

Alteration of the C-terminal Amino Acid of Tubulin Specifically Inhibits Myogenic Differentiation*

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Detyrosination is an evolutionarily conserved post-translational modification of microtubule polymers that is known to be enhanced during early morphological differentiation of cultured myogenic cells (Gundersen, G. G., Khawaja, S., and Bulinski, J. C. (1989) *J. Cell Biol.* 109, 2275–2288). We proposed that altering the C terminus of α -tubulin by detyrosination plays a role in morphological differentiation. To test our hypothesis, we treated L6 myoblasts with 3-nitrotyrosine (Eiserich, J. P., Estevez, A. G., Bamberg, T. V., Ye, Y. Z., Chumley, P. H., Beckman, J. S., and Freeman, B. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 6365–6375), a nontoxic inhibitor that resulted in high level inhibition of microtubule detyrosination and low level incorporation of nitrotyrosine into microtubules. Even though microtubule stabilization or modification by acetylation still occurred normally, morphological differentiation was blocked; myoblasts neither elongated significantly nor fused. Nitrotyrosine treatment prevented synthesis or activation of markers of myogenic differentiation, including muscle-specific myosin, α -actin, integrin α_7 , and myogenin. Consistent with this, myoblast integrin β_{1A} remained highly expressed. In contrast, the increase in β -catenin level characteristic of early myogenesis was unaffected by treatment. These results show that the identity of the C-terminal residue of α -tubulin modulates microtubule activity, possibly because binding to or signaling from modified microtubules is required for the myogenic program.

Microtubules (MTs)¹ participate in cell division, motility, transport, and morphogenesis. The ability of MTs to quickly polymerize and depolymerize, a process known as dynamic instability, places regulation of MT dynamics at the center of active research (1). Tubulin undergoes a host of post-translational modifications, including detyrosination, acetylation,

generation of $\Delta 2$ -tubulin, phosphorylation, polyglutamylation, and polyglycylation (2). Of these, detyrosination has been most extensively studied, yet its functions remain to be determined.

Detyrosination is a unique modification involving cleavage of the C-terminal tyrosine residue of α -tubulin within MTs by a tubulin-specific carboxypeptidase (TCP), leaving Glu tubulin, named for its newly exposed C-terminal residue. Detyrosination is reversible: tubulin tyrosine ligase (TTL) adds a tyrosine residue to the C terminus of protomeric Glu-tubulin, re-forming tyrosinated (Tyr) tubulin (2, 3). MTs enriched in Glu tubulin (called Glu MTs) have been shown to be enhanced in cellular longevity or stability (4–6). However, detyrosination is known to be insufficient to stabilize MTs (7, 8), rendering detyrosination an effect, not a cause, of MT stability.

Glu MTs are found in a distinct subset of MTs in undifferentiated cultured cells (9). Usually the Glu MT subset largely overlaps with the subset enriched in post-translationally acetylated subunits (10, 11), suggesting that post-translationally modified subunits within a stable MT may function to establish functionally distinct MT populations. For example, modified MTs may function in cellular morphogenesis or cell polarization (3). Antibody microinjections suggest that Glu tubulin subunits anchor vimentin filaments to MTs in migrating 3T3 fibroblasts (12) and may be involved in directed organelle transport (13, 14).

The hypothesis that stable Glu MTs are involved in cellular morphogenesis is suggested by their increased abundance during differentiative events (3, 15). However, to date no study has demonstrated that Glu MTs are necessary for differentiation. During myogenesis, cells undergo striking morphological changes; they elongate, align with neighbors, and finally fuse into multinucleated myotubes. In L6 cells, Glu MTs accumulate upon induction of myogenesis, coincident with formation of stable MTs and preceding myoblast fusion, accumulation of post-translationally acetylated MTs, and appearance of muscle-specific myosin (16). A test of the functional consequences of inhibiting MT detyrosination during myogenesis has not been straightforward, largely because no direct inhibitors of TCP are available.

Eiserich *et al.* (17) showed that a modified amino acid, 3-nitrotyrosine (NO₂Tyr), which is generated by reaction of tyrosine with nitric oxide-derived species, including nitrogen dioxide (NO₂) and peroxyxynitrite (ONOO⁻) (18–20), may act as an indirect inhibitor of TCP. That is, NO₂Tyr can be taken up by cells, where the tyrosinating enzyme, tubulin tyrosine ligase (TTL), can incorporate it into the C terminus of Glu tubulin to yield nitrotyrosinated tubulin (21). Eiserich *et al.* (17) found that, when NO₂Tyr was incorporated, Glu MTs were decreased,

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¹ The abbreviations used are: MT, microtubule; TCP, tubulin-specific carboxypeptidase; TTL, tubulin tyrosine ligase; Tyr, tyrosinated; DMEM, Dulbecco's modified Eagle's medium; NO₂Tyr, 3-nitrotyrosine; MOPS, 4-morpholinepropanesulfonic acid.

and *in vitro* studies with carboxypeptidase A suggested that the nitrotyrosinated MTs might work by blocking the generation of Glu MTs by TCP. In this study, we show that NO₂Tyr-MTs inhibit TCP; consequently, NO₂Tyr can be used as an indirect inhibitor of MT detyrosination during differentiation of L6 myoblasts. Perturbation of Glu MT formation prevents myogenic morphogenesis, and accumulation and activation of muscle-specific factors, revealing a link between MT modification and progression of the myogenic program.

EXPERIMENTAL PROCEDURES

Materials—Unless noted, tissue culture supplies were from Invitrogen, and sera were from HyClone, Inc. Other chemicals and antibodies were from Sigma Chemical Co., immunochemicals were from Organon Teknika, and 4-methyl-2-nitrophenol was from Aldrich Chemical Co.

Cell Culture and Myogenic Differentiation—Rat L6 myoblasts (ATCC), maintained in DMEM with 10% fetal bovine serum, were induced to differentiate by shifting to DMEM with 2% horse serum (heat-inactivated, 56 °C, 30 min), and the extent of differentiation was scored (16). To test media of various pH levels, proliferating cells were incubated in DMEM with serum, lacking sodium bicarbonate, containing 30 mM HEPES buffer at each pH. Note that the pH of each medium refers to the pH of the HEPES buffer added, and may not reflect the pH maintained in the incubator. The pH of unbuffered control media was ~7.3.

The pH shift protocol (see Fig. 1B) entailed adding pH 7.0 medium, at 37 °C (*i.e.* maximum Glu tubulin content, see Fig. 1A) to sub-confluent plates of L6 cells for 10 h to increase Glu MT level. Plates were rinsed once in cold pH 7.8 medium and placed at 4 °C for 1 h to depolymerize Glu MTs. Next, cells were incubated at 37 °C for 12 h at pH 7.8 (*i.e.* minimum Glu tubulin content) allowing TTL to re-tyrosinate Glu tubulin protomers, thus reducing Glu tubulin level significantly. Finally, differentiation medium was added. 400 μM NO₂Tyr was present in half the plates (+NO₂Tyr plates) throughout the experiment, and both control plates and those with added NO₂Tyr underwent the pH shift protocol. To prevent overgrowth of cultures during the time course, differentiation-defective cells were killed at 2 days with 18 μM β-D-arabinofuranoside, which has been shown not to interfere with myogenesis (22).

Antibodies—Antibodies were used at dilutions shown parenthetically: β-catenin, C2206 (1:6000); α-sarcomeric actin, 5C5 (1:1500); vimentin, V9 (1:400); desmin, D8281 (1:1000); and acetylated tubulin, 6-11B-1 (1:1000). MF20 hybridoma against muscle-specific myosin heavy chain was from the Developmental Studies Hybridoma Bank; undiluted culture supernatant was used. Anti-NO₂Tyr (1:800) was from Upstate Biotechnology, and anti-myogenin, M-225 (1:100) was from Santa Cruz Biotechnology. Anti-β-tubulin, 3F3 (1:2500), was generously provided by Dr. J. Lessard (University of Cincinnati) and anti-integrins β_{1A} (1:1000) and α_{7A} (1:500) were obtained courtesy of Dr. G. Tarone (University of Torino). Anti-Glu tubulin, SG (1:6000) was previously described (9).

Immunoblotting, Immunofluorescence, and Northern Blots—Preparation of cell lysates, SDS-PAGE, Western blotting, and immunostaining were described in Chapin and Bulinski (23). For enhanced chemiluminescence (ECL), blots were blocked with 1% bovine serum albumin; bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce). Northern blots were performed exactly as described in Gruber *et al.* (24), using actin and glyceraldehyde-3-phosphate dehydrogenase probes from Ambion (the latter for normalization of RNA loads).

Immunofluorescence was performed as described in Gundersen *et al.* (16). Antibodies were diluted as follows: anti-β-tubulin, 3F3, 1:100; anti-Glu, SG, 1:400; and secondary antibodies, 1:100. Images, captured with an Orca-cooled charge-coupled device camera (Hamamatsu Photonics) equipped with a Uniblitz Shutter (Vincent Associates), and attached to a Nikon Optiphot microscope, were processed with MetaMorph software (Universal Imaging).

In Vitro Assay of Tubulin Carboxypeptidase Activity—Preparation of brain TCP, [¹⁴C]tyrosine-labeled MT substrate, and the TCP assay, were described previously (25, 26). Briefly, [¹⁴C]tyrosine-labeled tubulin dimers were self-assembled in MHM (25 mM MOPS, 25 mM HEPES, pH 7.5, 5 mM MgCl₂) for 20 min, stabilized by Taxol (20 μM, 5 min), and centrifuged, all at 37 °C (436,000 × *g*, 4 min, Optima TL centrifuge; Beckman Instruments) to yield a MT substrate pellet. For the first incubation step, *i.e.* binding of TCP to MT substrate, the pellet from the previous step was incubated in MHM (10 min, 37 °C) with 5 volumes of

TABLE I
Inhibition of TCP activity by NO₂Tyr

A) NO ₂ Tyr does not inhibit binding of TCP to MT substrate ^a			
NO ₂ Tyr concentration		TCP activity	
	<i>mM</i>	<i>pmol/min/mg</i>	
	0	10.2 ± 0.3	
	10	10.5 ± 0.4	
B) NO ₂ Tyr does not inhibit activity of TCP bound to MT substrate ^b			
NO ₂ Tyr concentration		TCP activity	
	<i>mM</i>	<i>pmol/min/mg</i>	
	0	12.0 ± 0.8	
	1	11.4 ± 0.3	
	10	9.1 ± 1.1 ^c	
C) Nitrotyrosination of MT substrate inhibits TCP ^d			
Substrate	TCP activity		<i>N</i>
	<i>pmol/min/mg</i>		
Untreated MT substrate	8.3 ± 1.7		2
Solvent-treated MT substrate	7.7 ± 1.9		3
TMN-treated MT substrate ^e	0.20 ± 0.15 ^c		3

^a NO₂Tyr was added to TCP-substrate mixture during the first incubation (the binding step; see “Experimental Procedures”). *N* = 3 for all incubations.

^b NO₂Tyr was added to TCP-MT substrate mixture during the second incubation step, measuring TCP activity (see “Experimental Procedures”). *N* = 3 for all incubations.

^c Significant inhibition, using unpaired *t* test (*p* < 0.05).

^d See “Experimental Procedures” for details of the nitrotyrosinated MT substrate.

^e MT substrate was incubated in 4% ethanol for 1 h at 37 °C.

crude brain TCP and 20 μM Taxol (26), and the mixture was centrifuged as before, except at 25 °C. For the second incubation step, measuring TCP activity, the pellet from the previous step was resuspended in MHM (30 min, 37 °C), proteins were precipitated with 10% trichloroacetic acid (w/v) at 4 °C, centrifuged (245,000 × *g*, Optima TL), and radioactivity of trichloroacetic acid-soluble (*i.e.* containing [¹⁴C]tyrosine cleaved by TCP), and insoluble fractions were counted.

Nitrotyrosinated MT substrate was prepared by resuspending [¹⁴C]tyrosine MT substrate (above) in 100 mM Tris, pH 8.2 (alkaline pH to allow tyrosine nitration but no cysteine oxidation), and incubating with 4 mM tetranitromethane, 4% ethanol (1 h, 37 °C). [¹⁴C]Nitrotyrosinated MTs (NO₂Tyr content, 2 mol/mol tubulin dimer, quantified from A_{430 nm}), were centrifuged (436,000 × *g*, 4 min), washed with Tris buffer, centrifuged as before, and used in the TCP assay above. Release of [¹⁴C]NO₂Tyr into the supernatant during the second incubation was measured.

RESULTS

Nitrotyrosine (NO₂Tyr) Inhibits TCP Activity *In Vitro*—To ascertain the mechanism by which NO₂Tyr inhibits generation of Glu MTs, we assayed its effects on TCP activity *in vitro*. MT substrate containing [¹⁴C]tyrosine-labeled α-tubulin was first incubated with TCP preparations to allow binding of enzyme to MT substrate. TCP-MT substrate complex was then incubated, and TCP activity was measured as [¹⁴C]tyrosine released from the C terminus of the substrate’s α-tubulin (Refs. 24 and 26, see “Experimental Procedures”). We first tested whether free NO₂Tyr affected binding of TCP to MT substrate. Table I shows that adding NO₂Tyr to the initial incubation of MT substrate with TCP did not affect TCP activity measured, suggesting that free NO₂Tyr does not inhibit the binding of TCP to the MT substrate.

Next, we added NO₂Tyr during the second incubation, which quantified activity of TCP to generate Glu tubulin. We detected no difference without and with 1 mM NO₂Tyr, indicating that, at a concentration of 1 mM (*i.e.* 2.5 times that used *in vivo*), free NO₂Tyr did not interfere with activity of TCP once it was bound to MT substrate. However, in samples containing 10 mM NO₂Tyr, 25 times the concentration used *in vivo*, we did measure a small but significant (<25%, *p* < 0.05) decrease in TCP activity (Table I). Because TCP activity was inhibited only at

such high NO₂Tyr concentrations, and TCP-MT substrate binding was not inhibited, it is unlikely that free NO₂Tyr interferes significantly with detyrosination *in vitro* or *in vivo*.

Next, we tested whether nitrotyrosinated MT (NO₂Tyr-MT) substrate (*i.e.* MTs with incorporated NO₂Tyr) could inhibit detyrosination by TCP. NO₂Tyr-MT substrate, prepared by reacting ¹⁴C-labeled MT substrate with tetranitromethane (see "Experimental Procedures"), inhibited TCP activity effectively (Table I), and this inhibition was due to nitrotyrosination, rather than denaturation, of MT substrate, because activity was unaffected by solvent alone (Table I). The facts that 1) TCP activity bound to and sedimented with NO₂Tyr-MT substrate (data not shown) and 2) NO₂Tyr-MT-bound TCP cleaved neither ¹⁴C-labeled-NO₂Tyr nor ¹⁴C-labeled Tyr suggested that TCP was effectively sequestered on NO₂Tyr-MTs in the enzyme-substrate mixtures. One would expect similar inhibition *in vivo*; that is, TCP would bind NO₂Tyr-MTs tightly and would neither cleave NO₂Tyr from the MTs nor readily dissociate to cleave other Tyr-MTs in the cell. In addition, these results predict that the species inhibiting TCP *in vivo* would be NO₂Tyr-MTs rather than free NO₂Tyr.

NO₂Tyr Specifically Inhibits MT Detyrosination *In Vivo*—A suitable *in vivo* detyrosination inhibitor must satisfy three criteria: First, the inhibitor must be nontoxic to L6 cells during the experimental time course. Second, incorporation must be efficient enough either to block α -tubulin detyrosination completely or to prevent increased detyrosination during myogenesis. Third, the compound has to be incorporated only post-translationally and only into α -tubulin. Although all three criteria were met for use of NO₂Tyr in undifferentiated cells (17), satisfying these criteria in differentiating cells demanded further efforts.

For the first criterion, we examined possible toxicity by incubating either proliferating or differentiated L6 cells with NO₂Tyr in culture medium for 10 days. No detectable effects on mitosis or other deleterious effects such as cell death were observed (data not shown), and proliferation and/or differentiation rates were nearly normal ($\geq 90\%$ of controls; data not shown).

The second criterion, achieving efficient incorporation of the inhibitor, posed a significant challenge. Merely incubating L6 cells for 7 days in culture medium with 400 μ M or even 800 μ M NO₂Tyr did not result in diminution of Glu tubulin level, or incorporation of NO₂Tyr, as detected with Glu or NO₂Tyr antibodies, respectively (data not shown). The inefficiency of NO₂Tyr to perturb detyrosination was not surprising, because proliferating mammalian cells possess highly dynamic MTs (10), Glu MTs compose only a small subset (<5%), and Glu tubulin resides exclusively in polymer (5, 23), where it is unavailable to TTL. Thus, in proliferating cells <5% of the tubulin is available for detyrosination and subsequent re-tyrosination by TTL. To prevent the ~20-fold increase in Glu MTs during myogenesis, we had to improve NO₂Tyr incorporation into MTs and, thus, inhibition of TCP before inducing differentiation.

One strategy for effecting increased NO₂Tyr incorporation would be to perturb *in vivo* MT dynamics in NO₂Tyr-containing culture medium, allowing TTL to incorporate NO₂Tyr, in lieu of tyrosine, into Glu protomers (21). For example, transiently stabilizing MTs would generate Glu MTs (5); destabilizing these Glu MTs in the presence of NO₂Tyr would release Glu tubulin protomers that could be nitrotyrosinated by TTL.

We initially attempted to develop a MT stabilization-destabilization protocol using Taxol to stabilize MTs (5); however, Taxol treatments proved irreversible. Instead, we explored using pH to influence MT stability, based on reports that pH

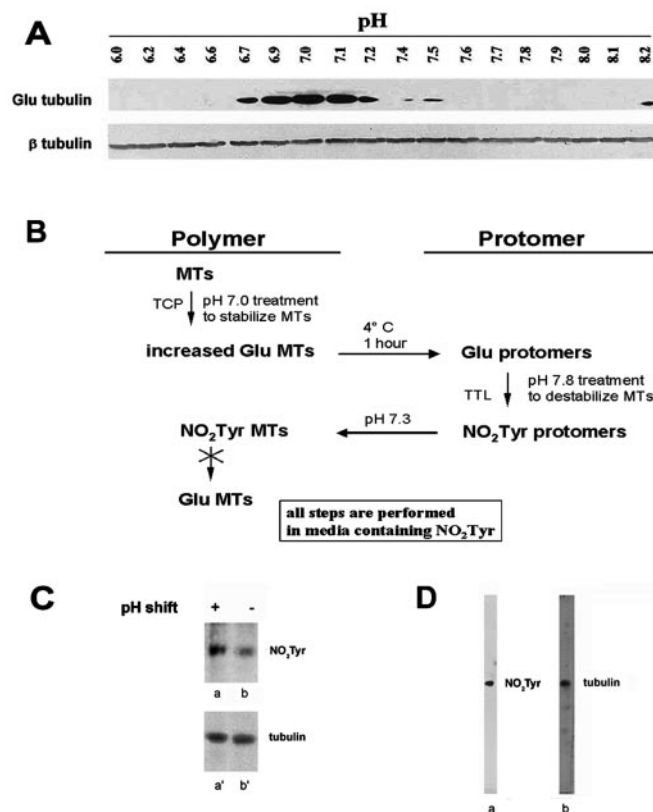


FIG. 1. Enhanced incorporation of NO₂Tyr into cellular MTs. A, Glu tubulin level of L6 myoblasts can be manipulated by altering extracellular pH. Proliferating L6 myoblasts, grown to half confluency in bicarbonate-buffered medium at pH 7.3, were incubated (10–12 h) in bicarbonate-free media containing 30 mM HEPES at each pH level. Cells reached steady-state Glu tubulin levels after ≤ 10 h of incubation at each pH (not shown). Cell extracts (30 μ g) were blotted, and detyrosinated α (Glu tubulin) and β tubulin were immunolabeled. Note that, while Glu tubulin content varied, total tubulin (β -tubulin) was constant at all pH levels. B, pH shift protocol promotes efficient incorporation of NO₂Tyr into L6 myoblasts. To maximize NO₂Tyr incorporation into MTs, L6 cells were subjected to a pH shift protocol, as depicted (see "Experimental Procedures" for details). The pH shift scheme was carried out with or without NO₂Tyr; only the +NO₂Tyr version is shown. First, the level of cellular Glu MTs was raised by exposure to medium buffered at pH 7.0, an MT-stabilizing pH that increases Glu MTs (A), then cells were chilled to depolymerize the resulting Glu MTs. This releases Glu tubulin protomer, which is a substrate for TTL. Next, cells were re-warmed and incubated in medium buffered at pH 7.8, an MT-destabilizing pH (Fig. 1A) at which Glu tubulin content reaches its nadir. Subsequently, cells were re-equilibrated in unbuffered medium and used immediately in differentiation experiments. C, NO₂Tyr is incorporated into tubulin efficiently, following the pH shift protocol. L6 cell extract (60 μ g) was immunoblotted with anti-NO₂Tyr antibody (a and b), and anti- β -tubulin antibody (a' and b'). Lanes a and b are from cells that were (+) and were not (-) subjected to the pH shift protocol, which increases NO₂Tyr incorporation (compare a and b) but does not alter tubulin content (compare a' and b'). D, NO₂Tyr is incorporated specifically into tubulin. Differentiated L6 cell extract (60 μ g) was blotted and immunolabeled with a, anti-NO₂Tyr antibody, and overexposed via ECL. b shows anti-tubulin immunolabeling of the same lane. Note in lane a that no nontubulin bands incorporated detectable NO₂Tyr. From densitometric scanning we conclude that >99% of NO₂Tyr was incorporated into tubulin, because even a species that incorporated 1% as much NO₂Tyr would have been detected.

affects MT polymerization *in vivo* (27). As shown in Fig. 1A, lower extracellular pH enhanced detyrosination, reaching a maximum Glu tubulin level at pH 7.0. Glu tubulin level was lower at media pH levels of 7.2–7.5 and was not detected at media pH 7.6–8.0 (Fig. 1A). Overexposure of blots revealed a nadir in Glu tubulin level at pH 7.8 (data not shown). Staining of blots with anti-acetylated tubulin, a standard indicator

of stable MTs (10), suggested that changes in Glu tubulin level were correlated with changes in MT stability (data not shown).

Fig. 1B shows the pH shift protocol devised from these data, which satisfied the second criterion for use of NO₂Tyr, that is, maximizing its incorporation into α -tubulin. Briefly, we first exposed L6 cell cultures to pH 7.0 medium for 10–12 h to stabilize MTs and increase Glu MT level. We then cold-treated the cells and incubated them in pH 7.8 medium for 12 h to destabilize MTs. Finally, we applied differentiation medium to start myogenesis. With or without added NO₂Tyr, all cells underwent the pH shift protocol, which markedly improved NO₂Tyr incorporation (Fig. 1C, lane a), compared with simply incubating cells in NO₂Tyr (Fig. 1C, lane b). In fact, densitometry of anti-NO₂Tyr-labeled blots revealed >8-fold more NO₂Tyr incorporation in lane a than in lane b. Moreover, other means of reversibly stabilizing MTs, e.g. transient sodium azide treatment, also increased NO₂Tyr incorporation into tubulin, and myogenesis was altered identically (data not shown).

The third criterion, that the inhibitor must be incorporated strictly post-translationally, and only into α -tubulin, was met in nonmuscle cells (17). Likewise, in L6 cells we could not detect translational incorporation of NO₂Tyr. However, concerned that the slightly slower cell growth rate (<10%) we observed in NO₂Tyr-treated *versus* control cells could result from translational incorporation of NO₂Tyr, albeit at a level not detectable even on the most sensitive Western blots (e.g. Fig. 1D), we treated cultures with an identical concentration (400 μ M) of 2-nitro-*p*-cresyl (4-methyl-2-nitrophenol). This deaminated/decarboxylated version of NO₂Tyr can neither be used in protein synthesis nor serve as a TTL substrate, and it was similarly nontoxic; we observed no mitotic defects or apoptosis. 2-Nitro-*p*-cresyl treatment yielded the same paltry inhibition of cell growth rate (<10%) as other nitrated phenol compounds that are incapable of translational incorporation (28). Thus, NO₂Tyr appears to be incorporated only post-translationally.

Exposing L6 cells to transient pH shifts in the continuous presence of NO₂Tyr yielded incorporation only into α -tubulin; even the most sensitive Western blots revealed no other NO₂Tyr-labeled species (Fig. 1D). We also noted that the severity of NO₂Tyr effects on cell differentiation (see below) was strictly dependent upon the efficiency of NO₂Tyr incorporation into tubulin. For example, proliferating or differentiated myoblasts or mature myotubes were all unaffected by a 10-day incubation with NO₂Tyr if they were not first subjected to the pH shift protocol, and little NO₂Tyr incorporation could be detected in cells that were NO₂Tyr-treated without pH shift (Fig. 1C, lane b). The pH shift procedure changes MT dynamics (Fig. 1B), heightening the incorporation of NO₂Tyr by TTL into protomeric Glu tubulin; however, it is unlikely that changing the dynamics of MTs would change incorporation of NO₂Tyr into nontubulin proteins. Taken together, these data strongly suggest that in our experiments NO₂Tyr was added only post-translationally and only to α -tubulin.

NO₂Tyr Inhibits Detyrosination, but Not MT Stabilization, during Myogenesis—To inhibit Glu MT formation during myogenesis, we first subjected L6 myoblasts to the pH shift protocol and then placed them in differentiation medium and monitored their myogenesis for a 10-day period. Fig. 2 documents the depletion of myogenesis-induced detyrosination. Note that a high level of Glu tubulin persisted throughout the pre-treatments with cold and pH 7.8, such that the Glu tubulin level was still elevated and NO₂Tyr incorporation was undetectable at the time differentiation was induced (Fig. 2, lanes marked 0). However, by the 2-day time point, NO₂Tyr incorporation could

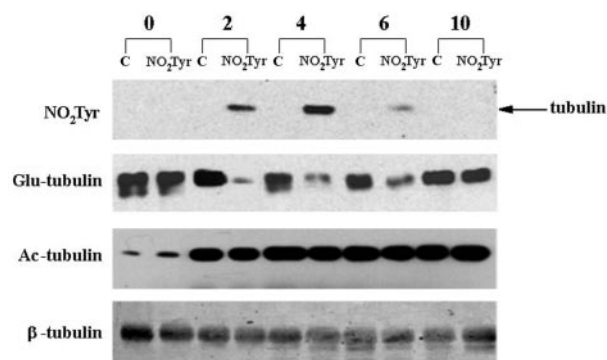


FIG. 2. **NO₂Tyr incorporation reduces detyrosination but not acetylation.** Extracts of pH-shifted L6 cells (see “Experimental Procedures”) without (control lanes, C) or with NO₂Tyr (NO₂Tyr lanes) and placed in differentiation medium for 0–10 days were blotted to reveal NO₂Tyr, Glu-tubulin, acetylated tubulin (Ac-tubulin), and total tubulin (β -tubulin). A hapten antibody against NO₂Tyr was used to visualize all proteins with covalently incorporated NO₂Tyr. As shown in Fig. 1C for a single time point, no proteins other than α -tubulin were labeled with anti-NO₂Tyr. The quantities of total protein loaded to detect each antigen were: NO₂Tyr, 60 μ g; Glu-, Ac-, and β -tubulin, 30 μ g.

easily be detected and Glu tubulin level was greatly diminished (Fig. 2, lanes marked 2). Fig. 2 shows that NO₂Tyr dramatically reduced Glu tubulin level (NO₂Tyr lanes). In contrast, Glu tubulin was abundant in control cells (Fig. 2, C lanes) or cells exposed to NO₂Tyr without prior pH shift (not shown). With or without NO₂Tyr, cells commencing differentiation (Fig. 2, 0 lanes) often contained a high Glu tubulin level, albeit markedly reduced in NO₂Tyr-treated cells.

Although NO₂Tyr treatment markedly decreased Glu tubulin level for the first week of differentiation, the level of Glu tubulin increased during the time course, such that its level was similar in cells with or without NO₂Tyr at the 10-day time point (Fig. 2, 10 lanes, compare C and NO₂Tyr). Eventual failure of the inhibitor could arise in one or both of two ways: First, even optimal incorporation efficiency would not result in nitrotyrosination of all tubulin molecules. Remaining Tyr tubulin, polymerized into Tyr MTs, could be detyrosinated during the differentiation time course. Second, Tyr tubulin synthesized and polymerized into Tyr MTs during the 10 days could also be detyrosinated.

Detyrosination is a post-polymerization modification (5); Glu MTs that appear during myogenesis in L6 cells represent a stable MT population, compared with their dynamic Tyr MT counterparts (16). We used antibodies specific for another modification enriched in stable MTs, acetylated α -tubulin (29), a standard indicator of stable MTs (10), to ask whether NO₂Tyr treatment not only decreased Glu content but also stability of MTs during differentiation. Fig. 2 shows that acetylation increased equivalently during differentiation, regardless of NO₂Tyr treatment, suggesting that MTs were stabilized normally in NO₂Tyr-treated cells, despite the reduction in Glu tubulin level.

NO₂Tyr Inhibits Glu MT Accumulation and Myogenic Morphogenesis—L6 cells treated with NO₂Tyr for up to 10 days did not differentiate, unlike untreated cells (Fig. 3A) or cells treated with NO₂Tyr without prior pH shift (not pictured). The low magnification micrographs in Fig. 3A show that, initially, with or without NO₂Tyr treatment, L6 myoblasts were largely unpolarized, and many mitotic cells were seen (Fig. 3A, panels a and b). At 2 days, untreated L6 cells had elongated and begun to align with one another (Fig. 3A, panel c, arrows), although little fusion had yet occurred. In contrast, few NO₂Tyr-treated cells had become polarized, even fewer appeared to be aligned with one another (Fig. 3A, panel d, arrow), and none had fused

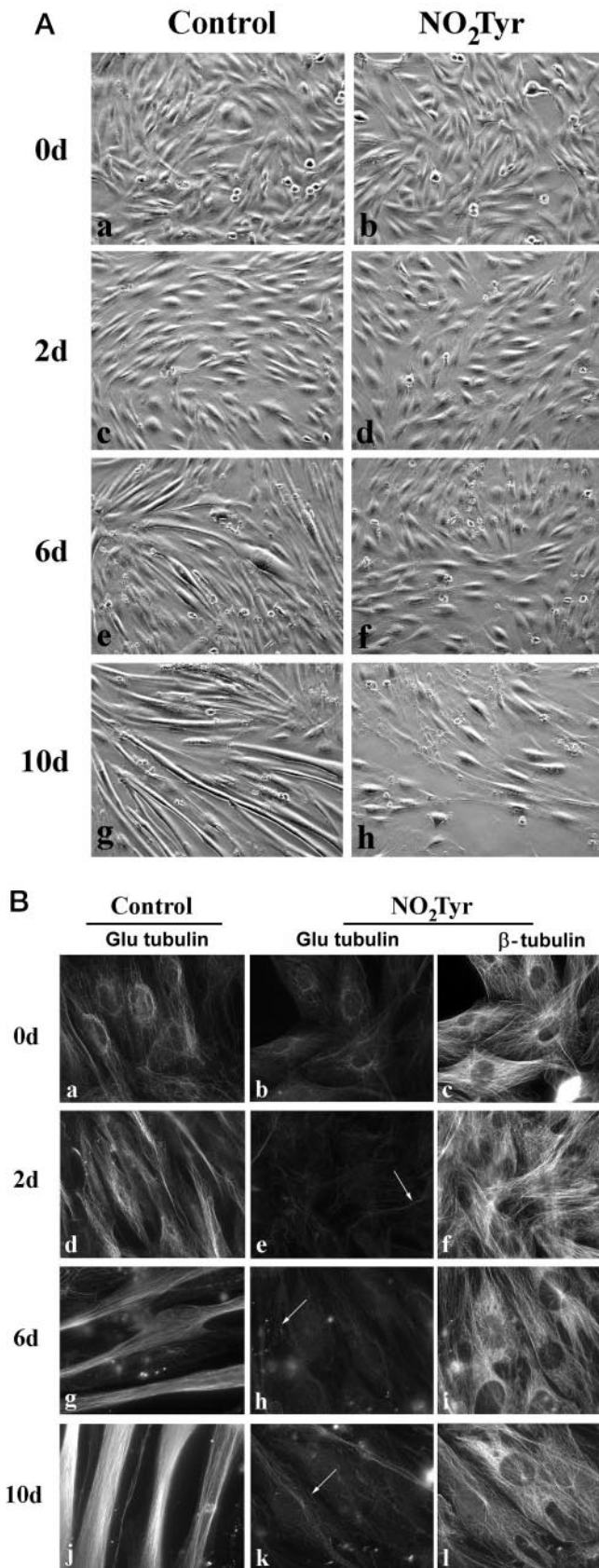


FIG. 3. A, NO_2Tyr treatment blocks early morphogenetic events of myogenesis. L6 cells were pH shifted without (a, c, e, and g) or with NO_2Tyr (b, d, f, and h), allowed to differentiate for 0–10 days, and photographed under phase illumination. Note the paucity of cells elongated and aligned parallel to one another in NO_2Tyr -treated, compared with control cells at 2 days (arrows, d and c). Note that 6 days and 10 days after induction of differentiation, myoblasts had fused to form

TABLE II
 NO_2Tyr inhibits myogenesis of L6 cells

Time of differentiation days	% nuclei in myotubes	
	Control ^a	NO_2Tyr
0 ^a	6.9	2.5
2	6.6	1.8
4	22	0.70
6	28	1.6
10	60	5.2

^a Cells grown in proliferation medium for 2 days, without pH shift.

(Fig. 3A, panel d). After 6 days and 10 days of differentiation, untreated L6 cells had formed long, compact myotubes that were often branched tubes. Many contained as many as 100 nuclei. In contrast, NO_2Tyr -treated cells were well-spread and unpolarized; they had not changed in morphology significantly during the time course (Fig. 3A, compare panels e and g to f and h). We quantified the extent of myogenic differentiation by scoring the percentage of fused cells (Table II). In control cultures at 10 days, a majority of cells (60%) had fused with their neighbors, whereas in NO_2Tyr -treated cultures fusion was insignificant at any time point (~5%, not statistically different from undifferentiated control cells).

We used immunofluorescence to monitor Glu MT distribution during myogenesis. Before differentiation, both NO_2Tyr -treated and untreated cells contained a significant number of Glu MTs (Fig. 3B, panels a–c), but these were fewer in number and dimmer in NO_2Tyr -treated than in control cells (Fig. 3B, panels a and b). Glu MTs in undifferentiated cells were frequently curly, and they clustered nearby or circumscribed the nucleus. By 2 days of myogenesis, Glu MTs in untreated cells had begun to form small, straight bundles aligned parallel to the long axis of the cell (Fig. 3B, panel d). At 6 days and 10 days (Fig. 3B, panels g and j), larger bundles of Glu MTs coursed along the length of highly developed myotubes. In contrast, at 2 days, NO_2Tyr -treated cells contained almost no Glu MTs, and the total MT array was neither aligned nor polarized (Fig. 3B, panels e and f, respectively). By 6 and 10 days, NO_2Tyr -treated cells showed only a few MTs dimly labeled with Glu antibody (Fig. 3B, panels h and k, arrows). NO_2Tyr treatment did not change the total cellular array of MTs in proliferating cells (not shown) or in differentiating cells (Fig. 3B, compare panels i and l to c). Thus, the paucity of Glu MTs did not stem from a decrease in the total MT array, of which they represent a subset.

Lack of Glu MTs Blocks Expression of Muscle-specific Structural and Regulatory Proteins—As major components of assembling myofibrils, muscle-specific myosin and sarcomeric α -actin are abundantly expressed at late stages of muscle differentiation, concomitant with myoblast fusion (30). Because NO_2Tyr -treated L6 cells showed morphological deficits early in myogen-

highly developed, multinucleated myotubes in control samples (e and g), whereas in NO_2Tyr -treated cells morphological differentiation was perturbed; that is, no fusion was observed. Note the flat, highly spread single cells (f and h), in contrast to the narrow myotubes (e and g). Bar in h, 100 μm for all panels. B, NO_2Tyr treatment blocks elaboration of Glu MTs during myogenesis. L6 cells were pH shifted without (control: a, d, g, and j) or with NO_2Tyr (b, c, e, f, h, i, k, and l), differentiated for 0–10 days, and immunostained to visualize Glu-enriched MTs (Glu tubulin: a, b, d, e, g, h, j, and k) or total tubulin (β -tubulin: c, f, i, and l). At 0 days, Glu MTs were abundant in control (a) but less so in NO_2Tyr -treated (b) cells. Although Glu MTs increased in abundance and staining intensity from 2–10 days in controls (d, g, and j), little staining was seen in NO_2Tyr -treated cells (e, h, and k). In contrast, the total MT array was unaffected by NO_2Tyr treatment (β -tub: c, f, i, and l). Note that staining intensity with anti-Glu tubulin antibody here correlates with Western blots (Fig. 2). Arrows indicate a few Glu MTs visible in NO_2Tyr -treated cells at 2, 6, and 10 days (e, h, and k). Bar in l, 20 μm .

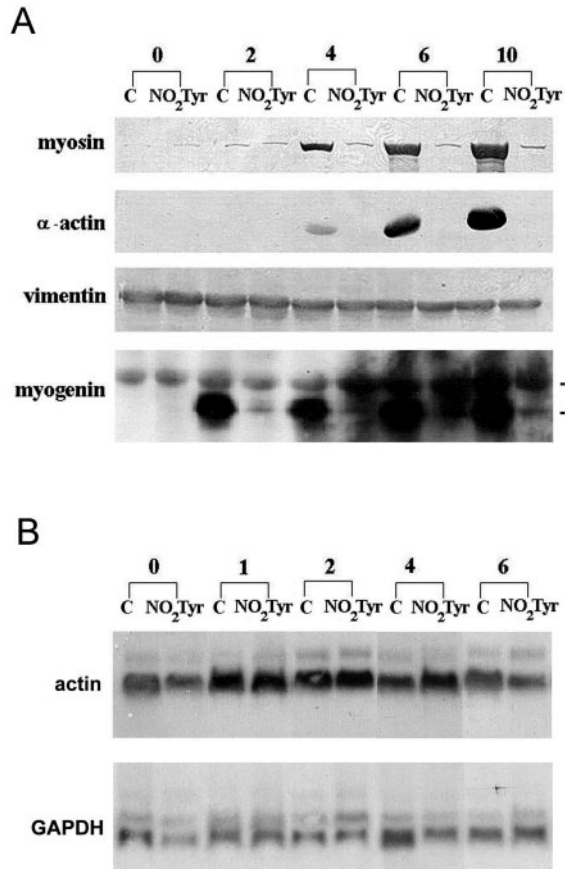


FIG. 4. A, NO_2Tyr blocks accumulation of muscle-specific proteins. Extracts of pH-shifted L6 cells without (control lanes, C) or with NO_2Tyr , differentiated for 0–10 days, were immunoblotted with antibodies to muscle-specific myosin heavy chain (*myosin*), sarcomeric α -actin, vimentin, and myogenin. 60, 60, 65, and 65 μg of extract protein were used to detect each antigen, respectively. *p* and *h* mark the electrophoretic position of phosphorylated and hypophosphorylated (active) myogenin, respectively. B, NO_2Tyr treatment does not affect accumulation of muscle-specific actin transcript. Total RNA (20 μg) from pH-shifted L6 cells without (control lanes, C) or with NO_2Tyr , differentiated for 0–10 days, were hybridized with a muscle actin probe (*actin lanes*) and a glyceraldehyde-3-phosphate dehydrogenase probe, as a loading control. Notice that NO_2Tyr treatment does not change the muscle actin transcript level detectably, in contrast to effects of the treatment on actin protein (compare B and A).

esis, we tested whether these late myogenic markers were properly expressed. As shown in Fig. 4, in control cells, accumulated muscle-specific myosin increased from a low, basal level in undifferentiated cells to a high level by 4 days (C lanes). In NO_2Tyr -treated cells, myosin expression remained at basal levels throughout the 10-day time course (Fig. 4, NO_2Tyr lanes). Similarly, sarcomeric α -actin was first detected at 4 days of differentiation in control cells (Fig. 4, C lanes), whereas it was not detected at any stage in NO_2Tyr -treated cells (Fig. 4, NO_2Tyr lanes). From these gene expression results and those of Fig. 3, which showed NO_2Tyr inhibition of early morphological changes, we conclude that NO_2Tyr treatment prevented both early and later events of myogenesis.

Fig. 4B shows that actin transcript accumulation, which precedes accumulation of α -actin, was not inhibited by NO_2Tyr treatment, in contrast to the striking inhibition of accumulation of muscle actin protein. NO_2Tyr most likely inhibits actin expression by inhibiting its translation or its accumulation, rather than its transcription. Because NO_2Tyr prevents myofibril assembly, actin turnover could conceivably be affected by its assembly state.

The intermediate filament protein, vimentin, was previously

shown to colocalize with Glu MTs in fibroblasts and to depend upon this MT subset for extension into peripheral areas of the cell (12). To determine whether a decrease in Glu MT level affected the level of vimentin, we probed blots of differentiating L6 cells with anti-vimentin (Fig. 4). The level of accumulated vimentin remained constant during differentiation regardless of treatment, indicating that vimentin level was insensitive to either the increase in Glu MTs during differentiation (in control cells) or the absence of Glu MTs (in NO_2Tyr -treated cells). This result presents the possibility that vimentin filaments may not utilize Glu MTs as interaction partners in muscle cells.

During differentiation of muscle tissue, expression of desmin intermediate filaments is induced. We hypothesized that desmin, which is highly homologous to vimentin, might be affected by NO_2Tyr depletion of Glu MTs. However, L6 cells were reported not to express desmin (31). Consistent with this finding, we were unable to detect either desmin protein or mRNA on Western or Northern blots, with or without NO_2Tyr treatment.

Our findings, that altering a cytoskeletal protein affected expression of muscle-specific cytoskeletal proteins, actin and myosin, prompted us to ask whether expression of a regulatory protein might be similarly affected. If transcription factors were affected by NO_2Tyr , expression of a subset of muscle-specific genes might then be blocked. Myogenin is the most relevant transcription factor in our system. Of the known myogenic basic helix-loop-helix transcription factors, L6 muscle cells express abundant myogenin, a low level of Myf-5, and no detectable MyoD (32, 33). In addition, myogenin synthesis is known to up-regulate its expression level, whereas myogenin phosphorylation negatively regulates its DNA binding activity (34). As shown in Fig. 4, we were able to assay both active and inactive forms, because myogenin separates electrophoretically into two bands: the active, hypophosphorylated form that migrates more rapidly (labeled *h*) than the inactive, phosphorylated form (labeled *p*) (35–37).

In control cultures (C lanes), myogenin expression was up-regulated at the onset of myogenesis (Fig. 4, compare 2d to 0d). Blots revealed that the active, hypophosphorylated species was more abundant than the phosphorylated species (Fig. 4, compare *h* and *p* bands in lanes C), and the intensity of both *h* and *p* bands increased during differentiation (C lanes). In contrast, NO_2Tyr treatment lowered the level of the lower molecular weight *h* band (i.e. active myogenin), throughout differentiation, whereas it did not have a consistent effect on the level of inactive phosphorylated myogenin (Fig. 4, NO_2Tyr lanes, *p* band). The finding that NO_2Tyr -treated cells possessed less of the active form of myogenin suggested that Glu MTs are necessary for synthesis or accumulation of a muscle-specific transcription factor.

Expression of Cell Adhesion Molecules Is Perturbed by NO_2Tyr Treatment—Because inhibition of Glu MT formation impacted upon both muscle-specific structural and regulatory proteins, as well as on morphological changes in myoblasts, we asked whether the lack of Glu MTs might also affect cell adhesion molecules involved in myogenic morphogenesis or signaling of myogenic gene expression. Integrins α_{7A} and β_{1A} form transmembrane heterodimeric receptors that mediate association of the extracellular matrix protein, laminin, to the intracellular actin cytoskeleton in myotubes (38, 39). α_{7A} integrin level increases during skeletal muscle differentiation (40, 41), whereas β_{1A} integrin level decreases as myoblasts switch to expressing integrin β_{1D} (42).

Accordingly, we examined expression of integrins α_{7A} and β_{1A} during differentiation of control and NO_2Tyr -treated L6 cells. Fig. 5 shows that, in control L6 cells, α_{7A} integrin level

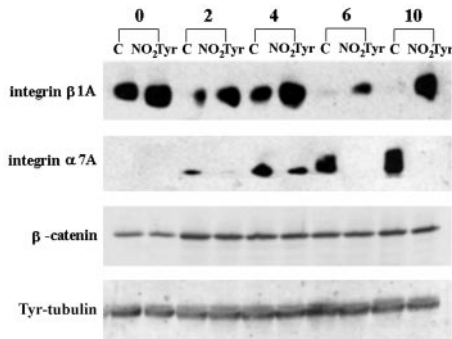


FIG. 5. Expression of cell surface adhesion molecules in NO₂Tyr-treated cells. Extracts of pH-shifted L6 cells without (control lanes, C) or with NO₂Tyr (NO₂Tyr lanes) and differentiated for 0–10 days were immunoblotted with antibodies to integrin β_{1A} , integrin α_{7A} , and β -catenin. 65 μ g of extract protein was loaded for integrin detection, 60 μ g for β -catenin. Values were normalized by labeling each blot with anti-Tyr tubulin antibody, as shown.

increased dramatically as myoblasts underwent differentiation (α_{7A} blot, C lanes); conversely, β_{1A} level decreased from high to undetectable during differentiation (Fig. 5, β_{1A} blot, C lanes) in agreement with Belkin *et al.* (42). NO₂Tyr-treated cells did not exhibit this integrin switch (Fig. 5, NO₂Tyr lanes): α_{7A} integrin was not abundant in NO₂Tyr-treated cells at any time from 0 to 10 days, with only a minor amount detected at 4 days. At the same time, β_{1A} level remained high at all stages examined. Constitutive expression of integrin β_{1A} was previously reported to be sufficient to block the cell cycle withdrawal requisite for myogenic differentiation in primary quail muscle cells (43). In contrast, our NO₂Tyr-treated L6 cells withdrew from the cell cycle (*i.e.* they survived β -D-arabinofuranoside treatment) even though they continued to express abundant integrin β_{1A} . Myogenin up-regulation has been correlated with up-regulation of integrin α_7 (31, 44), and lack of myogenin activity in NO₂Tyr-treated cells (Fig. 4) might contribute to their failure to induce integrin α_7 . Thus, proper switching of integrins was perturbed in post-mitotic L6 myoblasts in which Glu MT formation was inhibited.

N-cadherin, a cell-cell adhesion molecule expressed in differentiating skeletal muscle cells, forms cell surface clusters that are thought to promote activation of myogenesis by helping align cells for fusion (45, 46). N-cadherin-dependent adhesion was reported to up-regulate myogenin (47), and exogenously expressed N-cadherin was shown to increase myogenesis and abundance of the N-cadherin binding partner, β -catenin, which functions in intracellular signaling (45). Because inhibition of Glu MT formation by NO₂Tyr prevented myogenin up-regulation, we asked whether the inhibitor altered β -catenin level. However, the β -catenin level increased ~2-fold during myogenesis, and this increase was not affected by NO₂Tyr treatment (Fig. 5).

DISCUSSION

In this study, we tested the role of post-translational detyrosination during myogenesis. Previous studies had been hampered by the lack of direct inhibitors of TCP, which has not yet been purified or cloned. In other systems, Glu MT function had been inhibited by microinjecting anti-Glu antibodies (12, 14) or Glu tubulin protomers (13), but neither approach was amenable to studies of myogenic cells. A report by Eiserich *et al.* (17), that carboxypeptidase A could not cleave NO₂Tyr from tubulin *in vitro*, suggested that TCP might possess similar substrate specificity. Our demonstration that, indeed, NO₂Tyr-MT substrate inhibits TCP *in vitro* pointed to NO₂Tyr-MTs as an effective inhibitor of Glu MT formation in muscle cells. Successfully obtaining enough NO₂Tyr incorporation into MTs

to inhibit detyrosination effectively was predicated on the use of a pH shift protocol that altered cells' Glu tubulin content reversibly.

The fact that Glu tubulin level was altered in response to extracellular pH suggested that MT stability was also altered. Analysis of acetylated tubulin and examination of MT behavior *in vivo* by time-lapse microscopy corroborated this hypothesis (data not shown). Although it is unclear how extracellular pH affects *in vivo* MT stability, a putative mechanism is pH-induced changes in tubulin polymerization. The effects we measured coincide with evidence from other investigators that the quantity and, thus presumably, the stability of MTs polymerized is exquisitely sensitive to pH *in vitro* (48, 49) and *in vivo* (27). Instead of or in addition to, pH could affect activity of stathmin/Op18, an MT-destabilizer. For example, pH 6.8 enhances complexes of stathmin/Op18 with tubulin protomer, rather than with MT protofilaments, favoring persistence of stable MTs. In contrast, alkaline pH increases interaction of stathmin/Op18 with protofilaments, thus destabilizing MTs (50–52). Future work will elucidate how extracellular pH modifies MT dynamics; at present, we merely exploited this phenomenon as a convenient means of optimizing NO₂Tyr incorporation.

NO₂Tyr specifically inhibits detyrosination, as judged by several criteria: First, the degree of NO₂Tyr incorporation into MTs, rather than the length of treatment or concentration of NO₂Tyr applied, predicted the severity of myogenic inhibition. Furthermore, 2-nitro-*p*-cresyl, a nitrophenol compound similar to NO₂Tyr, neither affected myogenesis nor became incorporated into tubulin, further supporting our conclusion that NO₂Tyr was effective only when incorporated into MTs (see above). Third, NO₂Tyr was detected only in tubulin, suggesting that its incorporation was catalyzed only by the tubulin-specific enzyme, TTL. Fourth, with or without NO₂Tyr, MT acetylation occurred normally. Thus, NO₂Tyr administration inhibits only a single post-translational modification, detyrosination.

Our data corroborated results of Khawaja *et al.* (7) and Webster *et al.* (8), who showed that detyrosination is a result of, rather than a contributor to, MT stabilization. The specificity of NO₂Tyr allowed us to test independently the role of a single post-translational modification. Acetylation and detyrosination occur along distinct but overlapping populations of stable MTs (10, 11, 29), and both are up-regulated during myogenesis (16). Myogenesis was prevented when MT detyrosination was inhibited and acetylation was unaffected; therefore, the two modifications are not functionally redundant. No experiments to date address whether MT acetylation is also required for myogenesis. That different post-translational modifications affect functions or localizations of different MT subsets was suggested by Moreno and Schatten (53), who showed that post-translationally glutamylated MTs (54) and acetylated MTs were found in different compartments of developing spermatids.

Our finding that NO₂Tyr treatment of L6 cells irreversibly inhibited early myogenic events was not surprising, because the early elaboration of Glu MTs had implicated them in events prior to fusion (16). Our data showing early defects in cell elongation suggest that inhibition of Glu MTs inhibits signaling required for myogenesis rather than physically inhibiting fusion. Like myogenesis, early morphogenesis during neurite outgrowth occurs concomitant with MT stabilization and detyrosination (55, 56). Thus, by analogy, Glu MTs may be required for neurite outgrowth, as well.

Our experiments demonstrate that Glu MTs are *necessary* for myogenic differentiation. Other data, though, suggest that they may not be *sufficient*. For example, although the MT-stabilizing drug, Taxol, increases Glu MT level (5), attempts to

use low doses of Taxol to re-induce myogenesis after NO₂Tyr treatment were unsuccessful (data not shown). Failure to rescue NO₂Tyr-treated cells implies that Glu MTs are not sufficient for myogenic differentiation. Alternatively, the failure to restart some events of myogenesis with other interdependent events already in progress might have precluded differentiation that could have otherwise been induced by Glu MTs.

NO₂Tyr treatment of L6 myoblasts generated pleiotropic effects upon differentiation. For example, NO₂Tyr severely compromised accumulation of active myogenin, which functions in early myogenic transcription; this might lead to defects in protein expression later in the myogenic time course. Although NO₂Tyr treatment had no discernible effect on the early accumulation of muscle actin mRNA transcript, it prevented accumulation of actin protein, along with myosin, another late myogenic marker assembled into sarcomeres. A switch in integrins thought to mediate cell adhesion changes was also prevented, in this case, resulting from either a transcriptional or post-transcriptional block. Taken together, our results suggest that Glu MTs play a critical primary role early in the myogenic pathway and exert secondary effects at later myogenic stages.

Previous studies have focused on signal transduction events leading to the generation of Glu MTs (57, 58). In contrast, our data implicate a signaling pathway that is activated by generation of Glu MTs; failure of this signal perturbs myogenesis. Possible mechanisms by which Glu MTs could signal their presence include changes in motor activity, *i.e.* if Glu MTs change kinesin-based transport functions (59), or changes in binding of signal transduction molecules, *i.e.* if Glu MTs alter signaling to the nucleus. In the latter scenario, if detyrosination were prevented, Glu MT-binding molecules would fail to bind MTs, and changes in their targeting and/or activity would result. Alternatively, without Glu MTs, molecules bound specifically to Tyr MTs might fail to dissociate from MTs. The latter is unlikely, though, because the increase in Glu subunits within the MTs is more dramatic than the decrease in Tyr subunits.

It is a formal possibility that the presence of NO₂Tyr-MTs may exacerbate the effects of inhibiting Glu MTs. Incorporation of a novel residue, NO₂Tyr, may structurally perturb binding of molecules that normally bind either to Glu- or Tyr-MTs but not to NO₂Tyr-MTs. This will be tested in future experiments. Alternatively, NO₂Tyr-tubulin could have downstream effects by blocking further modification, *i.e.* removal of the C-terminal residue of Glu tubulin to form Δ 2-tubulin (54). The latter possibility is unlikely, because Δ 2-tubulin accumulates mainly in neuronal MTs and has not been detected in muscle (60). In any case, Western blots (Fig. 2), performed with equally reactive antibodies (not shown) document that NO₂Tyr treatment resulted in a Glu-MT decrease that was more significant than the NO₂Tyr-MT increase. This is probably because, as shown *in vitro* in TCP assays, NO₂Tyr-MTs bind tightly to TCP and prevent its subsequent activity to detyrosinate Tyr subunits. Even if a minuscule amount of NO₂Tyr-MTs is somehow responsible for potentially affecting myogenic MTs, our work shows that changing the C-terminal residue from Glu to NO₂Tyr, or to Tyr, abrogates functions required for myogenesis.

Our data suggest the existence of Glu MT-binding molecules; candidates for these include proteins that show muscle-specific expression and/or specific interaction with stable MTs. An interesting candidate is MURF, a muscle-specific RING-finger protein required for myoblast differentiation, which has been shown to interact with stable MTs (61). Involvement of MURF in transcription may provide an important link between MT properties and myogenic gene expression. Other proteins such as CLASPS, APC, and EB1, whose expression is not limited to

muscle, bind to MTs stabilized by plus-end capping (62–64). Plus-end binding species may exert MT-stabilizing effects early in the myogenic pathway, possibly upstream of MT detyrosination. These may be distinct from Glu MT-binding molecules that signal the stabilization of certain MTs. Future studies will be needed to identify mechanisms by which Glu MTs function in the signaling of myogenic events.

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