Eur. J. Biochem. **269**, 5037–5045 (2002) © FEBS 2002 doi:10.1046/j.1432-1033.2002.03220.x

Incorporation of 3-nitrotyrosine into the C-terminus of α-tubulin is reversible and not detrimental to dividing cells

C. Gastón Bisig, Silvia A. Purro, María A. Contín, Héctor S. Barra and Carlos A. Arce

Centro de Investigaciones en Química Biológica de Córdoba, Departamento de Química Biológica, Universidad Nacional de Córdoba, Argentina

The C-terminus of the α-chain of tubulin is subject to reversible incorporation of tyrosine by tubulin tyrosine ligase and removal by tubulin carboxypeptidase. Thus, microtubules rich in either tyrosinated or detyrosinated tubulin can coexist in the cell. Substitution of the terminal tyrosine by 3-nitrotyrosine has been claimed to cause microtubule dysfunction and consequent injury of epithelial lung carcinoma A549 cells. Nitrotyrosine is formed in cells by nitration of tyrosine by nitric oxide-derived species. We studied properties of tubulin modified by *in vitro* nitrotyrosination at the C-terminus of the α-subunit, and the consequences for cell functioning. Nitrotyrosinated tubulin was a good substrate of tubulin carboxypeptidase, and showed a similar capability to assemble into microtubules *in vitro* to that of tyrosinated tubulin. Tubulin of C6 cells cultured in F12K medium in the presence of 500 μM nitrotyrosine became fully nitrotyrosinated. This nitrotyrosination was shown to be reversible. No changes in morphology, proliferation, or viability were observed during cycles of nitrotyrosination, denitrotyrosination, and re-nitrotyrosination. Similar results were obtained with CHO, COS-7, HeLa, NIH-3T3, NIH-3T3(TTL⁻), and A549 cells. C6 and A549 cells were subjected to several passages during 45 days or more in the continuous presence of 500 μM nitrotyrosine without noticeable alteration of morphology, viability, or proliferation. The microtubular networks visualized by immunofluorescence with antibodies to nitrotyrosinated and total tubulin were identical. Furthermore, nitrotyrosination of tubulin in COS cells did not alter the association of tubulin carboxypeptidase with microtubules. Our results demonstrate that substitution of C-terminal tyrosine by 3-nitrotyrosine has no detrimental effect on dividing cells.

Keywords: tubulin; microtubules; tyrosination state; nitrotyrosine and cell injury.

One of the most studied post-translational modifications of tubulin is the addition or removal of a tyrosine residue at the C-terminus of the α-subunit [1–3]. Two enzymes, tubulin tyrosine ligase and tubulin carboxypeptidase, are involved in a cycle that renders two types of microtubules coexisting in cells: those enriched in tyrosinated tubulin (Tyr-microtubules) and those enriched in detyrosinated tubulin (Glu-microtubules) [4]. The carboxypeptidase selectively removes the C-terminal tyrosine from tubulin producing Glu-tubulin which, in turn, can be retyrosinated by the ligase [3,5–7]. The ligase acts rapidly on nonassembled tubulin but not on microtubules. On the other hand, the carboxypeptidase slowly releases tyrosine from tubulin while being assembled into microtubules. Therefore, dynamic microtubules, characteristic of dividing cells, remain mainly tyrosinated whereas stable, long-lived microtubules are mainly detyrosinated because they can accumulate Glu-tubulin before being disassembled. Artificial stabilization of microtubules with the drug taxol allows rapid accumulation of Glu-tubulin in microtubules of living cells [7]. In fact, differentiated cells contain a subset of stable microtubules which are highly detyrosinated [8,9] and, further, contain a low although significant amount of Δ2tubulin, an isospecies lacking also the ultimate glutamic acid residue and that cannot be retyrosinated by the ligase [5,10]. This tubulin form has not been detected in dividing cells.

Despite many studies, the physiological role of this reaction remains unclear. As an experimental strategy, we are studying tyrosine analogues capable of being incorporated into the C-terminus of α-tubulin, and also able to alter biochemical properties of the tubulin molecule; such modification is expected to produce anomalous microtubules that affect normal cell functioning. We first chose 3-nitrotyrosine because of a report [11] that it is post-translationally incorporated into tubulin in epithelial lung carcinoma A549 cells, affecting microtubules and some cellular functions. Recombinant ligase catalyzes incorporation of nitrotyrosine into tubulin *in vitro* [12]. This tyrosine derivative is elevated in many human diseases and clinical disorders [13–16]. It is formed by nitration of tyrosine by nitric oxide-derived species, which originate from L-arginine via the catalytic action of NO synthases [17]. Among these NO-derived species, anionic peroxinitrite ($ONOO^-$) causes nitration of free- and protein-bound tyrosine. The presence of this tyrosine analogue is used as a marker for oxidative

Correspondence to C. A. Arce, Departamento de Química Biológica, Facultad de Ciencias, Químicas, Ciudad Universitaria, 5000-Córdoba, Argentina. Fax: +54 351433–4074, Tel.: +54 351433–4168, E-mail: caecra@dqb.fcq.unc.edu.ar

Abbreviations: Tyr-tubulin, tubulin whose α-subunit has a C-terminal tyrosine residue; Glu-tubulin, tubulin whose α-subunit lacks the C-terminal tyrosine residue; nitrotyrosinated tubulin, tubulin whose α-subunit has a C-terminal nitrotyrosine residue; Tyr-microtubules, microtubules composed mainly of Tyr-tubulin; Glu-microtubules, microtubules composed mainly of Glu-tubulin.

(Received 17 April 2002, revised 12 July 2002, accepted 29 August 2002)

events in cells and tissues. Oxidative processes involving peroxinitrite and other NO-derived species are assumed to cause protein modification and/or local cellular DNA damage, with consequent cellular injury. Studies on post-translational nitrotyrosination of tubulin and its possible link to cellular injury [11] were the first attempt to describe at the molecular level the role of free 3-nitrotyrosine as a cytotoxic agent. In this case, morphological alterations of cells as well as microtubular networks were reported. Based on the observation that pancreatic carboxypeptidase A (which *in vitro* efficiently removes C-terminal tyrosine from tubulin) cannot release previously incorporated 3-nitrotyrosine, it was proposed [11] that the irreversible incorporation of nitrotyrosine into tubulin and its effects on properties of microtubules represent a distinct mechanism of cellular or tissue injury during pathological processes. Similarly, another group showed [18] that nitrotyrosine elicits neurodegenerative effects *in vivo*, independent of peroxinitrite-mediated oxidative and/or protein nitration events. The interest in oxidative biology in many areas of health care has reinforced efforts to understand mechanisms of cell injury and death during pathological conditions. Idriss hypothesized [19,20] that the selective cytotoxic effect of TNFα (tumor necrosis factor) on tumors is related to the capability of tumor cells to escape (or not) α-tubulin nitrotyrosination. However, results presented here suggest that (a) nitrotyrosination of tubulin is not irreversible as originally reported; and (b) nitrotyrosination of tubulin is not a mechanism for cellular injury or death, at least in the cell lines we studied.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, chemicals and culture media were purchased from Sigma. L-[U-^{14}C]Tyrosine (specific activity 450 μCi·μmol^{-1}) was from New England Nuclear. L-[U-^{14}C]-3-Nitrotyrosine (specific activity 450 μCi·μmol^{-1}) was obtained by nitration of [U-^{14}C]tyrosine using sodium nitrite and oxygen peroxide [21], and purified by two-dimensional TLC.

Soluble rat brain preparation

Brains from 15- to 30-day-old-rats were homogenized in one volume (w/v) MEM buffer (100 mM Mes adjusted with NaOH to pH 6.7, containing 1 mM EGTA and 1 mM $MgCl_2$). The homogenate was centrifuged at 100 000 ***g*** for 1 h and, when indicated, the supernatant solution was passed through a column of Sephadex G-25–80 equilibrated with MEM buffer to eliminate low molecular weight compounds. Tubulin concentration in this preparation is approximately 2 mg·mL^{-1}.

In vitro incorporation of tyrosine or 3-nitrotyrosine into tubulin

Except when otherwise specified, the incubation medium contained, per ml, 0.9 mL soluble brain extract, 2.5 μmol ATP, 12.5 μmol $MgCl_2$, 30 μmol KCl, 100 μmol Mes buffer, pH 6.7, and 3 μCi (6.7 nmol) [^{14}C]tyrosine or 3-nitro-[^{14}C]tyrosine. Incubation temperature was 37 °C. At the stated times, aliquots were inactivated by addition of 2 mL 5% trichloroacetic acid and heated at 90 °C for 15 min. Radioactivity bound to protein was measured in hot-trichloroacetic acid-insoluble material as described previously [22].

Antibodies

Rabbit polyclonal antibody specific to Glu-tubulin (anti-Glu) was prepared in our laboratory as described previously [4]. Polyclonal antibody specific to nitrotyrosinated tubulin (antinitro) was raised in rabbits following the technique described for antibodies specific to catecholamines [23]. In brief, 3-nitrotyrosine was bound through its amino group to keyhole limpet hemocyanin using glutaraldehyde as cross-linker. The resulting protein, after being mixed 1 : 1 with complete adjuvant, was injected subcutaneously every 15 days. The antisera were usually collected 15 days after each injection and tested for affinity and specificity and stored at −20 °C. Mouse monoclonal antibodies against Tyr-tubulin (Tub1A2) and total α-tubulin (DM1A), peroxidase-conjugated rabbit antimouse IgG, rhodamine-conjugated goat antirabbit, and fluorescein-conjugated goat antimouse secondary antibodies were from Sigma.

Cell culture

C6, COS-7, NIH 3T3, NIH 3T3 (TTL$^-$), HeLa, CHO, and A549 cells were grown in Ham's F12K medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) at 37 °C in an air/CO_2 (19 : 1) incubator. When indicated, the culture medium was Ham's F12 which contains 30 μM tyrosine as opposed to 60 μM in F12K medium. Cells were plated on plastic Petri dishes (60 mm diameter) or 24-well plates and grown for 2 days until reaching the desired final density. Culture medium was renewed every 24 h. Treatments involving cells were performed at 37 °C unless stated otherwise. Stock solution of nitrotyrosine (10 mM) was prepared in 10 mM HCl.

Isolation of cytoskeletons

Cells were washed with microtubule-stabilizing buffer (90 mM Mes pH 6.7; 1 mM EGTA; 1 mM $MgCl_2$; 10% (v/v) glycerol), then extracted with 2.5 mL microtubule-stabilizing buffer containing 10 μM taxol, 0.5% (v/v) Triton X-100, and proteases inhibitors (10 μg·mL^{-1} aprotinin, 0.5 mM benzamidine, 5 μg·mL^{-1} *o*-phenanthroline, 0.2 mM phenylmethanesulfonyl fluoride) at 37 °C for 3 min with gentle agitation. The detergent extract was removed by suction, and the cytoskeleton fraction (which remained bound to the dish) was washed twice with 5 mL prewarmed microtubule-stabilizing buffer. Isolated cytoskeletons were immediately subjected to immunoblotting or incubated to determine carboxypeptidase activity associated with microtubules as described [24].

Immunoblotting

Cytoskeleton fractions were dissolved in 100 μL sample buffer and subjected to SDS/PAGE [25], and the proteins were transferred to nitrocellulose sheets. The sheets were reacted overnight at 4 °C with either antinitro, anti-Glu,

Tub1A2, or DM1A antibody (diluted 1 : 600, 1 : 200, 1 : 1000, or 1 : 1000, respectively). Sheets treated with Tub1A2 or DM1A were incubated, after washing, with peroxidase-conjugated rabbit antimouse IgG (dilution 1 : 600), and then incubated for 1 h at room temperature in the presence of horseradish peroxidase conjugate to protein A (1 $\mu g \cdot mL^{-1}$). Color was developed using 4-chloronaphth-1-ol.

Quantification of nitrotyrosinated and Glu-tubulin

After color development, immunoblots were dried and scanned with a Duoscan T1200 (Agfa) connected to a PC, and optical density values determined using the Scion Image program. Experimental values were standardized relative to total tubulin loaded, by dividing the optical density of each band stained with antibodies to Glu- and nitrotyrosinated tubulin by that of an identical sample stained with DM1A antibody.

Capabilities of nitrotyrosinated and Tyr-tubulin to assemble into and to disassemble from microtubules

[^{14}C]Tyrosine (0.1 mM; 15 $\mu Ci \cdot \mu mol^{-1}$) or 3-nitro-[^{14}C] tyrosine (1 mM, 3.3 $\mu Ci \cdot \mu mol^{-1}$) was incorporated into tubulin from soluble brain extract. This preparation was filtered through Sephadex G-25 and mixed with three volumes of a similar soluble brain preparation kept at 0 °C. The mixture was incubated at 37 °C under assembly conditions (0.2 mM GTP, 40% glycerol) for 30 min, then centrifuged at 100 000 ***g*** for 10 min at 27 °C. The pellet and supernatant fractions were processed to measure radioactivity bound to protein, and for immunoblot using antibodies to tyrosinated, nitrotyrosinated and total tubulin. Pellets from parallel experimental tubes were resuspended in the original volume with MEM buffer and kept cold (0 °C for 30 min) with gentle stirring. The samples were then centrifuged at 100 000 ***g*** for 10 min at 0 °C, and the pellet and supernatant fractions were processed to measure radioactivity bound to protein and for immunoblot as above. After immunostaining, bands corresponding to each tubulin species were quantified by densitometry. Percentage of tubulin assembly was calculated as the optical density value of the pellet (sedimented microtubules) divided by the sum of optical density values for pellet and supernatant, multiplied by 100. The same formula was used to calculate percentage of assembly from radioactivity values.

Immunofluorescence

Cells cultured on coverslips were treated as described for isolation of cytoskeletons, and fixed with anhydrous methanol at −20 °C. The samples were washed, incubated with 2% (w/v) BSA in NaCl/P_i for 30 min, and stained by double indirect immunofluorescence using anti-nitro and DM1A Igs (1 : 600 and 1 : 1000 dilution in NaCl/P_i containing 1% goat serum, respectively). Fluorescein-conjugated antimouse IgG and rhodamine-conjugated goat anti-rabbit IgG were used simultaneously as secondary antibodies at 1 : 400 and 1 : 800 dilution, respectively. Coverslips were mounted in FluorSave and observed for epifluorescence on an Axioplan microscope (Zeiss, Germany).

Cell viability and proliferation

Percentage of viable cells was determined by Trypan Blue exclusion. To determine proliferation rate, cells in individual capsules were cultured in parallel for the stated times, and cell numbers were determined using Cell Titer 96 Aqueous One solution (Promega). When indicated, cells cultured in 60-mm dishes were scrapped off in 0.5 mL microtubule-stabilizing buffer, 30 μL-aliquots were plated on a 96-well plate, and 70 μL Cell Titer 96 Aqueous One solution was added to each well. Samples were incubated for 1 h at 37 °C, and optical density was measured at 455 nm to provide a direct estimate of cell number. Values represent means of triplicate determinations.

RESULTS

Characterization of *in vitro* nitrotyrosine incorporation

We showed previously that 100 000 ***g*** supernatant fraction from rat brain homogenate has the capability to incorporate [^{14}C]-tyrosine into endogenous tubulin [22,26]. Under similar conditions, [^{14}C]-3-nitrotyrosine (6.7 μM, 450 $\mu Ci \cdot \mu mol^{-1}$) was incorporated into protein (Fig. 1A). Inclusion of 1 mM nonradioactive nitrotyrosine in the incubation system led to reduced incorporation of radioactivity. In this case, as calculated from the specific radioactivity and tubulin content in the supernatant fraction, approximately 0.3 mol of nitrotyrosine was incorporated per mol of tubulin, at $t = 120$ min. The presence of nonradioactive tyrosine (0.1 mM) competed with incorporation of

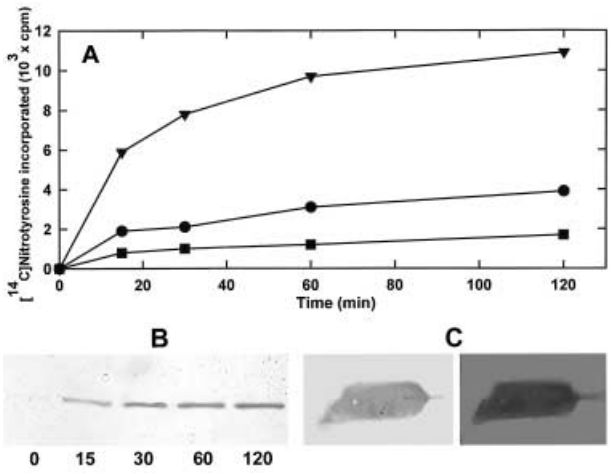

Fig. 1. Incorporation of 3-nitrotyrosine into tubulin. Soluble rat brain extract was passed through a Sephadex G-25 column and used to incorporate [^{14}C]nitrotyrosine with incubation conditions as described in Materials and methods. (A) Incorporation of radioactive nitrotyrosine into tubulin was determined in 0.1 mL-aliquots. (▼): 3 $\mu Ci \cdot mL^{-1}$ (6.7 μM) [^{14}C]nitrotyrosine; (●): 3 $\mu Ci \cdot mL^{-1}$ (1 mM) [^{14}C]nitrotyrosine; (■): 3 $\mu Ci \cdot mL^{-1}$ (6.7 μM) [^{14}C]nitrotyrosine plus 0.1 mM tyrosine. (B) Aliquots (2 μL) from (●) at the stated times were subjected to Western blot and immunostained with antibody specific to nitrotyrosinated tubulin. (C) An aliquot (20 μL) from (●) at 120 min was subjected to Western blot, immunostained with antibody specific to nitrotyrosinated tubulin (left), and radioactivity detected by autoradiography (right). Only α-tubulin band areas are shown.

radioactive nitrotyrosine, indicating a common mechanism for incorporation into protein. Western blot analysis after incorporation of nonradioactive nitrotyrosine, using antibody to nitrotyrosinated α-tubulin, showed that the tyrosine analogue was incorporated into a unique protein with mobility identical to that of α-tubulin (Fig. 1B). Similarly, radioactive nitrotyrosine (detected by autoradiography) was incorporated into a unique protein with mobility identical to that of nitrotyrosinated tubulin as revealed by immunostaining (Fig. 1C). Nitrotyrosinated tubulin was the only protein revealed by the antibody. No immunostaining was detected prior to incorporation of nitrotyrosine (Fig. 1B, $t = 0$) demonstrating the specificity of the antibody to nitrotyrosinated tubulin.

When tubulin in the 100 000 ***g*** supernatant fraction was blocked at the C-terminus by incorporation of nonradioactive tyrosine and subsequently incubated with radioactive 3-nitrotyrosine, there was essentially no incorporation of the radioactive analogue. Similarly, when tubulin was first blocked with nonradioactive nitrotyrosine and then incubated with radioactive tyrosine, no radioactivity was incorporated (data not shown). This mutual exclusion indicates that 3-nitrotyrosine and tyrosine are incorporated at the same site of the acceptor protein.

To determine whether [^{14}C]nitrotyrosine is incorporated as such or modified during incubation, proteins after incorporation of [^{14}C]nitrotyrosine were subjected to hydrolysis in 6 M HCl at 100 °C for 12 h, or treated at 37 °C with 10 μg·mL^{-1} pancreatic carboxypeptidase A. Products from both treatments were subjected to two-dimensional TLC. In each case, a single radioactive spot was found, coinciding in position and shape with authentic 3-nitrotyrosine (data not shown).

These results, cumulatively, indicate that nitrotyrosine is incorporated as such into the C-terminus of the α-tubulin subunit, by the same mechanism as tyrosine.

Capability of nitrotyrosinated tubulin to assemble into microtubules *in vitro*

Eiserich *et al.* reported previously [11] that incorporation of nitrotyrosine into tubulin of cultured A549 cells led to decreased length of microtubules, and increased perinuclear localization and aggregation with consequent alteration of cell morphology. We tested *in vitro* the possibility that substitution of C-terminal tyrosine of α-tubulin by 3-nitrotyrosine alters the ability of the molecule to form microtubules. For this purpose, we studied the behavior of nitrotyrosinated tubulin (and of tyrosinated tubulin for comparison) during reconstitution of microtubules from soluble rat brain extract. For monitoring the tubulin isospecies, we used radioactive amino acids as well as specific antibodies.

After incorporation of [^{14}C]nitrotyrosine or [^{14}C]tyrosine into tubulin, the protein preparations were processed to compare the abilities of tyrosinated and nitrotyrosinated tubulin to assemble into and to disassemble from microtubules, by monitoring radioactivity and immunoreactivity (see Materials and methods). For both monitoring methods, tyrosinated and nitrotyrosinated tubulin assembled into microtubules in a similar proportion (approximately 45%) (Table 1). Electron microscopy analysis did not reveal significant differences in either the aspect of microtubules or the amount of amorphous aggregates or microtubular structures (data not shown). Resuspension of pellets in cold buffer (0 °C for 30 min) caused solubilization of approximately 85% of either [^{14}C]tyrosinated or [^{14}C]nitrotyrosinated tubulin. These results indicate that, *in vitro*, the presence of a nitrotyrosine residue in place of tyrosine at the C-terminus of α-tubulin does not alter the ability of the protein to assemble into or disassemble from microtubules.

Kinetics of release of 3-nitrotyrosine from nitrotyrosinated tubulin

Two carboxypeptidases were used to study release of nitrotyrosine from nitrotyrosinated tubulin. One of them, carboxypeptidase A, catalyzes sequential release of the ultimate C-terminal amino acid (except basic residues) from peptides and proteins. The other, tubulin carboxypeptidase, participates in the physiological tyrosination/detyrosination cycle producing selective release of C-terminal tyrosine from the α-tubulin subunit [2,7,27]. Figure 2A shows time curves for release of [^{14}C]nitrotyrosine and [^{14}C]tyrosine from, respectively, [^{14}C]nitrotyrosinated and [^{14}C]tyrosinated tubulin, at two carboxypeptidase A concentrations. Low concentration of the enzyme (0.25 μg·mL^{-1}) produced almost no release of nitrotyrosine, whereas tyrosine was rapidly cleaved. A higher concentration of carboxypeptidase A (10 μg·mL^{-1}) was necessary to release a significant amount (approximately 60%) of nitrotyrosine in a 1-h period. Figure 2B shows time curves for release of the same radiolabeled species as in Fig. 2A, catalyzed, in this case, by endogenous tubulin carboxypeptidase. The carboxypeptidase present in the soluble protein fraction from rat brain was able to release approximately 30% of [^{14}C]nitrotyrosine and approximately 40% of [^{14}C]tyrosine in a 1-h period. These results indicate that tyrosinated and nitrotyrosinated

Table 1. Capability of nitrotyrosinated tubulin to assemble into and disassemble from microtubules. Assembly and disassembly were determined by monitoring each type of tubulin by immunoblot and by radioactivity. Results are the mean ± S.D. of three independent experiments. ND, not determined.

	Assembly (% as microtubules)		Disassembly (% as tubulin)	
Tubulin type	Immunoblot	Radioactivity	Immunoblot	Radioactivity
Nitrotyrosinated	42 ± 3	42 ± 6	85 ± 9	80 ± 10
Tyrosinated	46 ± 5	47 ± 6	83 ± 5	86 ± 9
Total	39 ± 5	ND	87 ± 8	ND

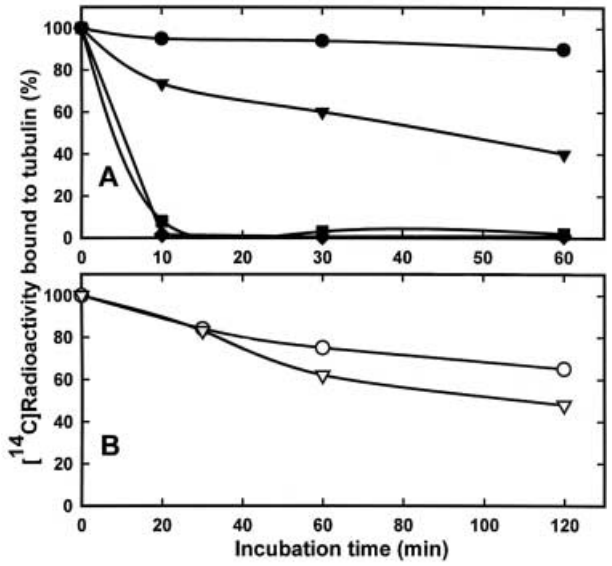

Fig. 2. Release of nitrotyrosine from nitrotyrosinated tubulin. Soluble brain extract passed through a Sephadex G-25 column was used to incorporate [^{14}C]nitrotyrosine or [^{14}C]tyrosine into tubulin as described in Materials and methods. After incubation, the mixture was passed through Sephadex G-25 and the protein fraction containing [^{14}C]nitrotyrosinated or [^{14}C]tyrosinated tubulin was collected. (A) Preparations containing [^{14}C]nitrotyrosinated (●,▼) or [^{14}C]tyrosinated (■,◆) tubulin were incubated at 37 °C in the presence of 0.25 μg·mL^{-1} (●,■) or 10 μg·mL^{-1} (▼,◆) carboxypeptidase A. (B) Preparations containing [^{14}C]nitrotyrosinated (○) or [^{14}C]tyrosinated (▽) tubulin were mixed with three volumes of a similar (unlabeled) brain preparation that had been kept at 0 °C. Both mixtures were incubated at 37 °C under assembly conditions (0.2 mM GTP and 40% glycerol). At the stated times, radioactivity bound to protein was measured.

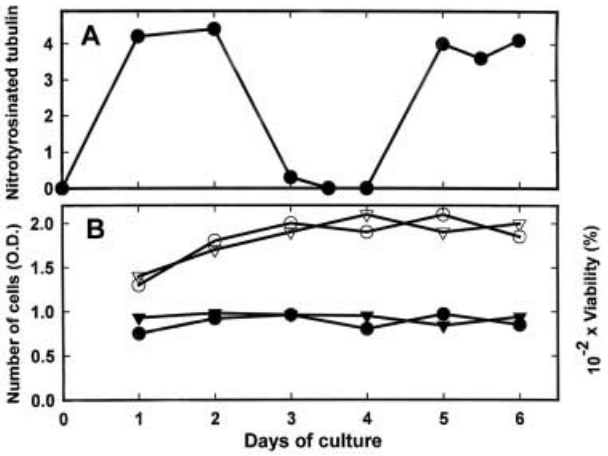

Fig. 3. Tubulin nitrotyrosination/denitrotyrosination cycle in cultured cells. C6 cells were grown in F12K medium supplemented with 500 μM nitrotyrosine. After 2 days, the medium was changed to F12K free of nitrotyrosine. At day 4, the medium was changed to F12K supplemented with 500 μM nitrotyrosine. As a parallel control, C6 cells were cultured in F12K free of nitrotyrosine. Some dishes were processed every 24 h to determine the amount of nitrotyrosinated and total α-tubulin in cytoskeleton fractions. (A) Nitrotyrosinated tubulin as a function of days in culture. Nitrotyrosinated tubulin values were standarized relative to total tubulin by dividing optical density of the band of nitrotyrosinated tubulin by that corresponding to an identical sample stained with DM1A antibody. (B) Number of cells (○,▽) and viability (●,▼) determined in experimental (○,●) and in control (▽,▼) cultures.

tubulin have similar capabilities to act *in vitro* as substrates of tubulin carboxypeptidase. In contrast, their susceptibility to the releasing action of carboxypeptidase A is quite different, consistent with previous reports [11,12].

Reversible incorporation of 3-nitrotyrosine into tubulin in living cells

When C6 cells were cultured in F12K medium (see Materials and methods) in the presence of 500 μM 3-nitrotyrosine, cellular tubulin became progressively nitrotyrosinated, with maximal value after 2 days of culture (Fig. 3A). When the culture medium was replaced by nitrotyrosine-free F12K, almost all the nitrotyrosinated tubulin disappeared during the first day without decrease of total tubulin. This result indicates rapid release of 3-nitrotyrosine from tubulin, presumably by tubulin carboxypeptidase activity. Maximal values of nitrotyrosination were obtained by changing back to F12K containing 500 μM nitrotyrosine (Fig. 3A). When nitrotyrosinated tubulin was maximal (days 1, 2, 5 and 6 in Fig. 3A), the amount of tyrosinated tubulin (as measured by immunoblot) was very low (data not shown), indicating that almost all C-terminal tyrosine was substituted by 3-nitrotyrosine. The possibility that the disappearance of nitrotyrosinated tubulin that occurred after elimination of nitrotyrosine from culture medium was due to protein degradation rather than to the tyrosination/detyrosination cycle, was evaluated. Under conditions in which protein synthesis was inhibited by more than 95%, the decay of nitrotyrosinated tubulin was identical to that shown in Fig. 3 (day 2–3), while the tyrosinated tubulin form increased to approximately 60% of a control without protein synthesis inhibitors (not shown). This indicates that although tubulin turnover seems to contribute significantly to the disappearance of nitrotyrosinated tubulin, the tyrosination/detyrosination at the C-terminus of α-tubulin is the main operating mechanism. Furthermore, when cells maximally nitrotyrosinated were cultured in the presence of taxol to stabilize microtubules, Glu-tubulin content was increased from 5 to 35% in a 4-h-period, indicating that active tubulin carboxypeptidase is present in the cell.

During the experiment shown in Fig. 3, number and viability of cells were determined every day. The number of cells increased during the first two days and then remained constant or increased slightly (Fig. 3B). At any given time, the majority of the cells (> 90%) were viable. These two parameters (viability and proliferation) were similar to those of control cells (not treated with nitrotyrosine) (Fig. 3B).

The distribution of nitrotyrosinated tubulin, compared with total tubulin, in cytoskeletons was determined by double immunofluorescence in cells cultured as in Fig. 3A. On day 0 (Fig. 4A), as expected, no structure was stained with antibody against nitrotyrosinated tubulin (antinitro), whereas bright staining of microtubules was seen with antibody to total tubulin (DM1A) (Fig. 4B). On day 2, brightly stained structures were observed with antinitro

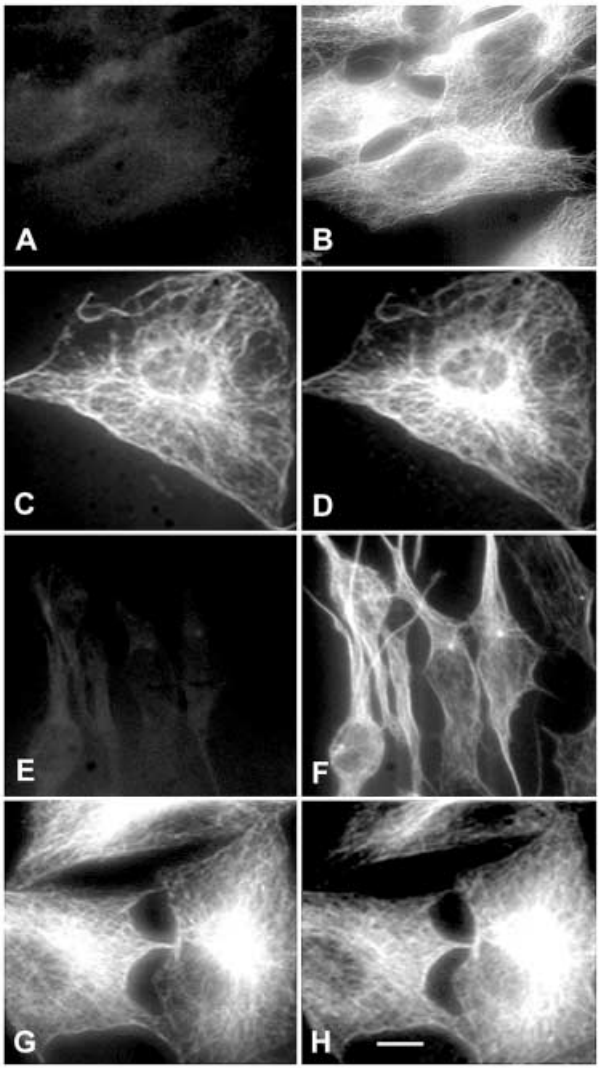

Fig. 4. Microtubular network of cells grown in the presence of nitrotyrosine. C6 cells were grown on coverslips under the protocol described for Fig. 3. Samples were processed every two days for double immunofluorescence using antibodies specific to nitrotyrosinated tubulin (A, C, E, G) or to total α-tubulin (DM1A) (B, D, F, H). A and B, day 0; C and D, day 2; E and F, day 4; G and H, day 6. Bar, 10 μm.

(Fig. 4C), coinciding with those revealed by DM1A (Fig. 4D). On day 4 (two days of de-nitrotyrosination), no staining was seen for antinitro (Fig. 4E), whereas DM1A produced strong staining (Fig. 4F). At day 6, immunostaining with antinitro and DM1A was coincident again (Fig. 4G,H).

The proportion of assembled vs. nonassembled tubulin (approximately 80 and 20%, respectively) in C6 cells (as estimated by immunoblot) was not altered by substitution of C-terminal tyrosine by nitrotyrosine. Furthermore, proportions of nitrotyrosinated tubulin and total tubulin were the same in assembled vs. nonassembled fractions (data not shown), indicating that nitrotyrosinated tubulin is indistinguishable from normal tubulin in the assembly process. Judging by these results, substitution of tyrosine by its analogue 3-nitrotyrosine at the C-terminus of α-tubulin is not relevant to the properties of microtubules involved in vital cell functions. This concept was supported by the observation that C6 cells survived, with normal morphology and proliferation rate, when cultured in F12K medium containing 500 μM nitrotyrosine, with successive passages, during several weeks (data not shown).

Association of tubulin carboxypeptidase with microtubules in cells cultured in the presence of 3-nitrotyrosine

Tubulin carboxypeptidase is known to be associated with microtubules in living cells [24]. Isolated cytoskeletons, freed of cytosolic components, show increased content of detyrosinated tubulin (Glu-tubulin) when incubated at 37 °C *in vitro*. We compared cytoskeletons isolated from COS cells cultured in F12K medium with or without 500 μM nitrotyrosine, in terms of the amount of tubulin carboxypeptidase associated with microtubules. Association of carboxypeptidase with microtubules has been extensively documented in these cells [24]. Rate of increase of Glu-tubulin during *in vitro* incubation was the same in both cases (data not shown), indicating that replacement of tyrosine by 3-nitrotyrosine at the C-terminus of α-tubulin is not relevant to the association of carboxypeptidase with microtubules.

Nitrotyrosination and denitrotyrosination of tubulin in different cell types

To determine whether the reversible incorporation of 3-nitrotyrosine into tubulin is restricted to C6 cells, we tested six other cell lines for the nitrotyrosination state of tubulin during a cycle of nitrotyrosination and de-nitrotyrosination. Variation of amount of nitrotyrosinated tubulin in each cell type (except 3T3/TTL$^-$) was similar to that of C6 cells (Fig. 5A). That is, in the presence of 500 μM nitrotyrosine (by day 3) tubulin became fully nitrotyrosinated, whereas in the absence of nitrotyrosine (from day 4–6) tubulin lost all its C-terminal nitrotyrosine. The six cell lines remained viable and divided normally during the cycle as compared with control cells cultured in the absence of nitrotyrosine (data not shown). 3T3/TTL$^-$ is a subclonal line of mouse NIH-3T3 cells which, through spontaneous mutation, are devoid of the tubulin tyrosine ligase [28]. Tubulin of TTL$^-$ cells could not be nitrotyrosinated (Fig. 5A) confirming the specificity of Tyr/nitrotyrosine incorporation into the C-terminus of α-tubulin.

Nitrotyrosination of tubulin is not detrimental for A549 cells

Presence of 500 μM nitrotyrosine in F12K culture medium led to nitrotyrosination of α-tubulin of A549 cells (Fig. 5). The incorporated nitrotyrosine was eliminated by changing to nitrotyrosine-free medium, confirming the reversibility of the reaction. Eiserich *et al.* reported that nitrotyrosination of tubulin is involved in A549 cell injury [11], but they used F12 medium (plus 10% fetal bovine serum), which has a tyrosine concentration of 30 μM. In contrast, we used F12K medium having a tyrosine concentration of 60 μM. Considering that this tyrosine concentration might be high enough to prevent full nitrotyrosination of tubulin and hence to avoid detrimental effects on the cells, we analyzed various cell parameters using F12 medium. We found that tubulin

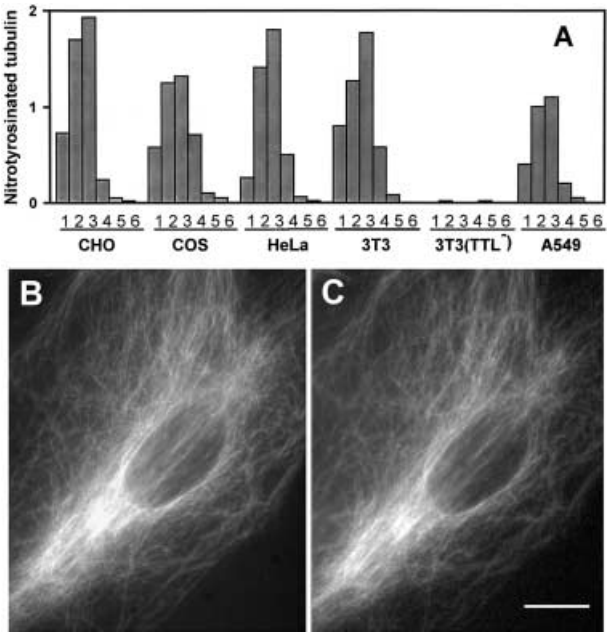

Fig. 5. Reversible incorporation of nitrotyrosine into tubulin occurs in different cell lines. (A) Cells were grown in F12K medium supplemented with 500 μM nitrotyrosine. On day 3, medium was changed to F12K without nitrotyrosine, and culture continued until day 6. Nitrotyrosinated tubulin in cytoskeleton fractions (standardized with respect to total α-tubulin) was determined daily. (B and C) A549 cells cultured on coverslips for 3 days in F12 medium containing 500 μM nitrotyrosine were processed for immunofluorescent visualization using double labeling with antibodies to nitrotyrosinated and total tubulin, respectively. Bar, 10 μm.

can be nitrotyrosinated and denitrotyrosinated without alteration of cell morphology or viability. Using double immunofluorescence, we examined microtubule networks of A549 cells cultured in F12 supplemented with 500 μM nitrotyrosine, as revealed with antinitro and DM1A antibodies. We examined many fields from several experiments, but observed no differences. In all cases, the images obtained with the two antibodies superimposed exactly. A representative example is shown in Fig. 5B,C. A549 cells survived without alteration of morphology or proliferation rate when cultured in F12 medium containing 500 μM nitrotyrosine for 45 days or more (data not shown).

DISCUSSION

Incorporation of 3-nitrotyrosine into the C-terminal position of α-tubulin was first described by Eiserich *et al.* [11]. In the present study, we used a radiolabeled tyrosine analogue to demonstrate that the unique acceptor protein is indeed tubulin, as radioactivity was bound to a single protein with the same mobility as tubulin (Fig. 1B,C). The nitrotyrosine molecule is bound to tubulin without modification before or after its incorporation, as it was recognized by an antibody specific to nitrotyrosine (Fig. 1B) and was recovered without alteration after enzymatic or acid hydrolysis of [^{14}C]nitrotyrosinated tubulin. The mutual exclusion by tyrosine and nitrotyrosine of their respective incorporations indicates clearly that the two compounds are incorporated into tubulin at the same site.

Another biochemical characteristic of tubulin is its ability to act as substrate of the detyrosinating enzyme, tubulin carboxypeptidase. Eiserich *et al.* reported [11] that the incorporation of nitrotyrosine into tubulin is irreversible. This was assumed based on the inability of 0.25 μg·mL^{-1} pancreatic carboxypeptidase A *in vitro* to release nitrotyrosine from nitrotyrosinated tubulin. This finding was confirmed in our study (Fig. 2A). However, tubulin carboxypeptidase not carboxypeptidase A is the physiological releasing enzyme in the post-translational tyrosination/detyrosination cycle. Activity of tubulin carboxypeptidase in both tyrosinated and nitrotyrosinated tubulin was quite similar (Fig. 2B). This suggests that the function of the tyrosination/detyrosination cycle in cells is not altered when tyrosine is replaced by nitrotyrosine at the C-terminus of α-tubulin. Furthermore, nitrotyrosinated tubulin can form microtubules *in vitro* as efficiently as tyrosinated tubulin (Table 1). These findings suggest that the presence of nitrotyrosinated tubulin instead of tyrosinated tubulin in living cells does not alter the normal assembly state of microtubules. Our experiments with living cells confirmed this assumption. Morphology, viability, and proliferation rate remained unaltered when cells were subjected to successive cycles of nitrotyrosination, de-nitrotyrosination, and re-nitrotyrosination (Fig. 3). This indicates strongly that substitution of C-terminal tyrosine by nitrotyrosine is reversible and does not affect microtubule properties, at least those involved in vital cell functions. Several other experimental observations support this concept: (a) the close similarity of immunofluorescent patterns of microtubular networks stained with antibodies to nitrotyrosinated and total tubulin, in cells grown in the presence of nitrotyrosine (compare Fig. 4C vs. D and G vs. H; Fig. 5B); (b) the normal appearance and proliferation of cells subjected to cycles of growth and passage for 45 days in the continuous presence of 500 μM nitrotyrosine; (c) the similar proportion of tubulin present in the assembled state (microtubules) in cells grown in the presence vs. absence of nitrotyrosine; (d) the similar proportion of nitrotyrosinated relative to total tubulin in assembled vs. nonassembled tubulin pools in cells cultured in the presence of nitrotyrosine and (e) the similar amount of tubulin carboxypeptidase activity associated with microtubules in cells grown in the presence vs. absence of nitrotyrosine.

The results presented here indicate that substitution of tyrosine by nitrotyrosine at the C-terminus of α-tubulin has no detrimental effects on normal cell function. In contrast, Eiserich *et al.* [11] presented data indicating that the same substitution leads to microtubule dysfunction and consequent damage to lung carcinoma A549 cells. Our results show that nitrotyrosination of tubulin does not cause cellular injury or death. This concept is supported by two facts: (a) the physiological concentration that nitrotyrosine can reach within cells or tissues is much lower than that of tyrosine; (b) the tubulin nitrotyrosination reaction is reversible and does not allow accumulation of nitrotyrosinated tubulin over time. It seems likely that the deleterious effects on cells and tissues observed by other authors [29–31] are due mostly to nitration of internal tyrosine residues of proteins, or other effects mediated by peroxinitrite and/or other secondary products of NO metabolism.

Recent studies have shown that the presence of a tyrosine residue at the C-terminus of α-tubulin is not necessary for survival and proliferation of cells. For example, when tubulin tyrosine ligase was inhibited by microinjection of its antibody, the cell cycle continued and cells divided even though the tubulin content was entirely Glu-tubulin [32]. NIH-3T3 (TTL^-) cells, in which the *ligase* gene is not expressed and tubulin is comprised entirely of Glu- and Δ2-isoespecies, showed normal cell division and tumor formation when injected in nude mice [28]. It appears that the presence of tyrosine at the α-tubulin C-terminus does not represent a 'switch on' for cell division or other vital functions, as cultured cells can live and divide well either with Glu-tubulin or with tubulin in which tyrosine is replaced by nitrotyrosine (present study). However, we cannot yet exclude the possibility that nitrotyrosination of α-tubulin leads to anomalous behavior of microtubules involved in subtle processes, which become important when cells differentiate and/or form part of a tissue. In a relevant recent study [18], stereotaxic injection of nitrotyrosine into mouse brain led to striatal neurodegeneration, although the nature of the amino acid bound at the C-terminus of α-tubulin in the striatum was not investigated. Further work is necessary to elucidate a possible relationship between nitrotyrosination of α-tubulin and cellular dysfunction.

ACKNOWLEDGEMENTS

We thank Drs Carlos E. Argaraña and Mario Guido for critical reading of the manuscript, Mrs S. N. Deza and Mrs M. G. Schachner for technical assistance and Dr Stephen Anderson for English editing of the manuscript. This work was supported partly by grants from Agencia Nacional de Promoción Científica y Tecnológica de la Secretaría de Ciencia y Tecnología del Ministerio de Cultura y Educación en el marco del Programa de Modernización Tecnológica (BID 802/OC-AR), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba y Agencia Córdoba Ciencia del Gobierno de la Provincia de Córdoba, Argentina.

REFERENCES

1. Barra, H.S., Arce, C.A., Rodríguez, J.A. & Caputto, R. (1974) Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule protein. *Biochem. Biophys. Res. Commun.* **60**, 1384–1390.
2. Hallak, M.E., Rodríguez, J.A., Barra, H.S. & Caputto, R. (1977) Release of tyrosine from tyrosinated tubulin. Some common factors that affect this process and the assembly of tubulin. *FEBS Lett.* **73**, 147–150.
3. Barra, H.S., Arce, C.A. & Argaraña, C.E. (1988) Post-translational tyrosination/detyrosination of tubulin. *Molec. Neurobiol.* **2**, 133–153.
4. Gundersen, G.G., Kalnoski, M.H. & Bulinski, J.C. (1984) Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently *in vivo*. *Cell* **38**, 779–789.
5. Barra, H.S., Arce, C.A. & Caputto, R. (1980) Total tubulin and its aminoacylated and non-aminoacylated forms during the development of rat brain. *Eur. J. Biochem.* **109**, 439–446.
6. Arce, C.A. & Barra, H.S. (1985) Release of C-terminal tyrosine from tubulin and microtubules at steady state. *Biochem. J.* **226**, 311–317.
7. Gundersen, G.G., Khawaja, S. & Bulinski, J.C. (1987) Post-polymerization detyrosination of α-tubulin: a mechanism for subcellular differentiation of microtubules. *J. Cell Biol.* **105**, 251–264.
8. Gundersen, G.G. & Bulinski, J.C. (1986) Microtubule arrays in differentiated cells contain elevated levels of a post-translationally modified form of tubulin. *Eur. J. Biol.* **42**, 288–294.
9. Kreis, T.E. (1987) Microtubules containing detyrosinated tubulin are less dynamic. *EMBO J.* **6**, 2597–2606.
10. Paturle-Lafanechère, L., Eddé, B., Denoulet, P., Van Dorsselaer, A., Mazarguil, H., Le Caer, J.P., Wehland, J. & Job, D. (1991) Characterization of a major brain tubulin variant which cannot be tyrosinated. *Biochemistry* **30**, 10523–10528.
11. Eiserich, J.P., Estévez, A.G., Bamberg, T.V., Ye, Y.Z., Chumley, P.H., Beckman, J.S. & Freeman, B.A. (1999) Microtubule dysfunction by post-translational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl Acad. Sci. USA* **96**, 6365–6370.
12. Kalisz, H.M., Erck, C., Plessmann, U. & Wehland, J. (2000) Incorporation of nitrotyrosine into alpha-tubulin by recombinant mammalian tubulin-tyrosine ligase. *Biochim. Biophys. Acta* **1481**, 131–138.
13. Fukuyama, N., Takebayashi, Y., Hida, M., Ishida, H., Ichimori, K. & Nakazawa, H. (1997) Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. *Free Radic. Biol. Med.* **22**, 771–774.
14. Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxigen species. *Arch. Biochem. Biophys.* **356**, 1–11.
15. Skinner, K.A., Crow, J.P., Skinner, H.B., Chandler, R.T., Thompson, J.A. & Parks, D.A. (1997) Free and protein-associated nitrotyrosine formation following rat liver preservation and transplantation. *Arch. Biochem. Biophys.* **342**, 282–288.
16. Tsuji, C., Shioya, S., Hirota, Y., Fukuyama, N., Kurita, D., Tanigaki, T., Ohta, Y. & Nakazawa, H. (2000) Increased production of nitrotyrosine in lung tissue of rats with radiation-induced acute lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **278**, L719–L725.
17. Michel, T. & Feron, O. (1997) Nitric oxide synthases: which, where, how and why? *J. Clin. Invest.* **100**, 2146–2152.
18. Mihm, M.J., Schanbacher, B.L., Wallace, B.L., Wallace, L.J., Uretsky, N.J. & Bauer, J.A. (2001) Free 3-nitrotyrosine causes striatal neurodegeneration *in vivo*. *J. Neurosci.* **21**, RC149 (1–5).
19. Idriss, H.T. (2000) Man to trypanosome: the tubulin tyrosination/detyrosination cycle revisited. *Cell Motil. Cytoskeleton* **45**, 173–184.
20. Idriss, H.T. (2000) Do TNF-alpha-insensitive cancer cells escape alpha-tubulin nitrotyrosination? *Nitric Oxide* **4**, 1–3.
21. Koppenol, W.H., Kissner, R. & Beckman, J.S. (1996) Syntheses of peroxinitrite: to go with the flow or on solid grounds? *Methods. Enzymol.* **269**, 296–302.
22. Barra, H.S., Rodríguez, J.A., Arce, C.A. & Caputto, R. (1973) A soluble preparation from rat brain that incorporates into its own proteins [^{14}C]arginine by a ribonuclease-sensitive system and [^{14}C]tyrosine by a ribonuclease-insensitive system. *J. Neurochem.* **20**, 97–108.
23. Mons, N. & Geffard, M. (1987) Specific antisera against the catecholamines: L-3,4-dihydroxyphenylalanine, dopamine, noradrenaline, and octopamine tested by an enzyme-linked immunosorbent assay. *J. Neurochem.* **48**, 1826–1833.
24. Contín, M.A., Sironi, J.J., Barra, H.S. & Arce, C.A. (1999) Association of tubulin carboxypeptidase with microtubules in living cells. *Biochem. J.* **339**, 463–471.
25. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**, 680–685.
26. Arce, C.A., Rodríguez, J.A., Barra, H.S. & Caputto, R. (1975) Incorporation of L-tyrosine, L-phenylalanine and L-3,4-dihydroxyphenylalanine as single units into rat brain tubulin. *Eur. J. Biochem.* **59**, 145–149.

27. Argaraña, C.E., Barra, H.S. & Caputto, R. (1978) Release of [^{14}C]tyrosine from tubulinyl-[^{14}C]tyrosine by brain extract. *Mol. Cell. Biochem.* **19**, 17–21.
28. Lafanechère, L., Courtay-Cahen, C., Kawakami, T., Jacrot, M., Rüdiger, M., Wehland, J., Job, D. & Margolis, R.L. (1998) Supression of tubulin tyrosine ligase during tumor growth. *J. Cell Sci.* **111**, 171–181.
29. Liu, J.S., Zhao, M.L., Brosnan, C.F. & Lee, S.C. (2001) Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am. J. Pathol.* **158**, 2057–2066.
30. Xu, J., Kim, G.M., Chen, S., Yan, P., Ahmed, S.H., Ku, G., Beckman, J.S., Xu, X.M. & Hsu, C.Y. (2001) iNOS and nitrotyrosine expression after spinal cord injury. *J. Neurotrauma* **18**, 523–532.
31. Moulian, N., Truffault, F., Graudy-Talarmain, Y.M., Serraf, A. & Berrih-Aknin, S. (2001) *In vivo* and *in vitro* apoptosis of human thymocytes are associated with nitrotyrosine formation. *Blood* **97**, 3521–3530.
32. Webster, D.R., Wehland, J., Weber, K. & Borisy, G.G. (1990) Detyrosination of alpha tubulin does not stabilize microtubules *in vivo*. *J. Cell Biol.* **111**, 113–122.