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Original Contribution

Peroxynitrite transforms nerve growth factor into an apoptotic factor for motor neurons

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Abstract

Nerve growth factor (NGF) overexpression and increased production of peroxynitrite occur in several neurodegenerative diseases. We investigated whether NGF could undergo posttranslational oxidative or nitrative modifications that would modulate its biological activity. Compared to native NGF, peroxynitrite-treated NGF showed an exceptional ability to induce $p75^{NTR}$-dependent motor neuron apoptosis at physiologically relevant concentrations. Whereas native NGF requires an external source of nitric oxide (NO) to induce motor neuron death, peroxynitrite-treated NGF induced motor neuron apoptosis in the absence of exogenous NO. Nevertheless, NO potentiated the apoptotic activity of peroxynitrite-modified NGF. Blocking antibodies to $p75^{NTR}$ or downregulation of $p75^{NTR}$ expression by antisense treatment prevented motor neuron apoptosis induced by peroxynitrite-treated NGF. We investigated what oxidative modifications were responsible for inducing a toxic gain of function and found that peroxynitrite induced tyrosine nitration in a dose-dependent manner. Moreover, peroxynitrite triggered the formation of stable high-molecular-weight oligomers of NGF. Preventing tyrosine nitration by urate abolished the effect of peroxynitrite on NGF apoptotic activity. These results indicate that the oxidation of NGF by peroxynitrite enhances NGF apoptotic activity through $p75^{NTR}$ 10,000-fold. To our knowledge, this is the first known posttranslational modification that transforms a neurotrophin into an apoptotic agent.

Keywords: Apoptosis; Motor neurons; NGF; $p75^{NTR}$; Nitrotyrosine; Peroxynitrite; Free radicals

Nerve growth factor (NGF), the prototypic member of the neurotrophin family, is critical for the differentiation and survival of specific neuronal populations during development and modulates neural plasticity in the mature nervous system [1,2]. Paradoxically, NGF also induces apoptosis of neurons during development and eliminates damaged neurons and glial cells under pathological conditions [3,4]. NGF can be a mediator of tissue inflammation [5] and accumulates in many diseases involving neuroinflammation [6–8]. NGF exerts its actions through two unrelated transmembrane receptors, the tyrosine kinase receptor TrkA and the p75 neurotrophin receptor ($p75^{NTR}$). TrkA activates well-characterized signaling pathways promoting neuronal survival, differentiation, and plasticity [2,9,10], whereas $p75^{NTR}$ is a member of the tumor necrosis factor receptor superfamily that can act as a death receptor, signaling apoptosis in several neuronal populations [4,11]. In addition, $p75^{NTR}$ can also act as a coreceptor for TrkA, B, and C or interact with other membrane receptors (sortilin, Nogo-R) to modulate diverse biological effects including survival, cytoskeleton rearrangement, and axonal elongation [3,4,12].

Abbreviations: ACN, acetonitrile; ALS, amyotrophic lateral sclerosis; BSA, bovine serum albumin; FGF, fibroblast growth factor; GDNF, glial-derived neurotrophic factor; HPLC, high-pressure liquid chromatography; MALS, multiangle light scattering; NGF, nerve growth factor; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; $p75^{NTR}$, p75 neurotrophin receptor; ROA, reverse-order addition; SOD1, superoxide dismutase 1; TNM, tetranitromethane.

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Embryonic motor neuron cultures represent an attractive model for studying the modulation of neuronal death induced by NGF. $p75^{NTR}$ is highly expressed in motor neurons at the embryonic stage, but its expression gradually declines after birth [13]. Neither TrkA nor $p75^{NTR}$ is expressed by adult motor neurons, although $p75^{NTR}$ can be reexpressed after axotomy [14–16] and under pathological conditions involving motor neuron degeneration, such as amyotrophic lateral sclerosis (ALS) [17,18]. Furthermore, $p75^{NTR}$ has been implicated in motor neuron death induced by axotomy [14,19,20]. Abnormal expression of $p75^{NTR}$ and NGF may contribute to adult motor neuron death in ALS transgenic mice overexpressing mutant Cu–Zn superoxide dismutase (SOD1) [18,21–25]. Pure motor neuron cultures are not sensitive to NGF/$p75^{NTR}$-induced apoptosis. However, exogenously generated nitric oxide at physiological concentrations (<50 nM) renders motor neurons sensitive to NGF-mediated apoptosis [25]. Therefore, we hypothesized that modulation of NGF-induced apoptosis by oxidative stress may additionally affect motor neuron fate.

Peroxynitrite ($ONOO^-$), the reaction product of nitric oxide and superoxide, is a potent oxidant and nitrating agent capable of reacting and modifying a wide variety of biomolecules including proteins, lipids, and DNA [26,27]. Peroxynitrite is formed *in vivo* mostly under pathological conditions associated with increased production of nitric oxide. Moreover, the production of superoxide and peroxynitrite could be catalyzed *in vivo* by altered redox properties of mutant SOD1 linked to ALS [28]. Tyrosine nitration (i.e., addition of a nitro group in the third position of the aromatic ring) is considered a footprint of oxidative damage mediated by peroxynitrite [27,29]. Although alternative pathways involving peroxidases using nitrite could be responsible for nitration of tyrosine residues under inflammatory conditions [30,31], peroxynitrite has been shown to be the predominant nitrating agent in motor neurons [32,33]. Peroxynitrite-dependent tyrosine nitration occurs by a free radical-mediated mechanism involving the simultaneous formation of tyrosyl radicals and $\cdot NO_2$, which combine at near diffusion-limited rates to form 3-nitrotyrosine [27,30]. Tyrosyl radicals may also dimerize to 3,3′-dityrosine, although this reaction can be limited by spatial and diffusional constraints [30]. Some tyrosine residues in proteins are particularly susceptible to nitration by peroxynitrite, inducing subsequent changes in protein function or turnover [31]. Known examples include protein surfactant A [34], neurofilament-L [35], cytochrome *c* [36], fibrinogen [37], protein kinase Cε [38], and glutathione *S*-transferase [39]. Because NGF is overexpressed in the oxidative environment linked to neurodegenerative processes [6–8,25], this protein could also be a target for peroxynitrite-mediated oxidative modifications.

NGF is a homodimer of approximately 26.5 kDa [40,41]. The mouse NGF monomer has 118 amino acids, although shorter chains truncated at both the N-and the C-terminus have also been identified [40,42]. Mouse NGF contains 2 tyrosine residues at positions 52 and 79 (Fig. 1) [43]. Conserved in all members of the neurotrophin family, Tyr52 participates in hydrophobic contacts at the dimer interface and is also engaged in $p75^{NTR}$ binding [41,44]. Site-directed mutagenesis studies revealed the structural importance of Tyr52 in determining a stable protein conformation [45]. On the other hand, Tyr79 is conserved in most NGFs, but not in other members of the neurotrophin family [43]. In mouse NGF, Tyr79 makes contact with residues of the same protomer and can also interact with the N-terminus of the second protomer (Fig. 1) [41]. Because these two tyrosine residues are highly conserved, peroxynitrite modification of these residues may have important consequences in NGF biological activity.

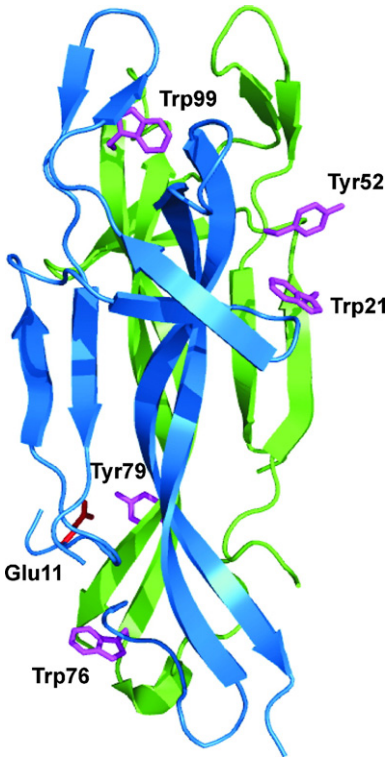

Fig. 1. Ribbon diagram of the NGF structure (PDB code 1BET). The two NGF monomers (green and blue) interact with each other through a largely hydrophobic interface [41]. The diagram shows the location of the two tyrosine and three tryptophan residues present in mouse NGF. The Tyr and Trp side chains from one monomer (green) are drawn in a stick representation and labeled (pink). Glu11 (red) from the opposite NGF monomer (blue) is also represented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the present study, we investigated whether NGF could undergo posttranslational oxidative or nitrative modification, altering its functional activity. Here we report that oxidation of NGF by peroxynitrite *in vitro* causes nitration and induces the formation of high-molecular-weight oligomers. Moreover, these oxidative modifications confer the exceptional ability to induce $p75^{NTR}$-dependent motor neuron apoptosis at physiologically relevant concentrations. Our data suggest that oxidative stress by peroxynitrite can critically modulate neurotrophin activity.

Materials and methods

Peroxynitrite treatment

Peroxynitrite synthesized as previously described [46] was generously provided by Dr. Rafael Radi (Universidad de la

República, Montevideo, Uruguay). Various batches were used and some experiments were performed with peroxynitrite from Upstate Biotechnology. Peroxynitrite concentration was determined by absorbance at 302 nm ($\varepsilon = 1700\ M^{-1}\ cm^{-1}$). Diluted stock solutions were freshly prepared in 0.01 M NaOH. The reaction of NGF (Harlan) with various concentrations of peroxynitrite (0.25 to 2 mM) was performed at a protein concentration of 0.2 to 1.0 mg/ml, obtaining similar results. The reaction was performed in 50 mM sodium phosphate buffer, pH 7.4, containing 20 mM sodium bicarbonate. NGF was subjected to 10 bolus additions of peroxynitrite (1 μl each) to reach the desired final concentration of peroxynitrite. One bolus of peroxynitrite stock solution was rapidly added on the top of the test tube and mixed by vortexing for 3 s. The procedure was repeated 10 times. To exclude a potential nonspecific effect of peroxynitrite treatment because of pH changes or contaminants, control experiments were performed using diluted NaOH or decomposed peroxynitrite (reverse-order addition, ROA). Treatment of bovine serum albumin (Sigma), FGF-1 (Sigma), and FGF-2 (R&D Systems) with peroxynitrite was performed as described above at a concentration of 0.2 mg/ml.

NGF nitration by tetranitromethane

The reaction of NGF (Harlan) with a 40-fold molar excess of tetranitromethane (Sigma) was performed at a protein concentration of 1 mg/ml in 0.1 M Tris–HCl buffer, pH 8.0, for 40 min. The reaction was finished by passing the reaction mixture through a Sephadex G-25 column equilibrated and eluted in 0.05 M ammonium bicarbonate, pH 8.0.

Purified motor neuron cultures

Media and sera were purchased from Gibco–Invitrogen. Motor neuron cultures were prepared from embryonic day 15 (E15) rat spinal cords by a combination of metrizamide gradient centrifugation and immunopanning with the monoclonal antibody Ig192 against $p75^{NTR}$ as previously described [47]. Motor neurons were plated at a density of 500 cells/cm^2 on four-well multidishes (Nunclon) precoated with polyornithine–laminin. Cultures were maintained in Neurobasal medium supplemented with 2% horse serum, 25 mM L-glutamate, 25 μM 2-mercaptoethanol, 0.5 mM L-glutamine, and 2% B-27 supplement (Gibco–Invitrogen). Motor neuron survival was maintained by the addition of GDNF (1 ng/ml; Sigma) to the culture medium. Motor neuron death induced by trophic factor deprivation (NONE, without GDNF) was determined in all experiments as a control and was never greater than 50%. Treatments with the different reagents were performed 3 h after motor neuron plating. Motor neuron survival was assessed after 48 h by direct counting of all neurons displaying intact neurites longer than four cell bodies in diameter, which typically express the motor neuron marker Islet-1/2 homeoprotein as determined by immunofluorescence using the 4D5 monoclonal antibody [25,48].

Antisense treatment

Treatment with antisense oligonucleotides to downregulate $p75^{NTR}$ expression was performed as described previously [49]. Briefly, high-pressure liquid chromatography (HPLC)-purified phosphorothioate antisense and missense oligonucleotides (5 μM; Integrated DNA Technologies) were added to the cell suspension of purified motor neurons with repeated pipetting before seeding. The oligonucleotides were present the whole time of culture. To determine the efficiency of uptake, cultures were incubated with $p75^{NTR}$ antisense oligonucleotides with a 5′ 56-FAM fluorescent label. Cells were transferred to the heated stage (37°C) of a Zeiss LSM510 confocal microscope with constant 5% CO_2. Fluorescence of live cells was imaged with a 63× oil immersion objective. Uptake efficiency was >96% in all experiments. Sequences used were $p75^{NTR}$ antisense, 5′-ACCTGCCCTCCTCATTGCA-3′, and $p75^{NTR}$ missense, 5′-CTCCCACTCGTCATTCGAC-3′ [49]. The antisense sequence used has been shown to be effective at inhibiting $p75^{NTR}$-dependent motor neuron death *in vivo* [20].

Electrophoretic and Western blot analyses

SDS–PAGE was performed in 15% polyacrylamide minigels under reducing conditions. Samples were boiled for 5 min in Laemmli buffer before running. Proteins were visualized by Coomassie blue or silver staining. For Western blot analysis proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham). Membranes were blocked for 2 h in blocking solution (5% BSA, 0.1% Tween 20 in Tris-buffered saline (TBS), pH 7.4) followed by an overnight incubation with the primary antibody diluted in blocking solution. After being washed with 0.1% Tween in TBS, the membrane was incubated with peroxidase-conjugated goat anti-rabbit antibody (1:4000; Bio-Rad) for 1 h and then washed and developed using the ECL chemiluminescence detection system (Amersham). Primary antibodies used were anti-NGF-β polyclonal antibody (1:3000; Chemicon) and anti-nitrotyrosine polyclonal antibody (1:250; Upstate Biotechnology).

Size-exclusion chromatography coupled to multiangle light scattering

Samples of NGF treated with peroxynitrite (1 mM) or decomposed peroxynitrite at a concentration of 0.5 mg/ml were injected onto a Biosuite 125 HPLC size-exclusion column (Waters) connected in line with a DAWN-EOS multiangle light-scattering detector and an OptilabRex refractometer (Wyatt Technology Corp.). The HPLC column was equilibrated with and developed in 50 mM sodium phosphate, 50 mM sodium sulfate buffer, pH 7.2. The light-scattering unit was calibrated following the manufacturer's instructions. A value of 0.185 ml/g was assumed for the refractive index increment (*dn/dc*) of the protein. The detector responses were normalized by measuring the signal for monomeric bovine serum albumin. The temperature of the light-scattering unit and of the refractometer was

maintained at 25°C. The column and all external connections were at ambient temperature (approximately 25°C). The flow rate was maintained at 0.5 ml/min throughout the experiments.

Mass spectrometry

Samples of native NGF or NGF treated with tetranitromethane or peroxynitrite (1 mM) at a concentration of 1 mg/ml were separated by reverse-phase HPLC. The stationary phase was a C_{18} reverse-phase Supelco column (15 cm×4.6 mm, 5 μM). The mobile phase was H_2O/CH_3CN and 0.1% trifluoroacetic acid (TFA). A linear gradient increasing 5 to 60% organic over 55 min was used to achieve separation. Samples were collected by a fraction collector, concentrated in vacuo, and resuspended to be analyzed by mass spectrometry to determine total molecular weight changes and mass changes from tryptic digests. To find total changes in molecular weight, collected fractions were resuspended in 30% acetonitrile with 0.1% formic acid and then directly injected into a Waters/Micromass LCT Classic electrospray time-of-flight (Tof) mass spectrometer. The mobile phase was 30% acetonitrile, 0.1% formic acid with 5 μl/min flow rate. The capillary voltage was 3.018 kV, the source temperature was 80°C, and sample cone voltage was 45 V. Tryptic digests were performed by resuspension in a 0.1% RapiGest SF (Waters) in 50 mM ammonium bicarbonate buffer and was carried out according to the manufacturer's instructions. Briefly, samples were reduced with dithiothreitol and blocked with IAA before being incubated with trypsin (1:50, μg trypsin:μg protein) overnight at 37°C. TFA was added to digested protein samples to a final concentration of 0.5% and samples were centrifuged at 13,000 rpm for 10 min and the supernatant was subjected to mass spectrometry. A NanoAcquity Waters HPLC system was used to inject samples onto the Waters Q-Tof Ultima Global mass spectrometer. Samples were loaded at 2 μl/min onto a Jupiter C_{18} trap and then washed for 6 min with water and 0.1% formic acid at 1 μl/min. Samples were eluted using a gradient (0.26 μl/min) which began at 2% acetonitrile, 0.1% formic acid and increased organic at ~2%/min over 45 min. Samples were separated over Waters BEH C_{18} material in a New Objective

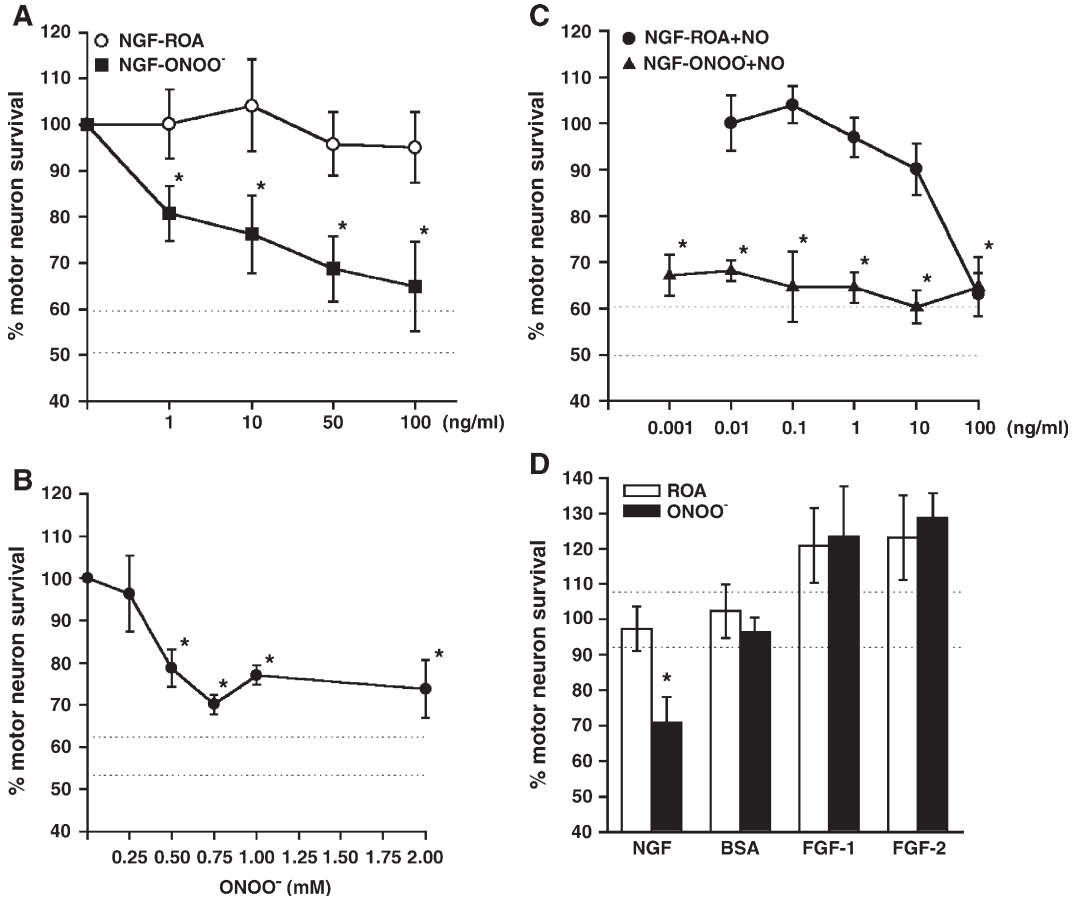

Fig. 2. Peroxynitrite treatment enhanced NGF apoptotic activity. (A) Pure motor neuron cultures maintained with GDNF (1 ng/ml) were exposed to increasing concentrations of NGF previously treated with peroxynitrite (1 mM; NGF-ONOO⁻) or decomposed peroxynitrite (NGF-ROA). Dashed lines represent the standard deviation (SD) of NONE (trophic factor deprivation). (B) Motor neuron cultures were exposed to NGF (10 ng/ml) previously treated with the indicated peroxynitrite concentrations. Dashed lines represent the SD of NONE. (C) Motor neuron cultures were treated with increasing concentrations of NGF-ROA or NGF-ONOO⁻ (1 mM) in the presence of the nitric oxide donor DETA-NONOate (10 μM, NO). Dashed lines represent the SD of NONE. (D) Motor neuron cultures were exposed to NGF (100 ng/ml), BSA (100 ng/ml), FGF-1 (10 ng/ml), or FGF-2 (10 ng/ml) previously treated with peroxynitrite (1 mM; ONOO⁻) or decomposed peroxynitrite (1 mM; ROA). Dashed lines represent SD of GDNF. Motor neuron survival was determined 48 h after treatment. Data are expressed as percentage of GDNF, mean±SD. *Significantly different from GDNF ($p \leq 0.05$).

Pico-Frit, 10-cm column and then injected using a Waters Nano Lockspray source with electrospray capillary voltage at 3.5 kV and source voltage of 70 kV. Data-dependent tandem mass spectrometry was performed with collision energy that was dependent upon the *m/z* of the parent ion. The MS^2 spectra were searched using a Mascot MS/MS ion search engine and the peptides reported received scores of identity or above.

Statistics

Each experiment was repeated at least three times and data are reported as means±SD. Comparison of the means was performed by one-way analysis of variance. Pair-wise contrast between means utilized the Student–Newman–Keuls test and differences were declared statistically significant if $p<0.05$. All statistics computations were performed using the SigmaStat Software (Jandel Scientific).

Results

Oxidation by peroxynitrite enhances NGF apoptotic activity

In cultures maintained with GDNF (1 ng/ml), motor neurons expressing $p75^{NTR}$ are not sensitive to NGF at concentrations up to 100 ng/ml. However, peroxynitrite-treated NGF induced motor neuron death at concentrations as low as 1 ng/ml (Fig. 2A). NGF treated with decomposed peroxynitrite (ROA) did not affect motor neuron survival (Fig. 2A). Peroxynitrite treatment enhanced NGF apoptotic activity in a dose-dependent manner, reaching a plateau at concentrations higher than 0.5 mM peroxynitrite (Fig. 2B). As previously reported [25], in the presence of a steady-state concentration of <50 nM nitric oxide, generated from the nitric oxide donor DETA-NONOate (10 μM), NGF-ROA significantly induced motor neuron loss at concentrations higher than 10 ng/ml (Fig. 2C). Moreover, in the presence of nitric oxide, peroxynitrite-modified NGF showed increased apoptotic activity, inducing a 33% loss of motor neurons at only 1 pg/ml (Fig. 2C). The addition of peroxynitrite-treated BSA, FGF-1, or FGF-2 to motor neuron cultures did not induce motor neuron death (Fig. 2D), suggesting a specific effect of peroxynitrite-treated NGF.

Motor neuron loss induced by peroxynitrite-treated NGF was blocked by the general caspase inhibitor DEVD-fmk (Fig. 3A), indicating the activation of an apoptotic mechanism. We have previously shown that NGF induces motor neuron apoptosis by signaling through $p75^{NTR}$ [25]. The apoptosis induced by peroxynitrite-treated NGF was also dependent on $p75^{NTR}$ activation because it was completely prevented by the addition of blocking antibodies to $p75^{NTR}$ (Fig. 3A) or the down-regulation of $p75^{NTR}$ expression by antisense treatment (Fig. 3B). As a control, antisense treatment also blocked motor neuron apoptosis induced by native NGF in the presence of nitric oxide

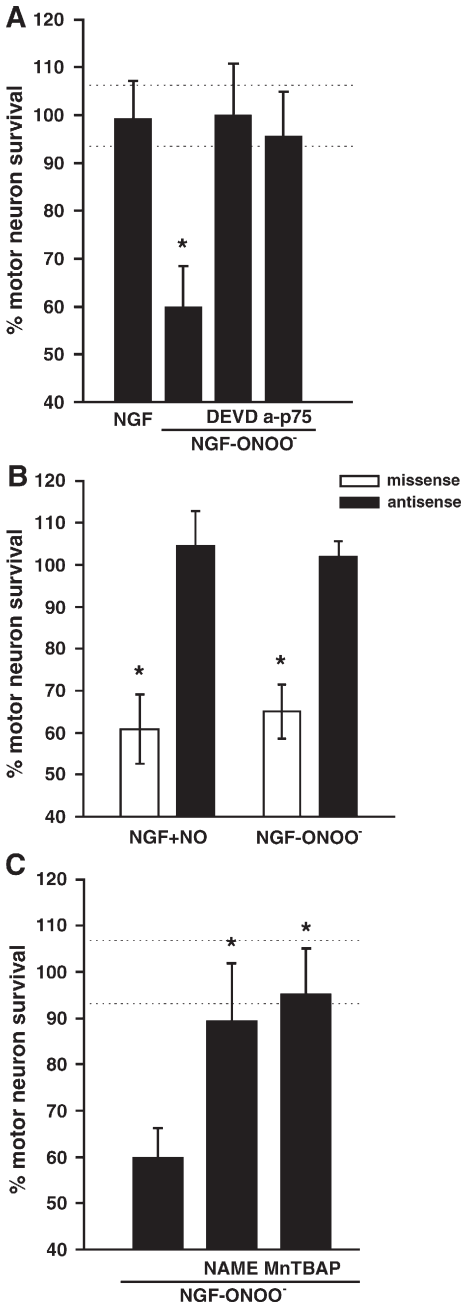

Fig. 3. The apoptosis mediated by peroxynitrite-treated NGF required $p75^{NTR}$. (A) Blocking antibodies to $p75^{NTR}$ (a-p75, 1:300, Chemicon AB1554) and the general caspase inhibitor DEVD-fmk (10 μM) prevented motor neuron death induced by NGF (100 ng/ml) previously treated with peroxynitrite (1 mM; $NGF\text{-}ONOO^-$). Antibodies to $p75^{NTR}$ were added once 3 h after motor neuron plating, whereas DEVD-fmk was added every 24 h. Data are expressed as percentage of GDNF, mean±SD. Dashed lines represent the SD of GDNF. *Significantly different from GDNF ($p \leq 0.05$). (B) Antisense and missense oligonucleotides were added to purified motor neuron cultures at the time of plating. 24 h later, cultures were exposed to $NGF\text{-}ONOO^-$ (100 ng/ml) or NGF (100 ng/ml) plus DETA-NONOate (10 μM) (NGF+NO). Antisense oligonucleotides completely blocked the loss of motor neurons induced by both treatments, whereas missense oligonucleotides had no effect on neuronal survival. Data are expressed as percentage of the respective GDNF, mean±SD. *Significantly different from the respective GDNF ($p \leq 0.05$). (C) Motor neuron apoptosis induced by $NGF\text{-}ONOO^-$ seemed to require the endogenous production of peroxynitrite. Motor neuron cultures were treated with 1 mM peroxynitrite-treated NGF (100 ng/ml) in the presence of L-NAME (1 mM) or MnTBAP (100 μM). Dashed lines represent the SD of GDNF. Data are expressed as percentage of GDNF, mean±SD. *Significantly different from $NGF\text{-}ONOO^-$ ($p \leq 0.05$). Motor neuron survival was determined in all cases 48 h after treatment.

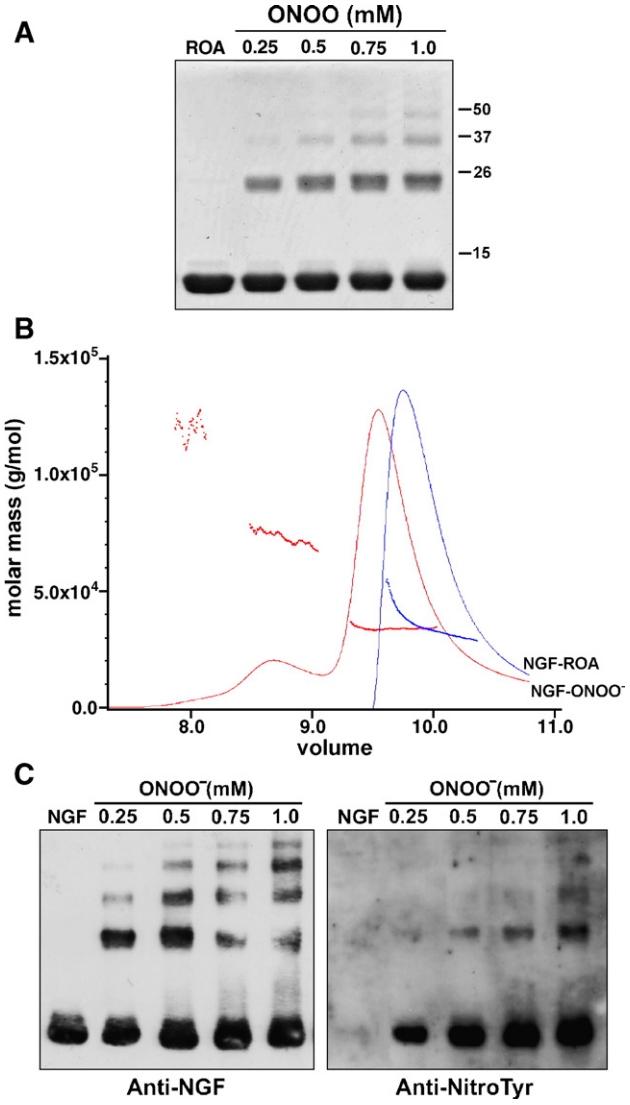

Fig. 4. Peroxynitrite induced NGF oligomerization and nitration. (A) SDS–PAGE showed the formation of high-molecular-weight species of NGF after treatment with increasing concentrations of peroxynitrite (0.25 to 1 mM). As a control, NGF was treated with decomposed peroxynitrite (1 mM; ROA). NGF was treated with peroxynitrite at a concentration of 0.2 mg/ml. 10 μg of protein was applied in each lane and electrophoretic separation was performed in 15% polyacrylamide gels under denaturing and reducing conditions. A representative gel stained with Coomassie blue is shown. (B) NGF treated with 1 mM peroxynitrite (NGF-ONOO⁻) or its degradation products (NGF-ROA) was analyzed by size-exclusion chromatography coupled to real-time multiangle light scattering. The absolute molar mass versus volume of elution was superimposed with the signals from the 90° LS detector. NGF-ROA (blue) eluted as a single peak with a mass corresponding to the dimer (33.0±1.2 kDa). In contrast, NGF-ONOO⁻ eluted as three peaks corresponding to dimer (33.2±0.6 kDa; 85% of the protein), tetramer (68.5±3.5 kDa; 13.1%), and octamer (125.0±10.0 kDa; 1.9%). (C) Western blots showed increased immunoreactivity for nitrotyrosine. 100 ng of NGF treated as in (A) was analyzed by immunoblotting using anti-nitrotyrosine (anti-NitroTyr) polyclonal antibodies. After being stripped, the membrane was developed with anti-NGF polyclonal antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3B). Motor neuron apoptosis induced by different apoptotic stimuli, including NGF, requires the endogenous production of peroxynitrite [25,32,33,50]. Motor neuron loss induced by peroxynitrite-treated NGF was prevented by the general nitric oxide synthase (NOS) inhibitor L-NAME (1 mM) or the SOD mimetic and peroxynitrite decomposition catalyst MnTBAP (100 μM) (Fig. 3C), further confirming the execution of a similar apoptotic mechanism.

Peroxynitrite induces NGF oligomerization and nitration

We then analyzed the modifications that peroxynitrite treatment induced in NGF. Exposure of NGF to successive bolus additions of peroxynitrite caused a dose-dependent appearance of three high-molecular-weight species as revealed by SDS–PAGE. Staining intensity of native NGF progressively diminished with the increase in peroxynitrite concentration (Fig. 4A). Treatment of NGF with decomposed peroxynitrite (ROA) failed to induce this migration shift. The formation of NGF oligomers was confirmed in solution by HPLC size-exclusion chromatography coupled to real-time multiangle light scattering (MALS) analysis (Fig. 4B). NGF treated with decomposed peroxynitrite (NGF-ROA) eluted from the size-exclusion column as a single peak with a mass corresponding to the dimer (33.0±1.2 kDa). In contrast, peroxynitrite-treated NGF eluted as three peaks, likely corresponding to dimer (33.2±0.6 kDa), tetramer (68.5±3.5 kDa), and octamer (125.0±10.0 kDa). Surprisingly, the peroxynitrite-treated NGF dimer eluted before the native dimer (NGF-ROA), probably reflecting the existence of conformational changes resulting in a reduction of the dimer compactness.

Peroxynitrite treatment also induced dose-dependent nitration of NGF, as revealed by reactivity with an anti-nitrotyrosine

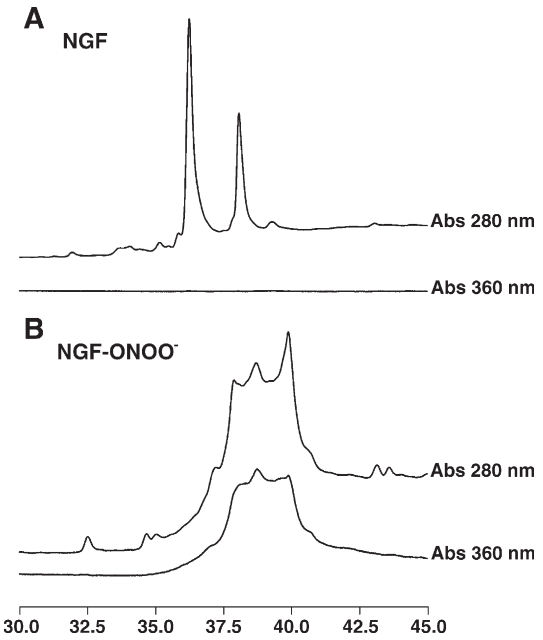

Fig. 5. Reverse-phase HPLC chromatograms of (A) native and (B) peroxynitrite-treated NGF. Native NGF eluted as two peaks at 36.3 and 38.1 min. Peroxynitrite treatment (1 mM) led to an incomplete separation of several products. Nonionized nitrotyrosine absorbs at 360 nm. Peroxynitrite-treated NGF had increased absorbance at 360 nm (B); however, no absorbance at 360 nm was observed in the native NGF chromatogram (A).

antibody (Fig. 4C). The specific sites of oxidative modifications induced in NGF by peroxynitrite were determined by mass spectrometry of purified oxidation products. Native NGF eluted as two peaks by reverse-phase HPLC, at 36.3 and 38.1 min (Fig. 5A), both identified as the NGF polypeptide chain by mass spectrometry. Chain B (eluting at 38.1 min) lacks the eight N-terminal residues present in Chain A and is known to be formed due to limited proteolysis during NGF purification [42]. Oxidation of NGF by peroxynitrite resulted in the incomplete separation of several products as eluted by reverse-phase HPLC (Fig. 5B). Mass spectrometry of the peroxynitrite-treated fraction collected at 38.6 min revealed several species of increased molecular weight compared to the mass spectrum of unmodified NGF (Fig. 6). As has been previously described [42], some of the unmodified NGF chains lacked the C-terminal arginine residue (Fig. 6A). In peroxynitrite-treated NGF, the smallest mass shift was an ~90-Da increase in Chain A (from 13,252 to 13,341 Da), suggesting the addition of two nitro groups (45 Da each; Fig. 6B). In addition, peroxynitrite treatment seemed to induce several additional modifications, suggesting up to five nitro groups and methionine oxidation. NGF contains three tryptophans and two tyrosines that might account for the five sites of nitration (Fig. 6B, table). A similar pattern of modifications was observed for chain B of NGF (data not shown).

To identify the specific residue(s) undergoing oxidative modification, HPLC-purified samples were digested and analyzed by Q-Tof mass spectrometry. Comparison of untreated and peroxynitrite-treated NGF digests revealed the nitration of Tyr52 and Trp99 (Fig. 7), which could account for the detected total molecular weight change of ~90 Da. Oxidation of NGF by peroxynitrite also resulted in a loss of tryptophan fluorescence, further supporting the nitration of tryptophan (data not shown). Oxidative modifications in other residues were not detected in the digested samples, possibly indicating that NGF with nitrated Tyr52 and Trp99 (ion $m/z = 13{,}341$) is the most abundant species. However, this could also be due to an increased efficiency of ionization compared to other peptides. Importantly, formation of 3,3′-dityrosine upon NGF oxidation was not observed by mass spectrometry. Moreover, no fluorescence emission from 3,3′-dityrosine was observed (excitation/emission 320/410 nm), although internal quenching by nitrotyrosine could prevent its detection (data not shown).

Does tyrosine nitration alter the biological activity of NGF?

To ascertain whether tyrosine nitration was critical for modifying NGF biological activity, we treated NGF with tetranitromethane (TNM; 40-fold excess). TNM is commonly

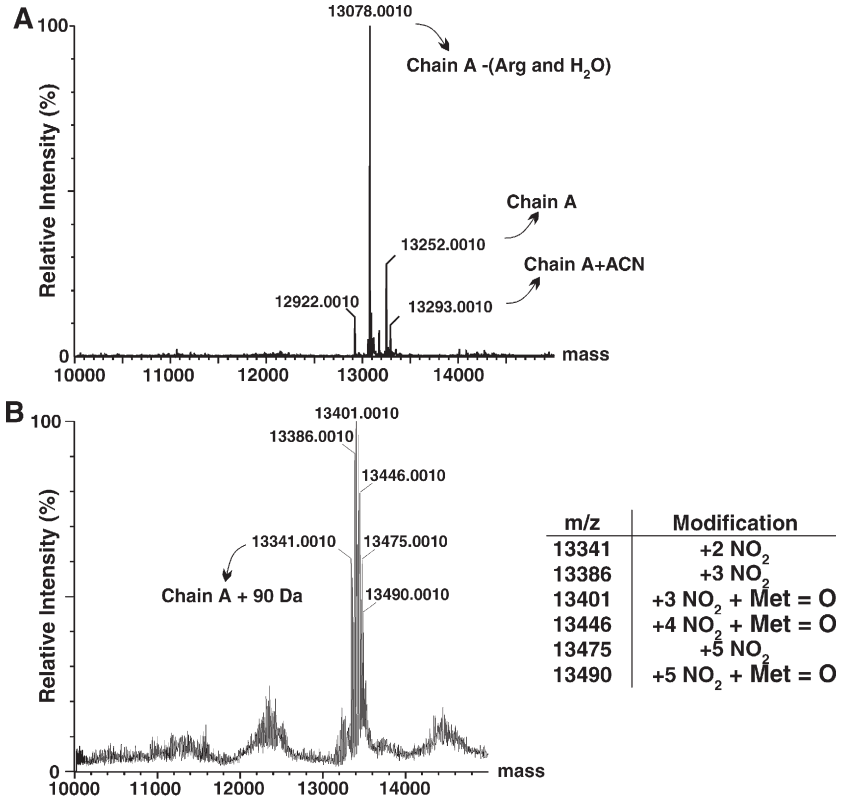

Fig. 6. Mass spectrometry of HPLC collected fractions. (A) Electrospray time-of-flight mass spectrometry of the eluent from native NGF at 36.3 min revealed a mass of 13,252 Da, corresponding to Chain A of NGF. The peak at 13,078 Da is consistent with NGF lacking the C-terminal arginine residue, and the peak at 13,293 Da is consistent with the formation of acetonitrile (ACN) adducts within the mobile phase. (B) Mass spectrometry of the eluent at 38.6 min from peroxynitrite-treated NGF showed an 89-Da increase in Chain A mass (from 13,252 to 13,341 Da). The eluent at 39.9 min corresponded to Chain B and also showed an increase of 90 Da (from 12,357 to 12,449 Da) (data not shown).

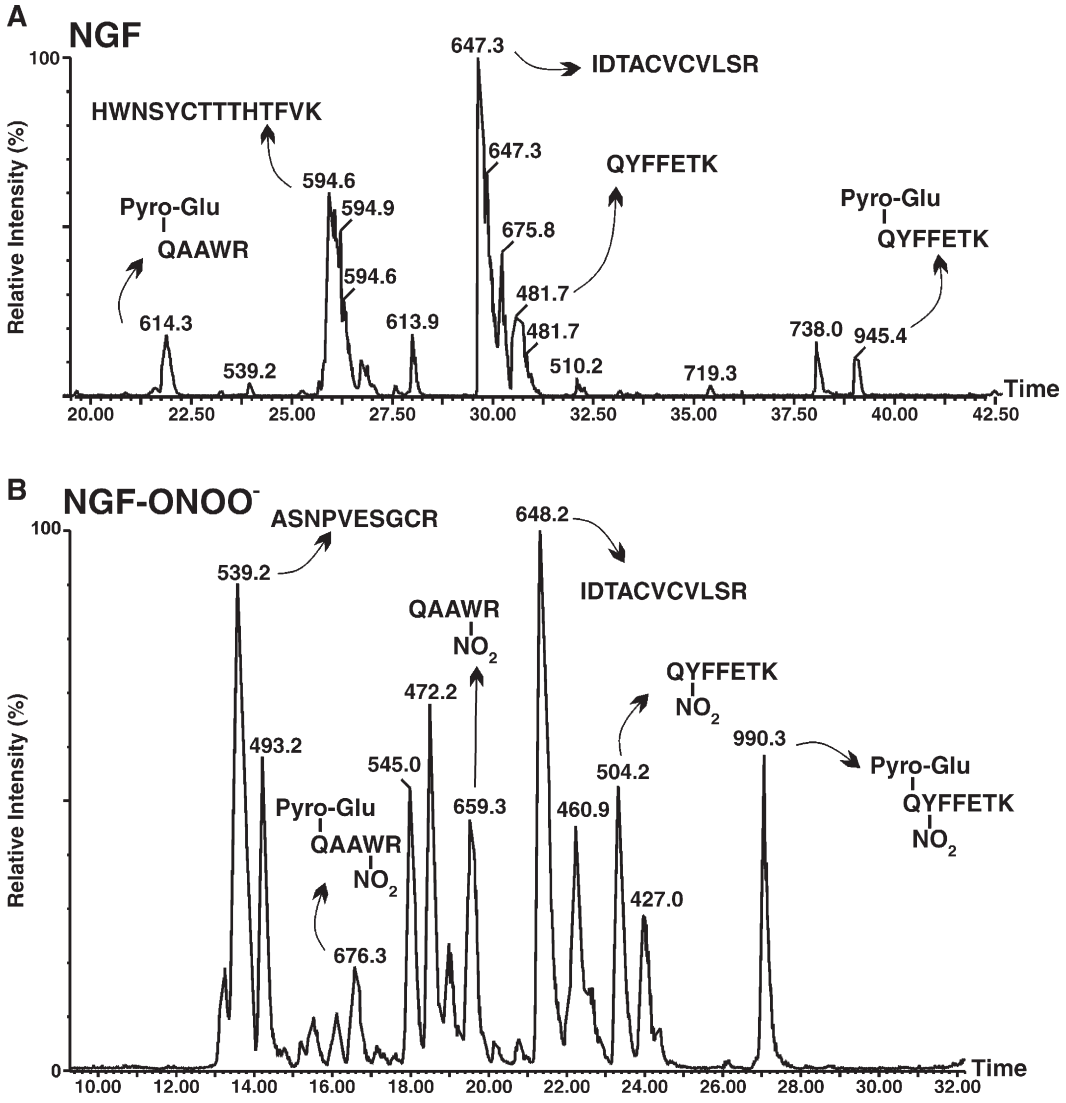

Fig. 7. Q-Tof mass spectrometry of trypsin-digested HPLC collected fractions. HPLC-purified samples were digested and analyzed by Q-Tof mass spectrometry, and subsequent MS/MS ion searches were performed by Mascot. (A) Native untreated NGF eluent at 36.3 min and (B) eluent from peroxynitrite-treated NGF at 38.6 min, showing modified peptides that indicate the nitration of Tyr52 and Trp99. Similar modifications were observed for the eluent at 39.9 min from peroxynitrite-treated NGF (data not shown). Pyro-glutamic acid (Pyro-Glu) is a common modification of glutamine (Q) at the N-terminus of a peptide.

used to form 3-nitrotyrosine in proteins at alkaline pH. Under the conditions used, TNM induced only tyrosine nitration as evidenced by mass spectrometry (see below). NGF treated with TNM separated into two products eluting at 37.9 and 39.9 min (Fig. 8A). As revealed by mass spectrometry, TNM treatment increased the molecular weight of NGF Chain A by 90 Da (from 13,252 to 13,342 Da; Fig. 8B), suggesting that the main product was a doubly nitrated species. However, the singly nitrated species could also be observed at 13,297 Da (Fig. 8B). The same pattern was observed for Chain B of NGF (data not shown). Tandem mass spectrometry of the digested products identified Tyr52 and Tyr79 as the nitrated residues (Fig. 8C), which accounted for the total molecular weight change of 90 Da (Fig. 8B). Tryptophan nitration or 3,3′-dityrosine formation was not observed in TNM-treated NGF. The electrophoretic pattern of NGF treated with 40-fold excess of TNM was comparable to that observed in peroxynitrite-treated NGF, revealing the formation of NGF oligomers (Fig. 8D).

We then analyzed the effects of TNM-treated NGF on motor neuron survival. Similar to peroxynitrite-treated NGF, TNM-treated NGF induced 32% motor neuron loss in the absence of nitric oxide (Fig. 9A). Motor neuron death induced by TNM-treated NGF was also prevented by the addition of blocking antibodies to $p75^{NTR}$ (Fig. 9B), suggesting the triggering of the same apoptotic mechanism.

Both nitration and NGF oligomerization were found as common modifications between TNM- and peroxynitrite-treated NGF. To further determine if nitration was conferring NGF with the capability of inducing motor neuron death, NGF was treated with peroxynitrite in the presence of urate (NGF-$ONOO^-$–urate). Urate is particularly effective at inhibiting nitration by peroxynitrite [51] and prevented tyrosine nitration and oligomerization of NGF in a dose-dependent manner

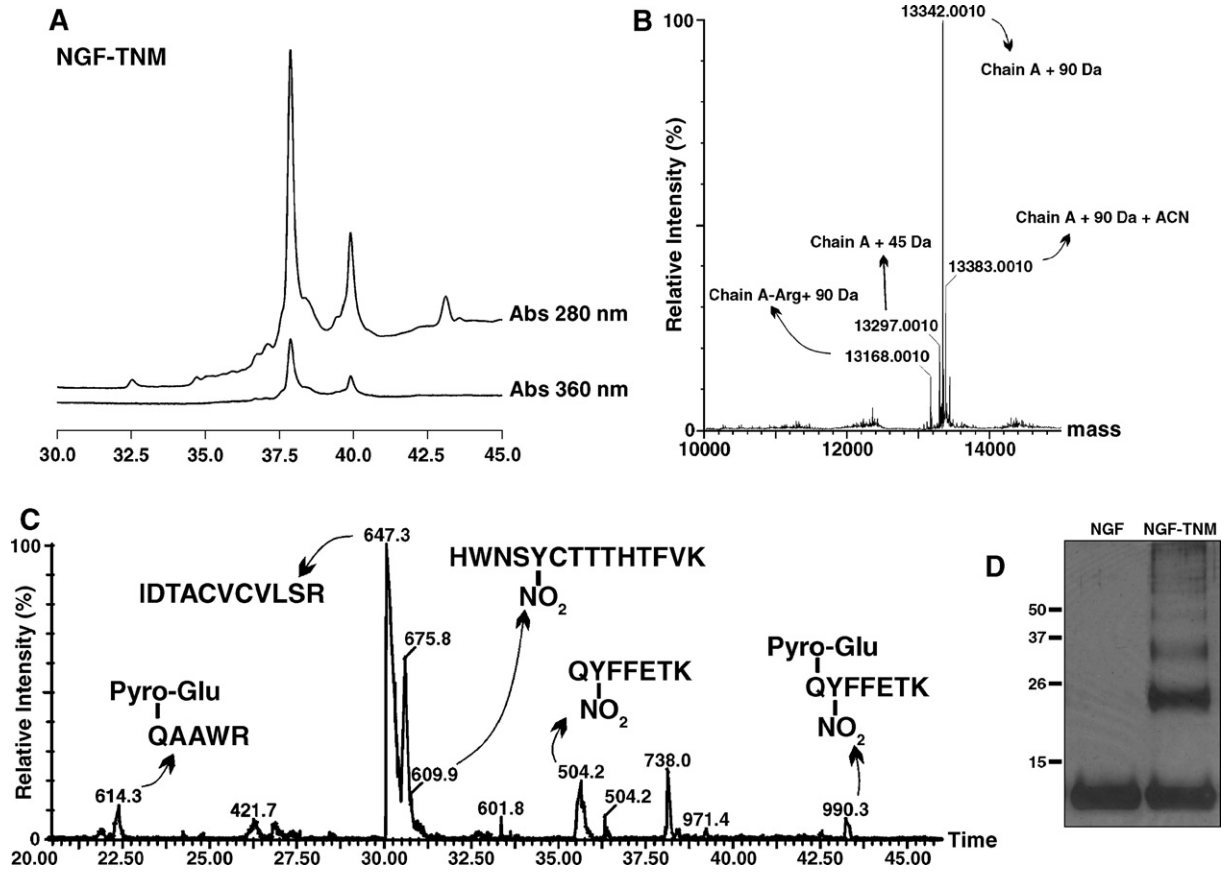

Fig. 8. Tetranitromethane treatment induced NGF nitration and oligomerization. (A) HPLC chromatogram of tetranitromethane (TNM)-treated NGF. NGF treated with 40-fold excess TNM eluted as two peaks at 37.9 and 39.9 min. Absorbance at 360 nm indicated the presence of nitrotyrosine in the eluted peaks. (B) Deconvoluted spectra of the eluent at 37.9 min showed a mass increase in NGF Chain A of 45 (to 13,297 Da) and 90 Da (to 13,342 Da) compared to that of native NGF (13,252 Da; Fig. 5A). The peak at 13,168 Da corresponded to doubly nitrated Chain A lacking the C-terminal arginine residue. The peak at 13,383 Da is consistent with the addition of two nitro groups to Chain A and the formation of ACN adducts. Deconvoluted spectra of the eluent at 39.9 min showed similar mass shifts for Chain B (data not shown). (C) Q-Tof mass spectrum of trypsin-digested eluent at 37.9 min indicated nitration of Tyr52 and Tyr79. Analysis of eluent at 39.9 min indicated the same modification on Chain B (data not shown). (D) SDS–PAGE showing the formation of high-molecular-weight species of NGF after the treatment with TNM (NGF-TNM). 100 ng of protein was analyzed in each lane and electrophoretic separation was performed in 15% polyacrylamide gels under denaturing and reducing conditions. A representative silver-stained gel is shown.

(Fig. 10A). Moreover, urate (200 μM) abolished the apoptotic effect of peroxynitrite-treated NGF in motor neuron cultures (Fig. 10B). As a control, 100 nM urate (the concentration expected to be present in the culture medium after adding 100 ng/ml NGF-$ONOO^-$–urate) did not prevent motor neuron loss induced by peroxynitrite-treated NGF (100 ng/ml) (Fig. 10B), implying that unreacted urate was not affecting motor neuron survival.

Discussion

Because motor neuron death and astrocyte reactivity in ALS have been associated with the increased production of reactive oxygen and nitrogen species [28,52,53], we investigated whether the oxidation or nitration of secreted NGF might enhance its apoptotic activity toward motor neurons. Oxidation of NGF by peroxynitrite *in vitro* increased the potency for inducing apoptosis of motor neurons by 10,000-fold in the presence of nitric oxide. To the best of our knowledge, this is the first report of a neurotrophin eliciting cell death in culture at physiologically relevant concentrations in the pg/ml range. Increased NGF levels have been implicated in the progressive death of motor neurons occurring in ALS [23–25]. We previously reported that spinal cord extracts from $SOD1^{G93A}$ ALS mice contain sufficient NGF to stimulate $p75^{NTR}$-dependent apoptosis of cultured motor neurons in the presence of an external source of nitric oxide [25]. However, the levels of NGF measured by ELISA in the degenerating spinal cord from $SOD1^{G93A}$ mice were in the range of pg/ml [25], a concentration 10,000 times lower than necessary for purified NGF to induce apoptosis in these motor neuron cultures and similar to the potency of peroxynitrite-treated NGF in the presence of an external source of nitric oxide.

In a previous study, nitration of NGF by TNM did not modify the biological activity of NGF as assessed by induction of neurite outgrowth in sensory ganglia [54]. The difference in the expression of NGF receptors may account for the apparent contradictory results. Sensory ganglia express both TrkA and $p75^{NTR}$ [55,56], whereas pure motor neuron cultures express $p75^{NTR}$ without detectable expression of TrkA [57]. Nitrated

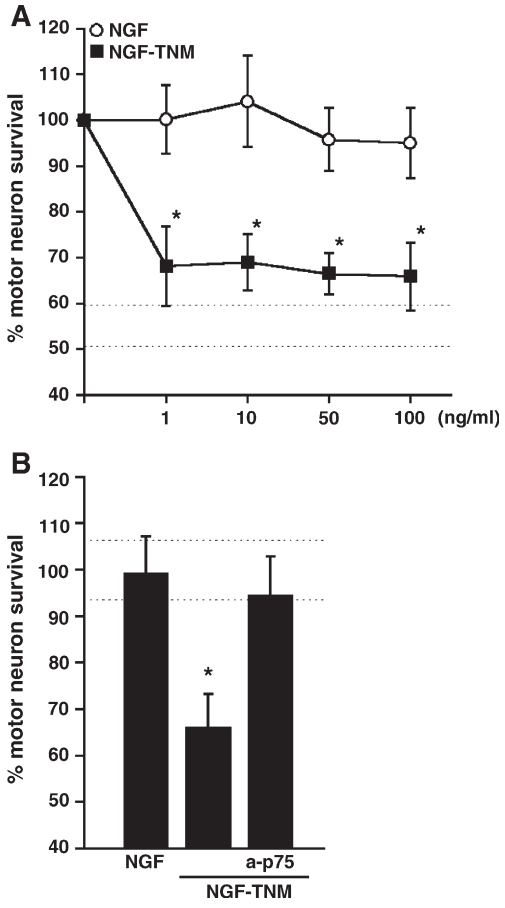

Fig. 9. Tetranitromethane-treated NGF induced $p75^{NTR}$-dependent motor neuron death. (A) Pure motor neuron cultures maintained with GDNF (1 ng/ml) were exposed to increasing concentrations of NGF previously treated with vehicle or 40-fold excess TNM (NGF-TNM). Dashed lines represent the SD of NONE (trophic factor deprivation). (B) Blocking antibodies to $p75^{NTR}$ (a-p75, 1:300; Chemicon AB1554) prevented motor neuron death induced by NGF-TNM (100 ng/ml). Antibodies to $p75^{NTR}$ were added with NGF-TNM 3 h after motor neuron plating. Dashed lines represent the SD of GDNF. Motor neuron survival was determined 48 h after treatment. Data are expressed as percentage of GDNF, mean±SD. *Significantly different from GDNF ($p \leq 0.05$).

NGF induced motor neuron apoptosis by a mechanism dependent on $p75^{NTR}$ signaling, as blocking antibodies to $p75^{NTR}$ or downregulation of $p75^{NTR}$ expression by antisense treatment completely prevented motor neuron death. Although peroxynitrite-treated NGF induced $p75^{NTR}$-dependent apoptosis in the absence of an external source of nitric oxide, motor neuron death was prevented by a general NOS inhibitor, as well as by the SOD mimetic and peroxynitrite decomposition catalyst MnTBAP. This is in agreement with our previous results showing that motor neuron death induced by native NGF requires increased nitric oxide production and peroxynitrite formation [25]. Furthermore, an external source of nitric oxide potentiates the apoptotic activity of peroxynitrite-treated NGF. Thus, peroxynitrite treatment most likely affects NGF apoptotic activity by altering the interaction with its receptors and not by affecting downstream events.

The gain of apoptotic activity by NGF was consistently associated with tyrosine nitration and abnormal oligomerization. Mouse NGF contains only two tyrosine residues at positions 52 and 79, both of which are solvent-accessible [43,54]. The hydroxyl group of Tyr79 is involved in hydrogen bonding interactions to Glu11 from the opposing NGF monomer (Fig. 1), which will reduce ionization of the hydroxyl group. Because ionization of the hydroxyl group enhances tyrosine oxidation to a radical, this may explain why Tyr79 was more resistant to nitration than Tyr52. The latter was readily nitrated by both peroxynitrite and TNM. Because Tyr52 is highly conserved and important for stabilizing NGF [45], its nitration may induce conformational changes in the protein that could facilitate aberrant protein interactions leading to oligomerization.

NGF oligomers ranged in size from dimers to octamers as determined by size-exclusion chromatography coupled to real-time MALS. When purified high-molecular-weight oligomers were subjected to SDS–PAGE, lower molecular weight oligomers and monomeric forms appeared, indicating non-covalent interactions (data not shown). These results can also explain the absence of the octamers observed by MALS in SDS–PAGE gels. However, both peroxynitrite and TNM treatments led to the formation of higher oligomers that were stable in SDS–PAGE gels, suggesting a non-thiol-dependent, covalent cross-linking of some subunits. Oligomerization of NGF was effectively prevented by urate. Urate is known to prevent peroxynitrite-induced tyrosine nitration by competing for carbonate and nitrogen dioxide radicals [58–60], suggesting radical formation is involved in the formation of oligomers. Although 3,3′-dityrosine cross-linking is one mechanism by which peroxynitrite can induce protein dimerization [61,62], larger oligomers would require at least two different tyrosine residues to be cross-linked. If cross-linking resulted only from the generation of tyrosine radicals, nitration of Tyr52 would inhibit oligomerization. Furthermore, we could not detect 3,3′-dityrosine formation. Therefore, oligomerization of NGF most likely involved other forms of cross-linking induced by peroxynitrite. In the presence of carbon dioxide, 30% of peroxynitrite forms carbonate radical plus nitrogen dioxide, which are both moderately strong oxidants that readily oxidize both tyrosine and tryptophan to form radicals [63]. Tyrosyl radicals can combine at near diffusion-limited rates with nitrogen dioxide to form 3-nitrotyrosine. Tryptophan oxidation yields multiple products, including *N*-formyl kynurenine and kynurenine, which can cross-link proteins [64]. On the other hand, tyrosyl radicals can also oxidize other amino acids, including tryptophan and cysteine, by intramolecular electron transfer reactions [64,65]. Within NGF, Tyr52 is spatially close to Trp21 (Fig. 1). Therefore, the oxidation of Tyr52 might transfer the radical to Trp21 and thereby facilitate covalent cross-links between NGF molecules.

Peroxynitrite-treated NGF potently stimulated $p75^{NTR}$-dependent apoptosis in motor neurons. Tyr52 and Trp21 in NGF are involved in the formation of the hydrophobic pocket that docks with the $p75^{NTR}$ cysteine-rich domain 2 [44]. Because Trp21 is a major part of the interface with $p75^{NTR}$, its oxidation products *in vivo* might form covalent adducts to $p75^{NTR}$ and facilitate aberrant apoptotic signaling. However, other receptors

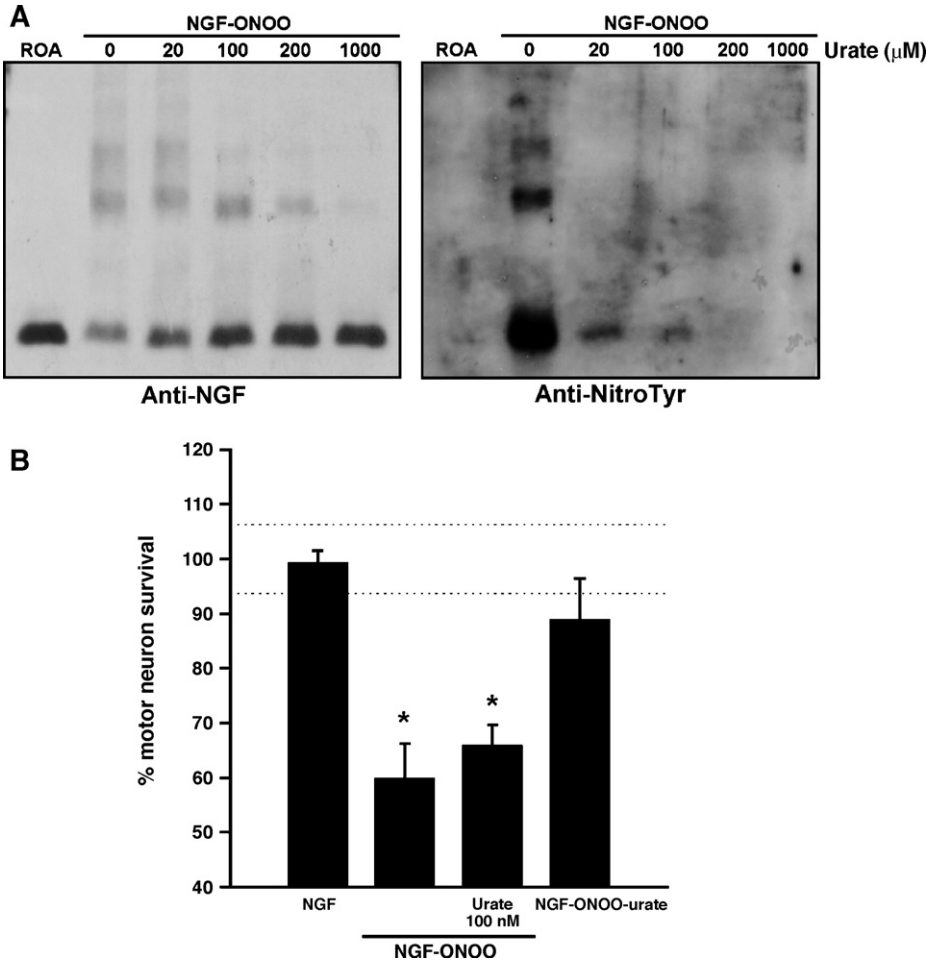

Fig. 10. Urate abolished the effects of peroxynitrite on NGF apoptotic activity. (A) Urate prevented, in a dose-dependent manner, tyrosine nitration and oligomerization of NGF. NGF was exposed to decomposed peroxynitrite (1 mM; ROA) or peroxynitrite (1 mM) in the presence of vehicle or increased concentrations of urate (20 to 1000 μM). Samples (100 ng) were analyzed by SDS–PAGE in a 15% polyacrylamide gel and Western blot using a polyclonal antibody to NGF (left) or nitrotyrosine (right). (B) Motor neuron cultures were exposed to NGF (100 ng/ml) previously treated with peroxynitrite (1 mM) in the presence of vehicle (NGF-$ONOO^-$) or urate (200 μM; NGF-$ONOO^-$–urate). To eliminate the possibility of a direct effect of unreacted urate on motor neuron survival, 100 nM urate, the concentration expected to be present in the culture medium after addition of NGF-$ONOO^-$–urate, was added to the cultures exposed to NGF-$ONOO^-$. Urate (100 nM) did not prevent motor neuron loss induced by NGF-$ONOO^-$ (100 ng/ml). Motor neuron survival was determined 48 h after treatment. Dashed lines represent SD of GDNF. Data are expressed as percentage of GDNF, mean ± SD. *Significantly different from GDNF ($p \leq 0.05$).

could also be involved in the induction of apoptosis by peroxynitrite-treated NGF. The precursor of NGF binds with lower affinity to $p75^{NTR}$ than NGF, but it forms a high-affinity signaling complex by simultaneously binding to $p75^{NTR}$ and sortilin [66]. In addition, $p75^{NTR}$ belongs to the TNFα receptor superfamily [67], and other members of this superfamily bind trimeric ligands and are known to require trimerization of their intracellular death domains for activation [68,69]. Similarly, NGF oligomers could recruit additional p75 receptors and thereby promote trimerization of its death domain and thus more strongly activate apoptotic signaling.

Because peroxynitrite treatment of NGF results in a gain of function, only a small fraction of nitrated protein is necessary to elicit apoptotic signaling. Peroxynitrite-oxidized NGF could be formed under pathological and inflammatory conditions under which NGF upregulation coincides with increased production of peroxynitrite and other nitrating species. Increased peroxynitrite formation may directly contribute to neuronal damage or inhibit NGF signaling by several mechanisms, including inactivation of TrkA receptors by nitration of their tyrosine residues [70]. However, the occurrence of oxidatively modified and nitrated NGF *in vivo* offers an exciting new mechanism by which neurotrophin signaling could be subverted under pathological conditions associated with increased oxidative stress.

Acknowledgments

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