Tao et al., 2013), vascular (Ai et al., 2008), and mouse heart (Bigelow & Qian, 2008; Zhang et al., 2010). (7) Tyrosine nitration is involved in kidney disease. Nitroproteins have been identified in a kidney disease patient's plasma (Piroddi et al., 2011). (8) Tyrosine nitration is involved in diabetes. Nitroproteins have been discovered in diabetic rats (Lu et al., 2010), diabetic mellitus patients (Safinowski et al., 2009), and a diabetic patient's urine (Kato et al., 2009). (9) Plants. Nitroproteins were discovered in plant disease (Cecconi et al., 2009; Chaki et al., 2009). (10) Others. Nitroproteins were discovered in sickle cell disease (Aslan et al., 2003), traumatic brain-injured rats (Reed et al., 2009), rat hippocampus after acute inhalation of combustion smoke (Lee et al., 2009a), Eosinophil granule toxins (Ulrich et al., 2008), hypertriglyceridemia (Casanovas et al., 2009), placenta/pre-eclampsia (Webster, Brockman, & Myatt, 2006), human plasma (Hamilton et al., 2008; Hui

**FIGURE 10.** Reaction scheme of the chemical-labeling method as exemplified with an N-terminal nitrotyrosine residue. All amines were blocked with acetylation with acetic acid N-hydroxysuccinimide ester (NHS-acetate). Nitrotyrosine was reduced to aminotyrosine with heme and dithiothreitol in a boiling-water bath. The reaction sequence was completed with biotinylation of aminotyrosine with NHS-biotin. Reproduced from Abello et al. (2010), with permission from Elsevier publisher, copyright 2009.
et al., 2012), murine liver (Zhu et al., 2008), rabbit muscle (Sharov et al., 2009), human hemoglobin associated with cigarette (Chen & Chen, 2012), human peripheral blood mononuclear cells (Ohama & Brautigan, 2010), mast cells (Sekar et al., 2010), and endothelial cells (Redondo-Horcajo et al., 2010).

VI. BIOLOGICAL ROLES OF NITROTYROSINE-CONTAINING PROTEINS

Nitrotyrosine-containing proteins and nitrotyrosine sites that have been identified with MS/MS must be further analyzed to elucidate biological roles of tyrosine nitration. Until now, several approaches have been used to analyze roles of nitrotyrosine-containing proteins: (1) literature data-based rationalization of biological function, (2) protein domain and motif analyses, (3) systems pathway analysis, and (4) structural biology analysis. Here, nitroproteins from pituitary control and adenoma (Table 2) are used as an example to address those analyses.

1. Literature data-based rationalization of biological function: Nine nitroproteins and 10 nitrotyrosine sites, and 3 non-nitrated proteins from a human pituitary adenoma...
were analyzed through a large number of literature data (Zhan & Desiderio, 2006). As a result, three non-nitrated proteins (glutamate receptor-interacting protein 2, ubiquitin, and interleukin 1 receptor-associated kinase-like 2) were recognized to interact with nitroproteins to form three nitroprotein–protein complexes (nitrated proteasome–ubiquitin complex, nitrated beta-subunit of cAMP-dependent protein kinase (pKa) complex, and nitrated interleukin 1 family member 6-interleukin 1 receptor-interleukin 1 receptor-associated kinase-like 2 (IL1F6-IL1R-IRAK2)) (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013). Moreover, those nine nitroproteins and three nitroprotein–protein complexes were rationalized into a corresponding functional system (Fig. 13) (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013). The nitrated proteasome–ubiquitin

**FIGURE 12.** Experimental flowchart to identify nitroprotein and nitroprotein–protein complexes with NTAC-based MALDI-LTQ MS/MS. The control experiment (without any anti-3-nitrotyrosine antibody) was performed in parallel with the NTAC-based experiments. Reproduced from Zhan and Desiderio (2006), with permission from Elsevier Science, copyright 2006; Reproduced from Zhan, Wang, and Desiderio (2013), with permission from Hindawi Publishing Corporation. Copyright 2013 remains with authors due to the open-access article under the Creative Commons Attribution License.
| Reference                        | Specimen                  | Methods                          | Nitroprotein and nitrotyrosine sites                                                                 | Remark                                      |
|--------------------------------|---------------------------|----------------------------------|------------------------------------------------------------------------------------------------------|---------------------------------------------|
| **(1) Tumor**                   |                           |                                  |                                                                                                      |                                             |
| Zhan and Desiderio, 2006        | Human nonfunctional pituitary adenoma tissue | NTAC-vMALDI-MS/MS              | Nine nitroproteins, 10 nitrotyrosine sites, and 3 nitroprotein-interacting protein were identified | Endogenous; Laser-induced decomposition     |
| Zhan and Desiderio, 2004        | Human pituitary post-mortem tissue | 2D-Western blot and vMALDI-MS/MS | Four nitroproteins and 4 nitrotyrosine sites were identified                                         | Endogenous; Laser-induced decomposition     |
| Zhan and Desiderio, 2007        | Human pituitary post-mortem tissue | 2D-Western blot and vMALDI-MS/MS | Four nitroproteins and 4 nitrotyrosine sites were identified                                         | Endogenous; Laser-induced decomposition     |
| Fiore et al., 2006              | Human glioma tissues      | Immunohistochemistry, 1D-Western blot and MALDI-PMF | Tubulin was nitrated at Tyr224 in glioma grade IV, but not in grade I and non-cancerous brain tissue | Endogenous; Laser-induced decomposition     |
| Nakagawa et al., 2007           | C6 rat glioma cell line   | HPLC, MALDI-PMF                  | Cytochrome c was nitrated at Tyr 48, Tyr67, and Tyr74                                               | Endogenous; Laser-induced decomposition     |

| **(2) Aging and aging-related diseases** |                               |                                  |                                                                                                      |                                             |
| Kanski, Hong, and Schoneich, 2005 | Skeletal muscle from 34-month-old Fisher 344/Brown Norway F1 rats | IEF, 1D-Western blot and ESI-MS/MS | Eleven nitroproteins and 12 nitrotyrosine sites were identified                                      | Endogenous                                  |
| Sharov et al., 2006             | Skeletal muscle from 6-month-old and 34-month-old Fisher 344/Brown Norway F1 hybrid rats | 1D-Western blot and HPLC-ESI-MS/MS | phosphorylase b was found the accumulation of 3-nitrotyrosine on Tyr113, Tyr161, and Tyr573. Nitration on Tyr 113 was detected in 6-month-old and 34-month-old rat, nitration on Tyr161 and Tyr573 was detected only in 34-month-old rat. | Endogenous. Nitration is accumulated with aging |
Kanski et al., 2005

Heart from 5-month-old and 26-month-old Fisher 344/BN F1 hybrid rats

1D- and 2D-Western blot, ESI-MS/MS

Forty eight putative nitrated proteins. Nitrination at Tyr105 of the electron-transfer flavoprotein was identified

Endogenous. Heart homogenate and heart mitochondria. Nitration is effects of biological aging. Not every protein was identified its nitrotyrosine site.

Marshall et al., 2013

Liver from young (19-22 weeks) and old (24 months) C57/BL6 male mice

1D-Western blot and LC-ESI-MS/MS

Six putative nitrated proteins were identified

Nitration is associated with aging. No nitrotyrosine site was identified.

(3) Inflammation-related disease

Lanone et al., 2002

Rectus abdominis muscle from the same control and septic patients

Western blot, MALDI-TOF-PMF, and molecular modeling

Inducible nitric oxide synthase (iNOS) was nitrated at Tyr299, Tyr336, Tyr446, and Tyr698

Analysis coupled with iNOS three-dimensional crystal model

Ghosh et al., 2002

Lung tissues from allergen-induced murine model of asthma

2D-Western blot and LC-ESI-MS/MS

Twenty seven putative nitrated proteins were identified

Inflammation-related disease. No nitrotyrosine site were identified.

Dhiman et al., 2008

Plasma from patients with Chagas’ disease

1D- and 2D-Western blot, MALDI-PMF and LC-ESI-MS/MS

Fifty differentially expressed/nitrated proteins were identified.

Inflammation-related disease. No nitrotyrosine site were identified.

Chatterjee et al., 2009

Spleens from LPS-induced systemic inflammation model of C57BL6/J mice

1D-Western blot and LC-ESI-MS/MS

Carboxypeptide B1 (CPB1) was nitrated at specific tyrosine sites

Inflammation-related disease

(4) Neurodegenerative diseases

Sacksteder et al., 2006

Brain from C57BL/6J SCX-LC-ESI-MS/MS

Twenty nine nitroproteins and 31 nitrotyrosine sites

Endogenous. Links to Neurodegenerative disease
| Reference                        | Tissue/Model                                                                 | Methodology                                                                 | Nitroproteins/Nitrotyrosine sites | Notes                                                                 |
|--------------------------------|------------------------------------------------------------------------------|------------------------------------------------------------------------------|----------------------------------|----------------------------------------------------------------------|
| Zhang et al., 2010*             | Brain from C57BL/6J mice                                                     | LC-ESI-MS/MS                                                                 | Endogenous                       |                                                                    |
| Casoni et al., 2005             | Spinal cord from Tg SOD1 G93A mice and Tg SOD1 WT mice                      | 2D-Western blot and MALDI-PMF                                                 | Thirty two nitroproteins and 16 nitrotyrosine sites                  | Endogenous, Laser-induced decomposition. Familial amyothrophic lateral sclerosis |
| Danielson et al., 2009          | dox-inducible MAO-B PC12 cells                                               | LC-ESI-MS/MS                                                                 | alpha-synuclein was nitrated at Tyr39                                 | Model of Parkinson's disease                                           |
| Yoon et al., 2010               | Mouse hippocampal cell line HT22                                            | 2D-Western blot and MALDI-PMF                                                 | Thirteen nitroproteins were detected                                 | Glutamate-treated HT22 cells                                           |
| (5) The neurovisual system      |                                                                              |                                                                              |                                  |                                                                    |
| Justilien et al., 2007          | Mouse posterior eye cups                                                    | NTAC, SDS-PAGE, LC-ESI-MS/MS                                                   | Eight nitroproteins and nine nitrotyrosine sites                    | Endogenous. SOD2 knockdown mouse model of early AMD                    |
| Palamalai et al., 2006          | Photoreceptor rod outer segments of cyclic light-reared rats treated or not with the antioxidant | 2D-Western blot and LC-ESI-MS/MS                                              | Ten putative nitroproteins were identified                          | Endogenous. No nitrotyrosine sites were identified.                    |
| Murdaugh et al., 2010           | Human Bruch's membrane                                                       | HPLC, ESI-MS/MS                                                              | A2E was nitrated                                                        | Endogenous. Nitro-A2E is a specific biomarker of nitrosative stress in Bruch's membrane and its concentration is directly related to tissue age. |
| (6) Cardiovascular system and related diseases |                                                                              |                                                                              |                                  |                                                                    |
| Liu et al., 2009                | Male C57BL/6 mice myocardial ischemia reperfusion injury (I/R) model        | 1D- or 2D-Western blot, LC-ESI-MS/MS                                         | Twenty three nitroproteins were identified. Ten of them were from mitochondria | Endogenous. No nitrotyrosine sites were identified.                     |
| Chen et al, 2008                | Sprague-Dawley rat in vivo myocardial regional ischemia-reperfusion model   | 1DE-LC-ESI-MS/MS                                                             | Flavin subunit is nitrated at Tyr56 and Tyr142                       | Endogenous. Mitochondrial complex II in the post-ischemic myocardium   |
| Author(s) and Year | Tissue or Sample | Technique | Findings |
|--------------------|-----------------|-----------|----------|
| Ai et al., 2008    | Endothelial cell of human coronary arteries | LC-ESI-MS/MS | LDL was nitrated. |
| **(7) Kidney disease** | | | |
| Piroddi et al., 2011 | Plasma from kidney disease patients | 2DE and LC-ESI-MS/MS | Fourteen tentative nitroproteins and seven nitrotyrosine sites were identified. |
| Kato et al., 2009 | Healthy and diabetic human urine | LC-ESI-MS/MS | Urine nitrotyrosine |
| **(8) Diabetes** | | | |
| **(9) Plant diseases** | | | |
| Chaki et al., 2009 | Sunflower hypocotyls | 2D-Western blot and LC-ESI-MS/MS | Twenty one putative nitroproteins were identified |
| **(10) Others** | | | |
| Aslan et al., 2003 | Liver and kidney from Sickle cell disease mouse | Western blot and precipitation, MALDI-PMF, LC-ESI-MS/MS | Actin was nitrated at Tyr91, Tyr198, and Tyr240 |
| Reed et al., 2009 | Traumatic brain-injured rats | 2D-Western blot and MALDI-PMF | Several nitroprotein such as GSH were identified |
| Lee et al., 2009 | Hippocampus from smoke inhalation rat model | 2D-Western blot and MALDI-PMF or MALDI-MS/MS | Five nitroproteins of mitochondrial proteins were identified |
| Ulrich et al., 2008 | Human lung tissues and blood samples, aminal granule protein preparation. | Western blot and MALDI-PMF | Six nitroproteins and nitrotyrosine sites at Tyr349 in eosinophil peroxidase (EPO) and Tyr33 in both eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) were identified. |
| Casanovas et al., 2009 | Lipoprotein lipase of bovine and rat | 2D-Western blot and LC-ESI-MS/MS | Lipoprotein lipase was nitrated at Tyr95, Tyr164, Tyr 316 |
| Webster, Brockman, and Myatt, 2006 | Human placenta | 1D-Western blot and MALDI-PMF | p38 MAPK was nitrated. |
| | | | No nitrotyrosine sites were identified |
| Reference               | Sample Description                                      | Methodology                     | Findings                                                                                                                                 |
|------------------------|---------------------------------------------------------|--------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Hamilton et al., 2008  | Human plasma                                           | LC-ESI-MS/MS                   | Low-density lipoprotein (LDL) was nitrated at Tyr276, Tyr666, and Tyr720 of LDL-alpha 1, Tyr2524 of LDL-alpha 2, Tyr4141 of LDL-alpha 3, Tyr3139, Tyr3205, and Tyr3489 of LDL-beta 2 |
| Zhu et al., 2008       | Liver from SOD1-/- and WT C57BL/6 mice                  | 1DE, LC-ESI-MS/MS              | Ten candidate nitrated proteins were identified                                                                                                                                                 |
| Sharov et al., 2009    | Rabbit muscle                                          | LC-ESI-MS/MS                   | Glycogen phophorylase b was nitrated at 28 nitrotyrosine sites                                                                                                                                   |
| Chen & Chen, 2012      | Human blood samples from smokers and nonsmokers         | LC-ESI-MS/MS under the selected reaction monitoring (SRM)mode | Homoglobin was nitrated at Tyr24 and Tyr42 (alpha-globin), Tyr130 (beta-globin)                                                                                                                     |
| Ohma & Brautigan, 2010 | Human peripheral blood monoluclear cells               | LC-ESI-MS/MS                   | Protein phosphatase 2A was nitrated at Tyr284                                                                                                                                                    |
| Sekar et al., 2010     | Mast cells                                              | 2D-Western blot and LC-ESI-MS/MS | Aldolase was nitrated                                                                                                                                                                              |
| Redondo-Horcajo et al., 2010 | Endothelial cells from bovine aorties and mouse lung | LC-ESI-MS/MS                   | Manganese superoxide dismutase (MnSOD) was nitrated at Tyr34 in vitro nitrated with cyclosporine A                                                                                                 |
| Protein name                                      | nY site | Protein name                                      | nY site |
|--------------------------------------------------|---------|--------------------------------------------------|---------|
| **Nitrate protein**                               |         | **Nitrate protein**                               |         |
| Rho-GTPase-activating 5 [Q13017] (ARHGAP5)        | Y^550   | Synaptosomal-associated protein (SNAP91)          | Y^237   |
| Leukocyte immunoglobulin-like receptor A4 [P59901]| Y^404   | Ig alpha Fc receptor [P24071] (FCAR)              | Y^223   |
| Zinc finger protein 432 [O94892]                  | Y^41    | Actin [P03996] (ACTA2, ACTG2, ACTC1)              | Y^296   |
| PKA beta regulatory subunit [P31321] (PRKAR1B)    | Y^20    | PKG 2 [Q13237] (PRKG2)                            | Y^354   |
| Sphingosine-1-phosphate lyase 1 [O95470]          | Y^356   | Mitochondrial co-chaperone protein HscB [Q8IWL3]  | Y^128   |
|                                                   | Y^366   |                                                   |         |
| Centaurin beta 1 [Q15027]                         | Y^485   | Stanniocalcin 1 [P52823] (STC1)                   | Y^159   |
| Proteasome subunit alpha type 2 [P25787] (PSMA2)  | Y^228   | Proteasome subunit alpha type 2 (PSMA2)           | Y^228   |
| Interleukin 1 family member 6 [Q9UHA7] (IL1F6)    | Y^96    | Progesterin and adipose receptor family member III [Q6TCH7] (PAQR3) | Y^33   |
| Rhophilin 2 [Q8IUC4] (RHPN2)                      | Y^258   |                                                   |         |
| **Nitroprotein-interacted protein**               |         | **Nitroprotein-interacted protein**               |         |
| Interleukin-1 receptor-associated kinase-like 2 (IRAK-2) [Q43187] (IRAK2) |         |                                                   |         |
| Glutamate receptor interacting protein 2 [Q9C0E4] (GRIP2) |         |                                                   |         |
| Ubiquitin [P62988] (UBB or UBC)                   |         |                                                   |         |

**Note:** nY, nitrotyrosine. Modified from Zhan & Desiderio, 2004, 2006, 2007, with permission from Elsevier Science, copyright 2004, 2006, and 2007.
complex is an important enzymatic complex that is involved in the intracellular non-lysosomal proteolytic pathway (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013). Nitrated leukocyte immunoglobulin-like receptor subfamily A member 4 (LIRA4) might be involved in the immune system. Nitrated sphingosine-1-phosphate lyase 1 (S1P lyase 1) participates in sphingolipid metabolism to regulate cell proliferation, survival, cell death, and immune system (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013). Nitrated centaurin beta 1 (CENT-b1) and nitrated cAMP-dependent protein kinase type I-beta regulatory subunit (PKAR1-b) are involved in the pKa signal pathway. IRAK-2 in the IL1-R complex and nitrated IL1-F6 are involved in the cytokine system. Nitrated zinc finger protein 432 (ZFP432) is involved in transcription regulatory systems. Nitrated Rho-GTPase-activating protein 5 (RHOGAP5) and nitrated rhophilin 2 are involved in the GTPase signal pathway (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013).

2. Protein domain and motif analyses: Identification of protein domain/motif and location of nitrotyrosine sites into a protein domain/motif will assist in accurate elucidation of biological activities of tyrosine nitration (Zhan, Wang, & Desiderio, 2013). Several software programs such as ScanProsite (http://us.expasy.org/tools/scanprosite/), Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), Inter-ProScan (http://www.ebi.ac.uk/InterProScan/), ProDom (http://prodom.prabi.fr/prodom/current/html/form. php), and Pfam (http://www.sanger.ac.uk/Software/Pfam/) were used to determine statistically significant domains/motifs of each nitroprotein and to locate each nitrotyrosine site within a protein domain to gain insight into the effect of tyrosine nitration on protein functions (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013). As a result, most nitrotyrosine sites occur within important protein domains and motifs (Zhan & Desiderio, 2006) (Fig. 14); that finding hints that tyrosine nitration alters protein functions. For example, sphingosine-1-phosphate lyase 1 (S1P lyase 1) (Fig. 14), nitrated in a human pituitary adenoma, is a key enzyme to catalyze decomposition of S1P. Two nitration sites (NO2-356Y and NO2-366Y) within the enzyme activity region could decrease the interaction intensity of enzyme: substrate (S1P lyase 1: S1P) to alter enzymatic activities of S1P lyase 1 (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013).

3. Systems pathway analysis: Each protein in a proteome does not work alone, but functions within a system of multiple, complex, and interacting systematic networks (Zhan & Desiderio, 2010a). It is necessary to determine effects of tyrosine nitration on those complex pathway systems networks. Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/) and MetaCore Pathway Analysis programs (http://www.genego.com/metacore.php) were used to elucidate signaling networks that involve nitroproteins from a human pituitary adenoma and control (Table 2) (Zhan & Desiderio, 2006; Zhan, Wang, & Desiderio, 2013). IPA pathway analyses (Zhan & Desiderio, 2010a) clearly indicated that those pituitary adenoma nitroproteins and their complexes are involved in the tumor necrosis factor (TNF) and interleukin 1 (IL1) signaling networks (Fig. 15A), which function in cancer, cell cycle, and reproductive system disease. Those control...
pituitary nitroproteins are involved in transforming growth factor beta 1 (TGFβ1) and actin cellular skeleton signaling networks (Fig. 15B), which function in gene expression, cellular development, and connective tissue development. Both networks include a beta-estradiol signal pathway; that factor indicates that hormone metabolism is involved in a normal pituitary and pituitary adenoma. Moreover, pathway analysis showed that tyrosine nitration was involved in three important signaling pathway network systems (oxidative stress, cell-cycle dysregulation, and MAPK-signaling abnormality) in a pituitary adenoma (Zhan & Desiderio, 2010a; Zhan, Wang, & Desiderio, 2013). Those network data clarify biological roles of tyrosine nitration in pituitary tumorigenesis.

4. Structural biology analysis: Tyrosine nitration decreases the electron density of a phenolic ring of a tyrosine residue to diminish interaction intensity between enzyme and substrate or between receptor and ligand (Zhan & Desiderio, 2004). Therefore, the spatial position of a nitrotyrosine would obviously affect functions and biological roles of tyrosine nitration. The three-dimensional spatial structure of a protein determines its biological functions. If the three-dimensional spatial structure of a nitroprotein can be reconstructed from X-ray crystallography data, then it would be very easy to interpret the effect of tyrosine nitration on the 3D structure of a nitroprotein. Meanwhile, based on the 3D structure and tyrosine nitration site and domain, it is possible for one to design a small drug towards the 3D structure and domain that

![Figure 14](image1.png)

**FIGURE 14.** Nitration site and functional domains of sphingosine-1-phosphate lyase 1. Reproduced from Zhan and Desiderio (2006), with permission from Elsevier Science, copyright 2006.

![Figure 15](image2.png)

**FIGURE 15.** Significant signaling pathway networks mined from nitroproteomic dataset. A: Network was derived from adenoma nitroproteomic data, and function in cancer, cell cycle, reproductive system disease. A gray node denotes an identified nitroprotein or protein that interacts with nitroproteins. B: Network is derived from control nitroproteomic data and function in gene expression, cellular development, and connective tissue development and function. A gray node denotes an identified nitroprotein. An orange solid edge denotes a direct relationship between two nodes (molecules: proteins, genes). An orange non-solid edge denotes an indirect relationship between two nodes (molecules: proteins, genes). The various shapes of nodes denote the different functions. A curved line means an intracellular translocation; a curved arrow means an extracellular translocation. Reproduced from Zhan and Desiderio (2010a), with permission from BioMed Central open-access journal, copyright remains with the authors.
contains tyrosine nitration (Zhan, Wang, & Desiderio, 2013). That study demonstrated that nitrated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could not bind nicotinamide adenine dinucleotide (NAD\(^+\))—as shown with an NAD\(^+\) binding assay (Palamalai & Miyagi, 2010). The X-ray crystal structure has been used to explain the effect of tyrosine nitration on the capability of NAD\(^+\) binding in GAPDH (Palamalai & Miyagi, 2010). MS analysis of nitrated GAPDH indicated that Tyr\(^{311}\) and Tyr\(^{317}\) were the only sites of nitration. The X-ray crystal structure revealed that the distances between Tyr\(^{311}\) and Tyr\(^{317}\) and the cofactor NAD\(^+\) were less than 7.2 and 3.7 Å, respectively; those data imply that nitration of these two residues might affect NAD\(^+\) binding (Palamalai & Miyagi, 2010). Another example is that the X-ray crystal structure of mammalian succinate ubiquinone reductase (SQR or Complex II) is used to effectively explain association of protein tyrosine nitration (Tyr\(^{142}\)) of the Flavin subunit with the S-glutathionylated cysteine residue Cys\(^{50}\) of mitochondrial complex II in a post-ischemic myocardium (Chen et al., 2008). Briefly, based on the X-ray crystal structure of SQR, the flavin subunit has a Rossman-type fold with four major domains. Tyr\(^{142}\) is located in the major helix (residues 136–158) of a floating subdomain (residues 105–196). Specifically, Tyr\(^{142}\) is highly surface-exposed and situated in the hydrophilic environment to suggest that this specific tyrosine is susceptible to nitration with OONO\(^-\). Moreover, Tyr\(^{142}\) is ~20 Å away from the isoalloxazine ring of flavin adenine dinucleotide (FAD). Cys\(^{50}\) is located within the part of the N-terminal beta barrel subdomain (residue 53–104) of the large FAD-binding domain. Cys\(^{50}\) is near the AMP moiety of FAD (~7.7 Å), where major catalysis of electron transfer and O\(_2\)\(^-\) production occurs. Therefore, S-glutathionylation of Cys\(^{50}\) seems likely to induce a conformational change near the floating subdomain (residues 105–196); that change might increase the shielding effect on Tyr\(^{142}\) to render Tyr\(^{142}\) less accessible to OONO\(^-\) oxidation (Chen et al., 2008). Therefore, the 3D structure of a protein can accurately explain effects of tyrosine nitration on that protein’s structure and functions.

VII. CONCLUSIONS

Protein tyrosine nitration is an important oxidative/nitrative-mediated modification that is associated with a wide range of different pathophysiological conditions (Zhan & Desiderio, 2004, 2006, 2007, 2009a and 2009b; Zhan, Wang, & Desiderio, 2013). Also, evidence suggests the presence of a denitrase in mammalian tissues; however, a denitrase has not been isolated and its enzymatic activity not confirmed. Therefore, tyrosine nitration can be considered as reversible. Tyrosine nitration is not only a result from oxidative damage, but it also participates in pathophysiological processes (Smallwood et al., 2007). Nitration dynamically alters protein function (Mani & Moore, 2005), including activation or inactivation (Lin et al., 2007, 2012; Yamakura & Kawasaki, 2010). MS-based identification of nitrotyrosine-containing proteins and nitrotyrosine sites is essential to understand biological roles of this modification (Kanski & Schoneich, 2005; Spickett & Pitt, 2012; Tsikas, 2012). However, it is analytically very challenging to identify endogenous nitroproteins and nitrotyrosine sites due to nitration’s low abundance in biological samples and its multiple mass spectrometric behaviors among MALDI UV-laser, ESI, CID, ECD, ETD, and MAD. Endogenous nitrotyrosine-containing proteins peptides must be enriched prior to MS analysis. Several enrichment methods have been developed, and include immuno-affinity enrichment, biotin-affinity enrichment, and COFRADIC. Nitrotyrosine sites have been found in many different pathophysiological conditions. TMT- or iTRAQ-based quantitative nitroproteomics are needed to quantify disease-key nitroproteins/peptides. Protein domain/motif analysis, systems pathway analysis, and structural biological analysis of nitrotyrosine-containing proteins are significantly needed to elucidate the biological roles of tyrosine nitration.

Moreover, one must clearly realize that no highly reliable, high-throughput, high-sensitivity, and high-reproducibility method exists to analyze the extremely challenging endogenous tyrosine nitration in a proteome; therefore, different approaches are under development. Nitrotyrosine antibody-based immunoaffinity methods such as 2D-Westen blotting and NTAC succeeded to identify endogenous nitrotyrosine sites; however an overwhelming amount of non-nitrated tryptic peptides negatively affects characterization of nitropeptides. For that reason, we suggest development of immunoaffinity enrichment of tryptic nitropeptides- not nitroproteins- prior to MS analysis. Until now, most methods based on chemical derivatization (as described above) are used only for in vitro experiments and not for endogenous nitrotyrosine sites. Although the COFRADIC-based characterization of nitropeptides succeeded in a serum proteome, throughput and sensitivity were very low, and it has not been used extensively in endogenous tissue nitroproteomes. Therefore, development of better nitrotyrosine analysis methods is necessary in the following aspects—alone or in combination: (i) derivatize a nitro to amino group to stabilize MS behaviors, (ii) develop specific amino group tags to enrich nitrotyrosine peptides, (iii) enrich nitrotyrosine- and aminotyrosine peptides is better than nitrotyrosine- or aminotyrosine proteins for sensitivity, (iv) improve liquid chromatography isolation, (v) develop super high-sensitivity mass spectrometers, (vi) choice of the appropriate ion source and collision model to fragment nitropeptide or aminopeptides, and (vii) develop reliable software for data analysis. The combined multiple aspects among items i–vii are recommended to maximize coverage of endogenous nitrotyrosine sites in a proteome.

VIII. ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| APPD         | (3R,4S)-1-(4-(aminomethyl)phenylsulfonyl)pyrrolidine-3,4-diol |
| BSA          | bovine serum albumin |
| CENT-β1      | centaurin beta 1 |
| CID          | collision-induced dissociation |
| COFRADIC     | combined fractional diagonal chromatography |
| cPILOT       | combined precursor isotopic labeling and isobaric tagging |
| ECD          | electron-capture dissociation |
| ESI          | electrospray ionization |
| ETD          | electron-transfer dissociation |
| FAD          | flavin adenine dinucleotide |
| FT-ICR       | Fourier transform ion-cyclotron resonance |
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