Analysis of Nitroproteome in Human Pituitary Adenomas

Xianquan Zhan¹ and Dominic M. Desiderio²

1 Introduction

Protein tyrosine nitration is an important reactive oxygen species/reactive nitrogen species (ROS/RNS)-related modification that is derived from the main in vivo peroxynitrite pathway and the secondary myeloperoxidase reaction pathway (Scaloni, 2006, Khan et al., 1998, Zhan & Desiderio, 2009a, 2009b, and 2009c, Dalle-Donne et al., 2005, Zhan, Wang, & Desiderio, 2013). Tyrosine nitration adds an electron-withdrawing nitro group (–NO₂) to the tyrosine phenolic ring (Zhan & Desiderio, 2006). Addition of this nitro group will decrease the electron density of the tyrosine phenolic ring, change the phenolic pKa value (from ~10 for tyrosine) into the physiological pH range (~7.1 for 3-nitrotyrosine), and affect chemical properties of the tyrosine residue (Zhan & Desiderio, 2006, Yee et al., 2003, Irie et al., 2003). Thus, the decreased electron density will negatively affect the interaction intensity between enzyme-substrate, receptor-ligand, or antigen-antibody when the nitration occurred within those interacting regions, and impact on functions of that protein (Zhan & Desiderio, 2006). Moreover, in vivo protein tyrosine nitration might be denitrated with a putative denitrase to result in a dynamically reversible process between nitration and denitration (Irie et al., 2003, Aulak et al., 2004, Koeck et al., 2004). Thus, protein nitration would have a biological function such as neurotransmission and redox signaling besides its pathological consequences. Tyrosine ni-

¹ Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, Hunan Engineering Laboratory for Structural Biology and Drug Design, National Local Joint Engineering Laboratory for Anticancer Drugs, Xiangya Hospital, Central South University, China
² The Charles B. Stout Neuroscience Mass Spectrometry Laboratory, Department of Neurology, University of Tennessee Health Science Center, USA
tration also occurs within a tyrosine phosphorylation motif ([R or K]-x2(3)-[D or E]-x3(2)-[Y]), which might compete with the tyrosine phosphorylation to affect cellular signaling (Zhan & Desiderio, 2011, Zhan et al., 2007, Mallozzi et al., 2012). Therefore, protein tyrosine nitration can alter protein functions, and is extensively associated with multiple pathophysiological processes including tumors, neurodegenerative diseases, and inflammatory diseases (Zhan & Desiderio, 2004, 2006 and 2009a, Haddad et al., 1994, Miyagi et al., 2002, Yeo et al., 2008, Halliwell et al., 1999).

A global nitroproteomics method, namely the use of proteomics to investigate protein tyrosine nitration, was used to analyze protein tyrosine nitration in human tissues. The challenging issue in nitroproteomic analysis of endogenous nitroproteins is its very low abundance (one nitrotyrosine per ~10^6 tyrosine residues) in a proteome (Haddad et al., 1994, Shigenaga et al., 1997) and limited sensitivity of a mass spectrometry (MS). Therefore, it is necessary to preferentially enrich endogenous nitroproteins or nitropeptides prior to MS analysis (Zhan & Desiderio, 2009a). The effective approaches that detect and preferentially enrich endogenous nitroproteins are anti-nitrotyrosine antibody-based enzyme-linked immunosorbent assay (ELISA) (Khan et al., 1998, Torreilles & Romestand, 2001), immunoprecipitation (Zhan & Desiderio, 2006), and one/two-dimensional gel electrophoresis (1DGE/2DGE)-based Western blot analyses (Zhan & Desiderio, 2004 and 2007, Aulak et al., 2001, Miyagi et al., 2002). The commercially available ELISA assay kit (Upstate Catalog No. 17–136) can measure nitrotyrosine content. 1DGE/2DGE-based Western blots can separate and preferentially enrich endogenous nitroproteins and also determine the relative level of nitrotyrosine. Immunoprecipitation can preferentially enrich endogenous nitroproteins from a complex proteome before MS analysis. Tandem mass spectrometry (MS/MS) can characterize a nitrotyrosine site in a nitroprotein (Zhan & Desiderio, 2004, 2006, 2007 and 2009a).

As a potential marker of oxidative/nitrative injuries, protein tyrosine nitration might be an important molecular event in human hypothalamic–pituitary–target organ axis systems (Zhan & Desiderio, 2004 and 2006). The up-stream molecules that promote formation of tyrosine nitration, nitric oxide (NO) and nitric oxide synthase (NOS), participate in those pituitary-mediated axis systems (Zhan & Desiderio, 2004, Lloyd et al., 1995, Ueta et al., 1998, Ceccatelli et al., 1993): luteinizing hormone (LH) (Ceccatelli et al., 1993, McCann et al., 2001, McCann et al., 2003, Pinilla et al., 2001), follicle-stimulating hormone (FSH) (McCann et al., 2001), adrenocorticotropic (ACTH) (Riedel, 2000), prolactin (PRL) (Duvilanski et al., 1995), and growth hormone (GH) (Bocca et al., 2000, Cuttica et al., 1997, Pinilla et al., 1999). The nitrotyrosine level was elevated in a pituitary adenoma. For human pituitary nitroproteomics studies, 2DGE-based nitrotyrosine Western blot analysis (Zhan & Desiderio, 2004 and 2007) and nitrotyrosine immunonoaffinity enrichment (Zhan & Desiderio, 2006) were used to separate and preferentially enrich endogenous nitroproteins from a complex human pituitary control and adenoma tissue. Enriched nitroproteins were subjected to trypsin digestion, followed by MS/MS analysis to identify nitroproteins and nitrotyrosine sites. Bioinformatics was used to determine structural/functional domains and motifs of a nitroprotein, and to locate the nitrotyrosine site within a protein domain/motif to clarify roles of tyrosine nitration in a protein (Zhan & Desiderio, 2006). Pathway analysis-based systems biology was used to
discover pathway networks that involved endogenous nitroproteins from a systematical and comprehensive angle (Zhan & Desiderio, 2010). In addition, MS characteristics of standard nitropeptides (Zhan & Desiderio, 2009c) were analyzed to obtain the fragmentation to assist interpretation of a MS spectrum of a tryptic peptide derived from an endogenous nitroprotein in a proteome. A total of eight nitrotyrosine-containing proteins (nitroproteins) in a human pituitary post-mortem tissue, and nine nitroproteins and three nitroprotein-interacting proteins in a human nonfunctional pituitary adenoma tissue (Zhan & Desiderio, 2004, 2006 and 2007), were identified with MS/MS. Nitrotyrosine sites located within important protein domains or motifs (Zhan & Desiderio, 2006) were involved in the tumor biological characteristics (Zhan & Desiderio, 2006) and important pathway network systems (Zhan & Desiderio, 2010).

2 Materials and Methods

2.1 Synthetic Standard Peptides

MS analysis of synthetic standard nitropeptide was described to assist interpretation of the MS spectrum of endogenous nitropeptide (Zhan & Desiderio, 2009c). Briefly, three synthetic standard peptides (leucine enkephalin acetate hydrate, LE1, Y-G-G-F-L, 555.1818 Da; nitro-Tyr-leucine enkephalin, LE2, (3-NO2)Y-G-G-F-L, 600.0909 Da; and d5-Phe-nitro-Tyr-leucine enkephalin, LE3, (3-NO2)Y-G-G-(d5)F-L, 605.1818 Da) were prepared as a series of dilutions including 5000, 1000, 500, 100, 50, 10, 5, and 1 fmol/µl, respectively. Each prepared peptide solution (4 µl) was mixed with 4 µl of the 5 mg/mlα-cyano-4-hydroxycinnamic acid (CHCA) matrix solution, and was loaded onto a vMALDI 96-well plate for MS characterization of each standard peptide with a vacuum matrix-assisted laser desorption/ionization-linear ion-trap mass spectrometer (vMALDI-LTQ). MS and MS/MS spectra were obtained for each synthetic standard peptide. Laser fluence and the normalized collision energy (NCE) were optimized. The spectrum (MS; MS²) of each scan (n = 30 scans) in each file was processed and accumulated (n = 30) to obtain a synthetic spectrum. The m/z value and peak intensity in the synthetic spectrum were used for data analysis and graph construction. Each experiment was carried out in triplicate.

2.2 Detection and Identification of Pituitary Nitroproteins with 2D-Western Blot and MS/MS

A 2D-Western blot-based MS/MS method was described to identify nitroproteins from a pituitary post-mortem control tissue (Zhan & Desiderio, 2004 and 2007). The brief experimental procedure includes: (1) 2DGE and Western blot. A human control pituitary post-mortem tissue (male, 45 year-old, drowning) was used for extraction of proteins. Extracted pituitary proteins (70 µg) were arrayed with isoelectric focusing (IEF; an 18-cm IPGstrip pH 3–10 NL) with an Amersham Multiphor II instrument, following by equilibration with the solution that contained dithiothreitol (DTT) or iodoacetamide,
and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a home-made 12% PAGE resolving gel (190 × 205 × 1 mm) on a vertical PROTEAN-plus Dodeca Cell (Zhan & Desiderio, 2003a). 2DGE-separated proteins were transferred onto a PVDF membrane (0.8 mA/cm²; 1 h, 40 min), incubated (1 h) with a primary antibody (rabbit anti-human nitrotyrosine antibody; Millipore product), and incubated (1 h, room temperature) with secondary antibody (goat anti-rabbit alkaline phosphate-conjugated IgG). The membrane was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Pierce, Rockford, IL, USA). A parallel negative-control experiment (only incubated with the secondary antibody but not primary antibody) was performed to observe any cross-reactivity of the secondary antibody. The 2DGE gel, after transfer of proteins to PVDF membrane, was silver-stained to determine efficiency of the protein transfer. Images of 2D gel and Western blot membrane were analyzed with PDQuest 2D gel image software. Detailed gel image analysis was described (Zhan & Desiderio, 2003b). (2) MS characterization of nitroprotein. Silver-stained 2D gel spots that corresponded to positive Western blot spots were excised, and proteins were subjected to in-gel trypsin digestion (Zhan & Desiderio, 2003a). The tryptic peptide mixture was purified with a ZipTipC18 micro-column, and analyzed with a capillary liquid chromatography-electrospray ionization-LCQDeca mass spectrometer (LC-ESI-Q-IT) to obtain MS/MS spectrum of each peptide. De novo sequencing was also used to independently and accurately determine each amino acid sequence. MS/MS data were used for protein database analysis with SEQUEST software with mass modifications [+45 kDa (+NO₂–H) at Tyr and of +57 kDa (+NH₂COCH₂–H) at Cys].

2.3 Identification of Pituitary Adenoma Nitroproteins with NTAC and MS/MS

A non-gel method, nitrotyrosine affinity column (NTAC)-based MS/MS approach (Figure 1) was described to identify nitroproteins from a pituitary adenoma tissue (Zhan & Desiderio, 2006). Briefly, The NTAC was prepared with a Pierce Seize X mammalian immunoprecipitation kit with a detailed procedure (Zhan & Desiderio, 2006). A portion (62 mg wet weight) of a nonfunctional human pituitary adenoma tissue (white male, 39 years old, negative expression of FSH, LH, GH, PRL, TSH, and ACTH) was used to extract the protein into a volume (600 µl) of protein extraction buffer (Pierce, Rockford, IL, USA), and was diluted (1:1, v/v) with binding/washing buffer. A volume (500 µl) of diluted sample was incubated (overnight, gently rocking, > 19 h, 4 °C) with the prepared NTAC to bind nitroproteins and nitroprotein–protein complexes. The bound nitroproteins and nitroprotein–protein complexes were eluted with 200 µl elution buffer (pH 2.8) that contained a primary amine (gently mix well, centrifuge 3,000 g, 1 min, 3×). The eluates that contained nitroproteins and nitroprotein–protein complexes were collected for trypsin treatment and MS/MS analysis. Moreover, another volume (500 µl) of diluted sample was added to the control column, followed by the same experiment steps listed above. MS/MS data were used to identify the protein and nitrotyrosine sites by searching the Swiss–Prot and NCBInr databases with Bioworks 3.2 software with mass modifications of +45 Da (+NO₂–H) at Tyr and of +57Da (+NH₂COCH₂–H) at Cys. Moreover,
Figure 1: NTAC-based experimental flow chart that was used to identify nitroprotein and its complexes. 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.
results derived from NTAC were compared to results derived from the control column to remove any nonspecific binding proteins. Protein domains and motifs analysis with ScanProsite software (http://us.expasy.org/tools/scanprosite) and MotifScan software (http://myhits.isb-sib.ch/cgi-bin/motif_scan) were used to locate the nitrotyrosine sites within protein domain/motif. An experimental data-based model of nitroproteins and their functions was proposed on the basis of the functional information of those nitroproteins on the Swiss-Prot annotation page and the related literature data.

2.4 Pathway Network Analysis of Identified Nitroproteins

Ingenuity pathway analysis (IPA) system (http://www.ingenuity.com) was used to mine the signal pathway networks that involved the nitroproteins from human pituitary control and pituitary adenoma tissues (Zhan & Desiderio, 2010). Briefly, the Swiss-Prot accession numbers of all nitroproteins were input to the IPA data upload workflow. The IPA system automatically searched the matched Gene/molecules, and generated a two-dimensional table-style format to show the matched proteins and the unmapped proteins. The Swiss-Prot accession number of unmapped protein was converted to the corresponding gene name by searching the ExPASY proteomics server. All Swiss-Prot accession numbers of matched proteins and gene names of unmatched proteins were input to the IPA data upload workflow to generate the final mapped list for next-step analysis. Five sub-datasets were generated including All IDs, Unmapped IDs, Mapped IDs, Network-eligible IDs, and Functions/Pathways/List-eligible IDs. Each sub-dataset contained ID, notes, molecules, description, location, type of biofunction, and drugs. The name of each molecule appeared in the pathway network nodes. The Network-eligible IDs were used as the pathway network analysis by comparing the network-eligible molecules with the IPA Knowledge Base (IPAKB) with a Fisher's exact test. The level of statistical significance was set to \( p < 0.05 \). Each Pathway analysis generated the top networks, biofunctions/Tox functions, and top canonical pathways with a statistical significance \( (p < 0.05) \). A toxicity pathway is defined as a canonical pathway that is significantly associated with toxicity lists that are functional gene groupings based on critical biological processes and key toxicological responses; and those toxicity lists describe adaptive, defensive, or reparative responses to xenobiotic insult, and could be used to understand biological responses.

3 Results and Discussion

3.1 Mass Spectrometric Behavior and Characteristics of a Synthetic Nitropeptide

The MS behavior of a nitropeptide is much different between MALDI and ESI (Zhan & Desiderio, 2009c, Petersson et al., 2001, Sarver et al., 2001). The MALDI UV laser commonly induces photochemical decompositions of the nitro group (-NO\(_2\)) to decrease the precursor-ion intensity of a nitropeptide and to complicate the MS spectrum (Zhan &
Desiderio, 2009c, Petersson et al., 2001, Sarver et al., 2001). ESI does not induce those decompositions (Zhan & Desiderio, 2009c, Yeo et al., 2008, Petersson et al., 2001, Sarver et al., 2001, Lee et al., 2007, Kim et al., 2011, Lee et al., 2009). In our experiments, MALDI MS and MS/MS were used to study the fragmentation pattern of in vitro nitropeptides (Zhan & Desiderio, 2009c) through analysis of synthetic LE1 (Y-G-G-F-L, 555.1818 Da), LE2 [(3-NO₂)Y-G-G-F-L, 600.0909 Da], and LE3[(3-NO₂)Y-G-G-(d5)F-L, 605.1818 Da] with a vMALDI-LTQ mass spectrometer.

The results showed that UV laser-induced photochemical decomposition (loss of one or two oxygen atoms of the nitro group to form the unique decomposition pattern of ions ([M+H]+ - 16 and [M+H]+ - 32) in LE2 and LE3 relative to LE1 (Figure 2) (Zhan & Desiderio, 2009c). A similar decomposition pattern ([M+H]+ + Na - 16 and [M+H]+ + Na - 32) occurred for the sodium adduct ([M+H]+ + Na) of LE2 and LE3 relative to the sodium adduct of LE1. A product ion ([M+H]+ - 30) was also observed in the LE2 and LE3 spectra, which could result from the reduction of the nitro group (-NO₂) to an amino group (-NH₂) (Sarver et al., 2001). Meanwhile, the base-peak intensity of the [M+H]+ ion of LE1 (NL = 1.01E5) was much higher than that of LE2 (NL = 3.25E4) and LE3 (NL = 9.09E4) because photochemical decomposition decreased ion intensity and complicated the MS spectrum. However, recognition of this unique decomposition pattern can unambiguously identify a nitrotyrosine.

For a vMALDI-MS/MS analysis, b- and a-ions were the most-intense fragment ions relative to y-ions (Figure 3) (Zhan & Desiderio, 2009c). Compared to unmodified LE1, more collision energy optimized fragmentation of the nitropeptides LE2 and LE3 (Figure 4A). However, higher collision energy increased the intensity of the a-ion and decreased the intensity of the b-ion (a-ion = the loss of CO from a b-ion) (Figure 4B). Also, the optimized laser fluence maximized fragmentation of the nitropeptides LE2 and LE3. Although MS³ analysis confirmed the MS²-derived amino acid sequence, MS³ analysis requires a higher amount of peptides relative to MS² (Zhan & Desiderio, 2009c). Thus, MS³ analysis might not be suitable for routine analysis of endogenous low-abundance nitroproteins. To detect an endogenous nitropeptide, the amount of peptide must reach the sensitivity of a mass spectrometer; for our synthetic nitropeptides, the sensitivity of vMALDI-LTQ was 1 fmol for MS detection and 10 fmol for MS² detection (Zhan & Desiderio, 2009c). The detailed results were described (Zhan & Desiderio, 2009c). Those MS data of synthetic nitropeptides would be useful to assist the interpretation of MS spectrum of an endogenous nitropeptide.

3.2 Enrichment of Endogenous Nitroproteins in Human Pituitary and Adenoma Tissues

Nitrotyrosine is formed from the reaction of free or protein-bound tyrosine with RNS including peroxynitrite (Beckman, 1996) and free-radical nitrogen dioxide (Squadrito & Pryor, 1998) and has a very low abundance (1 in ~10⁶ tyrosines) in an in vivo proteome (Haddad et al., 1994, Shigenaga et al., 1997). MS is the essential technique for identification of a nitroprotein/nitropeptide and nitrated site (Zhan & Desiderio, 2004, 2006 and 2007). However, MS is limited by it’s certain sensitivity (Zhan & Desiderio, 2009a).
Figure 2: MS spectra of LE1 (top), LE2 (middle), and LE3 (bottom) with vMALDI-MS analysis. nY = nitro-Tyr. F(d5) = Phe residue with five 2H (d) atoms. Reproduced from Zhan and Desiderio (2009c) with permission from Elsevier Science, copyright 2009.
Figure 3: MS² spectra of LE1 (top), LE2 (middle), and LE3 (bottom) with vMAL-DI-MS/MS analysis. nY = nitro-Tyr. F(d5) = Phe residue with five 2H (d) atoms. Reproduced from Zhan and Desiderio (2009c) with permission from Elsevier Science, copyright 2009.
Figure 4: 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 (2009c) with permission from Elsevier Science, copyright 2009.
Therefore, it is necessary to preferentially enrich nitroproteins/nitropeptides before MS analysis (Zhan & Desiderio, 2009a, Yeo et al., 2008, Kim et al., 2011, Lee et al., 2009). Two preferential enrichment methods were used to study human pituitary and adenoma nitroproteomes, including 2DGE-based nitrotyrosine Western blot (Zhan & Desiderio, 2004 and 2007) and NTAC-based enrichment (Zhan & Desiderio, 2006).

2DGE-based nitrotyrosine Western blot (Zhan & Desiderio, 2004 and 2007) involved the following experiment procedure. Briefly, proteins were extracted from a post-mortem control pituitary and arrayed according to pI and Mr. The arrayed proteins were transferred to a PVDF membrane, incubated with anti-nitrotyrosine antibody, and visualized (Figure 5). Ca. 1000 protein spots were detected in the silver-stained 2D gel with a range of pI 3–10 and Mr 10–100 kDa) (Figure 5A). Thirty-two nitrotyrosine-positive Western blot spots were detected (Figure 5C) by comparison of digitized Western blot image (Figure 5C) to the negative-control (Figure 5D). Also, each nitrotyrosine positive Western blot spot (Figure 5C) was matched to the corresponding silver-stained 2D gel-spots (Figure 5A) so that the silver-stained gel-spots were excised for MS analysis. Therefore, even though abundance of a nitroprotein in a human tissue proteome is very low, 2DGE can separate and enrich each nitroprotein in a 2D gel spot to improve its immunodetection and MS-characterization.

The NTAC method (Zhan & Desiderio, 2006) (Figure 1) was used to preferentially enrich nitroproteins from a human pituitary adenoma proteome. Briefly, anti-nitrotyrosine antibodies were cross-linked to protein G beads, and incubated with a pituitary adenoma protein sample. Nitroproteins and nitroprotein–protein complexes were bound to the cross-linked anti-nitrotyrosine antibodies. Bound nitroproteins and nitroprotein–protein complexes were eluted to provide an enriched nitroprotein sample, and were subjected to trypsin digestion and MS/MS analysis. For our experiment, NTAC was an effective method to enrich nitroproteins from a human pituitary adenoma proteome and improve MS/MS identification of endogenous nitroproteins. A total of nine nitroproteins and three proteins that interact with nitroproteins were identified from a pituitary adenoma proteome (Zhan & Desiderio, 2006).

However, one must realize that 2DGE-based method has obvious limitations in the detailed protein analysis including deficiencies in the coverage of proteome, dynamic range, sensitivity and throughput, especially when a limited sample is processed (Zhan & Desiderio, 2005). Also, 2DGE-based Western blot is to relatively enrich and detect the nitrotyrosine-containing proteins, the non-nitrated tryptic peptides are much more than nitrated tryptic peptides, which will hinder the MS/MS characterization of nitrated tryptic peptides. Advantages of 2DGE-based Western blotting are that it can provide directly visualized image of nitroproteins and detect protein isoforms. NTAC-based MS/MS method has more advantages to overcome those limitations of 2DGE method, but it cannot provide the visualized image of nitroproteins and detect protein isoforms. The use of NTAC method to enrich the tryptic nitrated peptides, but not nitroproteins, would maximize the coverage of nitrotyrosine sites in a proteome.
3.3 MS/MS Identification of Nitroproteins and Nitrated Sites in Human Pituitary and Adenoma

MS/MS is the essential method to obtain the amino acid sequence and nitration sites of a tryptic nitropeptide (Zhan & Desiderio, 2004, 2006 and 2007). A total of 32 2D gel spots were excised that corresponded to nitrotyrosine-positive Western blot spots from a post-mortem pituitary control tissue, and proteins were subjected to in-gel trypsin digestion and MS/MS analysis. Eight nitroproteins and eight nitrated sites were identified in pituitary control tissue (Table 1) (Zhan & Desiderio, 2004 and 2007), including actin, cGMP-dependent protein kinase 2, synaptosomal-associated protein, proteasome subunit alpha type 2, immunoglobulin alpha Fc receptor, stanniocalcin 1, mitochondrial cochaperone protein HscB, and progestin and adipQ receptor family member III. Those nitroproteins function in cellular immunity, neurotransmission, calcium and phosphate

Figure 5: 2DGE-based Western blotting analysis of nitrotyrosine immunopositive proteins in a human pituitary (70 µg protein per 2D gel). (A) Silver-stained image on a 2D gel before transfer of proteins onto a PVDF membrane. (B) Silver-stained image on a 2D gel after transfer of proteins onto a PVDF membrane. (C) Western blot image of nitrotyrosine immunopositive proteins (anti-3-nitrotyrosine antibodies + secondary antibody). (D) Negative control of a Western blot to show cross-reaction of the secondary antibody (only the secondary antibody; no anti-3-nitrotyrosine antibody). Reproduced from Zhan and Desiderio (2007) with permission from Elsevier Science, copyright 2007
<table>
<thead>
<tr>
<th>Nitroprotein</th>
<th>Pituitary Adenoma</th>
<th>nY site</th>
<th>Nitroprotein</th>
<th>Pituitary Control</th>
<th>nY site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho-GTPase-activating 5 [Q13017] (ARHGAP5)</td>
<td>Y550</td>
<td>Synaptosomal-associated protein (SNAP91)</td>
<td>Y237</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte immunoglobulin-like receptor A4 [P59901]</td>
<td>Y404</td>
<td>Ig alpha Fc receptor [P24071] (FCAR)</td>
<td>Y223</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc finger protein 432 [O94892]</td>
<td>Y41</td>
<td>Actin [P03996] (ACTA2, ACTG2, ACTC1)</td>
<td>Y296</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKA beta regulatory subunit [P31321] (PRKAR1B)</td>
<td>Y20</td>
<td>PKG 2 [Q13237] (PRKG2)</td>
<td>Y354</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centaurin beta 1 [Q15027]</td>
<td>Y485</td>
<td>Stanniocalcin 1 [P52823] (STC1)</td>
<td>Y159</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteasome subunit alpha type 2 [P25787] (PSMA2)</td>
<td>Y228</td>
<td>Proteasome subunit alpha type 2 (PSMA2)</td>
<td>Y228</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin 1 family member 6 [Q9UHA7] (IL1F6)</td>
<td>Y96</td>
<td>Progestin and adipoQ receptor family member III [Q6TCH7] (PAQR3)</td>
<td>Y33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhophilin 2 [Q8IUC4] (RHPN2)</td>
<td>Y258</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

metabolism, cellular structure and mobility, membrane receptor, co-chaperone in iron-sulfur cluster assembly in mitochondria, and the ATP/ubiquitin-dependent non-lysosomal proteolytic pathway.

The NTAC-enriched nitroprotein samples from a pituitary adenoma tissue were subjected to trypsin digestion and MS/MS analysis (Zhan & Desiderio, 2006). A total of nine nitroproteins and ten nitrated sites were identified (Table 1), including cAMP-dependent protein kinase type I-beta regulatory subunit, sphingosine-1-phosphate lyase 1, Rho-GTPase-activating protein 5, zinc finger protein 432, proteasome subunit alpha type 2, centaurin beta 1, leukocyte immunoglobulin-like receptor subfamily A member 4, interleukin 1 family member 6, and rhophilin 2. Three proteins including interleukin 1 receptor-associated kinase-like 2, ubiquitin, and glutamate receptor-interacting protein 2 were identified to interact with nitroproteins (Table 1), and form three nitroprotein–protein complexes, including nitrated interleukin 1 family member 6-interleukin 1 receptor-interleukin 1 receptor-associated kinase-like 2 (IL1F6-IL1R-IRAK2), nitrated proteasome-ubiquitin complex, and nitrated beta-subunit of cAMP-dependent protein kinase (PKA) complex (Zhan & Desiderio, 2006).

Those nine nitroproteins and three nitroprotein-protein complexes were rationalized into a corresponding functional system (Figure 6) (Zhan & Desiderio, 2006). The nitrated S1P lyase 1 participates in sphingolipid metabolism to regulate cell proliferation, survival, and cell death as well as the immune system (Zhan & Desiderio, 2006, Maceyka et al., 2002, Schwab et al., 2005, Hla, 2005). The nitrated proteasome-ubiquitin complex is an important enzymatic complex involved in the intracellular nonlysosomal proteolytic pathway (Zhan & Desiderio, 2006, Tamura et al., 1991, Kristensen et al., 1994). Nitrated CENT-beta 1 and nitrated PKA1-beta 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 LIRA4 might be involved in the immune system. Nitrated ZFP432 is involved in transcription regulatory systems. Nitrated RHOGAP5 and nitrated rhophilin 2 are involved in the GTPase signal pathway (Zhan & Desiderio, 2006).

3.4 Bioinformatics Recognition of the Functional Domains/Motifs of a Nitroprotein

Location of nitrotyrosine sites into a protein domain or motif would clarify biological activities of tyrosine nitration because functional and structural domains or motifs in a protein sustain a certain biological functions. Protein-domain analysis software 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 effective tools that detect statistically significant domains in a nitroprotein and to locate each nitrated site within a protein domain (Zhan & Desiderio, 2006). It was used to analyze protein domains and motifs in each nitroprotein in a human pituitary adenoma proteome (Zhan & Desiderio, 2006). An interesting result demonstrated most nitrated sites occurred within important protein domains and motifs. Four examples were taken here in detail.
First, sphingosine-1-phosphate lyase 1 (S1P lyase 1) that was nitrated in human pituitary adenoma (Zhan & Desiderio, 2006) (Figure 7A) is a key enzyme to catalyze decomposition of S1P. Two nitration (NO$_2$-356Y and NO$_2$-366Y) within the enzyme activity region would decrease the interaction intensity of enzyme:substrate (S1P lyase 1:S1P) and decrease the S1P decomposition because the nitro group (-NO$_2$) is an electron-withdrawing group that could decrease the level of enzyme-substrate binding. Study (Maceyka et al., 2002) demonstrates that sphingolipid metabolites including S1P, ceramide (Cer), and sphingosine (Sph) play an important role in the regulation of cell survival, proliferation, and cell death. Cer and Sph usually inhibit proliferation and promote apoptosis, whereas S1P stimulates growth and suppresses apoptosis. Because these metabolites are interconvertible, their relative levels determine cell fate. Nitration of S1P lyase 1 might increase the level of S1P relative to Cer and Sph, which would stimulate tumor cell proliferation and inhibit apoptosis.

Second, the Rho-GTPase-activating protein 5 (Figure 7B) contains four FF domains (Bedford & Leder, 1999) and one Rho-GAP domain. A nitration (NO$_2$-550Y) within the region between two domains (FF4 and Rho-GAP) might impact on Rho-GTPase signal transduction (Zhan & Desiderio, 2006).

Third, zinc finger protein 432 (Figure 7C), a transcript factor, includes 16 C2H2-type zinc fingers binding DNA and one Kruppel-associated box (KRAB) domain that functions as a transcriptional suppressor (Payre & Vincent, 1998, Witzgall et al., 1994, Margolin et al., 1994). Nitration (NO$_2$-41Y) within the KRAB domain might impair transcriptional suppression (Zhan & Desiderio, 2006).
Figure 7: Nitration site and functional domains of four nitroproteins. (a) Sphingosine-1-phosphate lyase 1. The site $^{353}$K is a pyridoxal phosphate-binding motif. (b) Rho-GTPase-activating protein 5. (c) Zinc finger protein 432. The KRAB domain is a transcriptional suppressor. The ZN-RING is a DNA-binding region. (d) cAMP-dependent protein kinase type I-beta regulatory subunit. Modified from Zhan and Desiderio (2006) with permission from Elsevier Science; reproduced from Zhan and Deiderio (2009a) with permission from Springer Science; and reproduced from Zhan, Wang and Desiderio (2013) with permission from Hindawi Publishing Corporation open access journal and copyright remains with the authors.
Fourth, The cAMP-dependent protein kinase type I-beta regulatory subunit (PKAR1-beta) (Figure 7D) contains one inhibitory region (pseudophosphorylation), one N-terminal dimerization domain, and two cAMP-binding domains. Each cAMP-binding domain contains two cAMP-binding sites. Nitrination (NO\textsubscript{2}O\textsuperscript{Y}) within the dimerization region might affect dimerization of two regulatory chains to impair the functions of PKA (Zhan & Desiderio, 2006).

So the location of nitrated sites within the important protein domains/motifs provides an in-depth insight into the biological effects of tyrosine nitration on a protein.

3.5 Systems Biological Analysis of Signaling Pathway Networks that Involve Nitroproteins

Systems biology is a comprehensive analysis of all functionally interacting components of a biological system over time (Hood, 2003, Aderem, 2005, Hood & Tian, 2012). Compared to the traditional molecular biology methods that were used to study the role of a single gene, single protein, or single small-molecule model, the high-throughput "-omic" technologies including genomics, transcriptomics, proteomics, and metabolomics, drive the rapid development of systems biology to study a multiple-factor model of disease and to address the network of interaction and regulatory events that contribute to a disease (Zhan & Desiderio, 2010). Pathway biology is one important component of systems used to extensively analyze "-omic" data and to mine significantly signaling pathway networks and to address the biological significance of those "-omic" data.

IPA pathway analysis software was used to analyze signaling pathway networks that involve nitroproteins from human pituitary control (Zhan & Desiderio, 2004 and 2007) and adenoma tissues (Zhan & Desiderio, 2006). Those eight nitroproteins from a pituitary control and nine nitroproteins and three non-nitrated proteins that interact with nitroproteins from a pituitary adenoma tissue (Table 1) were used for IPA pathway analysis (Zhan & Desiderio, 2010). The results clearly showed that those pituitary adenoma nitroproteins and their complexes were involved in the tumor necrosis factor (TNF) and interleukin 1 (IL1) signaling networks (Figure 8A), which function in cancer, cell cycle, and reproductive system disease. Those nitroproteins in that network include IL1F6, PRKAR1B, ARHGAP5, PSMA2, and RHPN2, and the non-nitrated proteins that interact with nitroproteins include ubiquitin, GRIP2, and IRAK2. Three nitroprotein–protein complexes were identified: nitrated proteasome-ubiquitin complex, nitrated beta-subunit of PKA complex, and nitrated IL1F6-IL1 receptor-IL1 receptor-associated kinase-like 2 (IL1F6-IL1R-IRAK2) complexes. Those control pituitary nitroproteins were involved in the transforming growth factor beta 1 (TGB1) and actin cellular skeleton signaling networks (Figure 8B), which function in gene expression, cellular development, and connective tissue development. Nitroproteins in that network include PRKG2, FCAR, actin, SNAP91, PAQR3, STC1, and PSMA2. Both networks (Figure 8A and B) include a beta-estradiol signal pathway, which indicates that hormone metabolism is involved in a normal pituitary and pituitary adenoma. It is consistent with the fact that NO is involved in pituitary hormone metabolism in normal physiology, and that a tumor interferes with hormone metabolism.
Figure 8: Significant signaling pathway networks mined from a nitroproteomic dataset. (A) Network is derived from adenoma nitroproteomic data and functions in cancer, cell cycle, reproductive-system disease. A gray node denotes an identified nitroprotein or protein that interacts with nitroproteins in our study. (B) Network is derived from control nitroproteomic data and functions in gene expression, cellular development, connective tissue development and function. A gray node denotes an identified nitroprotein in our studies. 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 intracellular translocation; a curved arrow means extracellular translocation. Reproduced from Zhan and Desiderio (2010) with permission from BioMed Central open access journal and copyright remains with the authors, and reproduced from Zhan, Wang and Desiderio (2013) with permission from Hindawi Publishing Corporation open access journal and copyright remains with the authors.
Moreover, twelve statistically significant canonical pathways were mined from those pituitary adenoma nitroprotein data (Zhan & Desiderio, 2010), including the protein-ubiquitination pathway, p38 MAPK signaling, sonic-hedgehog signaling, cell-cycle G2/M DNA damage-checkpoint regulation, Toll-like receptor signaling, GABA-receptor signaling, the phototransduction pathway, amyloid processing, sphingolipid metabolism, LXR/RXR activation, IL-10 signaling, hypoxia signaling, and PXR/RXR activation (Zhan & Desiderio, 2010); and three statistically significant toxicity pathways were mined, including PXR/RXR activation, hepatic cholestasis, and LXR/RXR activation. Among those control pituitary nitroprotein data, twelve statistically significant canonical pathways were mined (Zhan & Desiderio, 2010), including VEGF signaling, regulation of actin-based motility by Rho, clatrin-mediated endocytosis, Fcy receptor-mediated phagocytosis in NRF2-mediated oxidative-stress response, caveolar-mediated endocytosis, macrophages and monocytes, actin-cytoskeleton signaling, tight-junction signaling, leukocyte extravasation signaling, integrin signaling, and calcium signaling. No statistically significant toxicity pathways were mined.

Among four signaling pathway network systems (mitochondrial dysfunction, oxidative stress, cell-cycle dysregulation, and MAPK-signaling abnormality) that were tightly associated with pituitary adenomas (Zhan & Desiderio, 2010), three signaling pathway network systems (oxidative stress, cell-cycle dysregulation, and MAPK-signaling abnormality) are involved in protein nitration; for example, cell-cycle G2/M DNA damage checkpoint regulation, p38 MAPK signaling, and the NRF2-mediated oxidative-stress response were discovered from pituitary adenoma nitroproteomic data (Zhan & Desiderio, 2010). Therefore, pathway systems analysis revealed that tyrosine nitration plays important roles in the pituitary tumorigenesis.

4 Conclusions

Protein tyrosine nitration is an important molecular event in pituitary adenoma, and is extensively associated with pituitary pathophysiological processes. Identification of nitroproteins and nitrated sites is an essential step to elucidate the functional roles of tyrosine nitration in a biological system. 2DGE-based nitrotyrosine Western blot coupled with MS/MS was used to detect 32 nitrotyrosine-positive gel-spots and eight nitroproteins and nitrated sites from a pituitary post-mortem tissue (Zhan & Desiderio, 2004 and 2007). NTAC-based enrichment coupled with MS/MS was used to identify nine nitroproteins and modified sites and three nitroprotein-interacting proteins from a pituitary adenoma tissue (Zhan & Desiderio, 2006), and most nitrotyrosine sites were located within important protein domains/motifs. Tyrosine nitration participated in three pathway network systems, including oxidative stress, cell-cycle dysregulation, and MAPK-signaling abnormality, that are significantly associated with pituitary adenomas (Zhan & Desiderio, 2010). Moreover, MALDI UV laser caused photodecomposition (loss of one or two oxygen atoms) of a nitro group of a nitropeptide. Recognition of the photodecomposition pattern could assist the interpretation of an MS spectrum of an endogenous nitropeptides. Those data clearly indicate that protein tyrosine nitration plays
important roles in pituitary tumorigenesis.

However, the study of human pituitary adenoma nitroproteome is just at the beginning of our long-term program to elucidate the biological roles of tyrosine nitration in pituitary adenoma pathophysiology. Several aspects are worth studying: (1) Nitroproteomics of single-cell types of a pituitary adenoma will be necessary. A pituitary contains multiple cell types (Zhan & Desiderio, 2005). Those different cell types of pituitary adenomas could have not only a common mechanism in their formation but also some differences among different cell types of pituitary adenoma. Thus, it is important to study the same and different differentially expressed nitroproteins among the different cell types of pituitary adenomas, and to discover specific nitroprotein biomarkers for pituitary adenomas. (2) Quantitative nitroproteomics among different cell types of pituitary adenomas and controls are needed to determine nitroproteins that are unique to each cell type of pituitary adenoma. With precious LCM-enriched cell-types, isobaric tags for relative and absolute quantification (iTRAQ)-based quantitative proteomics and two-dimensional difference in-gel electrophoresis (2D-DIGE)-based quantitative proteomics would be the first method to analyze differentially expressed nitroproteins (DENPs) among different cell types of pituitary adenomas. (3) Structural biology is necessary to study three-dimensional spatial structure of a pituitary adenoma-related nitroprotein because it will be very easy to interpret the effect of tyrosine nitration on the 3D structure of a nitroprotein. Also, based on the 3-D structure and tyrosine nitration site and domain, it is possible for one to design a small drug towards the 3D structure and domain that contains tyrosine nitration. (4) A pituitary adenoma is a whole-body disease. In addition of pituitary adenoma tissue, cerebrospinal fluid (CSF) and blood plasma must be studied because some secreted proteins and peptides enter into the CSF and blood circulation in a pituitary adenoma patient (Zhan & Desiderio, 2010a). Also, CSF and blood specimens are much more accessible from patients and controls than pituitary tissues, and overcome the limitations of pituitary tissues (Zhan & Desiderio, 2010a). Any CSF and blood plasma nitroproteomic and nitropeptidomic variations would lead to the development of accurate biomarkers for predictive diagnosis, early-stage diagnosis, and measurement of prevention and therapy responses.

Acknowledgement

The authors acknowledge the financial supports from China “863” Plan Project (Grant No. 2014AA020610-1 to X.Z.), the Hunan Provincial Natural Science Foundation of China (Grant No. 14JJ7008 to X.Z.), the National Natural Science Foundation of China (Grant No. 81272798 and 81572278 to X.Z.), the Xiangya Hospital Funds for Talent Introduction (to X.Z.), and the National Institutes of Health, U.S.A. (RR16679 and NS 42843 to D.M.D.).
References


Mechanisms of Ageing and Development, 124, 9–16.


Tamura, T., Lee, D. H., Osaka, F., Fujiiwara, T., Shin, S., Chung, C. H., Tanaka, K., & Ichihara,


