Prevention of Peroxynitrite-induced Apoptosis of Motor Neurons and PC12 Cells by Tyrosine-containing Peptides*

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Although peroxynitrite stimulates apoptosis in many cell types, whether peroxynitrite acts directly as an oxidant or the induction of apoptosis is because of the radicals derived from peroxynitrite decomposition remains unknown. Before undergoing apoptosis because of trophic factor deprivation, primary motor neuron cultures become immunoreactive for nitrotyrosine. We show here using tyrosine-containing peptides that free radical processes mediated by peroxynitrite decomposition products were required for triggering apoptosis in primary motor neurons and in PC12 cells cultures. The same concentrations of tyrosine-containing peptides required to prevent the nitration and apoptosis of motor neurons induced by trophic factor deprivation and of PC12 cells induced by peroxynitrite also prevented peroxynitrite-mediated nitration of motor neurons, brain homogenates, and PC12 cells. The heat shock protein 90 chaperone was nitrated in both trophic factor-deprived motor neurons and PC12 cells incubated with peroxynitrite. Tyrosine-containing peptides did not affect the induction of PC12 cell death by hydrogen peroxide. Tyrosine-containing peptides should protect by scavenging peroxynitrite-derived radicals and not by direct reactions with peroxynitrite as they neither increase the rate of peroxynitrite decomposition nor decrease the bimolecular peroxynitrite-mediated oxidation of thiols. These results reveal an important role for free radical-mediated nitration of tyrosine residues, in apoptosis induced by endogenously produced and exogenously added peroxynitrite; moreover, tyrosine-containing peptides may offer a novel strategy to neutralize the toxic effects of peroxynitrite.

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Inflammatory pathological conditions, the increased production of nitric oxide and superoxide boosts the generation of reactive oxygen and nitrogen species, including peroxynitrite (1–10). Peroxynitrite is a strong oxidant formed by the diffusion-limited reaction of superoxide and nitric oxide (11, 12). It can oxidize biomolecules either by direct reactions or by peroxynitrite-derived radicals such as nitrogen dioxide and carbonate radicals (13–15). Peroxynitrite can affect cell metabolism by inducing lipid peroxidation (16), damaging DNA (17), and interfering with mitochondrial function (18). In addition, peroxynitrite affects protein activity, as illustrated by the inhibition of mitochondrial manganese superoxide dismutase (SOD)4 and tyrosine phosphatases (19–21), as well as the activation of Src kinase (22). The alterations in protein functions are probably caused by oxidative modifications of amino acid residues (23). In the case of the phosphatases and zinc thiolate-containing proteins, the oxidation of methionine and cysteine residues is critical for their inactivation (19, 20). In contrast, manganese SOD is inhibited by the nitration of a critical tyrosine residue near the active site (21, 24). Nitration has also been found in structural proteins such as neurlamin L, synuclein, actin, and tubulin, affecting their kinetic polymerization characteristics (25–30). Tyrosine nitration has attracted much attention as a particularly important modification of proteins by peroxynitrite (6, 14, 15, 31, 32), because it seems to be a universal marker for inflammation and has been detected in a large number of pathological conditions (6, 14, 15, 31, 32).

Deprivation of trophic factors induces motor neuron death by apoptosis both in vitro and in vivo (4, 33–46). Motor neuron apoptosis induced by trophic factor deprivation or Fas activation is mediated by the endogenous production of peroxynitrite (4, 35, 47). Apoptosis also results from incubation of PC12 cells with low concentrations of peroxynitrite (48–53). In motor
neuron studies, nitrotyrosine has often been used as a marker for peroxynitrite formation (4, 47); however, recent studies have shown that tyrosine nitration may occur independently of peroxynitrite (14, 15). Furthermore, it has also been suggested that physiologically relevant concentrations of peroxynitrite are not able to nitrate tyrosine residues (54–57), fueling further debate and further investigation over the mechanism underlying nitration and the role played by peroxynitrite and other reactive nitrogen species in normal and pathological conditions (58).

Although it is possible that the induction of apoptosis by peroxynitrite might be the result of general oxidative damage, growing evidence indicates that peroxynitrite interacts with specific intracellular pathways to induce apoptosis (47–50, 53, 59–61). In addition, the role of nitrotyrosine in peroxynitrite-mediated toxicity remains unknown. Using tyrosine-containing peptides, we investigated the role of peroxynitrite-mediated oxidation and tyrosine nitration in apoptosis induced in motor neurons by trophic factor deprivation and in PC12 cells by bolus addition of peroxynitrite. Tyrosine itself does not directly accelerate the rate of decomposition of peroxynitrite (23); therefore, the tyrosine-containing peptides should not affect the direct reactions of peroxynitrite but act instead as competitive targets for the nitrating radical intermediates derived from peroxynitrite (13, 62). Our results reveal an important role for free radical-dependent processes in protein nitration and apoptosis induced by endogenous and exogenous peroxynitrite.

**MATERIALS AND METHODS**

*Peroxynitrite Synthesis*—Peroxynitrite was synthesized by infusing acidified hydrogen peroxide with sodium nitrite (63). Residual hydrogen peroxide was eliminated with manganese dioxide, and peroxynitrite concentration was determined spectrophotometrically using $\varepsilon_{302} = 1700 \text{ M}^{-1} \text{ cm}^{-1}$. The concentration of peroxynitrite was measured before each experiment.

*PC12 Cell Culture and Treatment*—PC12 cells were cultured on collagen-coated dishes in RPMI medium (Invitrogen), supplemented with 10% horse serum, 5% FetalClone II (HyClone, Logan, UT), and antibiotics. For viability assays, the cells were plated in the same medium at a density of 1.44 $\times 10^5$ per 35-mm dish and incubated overnight. Following three washings with warm PBS, the cultures were incubated with peroxynitrite (0.5 mm) for 3 min in 1 ml of 50 mM phosphate-buffered saline (90 mM NaCl, 5 mM KCl, 0.8 mM MgCl$_2$, 1 mM CaCl$_2$, pH 7.2). Five minutes after the peroxynitrite was added, the buffer was replaced by complete RPMI medium. Viability was determined using fluorescein diacetate/propidium iodide (Molecular Probes, Eugene, OR) 18 h after exposure to peroxynitrite (48–50). For the peptide experiments, the cells were incubated with the peptides for 5 min before and during exposure to peroxynitrite. A number of tyrosine-containing peptides, and their controls replacing the tyrosine for phenylalanine, proline, and tryptophan, were used to perform the experiments. The RYEYA was designed to provide the maximum probability of tyrosine nitration after the analysis of the sequences nitrated in neurofilament L (29). The sequences EYTR and EYTA provide sequences with different net charge, which it is expected to result in different nitration efficiency (64). The dipeptide GY was used because free nitrotyrosine is known to induce motor neuron apoptosis (65). The di- and tri-tyrosine homopeptides (Sigma) were used to test whether increasing the number of tyrosine residues despite the sequence provides more protection against peroxynitrite.

*Caspase 3 Activity*—Caspase 3 activity was measured using the caspase 3 colorimetric assay kit from R&D Systems according to the manufacturer’s directions (R&D Systems, Minneapolis, MN). Briefly, $2 \times 10^7$ cells in a 100-mm dish were processed and incubated as described above. The cells were then collected in the culture media and centrifuged at $500 \times g$ for 5 min. After washing once with PBS, the cells were resuspended in lysis buffer to have a concentration of $10^6$ cells/25 $\mu$l. The protein concentration was measured using the BCA assay (Pierce). Two hundred $\mu$g of protein were used to perform the reaction. The product was measured at 405 nm after 2 h of incubation at 37 °C.

*Motor Neuron Isolation and Culture*—Rat embryo motor neurons were first purified using a combination of cushion centrifugation and immunoaffinity and were then cultured in Neurobasal medium enriched with glutamine, glutamate, $\beta$-mercaptoethanol, and B27 supplement (Invitrogen), as described previously (4, 33–35). More than 95% of the cells were immunoreactive for p75 neurotrophin receptor and Islet1, two early markers of motor neurons (66–68).

*Motor Neuron Peptide Treatment and Determination of Cell Survival*—For survival studies, 1500 motor neurons were plated in a mixture of 100 $\mu$l of PBS containing the peptides (Open Biosystems, Huntsville, AL; Sigma), the permeation agent Chariot (Active Motif, Carlsbad, CA), and 100 $\mu$l of Neurobasal medium (Invitrogen), according to the manufacturers’ instructions. Briefly, the peptides were prepared in 10 mM stock solutions in water and diluted in PBS to the concentrations indicated. Fifty $\mu$l of the peptide was incubated for 30 min at room temperature with 2 $\mu$l of a 1:10 dilution of Chariot and 48 $\mu$l of H$_2$O. The mixture was then added to 100 $\mu$l of motor neuron-containing Neurobasal medium and plated in well plates precoated with polyornithine/lamin. After 1 h of incubation at 37 °C in a humidified CO$_2$ incubator, supplements were added to 200 $\mu$l of the complete Neurobasal medium to reach a final volume of 400 $\mu$l. The cultures were then incubated for an additional 24 h, after which motor neuron survival was determined by counting all neurons with neurites longer than 3 soma diameters, as described previously (4). The survival of motor neurons cultured with a combination of brain-derived neurotrophic factor (BDNF, 1 ng/ml), glial-derived neurotrophic factor (GDNF, 0.1 ng/ml), and cardiotrophin 1 (CT-1, 10 ng/ml) was considered 100% and was used to standardize the experiments. The neurons determined to be alive by this method also were found to stain for the vital dye fluorescein di-acetate. For dot blot analysis, 50,000 motor neurons were plated in 200 $\mu$l of PBS and Chariot plus 400 $\mu$l of Neurobasal medium. After 1 h of incubation at 37 °C in a CO$_2$ (5%) humidified atmosphere, 1.4 ml of Neurobasal medium was mixed with enough supplement to reach the requisite volume and then was incubated for an additional 16 h. Proteins were harvested, and the dot blot was processed, as described previously (65).
Intracellular Delivery of Superoxide Dismutase—Human superoxide dismutase and pH-sensitive liposomes were prepared and incubated with motor neurons as described previously (35, 69). Two-day-old motor neurons were plated in 2-well chamber slides (Nalge Nunc International, Rochester, NY) and incubated with the liposomes by replacing the culture medium with 1.5 ml of fresh medium deficient in serum and glutamate but supplemented with penicillin and streptomycin. A liposome suspension (3.5 μl) corresponding to 50 mmol lipid/cm² was added to each well.

Nitrotyrosine Immunofluorescence in Motor Neurons—After brief fixation with a mixture of 4% paraformaldehyde, 0.1% glutaraldehyde in PBS on ice for 15 min, immunofluorescence for nitrotyrosine was performed and quantified in motor neuron cultures using arbitrary fluorescence units (AFU) (4). The cells were then rinsed with PBS, permeabilized with Triton X-100, blocked for 1 h with 10% goat serum, 2% bovine serum albumin plus 0.1% Triton X-100 in PBS, and incubated overnight at 4 °C with polyclonal nitrotyrosine antibody (1:500 (70 –72)). After being rinsed with PBS, the cells were incubated for 30 min with Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) at room temperature. The incubation with the secondary antibody was ended by rinsing with PBS and distilled water, followed by mounting the slides in Prolong antifade (Molecular Probes, Eugene, OR). The intensity of nitrotyrosine immunofluorescence was quantified in 100 –200 randomly selected motor neurons per condition. Images were captured using an IX70 Olympus microscope equipped with an OlymPix CCD camera connected to the UltraView system (PerkinElmer Life Sciences). The background immunofluorescence was determined as the fluorescence intensity detected in slides incubated with the nitrotyrosine antibody in the presence of 100 μM nitrotyrosine-containing peptide (alanyl-3-nitrotyrosyl-glycine) or 5 mM nitrotyrosine. The values were obtained from at least five independent experiments, and at least 15 neurons were quantified per slide.

Western and Dot Blotting Analysis—Western blots for caspase 3 were performed as described previously (53). Briefly, three 100-mm dishes were used per group. The cells were incubated with peroxynitrite (0.5 mM) as described above with or without peptides (0.5 μM). The cells were then harvested and rinsed once with PBS. Cold lysis buffer was added to the cell pellet, and the suspension was sonicated. The proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was cut at the 25-kDa level. The lower part was processed for cleaved caspase 3 (1:1000; Cell Signaling 9661) and the upper part for caspase 3 (1:2000; Cell Signaling 9506). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:40,000; Bio-Rad). Dot blot quantitation of nitrotyrosine in motor neurons was performed by harvesting total protein from 30,000 motor neurons plated in a 35-mm dish for 24 h. Samples containing equal amounts of protein were blotted on a nitrocellulose membrane by gravity flow using a Bio-Blot microfiltration apparatus (Bio-Rad). Membranes were then processed as described for Western blot using rabbit polyclonal primary antibody to nitrotyrosine as described previously (70). Immunoreactivity was visualized with SuperSignal® Western blot kit (Pierce) according to the manufacturer’s instructions. The intensity of immunoreactivity in the blots was quantified with a ChemiDoc gel documentation system with the Quantity One 1-D Analysis software (Bio-Rad). Brain homogenates (2 mg of protein/ml of PBS) were incubated with 0.5 mM peroxynitrite alone or together with peptides. Proteins (15 μg) were separated by electrophoresis in a 12% SDS-acylamide gel and then transferred to a nitrocellulose membrane. Western blot was performed as described previously (70) using the rabbit polyclonal anti-nitrotyrosine antibody (1:2000). Nitrotyrosine antibody was detected using a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad; 1:10,000), which was visualized using the SuperSignal® Western blot kit (Pierce) according to the manufacturer’s instructions. Nitrated proteins in PC12 cells were analyzed by incubating 2 × 10⁷ cells in 500 μl of PBS supplemented with 0.5 mM peroxynitrite. Nitrated proteins (7.5 μg) from the cells were processed using a slightly modified method already described for brain homogenates; the 1A2.9 mouse monoclonal anti-nitrotyrosine antibody was used and that antibody was detected with a horseradish peroxidase-conjugated secondary goat anti-mouse antibody (Bio-Rad; 1:10,000).

Immunoprecipitation and Mass Spectrometry Analysis of Proteins—The immunoprecipitation of nitrated proteins in PC12 cell (2 × 10⁷) homogenates was performed using polyclonal nitrotyrosine antibodies linked to AminoLink® gel (Pierce) immediately after incubation with 0.5 mM peroxynitrite. After separation of the proteins by SDS-PAGE (12%), the proteins were transferred to nitrocellulose membranes for Western blot analysis or stained in-gel using GelCode® Blue (Pierce). Stained gels were used for mass spectrometry analysis (MALDI-TOF) of trypsinized peptides at the University of Alabama at Birmingham Mass Spectrometry Facility. The protein was identified by analysis of the tryptic fragments and comparison with the NCBI data base. The procedure used was adapted from that developed at University of California, San Francisco. Briefly, the bands were excised from a polyacrylamide gel, washed three times with 50% acetonitrile, 25 mM ammonium bicarbonate, vacuum-dried, rehydrated in 25 mM ammonium bicarbonate, and digested overnight with trypsin, and the peptides were extracted with 50% acetonitrile, 5% formic acid (v/v). The extracts were concentrated and re-dissolved in 10 μl of 50% acetonitrile, 5% formic acid (v/v). The extracted peptides were mixed with the matrix α-cyano-4-hydroxyanilic acid (Sigma), spotted onto a MALDI plate, and analyzed with a Voyager DE-Pro mass spectrometer (Applied Biosystems, Foster City, CA). After de-isotoping, the peptide masses were entered into Mascot data base search engine, and the NCBI data base was searched to identify the protein. MOWSE scores above 73 were considered statistically significant.

Antibodies—Western blots and immunoprecipitations were performed using the following antibodies: mouse monoclonal β-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000 Western blot, 1:100 immunoprecipitation), rabbit polyclonal actin antibody (Santa Cruz Technology; 1:2000 Western blot, 1:100 immunoprecipitation), rabbit polyclonal antibody to heat shock protein 90 (Santa...
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Cruz Biotechnology; 1:1000 Western blot; 1:100 immunoprecipitation). The anti-nitrated HSP90 was prepared and characterized as described in the Supplemental Materials. All Western blots were developed using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad; 1:10,000) with the SuperSignal® Western blot kit.

Kinetic Studies—The kinetics of peroxynitrite decomposition as well as nitrotyrosine and nitrotryptophan formation were studied using a stopped-flow spectrophotometer set at 302, 430, and 400 nm, respectively (Applied Photophysics, SF 1.7MV, Leatherhead, UK) (73). Reactions were carried out in 0.1 M sodium phosphate buffer and 100 μM DTPA (pH 7.4) at 37 °C. The kinetic traces were fitted to a first-order reaction equation using the software provided with the spectrophotometer to determine the first-order kinetic constants (kobs (s⁻¹)). A typical run consisted of 400 points collected over 0.1–10-s range period, when more than 99.9% peroxynitrite had disappeared. Reported values are the averages of at least seven separate determinations. Time-dependent spectra were obtained with a fast-response photodiode array (Applied Photophysics, Leatherhead, UK) coupled to the stopped flow. In the kinetic experiments nitrotyrosine concentration was calculated using the absorbance coefficient of the phenolate (ε_{430} = 4400 M⁻¹ cm⁻¹) and the pKₐ = 7.5 of the phenol (74). Nitration yields were calculated with respect to the initial peroxynitrite concentration. Peptide nitration was further studied in 0.2 M sodium phosphate buffer and 100 μM DTPA at room temperature, and nitrotyrosine yields were determined by measuring the absorbance (ε_{430} = 4400 M⁻¹ cm⁻¹) at pH > 10 and subtracting the absorbance at pH < 4 (74). Nitrotryptophan formation was measured at 400 nm (ε_{400} = 5200 M⁻¹ cm⁻¹) (75). Nitration yields were calculated with respect to the initial peroxynitrite concentration. Dityrosine formation was studied obtaining the emission scan (λ = 370–430 nm) of the samples when excited at λ = 315 nm (Amino-Bowman Series 2 Spectrometer, Spectronic Unicam, Rochester, NY). Buffers containing bicarbonate were prepared immediately before the experiment to minimize diffusion of carbon dioxide out of the solution.

Quantification of Thiols—Thiols were quantified using 5,5’-dithiobis(2-nitrobenzoic acid) (ε_{412} = 1.36 × 10⁴ M⁻¹ cm⁻¹) (76). The reaction of peroxynitrite with thiols in vitro was performed by incubating 3 mM glutathione with 0.5 mM peroxynitrite in a 100 mM sodium phosphate buffer and 100 μM DTPA, pH 7.2, at room temperature. To test the effect of peroxynitrite on the cellular concentrations of reduced glutathione, PC12 cells were rinsed with PBS, before resuspending them at a density of 7 × 10⁵ cells in 100 μl of PBS. The cells were then incubated with 0.5 mM peroxynitrite and centrifuged at 500 × g for 1 min. Glutathione was extracted by resuspending the pellet in 200 μl of 20 mM sulfosalicylic acid and then centrifuged it at 13,000 × g for 10 min. Forty μl of each sample per triplicate was transferred to a well of a 96-well plate, and 160 μl of 0.2 M Tris, 2 mM EDTA, pH 8.0, was added. After 10 min of incubation with 10 μl of DTNB, the absorbance at 412 nm was measured using a microplate reader (77).

Statistical Analysis—For statistical analysis, analysis of variance (all groups compared by Bonferroni test) and the Kruskal-Wallis nonparametric tests (all pairs compared by Dunn’s test) were used. All tests were performed using the program Prism (GraphPad Software Inc., San Diego).

RESULTS

Prevention of Peroxynitrite-induced PC12 Cell Death by Tyrosine-containing Peptides—Peptides containing tyrosine greatly reduced apoptosis of PC12 cells induced by peroxynitrite, whereas peptides containing phenylalanine had no effect. To test the role of tyrosine-containing peptides on apoptosis induced by a bolus addition of peroxynitrite and other oxidants, we used PC12 cells because peroxynitrite induces apoptosis in this cell line (48–53). PC12 cells were incubated with the peptides in PBS for 5 min before and during exposure to peroxynitrite. The best protection was provided by the tetrapeptide Glu-Tyr-Thr-Arg (EYTR), which blocked peroxynitrite-induced apoptosis at a concentration of 250 μM with an EC₅₀ ~ 100 μM (Fig. 1C). When the carboxyl-terminal arginine of that peptide was replaced by alanine, the protection it afforded at a concentration of 250 μM was halved, although the EC₅₀ remained approximately the same (Fig. 1C). When tyrosine was replaced by tryptophan in this peptide sequence, maximum protection was provided at 500 μM with an EC₅₀ ~ 250 μM (Fig. 1C). When tyrosine was replaced by phenylalanine, no protection was afforded at all (Fig. 1C).

To further determine the relevance of tyrosine in the sequence, we next tested the effects of more complex peptides on peroxynitrite-induced apoptosis. The pentapeptide RYEYA prevented the death of ~50% of the cells after a bolus addition of peroxynitrite at a 500 μM concentration, with an EC₅₀ ~ 250 μM. When the tyrosine residues in the peptide were replaced by tryptophan, the maximum protection showed a tendency to decline, but the difference did not achieve statistical significance (Fig. 1B). Replacing the tyrosine residues with phenylalanine or proline resulted in peptides that provided no protection against peroxynitrite toxicity (Fig. 1B). When incubated with PC12 cells, tyrosine and the di- and tri-tyrosine homopeptides all provided about the same protection against peroxynitrite-induced cell death (Fig. 1A). Free tyrosine provided the maximum protection at 500 μM concentration, with an EC₅₀ ~ 100 μM. The maximum protection provided by the di- and tri-tyrosine peptides, which was achieved at a concentration of 250 μM with an EC₅₀ ~ 100 μM, still fell short of that afforded by tyrosine alone. The dipeptide GY provided maximum protection from peroxynitrite-induced PC12 cell apoptosis at concentrations between 500 μM and 1 mM with an EC₅₀ ~ 250 μM (Fig. 1A). The dipeptide GF and the phenylalanine tripeptide did not prevent PC12 cell death (Fig. 1A). Incubation of the cells with the peptides after a bolus addition of peroxynitrite in the same conditions did not prevent cell death (data not shown).

These results indicate that the protective character of the peptides resides in the tyrosine residues. In addition, the results showing that tryptophan is also protective indicate that the mechanism of protection should be some common characteristic shared by both amino acid residues. Interestingly, both amino acids react and are nitrated by peroxynitrite-derived...
radicals. Incubation of PC12 cells with 30 μM of hydrogen peroxide for 24 h reduced cell viability by ∼25%. Hydrogen peroxide toxicity was not affected by tyrosine-containing peptides at 500 μM concentrations (Fig. 2).

**Effect of Tyrosine-containing Peptide on Caspase 3 Activation**—Peroxynitrite can induce apoptosis or necrosis depending on the intensity of the insult (48, 78). To determine whether the effect of the peptides on peroxynitrite toxicity was mediated by inhibition of apoptosis, activation of caspase 3 was tested. The RYEYA, but not the RFEFA, peptide blocked peroxynitrite-induced caspase 3 cleavage (Fig. 3A) and activation (Fig. 3B). In contrast the peptides did not affect activation of caspase 3 by hydrogen peroxide (Fig. 3B) or staurosporine (Fig. 3C), indicating that the peptides were not directly inhibiting apoptotic pathways.

**Effect of Tyrosine-containing Peptides on Protein Nitration and Identification of Nitrated Proteins**—As concentrations of tyrosine-containing peptides increased, the levels of protein nitration by pure peroxynitrite decreased in PC12 cells (Fig. 4, A and B) and brain homogenates (Fig. 4C). The protection against nitration was not dependent in the sequence of the peptides but in the presence of tyrosine, which supports the concept that the peptides are competing with the target proteins for the nitrating species.

Incubation of PC12 cells with peroxynitrite resulted in the nitration of a discrete number of proteins (Fig. 4, A and B, and supplemental Fig. 1A). To identify the nitrated proteins, homogenates of PC12 cells exposed to peroxynitrite were immunoprecipitated using polyclonal antibodies against nitrotyrosine and separated by SDS-PAGE. The resulting bands were trypsinized, and the fragments were identified by MALDI-TOF mass spectrometry. Four proteins were identified as β-tubulin, actin, elongation factor 1, and heat shock protein 90 (HSP90) (Table 1). The nitration of β-tubulin, actin, and HSP90 was confirmed by immunoprecipitation followed by Western blot analysis (supplemental Fig. 1A). Because the peptides completely inhibited nitration by peroxynitrite (Fig. 4, A and B) and the identified nitrated proteins are intracellular (Table 1), it can be concluded that the tyrosine-containing peptides inhibited the nitration of intracellular proteins by peroxynitrite.
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**FIGURE 3. Effect of tyrosine-containing peptides on the activation of caspase 3 by peroxynitrite, hydrogen peroxide, and staurosporin.** A, Western blot analysis of caspase 3. PC12 cells were incubated with peroxynitrite as described previously and incubated in for additional 16 h. The protein was resolved by SDS-PAGE and analyzed by Western blot analysis for caspase 3 and cleaved caspase 3. Salt solution (Control); peroxynitrite (ONOO⁻, 0.5 mM); RYEYA (0.5 mM); RFEFA (0.5 mM). B, effects of tyrosine-containing peptides on caspase 3 activity. PC12 cells were exposed to peroxynitrite (1 mM) or hydrogen peroxide (150 μM) for 3 min in the presence or absence of the indicated peptides and incubated for 16 h. Control cultures (dotted bars) were incubated in the salt solution but received no treatment; peroxynitrite (1 mM) (filled bars); hydrogen peroxide (H₂O₂, 150 μM) (empty bars); RYEYA (0.5 mM); RFEFA (0.5 mM). *, p < 0.01 versus control; **, p < 0.01. The values are the mean ± S.D. of at least three independent experiments. C, effect of tyrosine-containing peptides on caspase 3 activity induced by staurosporin. PC12 cells were incubated for 16 h with staurosporin in the presence or absence of the indicated peptides. Staurosporin (STS, 1 μM; filled bars); RYEYA (0.5 mM); RFEFA (0.5 mM). Controls (dotted bars) were performed by adding the peptides to nontreated cultures. Values are the mean ± S.D. of at least three independent experiments. *, p < 0.001 versus control.

**TABLE 1 Identification of nitrated proteins by MALDI-TOF analysis of tryptic peptides**
Protein from peroxynitrite-treated PC12 cells were immunoprecipitated with an antibody to nitrotyrosine and separated by SDS-PAGE. The gels were stained with GelCode Blue as shown in supplemental Fig. 1. The protein bands were excised and subjected to in-gel trypsin digestion followed by MALDI-TOF mass spectrometry for peptide mass fingerprint analysis. The peptide masses were matched using the Mascot database search engine.

| Protein       | Score | Peptides matched |
|---------------|-------|-----------------|
| β-Tubulin     | 103   | 8               |
| β-Actin       | 123   | 7               |
| Elongation factor 1α-S | 122 | 8               |
| Heat shock protein 90 | 131 | 9               |

**FIGURE 4. Effect of tyrosine-containing peptides on protein-tyrosine nitration.** A, PC12 cells were incubated with peroxynitrite and the nitration analyzed by Western blot using the 1A2.9 mouse monoclonal anti-nitrotyrosine antibody. Decomposed peroxynitrite (H); peroxynitrite (ONOO⁻, 0.5 mM); peroxynitrite plus 0.1 mM peptide (lane 1); peroxynitrite plus 0.5 mM peptide (lane 2), and peroxynitrite plus 1 mM peptide (lane 3). B, PC12 cells were treated like in A, but the Western blot was developed using the 4709 rabbit polyclonal anti-nitrotyrosine antibody. C, brain homogenates were incubated with peroxynitrite alone and the indicated peptide at the indicated concentration. Peroxynitrite (ONOO⁻, 0.5 mM); peroxynitrite plus 0.1 mM peptide (lane 1); peroxynitrite plus 0.5 mM peptide (lane 2); peroxynitrite plus 1 mM peptide (lane 3) concentrations.

**Prevention of Motor Neuron Death by Tyrosine-containing Peptides**—To further investigate the role of peroxynitrite and potentially tyrosine nitration in motor neuron apoptosis,
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Trophic factor-deprived motor neurons were incubated with tyrosine-containing peptides in the presence of the membrane-permeating agent Chariot to facilitate intracellular access. Regardless of size or sequence, all peptides containing tyrosine prevented motor neuron apoptosis in a concentration-dependent manner (Fig. 5A), but sequence and size did factor into their efficacy. The most efficient of the peptides tested was the pentapeptide Arg-Tyr-Glu-Tyr-Ala (RYEYA), which prevented the death of approximately half of the motor neurons 24 h after plating at a concentration of 100 \(\mu M\) (Fig. 5A). In contrast, the tetrapeptide Glu-Tyr-Thr-Ala (EYTA) prevented the death of about 35% of the motor neurons at a 500 \(\mu M\) concentration (Fig. 5A). When the tyrosine residues of either of these peptides were replaced by phenylalanine, they ceased to have any effect on motor neuron survival. To test whether the protective effect was due only to the amount of tyrosine, homopeptides containing different numbers of tyrosines were used. Homopeptides containing two and three tyrosine residues prevented motor neuron apoptosis induced by trophic factor deprivation in a concentration-dependent manner (Fig. 5B), with the maximum effect achieved at a concentration of \(~250 \mu M\). Dipeptide glycyly-tyrosine (GY) but not glycyly-phenylalanine (GF) also prevented motor neuron apoptosis induced by trophic factor deprivation in a dose-dependent manner, with \(EC_{50} \approx 200 \mu M\) and maximum protection provided at a concentration of 500 \(\mu M\) (Fig. 5B). Neither Chariot nor the peptides alone protected motor neurons from apoptosis (not shown). No effect on cell survival was observed after the incubation of motor neurons with peptides of the same sequence containing nitrotyrosine instead of tyrosine (not shown). However, incubation of motor neurons with free nitrotyrosine induced motor neuron apoptosis as described previously (65). Because only peptides containing tyrosine, despite size or sequence, prevented motor neuron death, it can be concluded that the protective capacity of the peptides resides in the tyrosine.

Peroxynitrite-dependent Tyrosine Nitration in Motor Neurons—Motor neurons cultured without trophic factors present increased levels of protein nitrotyrosine (3). To test the mechanism of tyrosine nitration in trophic factor-deprived motor neurons, the intensity of nitrotyrosine immunofluorescence was measured in isolated motor neurons chosen at random under differential interference contrast. Intracellularly delivered SOD blocked the increase in nitrotyrosine immunoreactivity induced by trophic factor deprivation (Fig. 2A; 2.7 \pm 0.7 \(versus\) 16.5 \pm 3.7 AU; SOD \(versus\) trophic factor deprivation, respectively, mean \pm S.D.; \(p < 0.001\) Dunn's test). In contrast, the addition of much larger amounts of extracellular SOD (250 units/ml) did not affect nitrotyrosine levels (17.3 \pm 4.1 AU). The superoxide and peroxynitrite scavengers, manganese tetrakis(4-benzoic acid) porphyrin (100 \(\mu M\)) and iron 5,10,15,20-tetrakis-4-carboxyphenyl porphyrin (10 \(\mu M\)), prevented motor neuron death (4, 65) and decreased nitrotyrosine immunoreactivity triggered by trophic factor deprivation (Fig. 6A; 3.2 \pm 0.9 and 2.7 \pm 0.5 AU, respectively; \(p < 0.001\) scavengers \(versus\) trophic factor deprivation). Nitrotyrosine immunoreactivity can also be reduced by the nitric-oxide synthase inhibitor nitro-l-arginine methyl ester (Fig. 6B; l-NAME (100 \(\mu M\)); 2.72 \pm 0.52 AU; \(p < 0.001\) l-NAME \(versus\) trophic factor deprivation). The reduction of tyrosine nitration by l-NAME was reversed by low concentrations of exogenous nitric oxide (\(~100 \mathrm{mM}\) steady state concentration) produced by DETANONOate (20 \(\mu M\)) (Fig. 6B; 13.89 \pm 2.01 AU; \(p < 0.001\) DETANONOate \(versus\) l-NAME) but not by 40 \(\mu M\) nitrite alone or in combination with 40 \(\mu M\) of hydrogen peroxide (Fig. 6B). The results strongly suggest that the nitration of tyrosine residues in trophic factor-deprived motor neurons is mediated by peroxynitrite, as reported previously for the induction of apoptosis in similar conditions (4, 35) and not by intracellular peroxidases.

Inhibition of Tyrosine Nitration in Motor Neurons by Tyrosine-containing Peptides—The increase in tyrosine nitration in trophic factor-deprived motor neurons was reversed by incubation with either trophic factors or 500 \(\mu M\) RYEYA, but such reversal was not seen with a similar concentration of RFEFA (Fig. 7, A and B). These results support the observation that the presence of tyrosine in the peptide is important to prevent both nitration and cell death.
Because the role of heat shock proteins in regulating apoptosis is well established (79), and HSP90 was identified to be nitrated in PC12 cells after incubation with peroxynitrite, a monoclonal antibody that recognizes nitrated HSP90 but not HSP90 was developed (supplemental Figs. 2 and 3). Western blot analysis of protein from cultured motor neurons showed increased immunoreactivity for nitrated HSP90 in trophic factor-deprived motor neurons compared with motor neurons cultured with trophic factors (Fig. 8), indicating that at least one of the identified proteins is nitrated by both exogenously added and endogenously produced peroxynitrite in at least one common tyrosine residue.

**Mass Spectroscopic Analysis of the Peptide Modifications**

Mass spectroscopy analysis of the RYEYA and EYTR peptides before and after the addition of peroxynitrite in the presence of PC12 cells showed that the peptides were nitrated, but the evidence of 3,3'-dityrosine formation was not obvious (supplemental Figs. 4–7), suggesting that the resulting modification cannot be resolved by this method or, more likely, that the

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formation of dityrosine is a minor modification in the presence of the cells.

Kinetics of Peroxynitrite Decomposition and the Formation of Nitrated Products—To better understand the anti-apoptotic effects of tyrosine-containing peptides, the kinetics and product yield of the reaction of different peptides with peroxynitrite were investigated. At physiological pH and in the absence of target molecules, 30% of the peroxynitrite decays by proton-catalyzed homolysis to hydroxyl (·OH) and nitrogen dioxide radicals (NO2·) with the rest isomerizing directly to nitrate (NO3−) (2, 7, 16, 80). The kinetics of peroxynitrite decomposition in the presence of tyrosine-containing peptides was studied under pseudo-first-order conditions (pH 7.36 ± 0.03; 37 °C). Peroxynitrite (0.5 mM) decay followed first-order kinetics with a constant (kobs) rate of 1.13 ± 0.01 s−1. The rate of peroxynitrite decomposition was not increased by the addition of peptides containing tyrosine, proline, or phenylalanine, indicating that peptides do not react directly with peroxynitrite (Fig. 9A and Table 2) but rather with the radicals derived from its homolysis. Indeed, the incubation of tyrosine-containing peptides with peroxynitrite was accompanied by a first-order increase in the absorbance at 430 nm (Fig. 9B and Table 2), indicating the formation of 3-nitrotyrosine. Pseudo-first-order constants for the formation of 3-nitrotyrosine were equal to those for peroxynitrite decay, further confirming that there is no direct reaction between the tyrosine-containing peptides and peroxynitrite. The substitution of tyrosine by phenylalanine or proline in RYEYA suppressed the increase in absorbance at 430 nm, but in EFTA, EFTR, and GF, a smaller and slower increase in absorbance was also observed (Fig. 9B and Table 2). In the peptides containing tryptophan, an increase in absorbance at 400 nm was observed, indicative of nitrotryptophan formation (Table 2), that occurred with higher rates, in agreement with peroxynitrite direct reaction with tryptophan (75). Because carbon dioxide is known to enhance the rate of peroxynitrite decomposition as well as the rate and yield of the nitration of aromatics (81, 82), the spectra of the GY reaction with peroxynitrite were studied using stopped-flow spectrophotometry (Fig. 9, C and D). An increase in absorbance in the 400–450 nm range was observed by the addition of sodium bicarbonate (Fig. 9D).
Quantitation of this increase at 430 nm showed the absorbance was augmented by 2.5 times (Fig. 9, C and D). The efficiency of nitration of the peptides in phosphate buffer was ~6% for all the peptides containing tyrosine and yields where about 4% when tyrosine was substituted by tryptophan (Fig. 9E). Surprisingly, in the presence of bicarbonate the nitration efficiency of the tetrapeptide EYTA was ~25% less than when the carboxyl-terminal alanine was substituted by arginine (Fig. 9F). Fluorescence studies of peptides (1 mM) exposed to 1 mM peroxynitrite showed that in tyrosine-containing peptides dityrosine was also formed (not shown).

**Effect of Tyrosine-containing Peptides on Thiol Oxidation—** Under conditions where most of the oxidation (76%) occurs through a direct reaction between the thiol and oxidant (Fig. 10A), the oxidation of glutathione (3 mM) by peroxynitrite (0.5 mM) was not prevented by tyrosine homodipeptide, tripeptide, EYTR, EFTR, GY, or GF (3–20 mM). These findings are in agreement with the lack of direct reaction of peroxynitrite with the amino acids that compose the peptides (23). In addition, the peptides at 1 mM concentrations did not prevent the oxidation of glutathione in PC12 cells by 0.5 mM peroxynitrite (Fig. 10B).

**DISCUSSION**

It is well documented that reactive nitrogen species are produced both in human pathology and in animal models of human pathology (6, 14, 32, 83), and that they lead to the generation of stable modifications in amino acids, such as nitrotyrosine (6, 14, 32, 83). Tyrosine nitration has been proposed as a mediator of the activity of peroxynitrite and other reactive nitrogen species (1, 2, 84, 85). However, it remains unresolved whether tyrosine nitration plays a role in conditions of oxidative stress. In this study, we show for the first time that tyrosine-containing peptides prevented apoptosis in PC12 cells to which a bolus addition of peroxynitrite has been added and in motor neurons deprived of trophic support. Increased 3-nitrotyrosine immunoreactivity follows endogenous production of peroxynitrite and precedes motor neuron apoptosis induced by trophic factor deprivation (4, 35, 47). The peptides containing tyrosine and tryptophan prevented apoptosis induced by trophic factor deprivation in motor neurons and by a bolus addition of peroxynitrite in PC12 cells. Of the two, however, tryptophan-containing peptides were less effective at preventing cell death, most likely because of tryptophan’s lower reactivity with nitrogen dioxide, a key peroxynitrite-derived intermediate that mediates the final nitration step (15, 86). Mass spectrometric analysis of the peptides shows that the only detectable modification after exposure of the peptides to peroxynitrite in the presence of PC12 cells was the nitration of tyrosine. When tyrosine residues were replaced by phenylalanine or proline, no protection was afforded. In fact, the only important common characteristic of all the protection-providing peptides is the presence
of tyrosine or tryptophan. The protection afforded by the different peptides containing tyrosine was variable. The in vitro studies on the efficiency of nitration of the peptides by a bolus addition of peroxynitrite showed that some of the variability might be due to differences in the reactivity of the peptides with the nitrating species. However, these differences in reactivity are not large enough to explain the differences in anti-apoptotic effects. The difference in peptide protection seems to be independent of the number of tyrosine residues and the size of the peptide, suggesting the variability could also be due to differences in permeability, accumulation, and subcellular compartmentalization of the peptides in the cells. Trophic factor-deprived motor neurons were best protected by the pentapeptide with two tyrosines, with the homopeptides di- and tri-tyrosine providing considerably less protection. In contrast, the pentapeptide was less protective of PC12 cells exposed to exogenous peroxynitrite than the homopeptides and even free tyrosine. On the other hand, the tetrapeptide EYTR was 20% more effective in preventing peroxynitrite-induced PC12 cell death than the tetrapeptide EYTA. Similarly, EYTR was nitrated more efficiently by peroxynitrite in the presence of bicarbonate than EYTA; the difference in the nitration yield was about 25%, suggesting that the difference in protection could be at least in part due to differences in the peptide reactivity with the nitrating species.

Free tyrosine had no effect on motor neuron survival (not shown) but protected PC12 cells from apoptosis induced by peroxynitrite. Perhaps free tyrosine failed to protect motor neurons because the protection afforded by it was offset by the known toxic effects of free nitrotyrosine on motor neurons in culture (65). However, nitrotirosine-containing peptides have no effect on motor neuron survival (not shown) indicating that the toxicity was restricted to free nitrotyrosine. The prevention of cell death by the tyrosine- and tryptophan-containing peptides can be interpreted as competitive inhibition of the nitration of tyrosine residues in the cells. This interpretation is bolstered by a recent study using neuronal cultures that showed that the YVAD-selective caspase 1 inhibitor and two PARS inhibitors prevent the nonapoptotic cell death and protein nitration induced by the peroxynitrite donor SIN-1, probably by preventing tyrosine nitration rather than by their specific enzymatic inhibition activities (87).

The reactivity of the tested tyrosine- and phenylalanine-containing peptides with peroxynitrite correlates strongly to the reactivity of their constituent amino acids. The lack of direct reactivity for RYEYA, GY, tri-tyrosine homopeptide, RFEFA, and GF corresponds to the very low \( k_{\text{obs}} \) values reported for arginine, glutamate, alanine, and glycine (23), and the zero-order reaction with respect to tyrosine and phenylalanine (23, 88). The apparent decrease in the rate of peroxynitrite decomposition induced by the tyrosine homotripeptide and GY \( (k_{\text{obs}} \sim 0.8 \text{s}^{-1}) \) may be due to the formation of other 302 nm-absorbing species such as peroxynitrate (89). Moreover, the tested tyrosine-containing peptides were unable to protect glutathione from direct oxidation by peroxynitrite, either in vitro or in culture. On the other hand, whether or not carbon dioxide was present, the tested tyrosine-containing peptides reacted with radical species generated during peroxynitrite decomposition, forming nitrated adducts with kinetics and yields similar to those reported in the literature for nitrotyrosine formation (81, 82, 88, 90). These results further suggest that the peptides are not acting as peroxynitrite scavengers but rather as competitive targets for peroxynitrite-derived nitrating species, namely carbonate radical and nitrogen dioxide (14, 15).

The oxidation of intracellular thiols by a bolus addition of peroxynitrite was not sufficient to stimulate apoptosis in PC12 cells, because the peptides prevented cell death but not the oxidation of glutathione. These results suggest that the effects of peroxynitrite are mediated by one-electron oxidations, rather than the two-electron oxidations of the direct reactions of peroxynitrite. In addition, the analysis of the reactions of the peptides with peroxynitrite showed the formation of dityrosine in vitro. However, the mass spectroscopic analysis of the peptides exposed to peroxynitrite in the presence of PC12 cells showed clear evidence of tyrosine nitration and but only inconclusive evidence for 3,3’-dityrosine formation (not shown). All these results suggest the formation of tyrosyl radical in the reaction of the peptides with the nitrating agents derived from peroxynitrite. The effect of the tyrosine-containing peptides was limited to peroxynitrite-induced apoptosis, because they did not protect against hydrogen peroxide- and staurosporin-induced PC12 cell death, indicating they do not protect against nonspecific oxidative stress and apoptosis in general.

The analysis of PC12 cell nitrated proteins after peroxynitrite-induced nitration showed that a limited number of proteins were nitrated. By using mass spectrometry, we identified four of these proteins. Actin, \( \beta \)-tubulin, and the elongation factor 1 \( \alpha \) are among the more abundant proteins in any cell, which increase the chances of being randomly nitrated. Actin and tubulin have been shown to be nitrated in vivo in pathological conditions (14). A small proportion of nitrated actin is enough to disrupt its polymerization (25). Heat shock protein 90 (HSP90) is a ubiquitous chaperone protein with multiple functions in the cell (91–93). Heat shock proteins are important for the prevention of apoptosis (94). HSP90 is a very abundant protein that can account for 1–2% of total cytosolic protein (79). The clients of HSP90 include transcription factors, hormone receptors, and kinases, such as Akt (92). HSP90 is indispensable for cell survival. HSP90 has antiapoptotic activities (79). These activities involve the inhibition of Apaf-1 oligomerization and caspase activation (95). Motor neurons have a high threshold for the activation of the heat shock response, which is regulated by HSP90 (96). In addition, exogenous addition of HSP70 prevents motor neuron apoptosis induced by trophic factor deprivation (97), suggesting that alterations in HSP90 function by nitration may be responsible for motor neuron death. Although the full extent of the effects of nitration on HSP90 remain unknown, nitration of HSP90 results in decreased chaperone activity and intracellular release of the nitrated chaperone-induced PC12 cell death, and the nitrated protein is present in several pathological conditions, including heart disease, transplant kidney rejection, amyotrophic lateral sclerosis, and stroke.

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5 Y. Ye and A. G. Estévez, unpublished observations.
6 L. Viera, J. A. Thompson, M. Kiaei, J. C. Chavez, and A. G. Estévez, unpublished observations.
3-Nitrotyrosine was first believed to be a marker for peroxynitrite formation, but over time it became clear there are other mechanisms for the nitration of tyrosine (98, 99). In fact, it remains a subject of debate whether peroxynitrite can nitrate tyrosine under physiologically relevant conditions (54–58). Peroxynitrite can nitrate tyrosine by different biologically relevant chemical mechanisms (6, 14, 15, 32, 90, 100, 101), although myeloperoxidase may contribute as much as 50% of the nitration under severe inflammatory conditions (102). Formation of 3-nitrotyrosine in motor neurons has been shown to require the production of peroxynitrite (4, 35, 47). Our study confirmed these results when we found that inhibition of nitric oxide production did not prevent nitrotyrosine formation if low steady state concentrations of exogenous nitric oxide were applied; however, equivalent concentrations of nitrate, even in the presence of hydrogen peroxide, did not lead to nitrotyrosine formation, indicating that nitration in this model is not mediated by intracellular peroxidases. Intracellular superoxide dismutase and other superoxide and peroxynitrite scavengers also abolished tyrosine nitration, further supporting the conclusion that nitration is mediated by peroxynitrite. Although metalloporphyrins may increase nitration in the presence of peroxynitrite or nitrite plus hydrogen peroxide (103), reduced metalloporphyrins also can catalytically decompose peroxynitrite (104–106). In that case, the metalloporphyrins might be acting as the originally proposed superoxide dismutase mimics (107) or as peroxynitrite reductases (108).

In summary, although tyrosine-containing peptides do not protect biomolecules from direct oxidation reactions mediated by peroxynitrite, they clearly do scavenge peroxynitrite-derived radical species, namely nitrogen dioxide, hydroxyl radical, and carbonate radical. Furthermore, protein-tyrosine nitration is clearly inhibited by the presence of tyrosine-containing peptides in cells and tissue homogenates. The protective effects of the peptides seem to be linked to their ability to inhibit tyrosine nitration, as they were unable to prevent cell death induced by hydrogen peroxide and staurosporin. These results demonstrate for the first time the following: 1) a fundamental role for free radical-mediated oxidation, and most probably nitration of tyrosine residues in the induction of apoptosis by endogenously produced and bolus-added peroxynitrite; and 2) that cell incorporation of tyrosine-containing peptides is a novel strategy to neutralize the cytotoxic effects of peroxynitrite.

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