The nitration of τ protein in neurone-like PC12 cells

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Abstract Tyrosine nitration of proteins is emerging as a post-translational modification playing a role in physiological conditions. Looking for the molecular events triggered by nitric oxide in nerve growth factor-induced neuronal differentiation, we now find that nitration occurs on the microtubule-associated protein τ. In differentiated PC12 cells, we have identified as τ a nitrated protein that co-immunoprecipitates with α-tubulin and indicated that the modified protein is associated with the cytoskeleton but it is confined to a restricted cell region. This paper supplies the first evidence that nitration of τ occurs in a physiological process and suggests that it could play a role in neuronal differentiation.

Key words: Nitric oxide; Nitration; Tau; Cytoskeleton; Neural cell; Matrix-assisted laser desorption/ionization time of flight

1. Introduction

Nitric oxide (NO) is a signalling molecule involved in numerous physiological and pathophysiological events. The majority of NO actions in physiological conditions occur through the activation of soluble guanylate cyclase resulting from NO binding to its haem prosthetic group [1]. However, some actions of NO are mediated directly by protein modifications, including *S*-nitrosylation of reduced cysteine residues [2,3] and the nitration of tyrosine and tryptophan residues [4]. Although the accumulation of nitrated proteins correlates well with many disease states and is considered a marker of oxidative stress under pathological conditions, substantial evidence has accrued that protein tyrosine nitration is a post-translational modification playing a role in physiological processes, including signal transduction [5]. The biological implications of protein tyrosine nitration in the transduction of NO effects in cells have been suggested by the identification of in vivo substrates for nitration in neurones and astrocytes and in other cell types [6,7]. In addition, the evidence for a 'denitrase' enzyme [8] has suggested that protein nitration is a reversible and perhaps regulatable process.

NO mediates a variety of actions in the nervous system including neurotransmitter release, synaptic plasticity, neuronal differentiation and development. We have recently reported that tyrosine nitration of proteins is implicated in the signalling pathway triggered by NO during nerve growth factor (NGF)-induced neuronal differentiation [9]. In differentiating PC12 cells, the cytoskeleton becomes the main cellular fraction containing nitrotyrosinated proteins and α-tubulin is the major target. In accordance with our data, nitrated α-tubulin has been previously identified in a specific region of the nervous system in invertebrates [10] and in chorioallantoic membrane during chick embryo development [11]. Interestingly, in the attempt to disclose the physiological function of nitration of cytoskeletal proteins, recent papers proposed that it could be a natural mechanism of cytoskeletal protein turnover [10] or a novel event through which NO modulates the phosphorylation state of cytoskeletal elements [12]. Looking for cytoskeletal proteins undergoing nitration during NGF-induced differentiation, we now find that this post-translational modification occurs on the microtubule-associated protein τ. This paper supplies the first evidence that τ protein is nitrated in a physiological process and, in addition, that it could play a role in neuronal differentiation.

2. Materials and methods

2.1. Cell culture

PC12 cells were maintained in RPMI 1640 (HyClone) containing 10% horse serum and 5% foetal bovine serum (HyClone) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, at 37°C in a humidified atmosphere, 5% CO_2. For differentiation, cells were plated at $1.5\times10^4/cm^2$ 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) in 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.

2.2. Cell extracts, immunoprecipitation and immunoblotting

Cell extracts were prepared by incubating cell culture with ice-cold lysis buffer containing 1% v/v NP-40, 10% v/v glycerol, protease inhibitors and 25 mM Tris–HCl, pH 7.5, for 30 min on ice. Before immunoprecipitation, the samples were normalised to contain the same protein concentration (1 mg/ml). α-Tubulin immunoprecipitates were prepared as previously described [13], and solubilised in twice-concentrated sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer not containing β-mercaptoethanol. Nitrotyrosine-containing proteins were immunoprecipitated as previously described [12]. For preparation of whole cell extracts, cells from cultures exposed to NGF for 5 days were washed twice with phosphate-buffered saline (PBS) and scraped into SDS–PAGE sample buffer containing protease inhibitors. To separate cytosolic and cytoskeletal associated proteins, cells were rinsed twice in PEM buffer (85 mM PIPES, pH 6.94, 10 mM EGTA, 1 mM $MgCl_2$, 2 M glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1 μM pep-

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statin, 2 μg/ml aprotinin), extracted for 10 min at room temperature with PEM buffer containing 0.1% v/v Triton X-100, and rinsed briefly in PEM buffer. The obtained Triton X-100-soluble fractions were diluted 3:1 with 4×SDS–PAGE sample buffer. The insoluble material remaining attached to the dish was scraped into SDS–PAGE sample buffer containing protease inhibitors. Protein samples were separated by SDS–PAGE, Western blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon™-P, Millipore) and immunostained as previously described using monoclonal anti-α-tubulin antibody [13] and anti-nitrotyrosine antibody [9]. For the detection of τ protein, blocking was performed by incubating the membranes in 10% w/v non-fat dry milk in 50 mM Tris, pH 7.6, 200 mM NaCl with 0.1% v/v Tween 20 overnight at 4°C. Primary antibody incubations were for 2 h at room temperature using 0.4 μg/ml rabbit polyclonal anti-τ H-150 antibody (Santa Cruz Biotechnology), 0.4 μg/ml goat polyclonal anti-τ C-17 (Santa Cruz Biotechnology) or 2 ng/ml mouse monoclonal anti-human τ (Upstate Biotechnology). Membranes were washed for 30 min with five changes and incubated with horseradish peroxidase-linked goat anti-rabbit IgG diluted 1:40 000 (Pierce), horseradish peroxidase-linked mouse anti-goat IgG diluted 1:2500 (Santa Cruz Biotechnology) or horseradish peroxidase-linked goat anti-mouse IgG diluted 1:15 000 (Pierce), for 1 h at room temperature. After washing the reaction was developed using enhanced chemiluminescence (SuperSignal® West Pico Chemiluminescent, Pierce).

2.3. Protein identification by N-terminal sequence analysis

α-Tubulin immunoprecipitate was subjected to SDS–PAGE and either electroblotted on PVDF membrane for N-terminal sequencing of the unknown protein band [9] or blue colloidal stained and in-gel digested as follows. The gel band to be identified was excised, cut into smaller pieces, destained with 100 μl of 50% acetonitrile in ammonium bicarbonate 0.1 M (40 min at 25°C) and dried in a Speed Vac. Dried gels were reduced by 10 mM dithiothreitol at 56°C for 1 h. Following cysteine derivatisation by iodoacetamide at 25°C the gels were digested overnight with trypsin sequencing grade (Roche) at 37°C. The in-gel tryptic digest was extracted with 30 μl 50% acetonitrile in 0.1% trifluoroacetic acid and the peptide mixture was either analysed by mass spectrometry or separated for N-terminal sequence analysis. In the latter case purification of the extracted peptides was carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) using an Applied Biosystem 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 Biosystem). Solvents used were: (A) 0.1% (v/v) trifluoroacetic acid in water, (B) 0.075% (v/v) trifluoroacetic acid in acetonitrile. The peptides were eluted with a linear gradient from 0 to 50% B in 70 min at a flow rate of 75 μl/min. The amino acid sequence of each peptide was determined by a pulsed-liquid sequencer (Procise model 491 Applied Biosystems, Foster City, CA, USA). Similarities with the entries in the SwissProt, TrEMBL, and NCBI sequence databases were searched using the BLAST program.

2.4. Protein identification by matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) analysis

MALDI-TOF mass spectrometric analysis was carried out on the band to be identified following immunoprecipitation, SDS–PAGE, in-gel digestion and peptide extraction as indicated above. The extract was subjected to MALDI-TOF mass spectrometric analysis using a Bruker Daltonics Reflex IV instrument equipped with a nitrogen laser (337 nm) and operated in reflector mode with a matrix of α-cyano-4-hydroxy-cinnamic acid. External standard were used for calibration (Bruker peptide calibration standard) ranging from 1 to 3 kDa. Each spectrum was accumulated for 30 laser shots. Measured peptide masses were used to search the SwissProt, TrEMBL, and NCBI sequence databases for protein identifications by the Mascot program (http://www.matrixscience.com).

2.5. Fluorescence microscopy

For immunofluorescence analysis, cells were rinsed twice in PEM buffer, extracted at room temperature for 10 min with PEM buffer containing 0.1% v/v Triton X-100, and rinsed in PEM buffer. Cells were fixed and permeabilised for 10 min with methanol at −20°C, washed with PBS and blocked in PBS+5% bovine serum albumin (BSA) for 1 h at room temperature. Nitrotyrosine-containing proteins were detected by incubation with 10 μg/ml monoclonal anti-nitrotyrosine antibody (Alexis) in PBS+5% BSA, overnight at 4°C. To control for non-specific binding, primary antibody was excluded or anti-nitrotyrosine antibody was used in the presence of 10 mM nitrotyrosine. Tau protein was localised by incubation with 10 μg/ml rabbit polyclonal anti-τ H-150 antibody or 10 μg/ml goat polyclonal anti-τ C-17 (Santa Cruz Biotechnology) in PBS+5% BSA for 2 h at room temperature. As secondary antibodies we used Alexa Fluor™ 488 goat anti-rabbit IgG, Alexa Fluor™ 488 donkey anti-goat IgG and Alexa Fluor™ 594 donkey anti-mouse (Molecular Probes), 1:1000 in PBS+5% BSA for 45 min at 37°C. Nuclear staining was performed by incubation with DAPI (0.25 μg/ml in PBS) for 15 min at room temperature. The coverslips were mounted in Mowiol® (Calbiochem)-DABCO (Sigma-Aldrich) and examined with a Leica TCS NT laser scan microscope imaging system equipped with an argon–krypton and UV laser. Switching off the appropriate laser line using the AOTF in the confocal microscope showed that there was negligible 'bleed-through' between channels. Complete 'z' series optical sections were collected and projected onto a single plane using Leica TCS software.

3. Results and discussion

3.1. Identification of τ by N-terminal sequence and MALDI-TOF

During PC12 cell differentiation triggered by NGF, the cytoskeleton becomes the main cellular fraction containing nitrotyrosinated proteins and α-tubulin is the major target [12]. To identify other proteins functionally related to α-tubulin that are cellular targets of nitration in differentiated PC12 cells, we have looked at proteins that co-immunoprecipitate with α-tubulin. PC12 cells were incubated for 5 days with NGF and α-tubulin was immunoprecipitated, the proteins separated by SDS–PAGE and probed with anti-α-tubulin (Fig. 1A) and anti-nitrotyrosine antibodies (Fig. 1B). Besides α-tubulin, which was recognised by both antibodies, an additional band is clearly stained only in the blot probed with anti-nitrotyrosine antibody. The identification of this protein, which co-immunoprecipitates with α-tubulin and is nitrated in tyrosine, was initially addressed by N-terminal sequencing of the entire protein electroblotted on PVDF membrane but no sequence information was obtained suggesting that the N-terminus was blocked. Therefore, in order to identify the protein, in-gel tryptic digestion was carried out on six gel bands after staining with brilliant blue G-colloidal as detailed in Section 2. Peptide mixture was either analysed by mass spectrometry or separated by RP-HPLC (data not shown) in order to purify

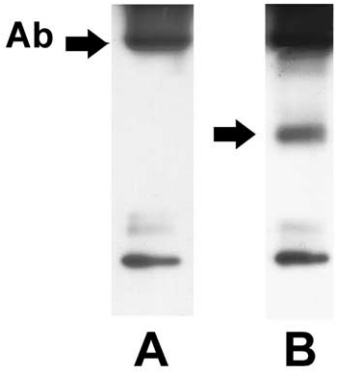

Fig. 1. α-Tubulin was immunoprecipitated from PC12 cells incubated 5 days with NGF, separated by SDS–PAGE and immunoblotted with anti α-tubulin (A) or anti-nitrotyrosine (B) antibodies. The molecular weight markers are not present since the SDS–PAGE was run without β-mercaptoethanol. The arrows indicates the antibody chains (Ab) and the band cut and identified by MALDI-TOF analysis.

Table 1
Peptides used as sequence tag for databank searches

72–78	APDKEATAQ
117–130	VEIFSQSLLVEPGR
179–193	SHPASELL(W)QESPQK
361–366	RVPQLK
585–590	VQIINK
592–604	LDLSNVQSK(C)GSK

The numbers in the first column refer to the sequence of the TAU-A isoform from rat. Amino acids in parentheses were not experimentally detected.

the peptides to be subjected to N-terminal sequence analysis. As indicated in Table 1, five peptides were successfully sequenced by Edman degradation and used as sequence tag to search in the SwissProt, TrEMBL, and NCBI sequence databases allowing the identification of the protein as τ. Consistent with this result, τ was also identified by peptide fingerprint using MALDI-TOF (Table 2). It should be pointed out that the presence of multiple τ isoforms has been previously described in PC12 cells [14–16] and in the SwissProt data bank eight isoforms are described for rat τ protein (entry P19332). Our results rule out the possibility that the nitrated band identified as τ corresponds to isoforms missing regions 33–90 and 113–366 since peptides within these sequence portions have been identified by both N-terminal sequence and MALDI-TOF analyses (Tables 1 and 2).

3.2. Immunodetection and localisation of nitrated τ

To further investigate the nitrated form of τ in neurone-like PC12 cells, we have analysed τ isoform expression in whole cell extracts from PC12 differentiated for 5 days with NGF (Fig. 2A). In accordance with previous studies [14–16], Western blot analysis performed on the total extract shows that τ migrates as several bands ranging from 45 to 70 kDa, together with high molecular weight τ around 110 kDa. To examine τ isoforms undergoing nitration in differentiated PC12 cells and to compare them with undifferentiated cells, nitrotyrosine-containing proteins were immunoprecipitated from PC12 cells incubated for 0 or 5 days with NGF, separated by SDS–PAGE and probed with anti-τ antibodies (Fig. 2B). We observe a clear τ staining for some proteins immunoprecipitated from differentiated PC12 cells whereas a weak signal is observed in unstimulated cells. These data indicate that some τ isoforms undergo nitration in differentiating PC12 cells and suggest that this post-translational protein modification could play a role during the progression of differentiation.

Nitrated proteins have been shown by confocal microscopy to localise into the cell body and neurites and to be mainly associated with the cytoskeleton in differentiated PC12 cells [9]. To investigate the localisation of nitrated τ and to test whether the nitration drastically affects the association of τ

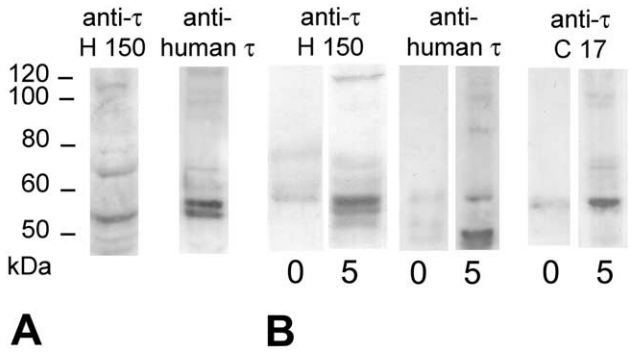

Fig. 2. Immunodetection of nitrated τ in PC12 cells. A: Immunoblot analysis showing τ protein in whole cell extracts was performed on PC12 cells treated for 5 days with NGF by using polyclonal anti-τ H-150 and monoclonal anti-human τ antibodies. B: Nitrotyrosine-containing proteins were immunoprecipitated from PC12 cells incubated for 0 or 5 days with NGF and immunoblotted with polyclonal anti-τ H-150 and anti-τ C-17 antibodies, and monoclonal anti-human τ antibody.

with the cytoskeleton fraction, differentiated PC12 cells following Triton X-100 extraction were initially analysed by double immunofluorescence labelling of τ and nitrotyrosine and observed by confocal microscopy (Fig. 3A,B). Tau immunoreactivity is distributed in the cell body in addition to the neurite as well as nitrotyrosine immunoreactivity. Looking for the colocalisation of τ and nitrotyrosine, we found that the immunoreactivities coexist only in the cell soma suggesting that nitration affects only a small fraction of τ protein in cells and that the modified protein is associated with the cytoskeleton but it is confined to a restricted cell region. In addition, immunoblotting was performed on cytoskeletal and cytosolic fractions obtained from differentiated PC12 cells (Fig. 3C). Tau staining as well as nitrotyrosine staining are almost exclusively found in the cytoskeletal fraction. These results confirm the evidence deriving from immunofluorescence experiments and suggest that nitrated τ maintains its association with the cytoskeleton.

3.3. Conclusions

The present study supplies the first evidence that: (i) nitration of τ protein occurs in differentiating PC12 cells and (ii) the modified protein is associated with the cytoskeletal fraction. Until now, there was a single report that τ protein could be nitrated and it was linked to a pathological event as the formation of filamentous τ inclusions in neurodegeneration [17]. The data presented here are consistent with the notion that nitration of τ may be a physiological process, in agreement with recent papers suggesting the physiological nitration of other cytoskeletal proteins in different model systems from invertebrates to mammalian cultured cells [9,11,12].

Table 2
Peptides found by MALDI-TOF analysis and used in data bank searches

		MW calculated	MW observed
117–130	VEIFSQSLLVEPGR	1572.85	1572.35
179–193	SHPASELLWQESPQK	1735.85	1737.48
194–200	EAWGKDR	860.92	860.32
491–504	TPPGSGEPPKSGER	1394.68	1394.20
592–604	LDLSNVQSKCGSK	1434.71	1435.25
658–663	DRVQSK	732.39	732.38

The numbers in the first column refer to the sequence of TAU-A isoform from rat. The MWs are reported as monoisotopic mass.

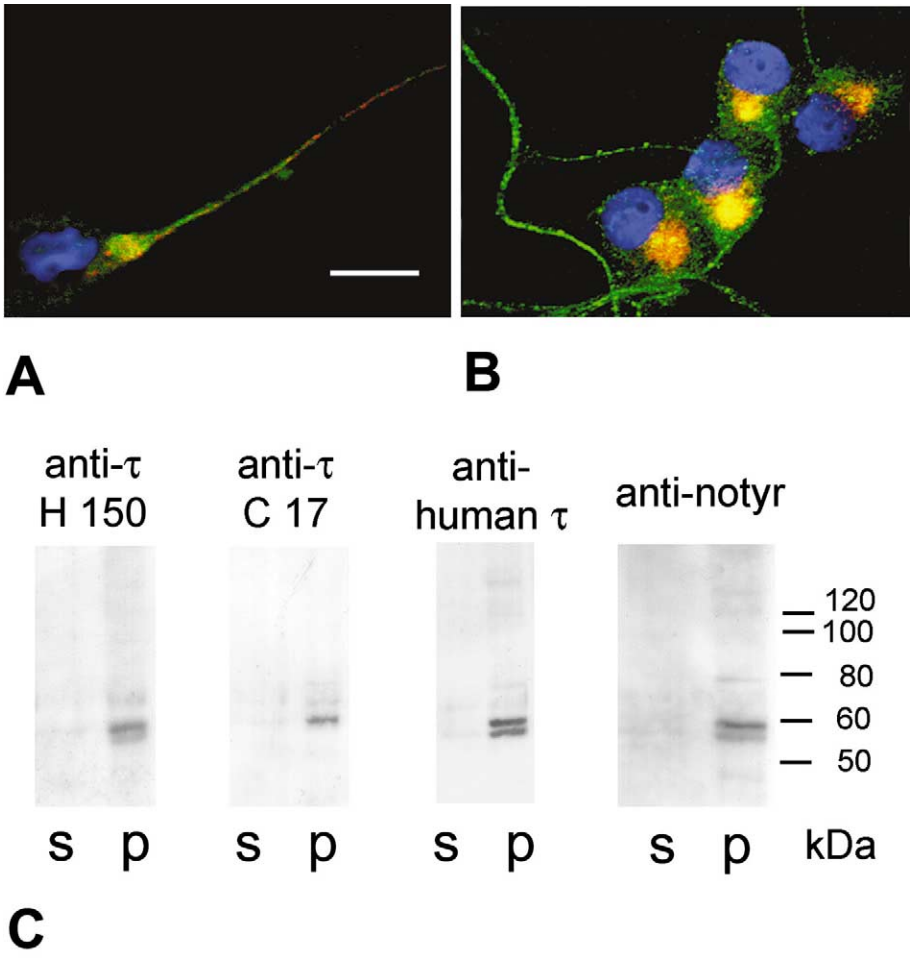

Fig. 3. Association of nitrated τ with the cytoskeleton in differentiated PC12 cells. A,B: Confocal analysis was performed on cells fixed and immunostained for τ (green), nitrotyrosine (red) and nuclear stained with DAPI (blue) after Triton X-100 extraction to show the association with the insoluble cytoskeletal fraction. Tau protein was stained with rabbit polyclonal anti-τ H-150 (A) and goat polyclonal anti-τ C-17 (B) antibodies. Bar, 10 μm. C: Immunoblot analysis was performed on cytosolic (s) and cytoskeletal (p) fractions using polyclonal anti-τ H-150 and anti-τ C-17 antibodies, monoclonal anti-human τ and anti-nitrotyrosine antibodies.

Tau is a member of the microtubule-associated proteins that modulate microtubule structure and dynamics, it is an abundant microtubule-associated protein in neuronal cells and it has been implicated in axonal growth and development of neuronal polarity [18]. A key role for τ in neurite outgrowth has been reported in cultured cells including PC12 cells induced to differentiate with NGF [19]. The present work, by showing that nitration of τ occurs in differentiating PC12 cells, suggests that nitration could be a novel post-translational modification that regulates τ function in neuritogenesis and differentiation. Looking for mechanisms of τ regulation in neuronal cells, activation of protein kinases and phosphatases plays a primary role [20]. Next to the well known serine/threonine kinases, the tyrosine kinase Fyn has been reported to be associated with tyrosine phosphorylation of τ [21]. A recent paper has suggested that τ is indeed a substrate of Fyn and is tyrosine phosphorylated in neuronal cells exposed to the amyloid-β peptide [22], but failed to demonstrate that tyrosine phosphorylation occurs on normal adult brain τ. An intriguing hypothesis, in our opinion, is that nitration of τ in physiological conditions might mimic or interfere with other post-translational modifications occurring on tyrosine residues such as phosphorylation, as emerging for some cytosolic and cytoskeletal proteins [23–25]. If this can be demonstrated to be the case, nitration of τ might have a crucial role in the signal transduction pathway triggered by NO during neuronal differentiation.

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