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REGULAR ARTICLE

Characterization of nitroproteome in neuron-like PC12 cells differentiated with nerve growth factor: Identification of two nitration sites in α-tubulin

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Nitric oxide (NO) is a precursor of reactive nitrating species, peroxynitrite and nitrogen dioxide, which modify proteins to generate oxidized species such as 3-nitrotyrosine that has been used as a hallmark of peroxynitrite-mediated oxidative stress on proteins. In the last few years however, a growing body of evidence indicates that NO also regulates a myriad of physiologic responses by modifying tyrosine residues. Looking for the molecular event triggered by NO in nerve growth factor (NGF)-induced neuronal differentiation, we recently reported that in differentiating PC12 cells, the cytoskeleton becomes the main cellular fraction containing nitrotyrosinated proteins, and α-tubulin is the major target. In the present work, we focus on the investigation of the sites of tyrosine nitration in α-tubulin purified by two-dimensional gel electrophoresis following anti-α-tubulin immunoprecipitation of protein extract from NGF-treated PC12 cells. Using Western blotting and matrix-assisted laser desorption/ionization-time of flight analysis, we show for the first time, both *in vivo* and *in vitro*, that nitration can occur on α-tubulin at sites other than the *C*-terminus and we positively identify Tyr 161 and Tyr 357 as two specific amino acids endogenously nitrated.

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1 Introduction

Nitric oxide (NO) is a relatively stable radical that diffuses from the site of production and interacts with targets without the need for special transporters or receptors. It is generated in eukaryotes by three isoforms of NO synthase (NOS) [1, 2], and it is involved in a growing number of biological and pathological functions [3–8]. NO may have direct effects by interacting with soluble guanylate cyclase leading to vasodilation and causing alterations in gene expression and it may also interact with other oxidants (superoxide in particular) to generate more reactive species that induce a series of oxidative protein modifications [9]. One such reactivity results in the formation of 3-nitrotyrosine in proteins. The mechanism, regulation and role of protein tyrosine nitration are still controversial [10, 11]. It has mainly been considered as a disease marker in vascular and neurological pathologies and it has recently been suggested to be the best indicator for cardiovascular diseases [12]. 3-Nitrotyrosine has been detected in several diseases such as atherosclerosis, Alzheimer's and Parkinson's, at sites of inflammation, in some forms of retinopathies and in cancer [13]. However, there is a growing body of evidence on the possible involvement of protein

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nitration in physiological processes, including signal transduction [14]. This has been suggested by the identification of substrates for nitration in neurones, astrocytes and other cell types [15, 16]. In addition, the evidence for a "denitrase" enzyme [17] has suggested that protein nitration is a reversible and perhaps regulatable process. NO mediates a variety of actions in the nervous system including neurotrasmitter release, synaptic plasticity, neuronal differentiation and development. We recently reported that tyrosine nitration of proteins is implicated in the pathway triggered by NO during nerve growth factor (NGF)-induced neuronal differentiation [18]. In differentiating PC12 cells, the cytoskeleton becomes the main cellular fraction containing nitro-proteins, and we identified α-tubulin and the microtubule-associated protein tau as two targets of this modification [18, 19]. In particular, we reported that the band corresponding to α-tubulin becomes more nitrated following up to 7 days exposure to NGF [18]. In accordance with our data, nitrated α-tubulin has been previously identified in a specific region of the nervous system in invertebrates [20] and in the chorioallantoic membrane during chick embryo development [13]. In an attempt to disclose the physiological function of nitration of cytoskeletal proteins, recent studies proposed that it could be a natural mechanism of cytoskeletal protein turnover [20] or a novel event through which NO modulates the phosphorylation state of cytoskeletal elements [21].

In the present work, we focus on the investigation of the sites of tyrosine nitration in α-tubulin. α-Tubulin takes part in microtubule assembly, which is critical for cell division, directed intracellular transport and dynamic organization of cellular morphology, including maintenance of neuronal form. This protein undergoes a host of PTMs [22]. Of these, tyrosination/detyrosination has been most extensively studied. It is a unique modification involving removal or addition of a tyrosine residue at the *C*-terminus of α-tubulin, promoted by tubulin-specific carboxypeptidase and tubulin tyrosine ligase, respectively [23]. It has been reported that cultured cells incubated in the presence of free 3-nitrotyrosine selectively incorporated this modified amino acid into the extreme *C*-terminus of α-tubulin resulting in microtubule dysfunction and alteration in cell morphology [24] or microtubule destabilization and prevention of complete myogenic differentiation [25]. These studies suggest that nitration of α-tubulin in the *C*-terminus could be deleterious for cell function. Our previous finding that α-tubulin is subjected to tyrosine nitration during the progression of neuronal differentiation [18] is of particular interest, since very little is known regarding the occurrence of this modification on α-tubulin *in vivo* and its possible functions. Moreover, to date, no information is available regarding the identification of the sites of nitration. In addition, besides the incorporation at the *C*-terminus, no other evidence, both *in vitro* and *in vivo*, has been supplied showing that α-tubulin could be nitrated in a different site. Therefore, all the studies carried out on nitrated α-tubulin by means of antibodies presumed that the protein was modified at the *C*-terminus.

Recently, the concomitant application of proteomic-based strategies to the analysis of nitrated proteins *in vitro* has yielded more details on the extent of nitration of specific amino acids, and increasing attention has been focused on the study of specific sites of nitration and their impact on protein structure and function. However, very few successful identifications of *in vivo* sites of nitration have been achieved to date [26, 27]. An attempt was also made on rat retinal nitroproteome using MS. Unfortunately, the authors reported that all nitrotyrosine-containing peptides were below the detection limits [28]. In the present study, we address this issue on endogenously nitrated α-tubulin and we successfully identify Tyr 161 and Tyr 357 as two residues nitrated in differentiated PC12 cells.

2 Materials and methods

2.1 Materials

Sequence grade trypsin and chymotrypsin were obtained from Roche (Monza, Italy) recrystallized CHCA was purchased from Bruker Daltonics (Milan, Italy), and all other chemicals were of analytical or HPLC grade. Antibody to nitrotyrosine at the *C*-terminus (anti-nitro antibodies) was a gift from Professor Arce (Universitad Nacional de Cordoba, Argentina) [29].

2.2 Cell culture

PC12 cells were maintained in RPMI 1640 (HyClone, Logan, UT, USA) containing 10% horse serum and 5% foetal bovine serum (HyClone) supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. For differentiation, cells were plated at $1.5 \times 10^4/cm^2$ onto poly-L-lysine (0.1 g/L in double distilled water)-coated petri dishes and exposed to 50 ng/mL human β-NGF (PeproTeck, London, UK) in a low-serum medium (RPMI 1640 supplemented with 1% horse serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin). Medium and NGF were replenished every 48 h.

2.3 Immunoprecipitation and isolation of nitrated α-tubulin by SDS-PAGE

Cell extracts were prepared by incubating the cell culture with ice-cold lysis buffer containing 1% v/v NP-40, 10% v/v glycerol, protease inhibitors (1 mM PMSF, 0.1 mM leupeptin, 1 μM pepstatin, 2 μg/mL aprotinin) and 25 mM Tris-HCl, pH 7.5, for 30 min on ice. Before immunoprecipitation, the samples were normalized to contain the same protein concentration (1 mg/mL) by bicinchoninic acid reagent assay (Micro BCA; Pierce, Rockford, IL, USA). α-Tubulin immunoprecipitates were prepared as previously described [30], and solubilized in twice-concentrated SDS-PAGE sample buffer (2% w/v SDS, 10% v/v glycerol, 0.001% w/v bromophenol

blue and 62.5 mM Tris, pH 6.8) containing protease inhibitors but without β-mercaptoethanol. Protein samples were separated by SDS-PAGE, Western blotted onto PVDF membranes (Immobilon™-P; Millipore, Billerica, MA, USA) and immunostained as previously described by using monoclonal anti-α-tubulin antibody [30] and anti-nitrotyrosine antibody [18]. The band positively stained with anti-α-tubulin antibody was subjected to *N*-terminal sequence analysis following electroblotting on PVDF as detailed in [31].

2.4 2-DE of the anti-α-tubulin immunoprecipitate and Western blotting analysis

2-D PAGE was carried out on the α-tubulin immunoprecipitate by performing the reduction and the alkylation before the first dimension as suggested by Castagna *et al.* [32]. A procedure for the solubilization of the proteins was set up in order to overcome the insolubility of the sample following immunoprecipitation. The sample was solubilized in 50 mM Tris-HCl pH 6.8, 4% SDS and 5% β-mercaptoethanol, heated at 70°C, sonicated for 1 min and centrifuged at 10 000 × *g* for 5 min. The supernatant was treated with 5 mM tributylphosphine in NH_4HCO_3 0.1 M as a reductant and stored at room temperature for 1.5 h. After this step, the sample was incubated with 20 mM iodoacetamide at room temperature for 1.5 h. The sample was then precipitated in an anhydrous solution of acetone and methanol (8:1 v/v). The precipitate was resuspended in the 2-D PAGE sample buffer (7 M urea, 2 M thiourea, 2% NP-40, 0.5% Resolyte 3.5–10 NL, bromophenol blue) and sonicated for 1 min. For the first dimension, 300 μg of protein was applied to a rehydrated IPG strip (110 mm, pH 3–10 NL; Amersham Biosciences, Calogno Monzesa, Italy) and IEF was carried out at 15°C, 20 000 V total voltage, for 6 h. Before the second dimension, the strip was rinsed with buffer (6 M urea in 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol, bromophenol blue). The second dimension was performed on a homemade 10% SDS minigel (8.5 × 6.5 × 0.15 cm) at 180 mA, 4°C for 1 h. Finally, the gel was stained with colloidal CBB G (Sigma, Milan, Italy) before MALDI-TOF analysis. To reveal nitrated proteins, one strip was loaded with 100 μg of protein and after 2-DE the gel was blotted onto a PVDF membrane as previously described [31]. Immunodecoration with anti-nitrotyrosine antibody was performed as described in [18].

2.5 Separation of the peptide mixture after in-gel proteolytic digestion

Proteolytic digestion of the 2-D spot corresponding to nitrated α-tubulin was performed as previously described using chymotrypsin or trypsin [18]. Following extraction by 50% ACN in 0.1% TFA, purification of the peptide mixture was carried out by RP-HPLC using an Applied Biosystems instrument equipped with a UV detector set at 220 nm and an Aquapore C-8 RP-300 microbore column (0.1 × 10 cm, 0.7 μm; Applied Biosystems, Foster City, CA, USA). Solvents used were: (A) 0.1% v/v TFA in water, (B) 0.075% v/v TFA in ACN. The peptides were eluted with a linear gradient from 0 to 50% B in 70 min at a flow rate of 75 μl min^{-1}. Aliquots of the isolated peaks were spotted on a PVDF membrane and immunostained with anti-nitrotyrosine antibody. Nitrated peaks were subjected to *N*-terminal sequence and MALDI-TOF analyses. The amino acid sequence was determined by a pulsed-liquid sequencer (Procise model 491; Applied Biosystems) as previously described [31]. The mass analysis was performed following the same procedure detailed in Section 2.6 for the MALDI-TOF analysis of the mixture extracted after in-gel protease digestion of the 2-D spots.

2.6 *In situ* digestion of 2-D spots and protein identification by MALDI-TOF analysis

MALDI-TOF analysis was carried out on the spot to be identified following immunoprecipitation, 2-DE, in-gel digestion and peptide extraction. The spot was excised, cut into smaller pieces, destained with 100 μL of 50% ACN in ammonium bicarbonate 0.1 M (40 min at 25°C) and dried in a SpeedVac. The gels were soaked with ammonium bicarbonate 0.1 M and digested overnight both with sequencing grade trypsin and chymotrypsin (Roche) at 37°C. The in-gel tryptic or chymotryptic digest was extracted with 30 μL 50% ACN in 0.1% TFA and the peptide mixture was subjected to MALDI-TOF analysis by using a Bruker Daltonics Reflex IV instrument equipped with a nitrogen laser (337 nm) and operated in reflector mode with a CHCA matrix. The peptide mixture was loaded onto an AnchorChip plate (Bruker Daltonics). External standards were used for calibration (Bruker peptide calibration standard). Each spectrum was accumulated for at least 200 laser shots. In order to improve the precision of the results, each spectrum was analyzed using two sets of acquisition parameters, one for masses in the range 0.5–2 kDa and the second for masses ranging from 2 to 3 kDa. Measured peptide masses were used to search the Swiss-Prot, MSDB, TrEMBL, and NCBI sequence databases for protein identifications with the MASCOT program (http://www.matrixscience.com).

2.7 Detection of nitrotyrosine at the *C*-terminus

For preparation of whole cell extracts, cells from cultures exposed to NGF for 5 days were washed twice with PBS and scraped into SDS-PAGE sample buffer containing protease inhibitors. In addition, whole cells extracts were prepared from PC12 not treated with NGF but incubated for 48 h in Ham's F12 (Sigma) in the presence and in the absence of 500 μM 3-nitrotyrosine. Protein samples were subjected to SDS-PAGE using Laemmli buffers and Western blotted onto nitrocellulose membranes (Millipore). For the detection of nitrotyrosine at the *C*-terminus of proteins, blocking was performed by incubating the membranes in 6% w/v non-fat dry milk in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) with 0.05% v/v Tween 20 overnight at 4°C. Primary antibody

was incubated for 2 h at room temperature with rabbit polyclonal anti-nitro antibody diluted 1:600 [29] kindly supplied by Professor Arce (Universitad Nacional de Cordoba, Argentina). Membranes were washed for 30 min with four changes and incubated with horseradish peroxidase-linked goat anti-rabbit Ig diluted 1:40 000 (Pierce) for 1 h at room temperature. After washing, the reaction was developed using enhanced chemiluminescence (SuperSignal® West Pico Chemiluminescent; Pierce). For the detection of tyrosine at the *C*-terminus of α-tubulin, immunoblots were performed using mouse monoclonal antibodies against tyrosinated tubulin diluted 1:1000 (clone TUB-1A2; Sigma) according to the procedure described for α-tubulin immunoblot [30]. Further immunoblots with monoclonal anti-α-tubulin and anti-nitrotyrosine antibodies were performed as previously described.

3 Results

3.1 Identification of two nitrotyrosine residues in endogenously nitrated α-tubulin

We have previously reported on the presence of nitrated α-tubulin in PC12 cells following differentiation with NGF [18]. In particular, our experiments suggested that there is a positive correlation between the progression of neuronal differentiation and nitration of proteins. A change in the relative concentration of two major nitrated bands of 50 and 53 kDa could be observed. The latter was identified as α-tubulin by *N*-terminal sequence and MS analyses.

In order to identify the sites of nitration on this protein, we analyzed α-tubulin in PC12 cells after 5 days of NGF treatment, when the level of α-tubulin nitration reaches its maximum. All experiments described in this section were repeated twice, starting from freshly prepared material, with highly reproducible results.

The complex protein mixture prepared from PC12 cells after 5 days of NGF treatment was enriched for α-tubulin by immunoprecipitation with anti-α-tubulin antibodies and the precipitate was separated by SDS-PAGE (Fig. 1A). In addition to the polypeptide identified as α-tubulin by Western blot and *N*-terminal sequence analyses, multiple bands of other proteins functionally related to α-tubulin were detected. Endogenously nitrated proteins were stained by anti-nitrotyrosine immunodecoration as shown in Fig. 1B and described in [19], where the identification of one of them as tau is reported. Therefore, to isolate the endogenously nitrated form of α-tubulin and to examine the sites of nitration, it was advisable to separate all the proteins precipitated with anti-α-tubulin antibodies by 2-DE (Fig. 1C).

One major problem we had to deal with was the insolubility of the immunoprecipitated material in the 2-D sample buffer. This was overcome by solubilizing the sample in the presence of SDS following heating and sonication. The sample was then reduced and alkylated before the first dimension, as suggested by Castagna *et al.* [32], and precipitated in organic solvent as described in Section 2.

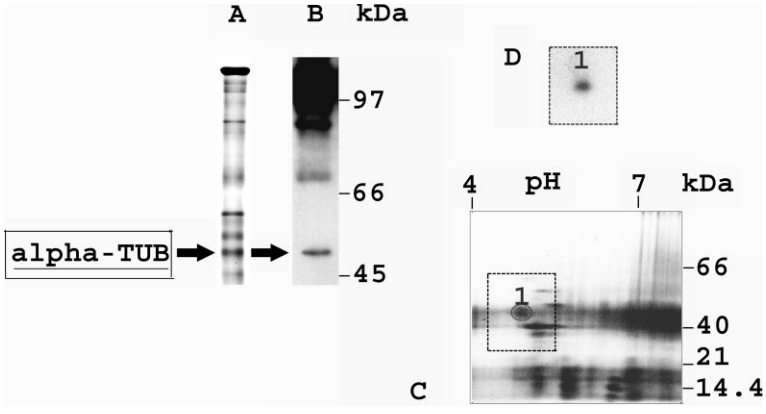

Figure 1. Isolation and detection of nitrated α-tubulin in NGF-differentiated PC12 cells. α-Tubulin was immunoprecipitated with anti-α-tubulin antibodies from PC12 cells incubated for 5 days with NGF. The immunoprecipitated proteins were separated by SDS-PAGE, silver stained (A) or blotted onto PVDF and immunostained with anti-nitrotyrosine antibodies (B). α-Tubulin is indicated by arrows. The SDS-PAGE was run without β-mercaptoethanol. (C) 2-DE of the immunoprecipitate with anti-α-tubulin antibodies stained with silver. The figure shows the area between pH 4 and 7 only, where nitrated α-tubulin spot is detected. The box marks the image area that corresponds to the area presented in (D). Spot 1 corresponds to α-tubulin. (D) Western blot analysis. After 2-DE and blotting onto PVDF, the membrane was immunodecorated with anti-nitrotyrosine antibodies and positive spots were detected by enhanced chemiluminescence. Only the area corresponding to the one boxed in (C) is reported in (D).

Identification of α-tubulin was achieved by immunostaining with anti-nitrotyrosine antibodies and confirmed by proteolytic digestion followed by MS peptide mapping using MALDI-TOF. As shown in Fig. 1C, spot 1 was identified as α-tubulin. The same spot was also stained following Western blot analysis using anti-nitrotyrosine antibodies as shown in Fig. 1D. We concentrated on this spot to study the sites of nitration of nitrated α-tubulin. In order to obtain as many tyrosine-containing peptides as possible, the spot was subjected to two different in-gel digestions, using either trypsin or chymotrypsin, because when using only trypsin some of the expected peptides deriving from the tryptic digestion were too long to be efficiently extracted from the gel. The tryptic and chymotryptic peptide mixtures were studied by MS, searching for tyrosine-containing peptides. Many spectra were taken for the same sample applying different acquisition parameters in order to obtain reliable data in different *m/z* ranges. Putative nitrated peaks were identified by an increase in mass of 45 units (+45), due to the nitro group, and/or an increase of 16 and 32 units lower than the one representing nitration (+29, +13), which correspond to products from prompt fragmentation caused by the immediate loss of an oxygen molecule to form a nitroso species, followed by loss of a second oxygen molecule possibly to form nitrene or dehydroazepine species, as outlined by Sarver *et al.* [33]. These authors report that such characteristic addition to the molecular ion of the modified peptide containing the nitro-substituted tyrosine group provides the unequivocal evidence for the presence of this modification.

Some of the results obtained from the analysis of the nitrated α-tubulin digests are shown in Fig. 2, which reports two representative mass spectra of tryptic (A) and chymotryptic (B) peptide mixtures. For clarity, only a few peaks are labeled with the corresponding monoisotopic mass. In particular, in spectrum 2A, 1023.41, 1085.54, 1457.75, 1718.19, 1756.86, 1779.76, 2329.89 and 2409.03 correspond to peptides 97–105 (expected *m/z* 1023.45), 113–121 (expected *m/z* 1085.62), 230–243 (expected *m/z* 1457.87), 216–229 (expected *m/z* 1718.88), 265–280 (expected *m/z* 1756.96), 97–112 (expected *m/z* 1779.84), 403–422 (expected *m/z* 2330.02) and 244–264 (expected *m/z* 2409.21), respectively. In spectrum 2B, 1083.47, 1259.62, 1471.81, 1455.87, 1627.59, 2030.04 and 2255.19 correspond to peptides 409–418 (expected *m/z* 1083.43), 303–312 (expected *m/z* 1259.63), 150–161 containing NO_2Tyr (expected *m/z* 1471.72), 150–161 containing NOTyr (expected *m/z* 1455.72), 419–432 (expected *m/z* 1627.73), 256–272 (expected *m/z* 2030.09) and 2–21 (expected *m/z* 2255.08), respectively.

α-Tubulin contains 18 or 19 tyrosine residues depending on the presence of the *C*-terminal tyrosine which can be lost as a PTM of the protein [34]. Combining the results obtained from the tryptic and the chymotryptic digestion and from all the spectra produced with different acquisition parameters, 11 tyrosines were found which do not show the characteristic pattern of nitration (Table 1).

Of interest, the analysis of spectra related to the chymotryptic digestion revealed the presence of peaks originated from the nitration of Tyr 161 and Tyr 357. Two peaks (1455.87 and 1471.81) were found which correspond to peptide 150–161 containing NOTyr 161 and NO_2Tyr 161, respectively, as shown in Fig. 2B and in Table 2.

In accordance with this finding, it was possible to detect a peak at *m/z* 881.40 which corresponds to the native peptide 155–161, and peaks occurring 45, 29 and 13 units higher, suggesting that tyrosine 161 is modified (Table 2). Similar

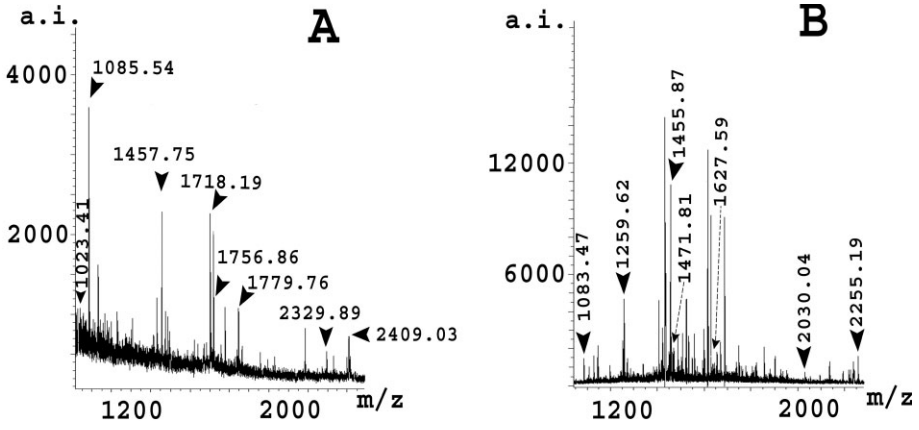

Figure 2. MALDI-TOF analysis of nitrated α-tubulin. After 2-DE, the spot corresponding to nitrated α-tubulin was digested with trypsin (A) or chymotrypsin (B) and the peptide mixture was analyzed by MS using a Bruker Daltonics Reflex IV instrument equipped with a nitrogen laser (337 nm) and operated in reflector mode with a CHCA matrix. The peptide mixture was loaded onto an AnchorChip© plate. External standards were used for calibration (Bruker peptide calibration standard). Each spectrum was accumulated for at least 200 laser shots. For clarity, only a few peaks are labeled in each spectrum. Molecular masses are reported as monoisotopic mass.

Table 1. MS analysis of the tryptic and chymotryptic peptide mixtures of nitrated α-tubulin in NGF-differentiated PC12 cells. Only peptides containing unmodified tyrosine residues and obtained after *in situ* tryptic or chymotryptic digestion are reported. After 2-DE, the spot corresponding to nitrated α-tubulin was cut into smaller pieces and digested overnight either with sequencing grade trypsin or with chymotrypsin (Roche) at 37°C. The in-gel tryptic or chymotryptic digest was extracted with 30 μL 50% ACN in 0.1% TFA and the peptide mixture was subjected to MALDI-TOF analysis. Combining the results obtained from the tryptic and the chymotryptic digestion, 11 tyrosines were found that do not show the characteristic pattern of nitration.

Tyr	Digestion	Peptide	Sequence	*m/z* calc.[a] (Da)	*m/z* obs[a] (Da)
103	tryp	97–105	EDAANN**Y**AR	1023.45	1023.41
	tryp	97–112	EDAANN**Y**ARGH**Y**TIGK	1779.84	1779.76
108	tryp	97–112	EDAANN**Y**ARGH**Y**TIGK	1779.84	1779.76
210	chy	203–210	MVDNEAI**Y**	954.42	954.22
224	tryp	216–229	NLDIERPT**Y**TNLNR	1718.88	1718.79
	chy	218–224	DIERPT**Y**	893.43	893.35
262	tryp	244–264	FDGALNVDLTEFQTNLVP**Y**PR	2409.20	2409.03
	chy	256–272	QTNLVP**Y**PRIHFPLAT**Y**	2030.08	2030.04
272	tryp	265–280	IHFPLAT**Y**APVISAEK	1756.96	1756.86
	chy	256–273	QTNLVP**Y**PRIHFPLAT**Y**	2030.08	2030.04
312	chy	303–312	VKCDPRHGK**Y**	1259.63	1259.62
319	chy	313–319	MACCLL**Y**	930.39	930.31
399	tryp	395–402	FDLM**Y**AKR	1043.53	1043.50
	chy	396–404	DLM**Y**AKRAF	1114.57	1113.97
408	tryp	403–422	AFVHW**Y**VGEGMEEGEFSEAR	2330.02	2329.89
432	chy	419–432	SEAREDMAALEKD**Y**	1627.73	1627.59

a) The *m/z* are reported as monoisotopic mass

data referring to the nitration of tyrosine 357 were found from the analysis of the same peptide mixture, as reported in Table 2.

To confirm the nitration of α-tubulin at tyrosine 161 and 357 as a PTM occurring in NGF-differentiated PC12 cells, different sample aliquots were subjected to 2-DE, the spot of nitrated α-tubulin was digested with either trypsin or chymotrypsin, and the two peptide mixtures were separated by HPLC (data not shown). In both cases the chromatographic analysis was carried out with an unspecific detection at 220 nm, since the amount of the peptide mixture was too low to allow detection at 354 nm, a wavelength at which the increase in absorbance most likely reflects the presence of nitrotyrosine [35]. Therefore, the peaks containing nitrated peptides were recognized by immunostaining. An aliquot of each peptide was spotted onto a PVDF membrane and immunodecorated with anti-nitrotyrosine antibodies. A few of them were positively immunostained and were identified by MS and *N*-terminal sequence analyses for the unequivocal structural determination of the nitration sites. From the chymotryptic digestion, four nitro-peptides were identified: peptide 155–161 (expected *m/z* for the NO_2 form 926.44) and peptide 150–161 (expected *m/z* for the NO_2 form 1471.72) that confirm the nitration of Tyr 161, and peptide 352–357 (expected *m/z* for the NO_2 form 738.39) and peptide 347–357 (expected *m/z* for the NO_2 form 1300.61), which refer to the nitration of Tyr 357. The mass spectra of these peptides present the characteristic pattern of nitration, as shown in Fig. 3 and Table 2. *N*-terminal sequence analyses of the corresponding peaks confirmed the presence of these peptides although, as expected, it was impossible to detect the unmodified tyrosine residue.

The same approach was applied to the tryptic digestion mixture allowing the identification of two nitrated peptides: peptide 353–370 (expected *m/z* for the NO_2 form 1869.98) and peptide 157–163 (expected *m/z* for the NO_2 form 826.4), suggesting that Tyr 161 and Tyr 357 are nitrated in perfect agreement with the results reported here. The corresponding mass spectra are presented in Fig. 4.

3.2 Analysis of the *C*-terminus of α-tubulin

One of the most studied PTM of tubulin is the addition or removal of a tyrosine residue at the *C*-terminus of the α subunit [34]. The incorporation of 3-nitrotyrosine at this site was first described by Eiserich *et al*. [24], who suggested that nitration of α-tubulin causes microtubule dysfunction, whereas Bisig *et al*. reported that substitution of *C*-terminal tyrosine by 3-nitrotyrosine has no detrimental effect on dividing cells [29]. Therefore, in our study it was of interest to

Table 2. MS analysis of the chymotryptic peptide mixture and the HPLC-purified nitrated peptides. The chymotryptic peptide mixture and the HPLC-purified nitrated peptides were studied by MS searching for tyrosine-containing peptides. Putative nitrated peaks were identified by an increase in mass of 45 units (+45), due to the nitro group, and/or an increase of 16 and 32 units lower than those representing nitration (+29, +13), which corresponds to products from prompt fragmentation caused by the immediate loss of an oxygen molecule to form a nitroso species, followed by loss of a second oxygen molecule possibly to form nitrene or dehydroazepine species. Only peptides containing nitrated tyrosine residues found in the chymotryptic mixture (Mixture column) or in the HPLC purified peaks (HPLC column) are reported.

Tyr	Peptide	Sequence	*m/z* calc.[a]	*m/z* obs.[a] Mixture	*m/z* obs.[a] HPLC	Type
161	155–161	ERLSD**Y**	881.44	881.40	881.27	//
			926.44 (+45)	926.25	926.00	NO_2
			910.44 (+29)	910.28	910.16	NO
			894.44 (+13)	894.31	894.25	N
	150–161	TSLLMERLSD**Y**	1426.72	1426.20	N.D.[b]	//
			1471.72 (+45)	1471.81	N.D.[b]	NO_2
			1455.72 (+29)	1455.87	1455.80	NO
			1439.72 (+13)	N.D.[b]	1439.51	N
357	352–357	KVGIN**Y**	693.39	693.12	693.32	//
			738.39 (+45)	N.D.[b]	N.D.[b]	NO_2
			722.39 (+29)	722.10	722.30	NO
			706.39 (+13)	706.14	706.34	N
	347–357	CPTGFKVGIN**Y**	1255.61	1255.10	N.D.[b]	//
			1300.61 (+45)	1300.67	1300.26	NO_2
			1284.61 (+29)	1284.90	1284.3	NO
			1268.61 (+13)	N.D.[b]	N.D.[b]	N

a) The *m/z* are reported as monoisotopic mass in Da
b) Not detected

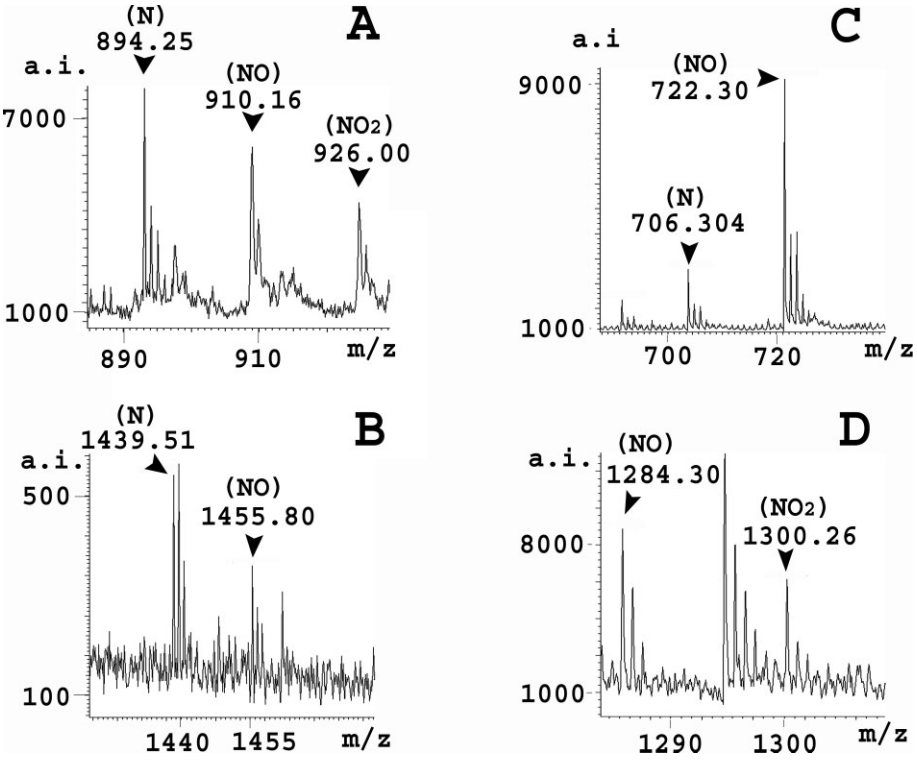

Figure 3. Mass spectra relative to nitrated peptides obtained after HPLC separation of the chymotryptic digestion of α-tubulin. (A), (B), (C) and (D) show the mass spectra of peptides 155–161, 150–161, 352–357 and 347–357, respectively. Molecular masses are reported as monoisotopic mass.

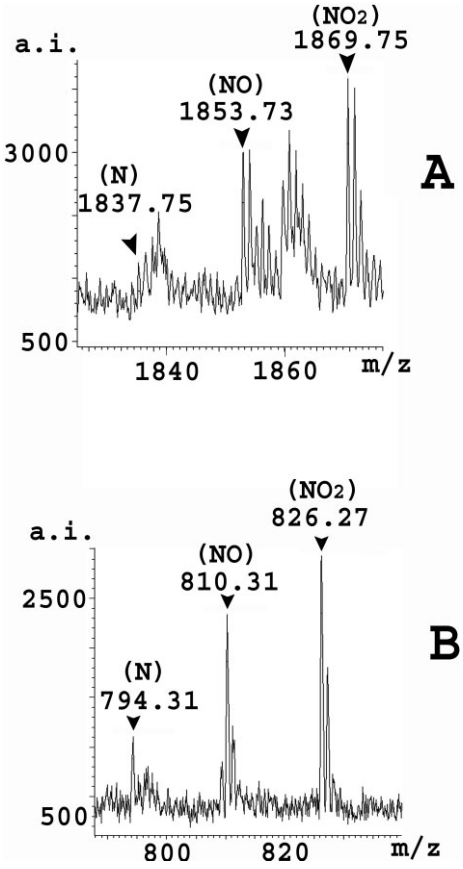

Figure 4. Mass spectra relative to nitrated peptides obtained after HPLC separation of the tryptic digestion of α-tubulin. (A) and (B) show the mass spectra of nitrated peptides 353–370 and 157–163, respectively. Molecular masses are reported as monoisotopic mass.

investigate whether nitration of α-tubulin in differentiated PC12 cells occurred at the *C*-terminus. We addressed the question by using anti-nitro antibodies that are specific to *C*-terminal 3-nitrotyrosine of all proteins [29] (anti-nitro), anti-tyrosinated α-tubulin antibodies that are specific for α-tubulin tyrosinated at the *C*-terminus (anti-tyr-tub) and anti-nitrotyrosine antibodies that recognize all proteins nitrated at Tyr (anti-notyr). Whole cell extracts from differentiated PC12 cells were separated by SDS-PAGE and immunoblotted with anti-nitro, anti-α-tubulin, anti-tyr-tub and anti-notyr antibodies (Fig. 5A). The results show that α-tubulin is tyrosinated at the *C*-terminus in differentiated cells, since it is stained by anti-tyr-tub antibodies, and it is nitrated because it is recognized by anti-notyr antibodies. However, it failed to be stained by anti-nitro antibodies, suggesting that the Tyr at the *C*-terminus of α-tubulin is not nitrated. PC12 cells incubated for 48 h in the presence or in the absence of free 3-nitrotyrosine were loaded as positive and negative controls, respectively. In accordance with previous data [29], cells incubated with free 3-nitrotyrosine show a unique protein that incorporates the modified amino acid at the *C*-terminus, displays an apparent mass of 53 kDa and corresponds to α-tubulin. The lack of staining by anti-nitro antibodies in differentiated PC12 cells suggests that nitration of the *C*-terminus of proteins does not occur. The same result was observed when α-tubulin immunoprecipitated from differentiated PC12 cells was immunoblotted with anti-nitro and anti-tyrosinated α-tubulin antibodies (Fig. 5B). Tyrosinated α-tubulin was present in the immunoprecipitate but failed to be stained by anti-nitro antibodies. These results strongly suggest that α-tubulin is not nitrated at the *C*-terminus in NGF-differentiated PC12 cells under physiological conditions.

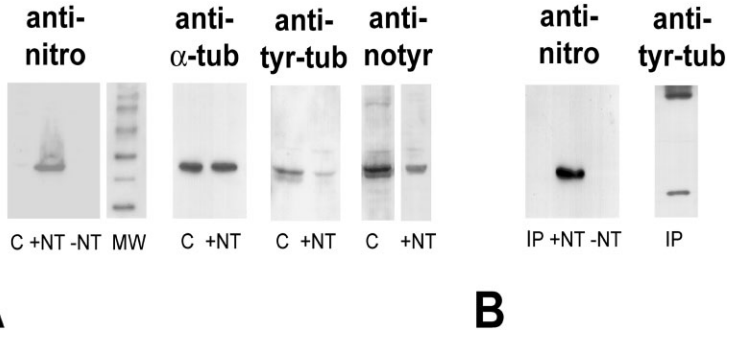

Figure 5. Immunochemical analysis of the C-terminus of α-tubulin. (A) Immunoblot analysis showing *C*-terminal 3-nitrotyrosine (anti-nitro), α-tubulin (anti-α-tub), *C*-terminal tyrosinated α-tubulin (anti-tyr-tub) and 3-nitrotyrosine (anti-notyr) in whole-cell extracts from PC12 cells treated for 5 days with NGF (S) or incubated in the presence (+NT) or in the absence (−NT) of free 3-nitrotyrosine. All lanes were loaded with 20 mg protein. MagicMark^{XP} Western Protein Standards (MW): 40, 50, 60, 80, 100 and 120 kDa. (B) Immunoblot analysis showing *C*-terminal 3-nitrotyrosine (anti-nitro) and tyrosinated α-tubulin (anti-tyr-tub) in α-tubulin immunoprecipitates from PC12 cells treated for 5 days with NGF (IP). The SDS-PAGE was run without β-mercaptoethanol.

4 Discussion

Tyrosine nitration of α-tubulin was first shown in cell cultures incubated with free nitrotyrosine, and it has been proposed that this modification could lead to conformational changes affecting microtubule organization as well as motor- and microtubule-associated proteins [24, 25]. These studies show that α-tubulin may incorporate 3-nitrotyrosine at the *C*-terminus, but nitration of other tyrosine residues has neither been suggested nor excluded. In the present report, for the first time, both *in vivo* and *in vitro*, we show that in physiological conditions nitration occurs on α-tubulin at sites other than the *C*-terminus and we positively identify Tyr 161 and Tyr 357 as two specific amino acids endogenously nitrated. However, 12 out of 19 tyrosines present in the sequence are not modified.

Selectivity of nitration of tyrosine residues has been previously studied in known proteins chemically modified by nitrating agents [35] and in histones nitrated *in vivo* in Mutatect tumours [26, 35–38]. These authors suggest that there are structural factors favoring tyrosine nitration: (i) the exposure of the aromatic ring to the surface of the protein or accessibility of the residue to the nitrating agent; (ii) the localization of tyrosine residue in a loop and the presence of a turn-inducing amino acid residue such as Pro or Gly within −5 to +5 from the tyrosine; (iii) the presence of tyrosine in hydrophobic pockets; (iv) a negative-charged amino acid at position −1 relative to tyrosine, Glu being more effective than Asp for this purpose; and (v) the lack of negative factors such as steric hindrance (disulfide bond) and alternative targets (Met and Cys above all) in proximity to tyrosine. However, the existence of a consensus sequence is controversial. According to Souza *et al.* [35], there is no apparent sequence homology recognized by nitrating agents. On the other hand, two different consensus sequences promoting tyrosine nitration have been suggested by Elfering *et al.* [37] and Lanone *et al.* [36], respectively.

In our study, the sites of α-tubulin nitration are positively identified at tyrosine 161 and tyrosine 357. The α-tubulin structure shown in Fig. 6 highlights those residues that are nitrated (in bold) and those that are not. It should be pointed out that in the absence of rat tubulin structure, Fig. 6 refers to the structure of bovine α-tubulin [39]. The sequence comparison, however, shows a very high similarity between all known tubulins and allows us to refer to the 3-D structure of bovine α-tubulin. Tyrosine 161 and tyrosine 357 are solvent exposed. The nitration of tyrosine 161 may be facilitated by the presence of Asp in position −1 and the lack of Met or Cys residues in the close vicinity, although this tyrosine is not located in a loop and the flanking region does not fit with any of the two consensus sequences proposed so far. The opposite can be observed considering tyrosine 357. There is no Glu or Asp residue in close proximity, but this tyrosine is located in a loop of a rather hydrophobic region that presents the pattern [LMVI]-X-[DE-[LMVI]-X(2,3)-[FVLI]-X(3,5)-Y, proposed by Elfering *et al.* [37], where X is any amino acid and Y is the target tyrosine. Regarding tyrosines not nitrated in our conditions, five out of 11 (Tyr 210, Tyr 272, Tyr 312, Tyr 319 and Tyr 432) are buried inside the protein. Therefore they are not accessible to the solvent and to the nitrating agent. Tyr 262, Tyr 103, Tyr 108, Tyr 224, Tyr 408 and Tyr 399, on the contrary, are solvent exposed. The absence of nitration may be assigned to the lack of a negative residue (Glu or Asp) at position −1 and, in the case of Tyr 399, also to the close proximity of a Met residue. Therefore, our data support the notion that also in physiological conditions: (i) nitration is a selective PTM; (ii) overall, a single factor is not sufficient to explain this selectivity; (iii) the local environment and a

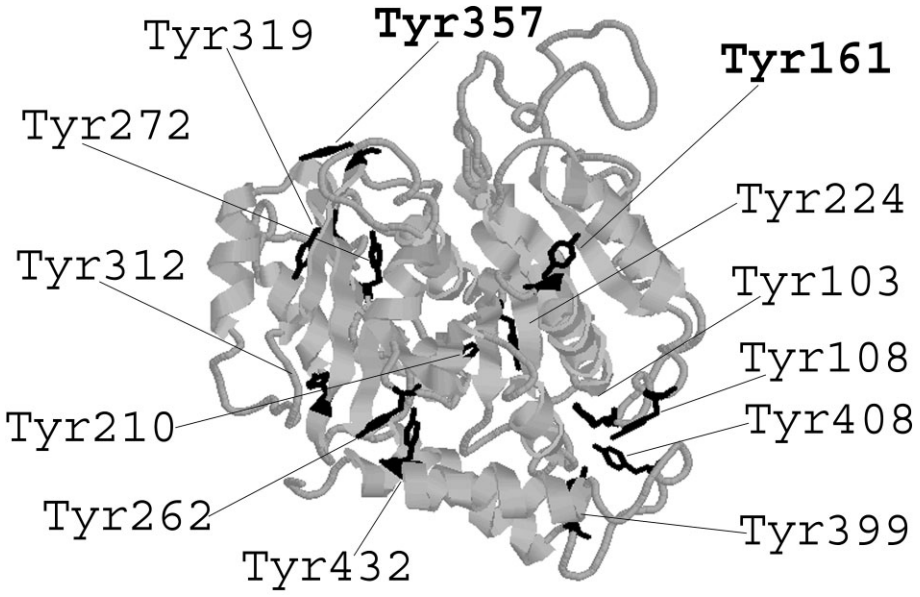

Figure 6. Three-dimensional structure of bovine α-tubulin (PDB entry: 1TUB). Nitrated tyrosine residues are labeled and highlighted in bold and those unmodified are labeled. The structure was drawn using RasMol software.

combination of factors may be responsible for the selective nitration, such as accessibility to the solvent, proximity to Asp or Glu, and a particular consensus sequence flanking the tyrosine residue. These observations are in accordance with the data reported by Ischiropoulos [38] and with the results described by Haqqani *et al.* [26], who report a positive identification of specific tyrosine residues nitrated *in vivo*.

One of the most studied tubulin modifications is the tyrosination/detyrosination cycle carried out by specific enzymes at the *C*-terminus of the protein. As mentioned previously, most recently it has been shown that α-tubulin can incorporate 3-nitrotyrosine *in vivo* in different cell types [24, 25, 29, 40]. To investigate this particular aspect, we used antibodies specific for nitrotyrosine at the *C*-terminus of proteins [29]. The results unambiguously show that in differentiated PC12 cells the *C*-terminus of α-tubulin contains Tyr 451 but this residue is not nitrated. Notably, the *C*-terminal domain of α-tubulin is formed by two helices and a disordered highly acidic segment lacking in the structure presented in Fig. 6, since its 3-D structure has not been solved [39]. Our proteomic approach indicates that all the tyrosines in this portion of the molecule (Tyr 319, Tyr 399, Tyr 408, Tyr 432 and Tyr 451) are not modified by NO, suggesting that the spatial arrangement of nitration in α-tubulin is quite different from that of other PTMs which occur in the *C*-terminal of the tubulin tails with the only exception of acetylation.

Tubulin undergoes a series of well-known PTMs, including detyrosination, acetylation, phosphorylation, polyglycylation, palmitoylation and polyglutamylation that can be potentially important for regulation of microtubule function. At present, we can only speculate on the physiological meaning of the nitration of α-tubulin at Tyr 161 and Tyr 357 and its overall effects on α-tubulin function in a physiological process like neuronal differentiation. However, some considerations can be pointed out when looking at the 3-D structure of α-tubulin. According to Nogales *et al.* [41], Tyr 161 is located in the H_4S_5 region (amino acids 160–163), which is involved in lateral interaction surfaces between protofilaments. These lateral contacts show a marked electrostatic character that can be possibly perturbed by the nitration of Tyr 161. In addition, Tyr 357 is located in the loop S_9–S_{10} on the inside surface of the microtubule. Based on these considerations, we can hypothesize that the nitration of these two residues might influence and modulate the assembly of microtubules, the organization of microtubules onto supramolecular structures or the dynamics of microtubules. Since the microtubule cytoskeleton plays an important role in controlling different spatial organizations in diversely differentiated cells, tyrosine nitration of α-tubulin might be an important event in the complex and dynamic organization of the cytoskeleton underlying neuritogenesis and differentiation. Identification of the modified sites represents one of the first and crucial steps for further investigations on the impact of this post-translationally modified form on cell and organ function.

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