A Role for the p75 Neurotrophin Receptor in Axonal Degeneration and Apoptosis Induced by Oxidative Stress*

Received for publication, March 10, 2014, and in revised form, June 10, 2014. Published, JBC Papers in Press, June 17, 2014, DOI 10.1074/jbc.M114.563403

Bradley R. Kraemer†, John P. Snow‡, Peter Vollbrecht§, Amrita Pathak‡, William M. Valentine§, Ariel Y. Deutch§, and Bruce D. Carter††

From the Departments of††Biochemistry, §Psychiatry, ‡Pharmacology, §Pathology, Microbiology, and Immunology, the Vanderbilt Brain Institute, Vanderbilt University School of Medicine, Nashville, TN 37232

Background: The p75 neurotrophin receptor (p75NTR) promotes neurodegeneration during development and in response to cellular injury.

Results: Reactive oxygen species promoted cleavage of p75NTR, leading to axonal degeneration and apoptosis.

Conclusion: Oxidative stress activates intracellular p75NTR signaling to induce neurodegeneration.

Significance: These results suggest a novel mechanism through which p75NTR contributes to neurodegeneration associated with cellular injury or pathological conditions.

The p75 neurotrophin receptor (p75NTR) mediates the death of specific populations of neurons during the development of the nervous system or after cellular injury. The receptor has also been implicated as a contributor to neurodegeneration caused by numerous pathological conditions. Because many of these conditions are associated with increases in reactive oxygen species, we investigated whether p75NTR has a role in neurodegeneration in response to oxidative stress. Here we demonstrate that p75NTR signaling is activated by 4-hydroxynonenal (HNE), a lipid peroxidation product generated naturally during oxidative stress. Exposure of sympathetic neurons to HNE resulted in neurite degeneration and apoptosis. These effects were reduced markedly in neurons from p75NTR−/− mice. The neurodegenerative effects of HNE were not associated with production of neurotrophins and were unaffected by pretreatment with a receptor-blocking antibody, suggesting that oxidative stress activates p75NTR via a ligand-independent mechanism. Previous studies have established that proteolysis of p75NTR by the metalloprotease TNFα-converting enzyme and γ-secretase is necessary for p75NTR-mediated apoptotic signaling. Exposure of sympathetic neurons to HNE resulted in metalloprotease- and γ-secretase-dependent cleavage of p75NTR. Pharmacological blockade of p75NTR proteolysis protected sympathetic neurons from HNE-induced neurite degeneration and apoptosis, suggesting that cleavage of p75NTR is necessary for oxidant-induced neurodegeneration. In vivo, p75NTR−/− mice exhibited resistance to axonal degeneration associated with oxidative injury following administration of the neurotoxin 6-hydroxydopamine. Together, these data suggest a novel mechanism linking oxidative stress to ligand-independent cleavage of p75NTR, resulting in axonal fragmentation and neuronal death.

The p75 neurotrophin receptor (p75NTR)2 is a multifunctional transmembrane protein originally identified by its ability to bind members of the neurotrophin family, which consists of NGF, BDNF, neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4). Although p75NTR has been studied for over 20 years, its signaling mechanisms remain poorly understood, primarily because of the complexity of ligands, coreceptors, and cytosolic interactors that regulate p75NTR in a cell-specific manner (1). However, one established mechanism of p75NTR signaling occurs through regulated proteolysis of the receptor (2–4). Within this process, p75NTR is first cleaved in its extracellular domain by the metalloprotease TNF-α-converting enzyme (TACE, also known as ADAM17). Subsequently, the remainder of the membrane-bound receptor, termed the p75NTR C-terminal fragment, is cleaved within its transmembrane region by the γ-secretase complex, thereby releasing the cytosolic intracellular domain (p75NTR ICD). These cleavage events promote a variety of downstream signals with differing cellular functions, including nuclear translocation of neurotrophin receptor-interacting factor (5) and prolonged activation of JNK (6) to promote apoptosis, activation of the small GTPase Rho to inhibit neurite outgrowth (7), and enhancement of Trk receptor signaling to promote cell survival (8).

Studies of p75NTR−/− animals have revealed that the receptor is critical for naturally occurring developmental apoptosis within the retina, superior cervical ganglia, spinal cord, and basal forebrain (9–12). In addition to promoting cellular apoptosis, more recent findings have demonstrated that p75NTR also causes the breakdown of mislocalized axons because the receptor has been found to promote degeneration of aberrantly sprouting septal cholinergic axons, thereby preventing the fibers from growing into myelinated tracts (13), and to mediate

* This work was supported, in whole or in part, by National Institutes of Health Grants R01NS038220 (to B. D. C.), R01MH077298 (to A. Y. D.), and R01ES019969 (to W. M. V.).

† To whom correspondence should be addressed: 625 Light Hall, Dept. of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232. Tel.: 615-936-3041; E-mail: bruce.carter@vanderbilt.edu.

© 2014 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

2 The abbreviations used are: p75NTR, p75 neurotrophin receptor; TACE, tumor necrosis factor α-converting enzyme; ICD, intracellular domain; ROS, reactive oxygen species; HNE, 4-hydroxynonenal; 6-OHDA, 6-hydroxydopamine; ECD, extracellular domain; DI, degeneration index; TH, tyrosine hydroxylase; TAPI, tumor necrosis factor alpha-processing inhibitor; DAPT, N-[3,5-difluorophenacetyl]-L-alanyl]S-phenylglycine t-butyl ester; ZLLLH, benzoylcarbonyl-leucyl-leucyl-leucinal; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide.
Oxidative Stress Activates p75<sup>NTR</sup>-mediated Neurodegeneration

developmental pruning of sympathetic axons projecting to the iris (14). Hence, p75<sup>NTR</sup> functions as a regulator of neurodevelopment, ensuring the removal of unsuitable neurons or neural projections. Apart from these roles, however, numerous studies have indicated that p75<sup>NTR</sup> also promotes neuronal apoptosis in response to cellular injuries or pathological conditions. The receptor is necessary for programmed cell death caused by seizures (15, 16), corticospinal axotomy (17, 18), and spinal cord injury (19). Additionally, p75<sup>NTR</sup> has been linked to neurodegeneration occurring in models of Alzheimer disease (20), amyotrophic lateral sclerosis (21), and ischemia (22–25). Therefore, p75<sup>NTR</sup> appears to function as a stress-activated receptor that promotes degeneration in response to neuronal injury.

Of the numerous pathological conditions in which p75<sup>NTR</sup> signaling has been implicated, nearly all are associated with oxidative stress (26–31). Therefore, we speculated that reactive oxygen species (ROS) activate p75<sup>NTR</sup>, leading to neurodegeneration. During conditions that promote oxidative stress, free radicals oxidize bioactive molecules such as proteins and nucleic acids, thereby disrupting enzymatic activities and signaling pathways, increasing protein degradation, and causing DNA damage. Additionally, unstable ROS attack polyunsaturated fatty acids within lipid membranes in a process termed lipid peroxidation, resulting in the production of more stable, yet still actively damaging molecules. A major end product of lipid peroxidation is 4-hydroxynonenal (HNE), a highly reactive lipid aldehyde, and some variability in toxicity was observed between different batches of the compound. Therefore, every effort was made to limit exposure of the HNE to oxygen, including its storage at −80 °C under inert gas. For experiments with 6-OHDA (Sigma), the chemical was dissolved in cold phosphate-buffered saline with 0.02% ascorbate immediately prior to each experiment. For assessment of neurotrophin involvement in HNE-induced neurite degeneration and apoptosis, C57BL/6J sympathetic neurons were pretreated for 30 min with control serum or with 9650 immune serum containing ligand-blocking antibody specific for the p75<sup>NTR</sup> extracellular domain (α-p75 ECD, 1:500) (42). The neurons were then exposed to 12 μM HNE for 20 h before fixation and quantification of neurite degeneration or cell survival.

Western Blot Analyses—After treating rat sympathetic neurons as indicated with HNE, 6-OHDA, DAPT (Millipore), or TAPI (Millipore), the neurons were lysed in Nonidet P-40 lysis buffer. Lysates were subjected to Western blot analysis using antibodies to cleaved caspase 3 (1:500, Cell Signaling Technology, catalog no. 9664S), nerve growth factor (1:200, Alomone, catalog no. AN-240), brain-derived neurotrophic factor (1:300, Millipore, catalog no. AB1534), neurotrophin 3 (1:1000, Alomone, catalog no. ANT-003), neurotrophin 4 (1:200, Alomone, catalog no. ANT-004), and tubulin (1:1000, Calbiochem, catalog no. CP06). To detect p75<sup>NTR</sup> cleavage fragments, neurons were treated with the proteasome inhibitor ZLLLH (Peptide Institute Inc., 10 μM) 45 min prior to cell lysis and analyzed by Western blot analysis using p75<sup>NTR</sup> ICD antibody (1:500, Alomone, catalogue no. ANT-005).

EXPERIMENTAL PROCEDURES

Sympathetic Neuron Culture—All experiments with animals were approved by the Animal Care and Use Committee at Vanderbilt University. Superior cervical ganglia were dissected from postnatal day 5/6 Sprague-Dawley rats, C57BL/6J mice, or C57BL/6J p75<sup>NTR</sup><sup>exonIII</sup>/+− mice and dissociated with 0.08% trypsin (Worthington) and 0.3% collagenase (Sigma). Dissociated cells were then plated at a density of 5000–7000 neurons/0.7 mm<sup>2</sup> on 8-well chamber slides (Thermo Scientific) or cell culture plates coated with poly-d-lysine (MP Biomedicals) and laminin (Invitrogen). All neurons were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 40 ng/ml nerve growth factor (Harlan), 2 mM l-glutamine (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). To inhibit the proliferation of non-neuronal cells, the neurons were treated with 5–10 μM cytosine arabinofuranoside (Sigma) 24 h after plating. Following 3 days of exposure, cytosine arabinofuranoside was removed for 24 h, and the neurons were then treated with the indicated concentrations of HNE or different pharmacological reagents. During pilot studies, we observed increased toxicity of HNE at lower cell densities, as has been reported for 6-hydroxydopamine (6-OHDA) (40), and, therefore, for all experiments, neuron plating densities and cytosine arabinofuranoside exposures were equivalent across all experimental conditions.

Cell Treatments—HNE was produced as described previously (41) as well as obtained from Calbiochem, and its concentration was determined by measuring the optical density at 224 nm and using a molar extinction coefficient of 13,750. HNE is a highly reactive lipid aldehyde, and some variability in toxicity was observed between different batches of the compound. Therefore, every effort was made to limit exposure of the HNE to oxygen, including its storage at −80 °C under inert gas. For experiments with 6-OHDA (Sigma), the chemical was dissolved in cold phosphate-buffered saline with 0.02% ascorbate immediately prior to each experiment. For assessment of neurotrophin involvement in HNE-induced neurite degeneration and apoptosis, C57BL/6J sympathetic neurons were pretreated for 30 min with control serum or with 9650 immune serum containing ligand-blocking antibody specific for the p75<sup>NTR</sup> extracellular domain (α-p75 ECD, 1:500) (42). The neurons were then exposed to 12 μM HNE for 20 h before fixation and quantification of neurite degeneration or cell survival.
clonal norepinephrine transporter antibody 43411 (provided by Randy Blakely, Vanderbilt University).

Quantification of Neurite Degeneration—Analyses of neurite degeneration were performed as described previously (43–45), with slight modifications. Following the indicated treatments, sympathetic neurons in 8-well chamber slides were fixed with 4% paraformaldehyde and visualized via a ×20 optical lens on a Leica inverted fluorescence microscope. Phase-contrast images of five fields of view per well were captured with 16-ms exposure by a Nikon DXM1200C digital camera. To ensure accurate measurement of neurites, images were captured from blindly selected regions with well separated axon tracts. Using an automated method of image analysis, the fragmentation of the neurites was then measured. Levels of neurite degeneration are reported as a degeneration index (DI), which is the ratio of the fragmented neurite area over the total neurite area. To process images for DI calculation, the auto-level function of the GNU image manipulation program software was first used to adjust image gray levels to objectively provide uniform background intensity to all of the images. ImageJ software was then utilized to binarize the image and to remove all cell bodies, rendering an image composed of black neurites on a white background. Although healthy neurites appear continuous, degenerating neurites have a disrupted, particulate structure because of blebbing and fragmentation. To measure the area of fragments from degenerating neurites, the Particle Analyzer algorithm of ImageJ was applied to identify regions of fragmentation on the basis of size (20–10,000 pixels) and circularity (0.2–1.0). The total area of these detected neurite fragments was then divided by the total black neurite area to determine the DI. In agreement with other studies (43), a DI of 0.2 or greater accurately indicated neurite degeneration, whereas a DI of 1.0 would theoretically represent neurites that have completely degenerated into fragmented particles.

Quantification of Neuronal Death—Following the indicated treatments, sympathetic neurons in 8-well chamber slides were fixed with 4% paraformaldehyde and immunostained with neuron-specific anti-TUJ1 primary antibody (neuronal class III β-tubulin, 1:500, Covance, catalog no. MMS-435P) and Alexa Fluor® 488 secondary antibody (1:1000, Invitrogen, catalog no. A11001). Slides were then mounted using Vectashield with 4,6-diamidino-2-phenylindole (Vector Labs), and the neurons were stained with the neuron-specific marker TUJ1, and labeled with the nuclear stain DAPI. TUJ1-positive neurons were scored blindly as apoptotic or non-apoptotic on the basis of the appearance of the nucleus, apoptotic nuclei being condensed or fragmented. At least 75 TUJ1-positive neurons were counted per condition in all experiments.

Measurement of Protein Carbonylation—Detection of protein carbonylation was performed using the Oxyblot kit (Millipore) following the instructions of the manufacturer. Briefly, cell lysates were treated with 2,4-dinitrophenylhydrazine to derivatize protein side chain carbonyl groups to 2,4-dinitrophenylhydrazone. Separately, as a negative control, aliquots of all cell lysates were treated with solution lacking 2,4-dinitrophenylhydrazine. Protein samples were then separated by polyacrylamide gel electrophoresis and analyzed by Western blotting using an anti-2,4-dinitrophenyl antibody.

RESULTS

P75NTR Is Required for HNE-induced Neuronal Apoptosis—Previous studies have demonstrated that sympathetic neurons exposed to the naturally produced oxidant HNE undergo caspase-dependent programmed cell death (47). To examine the effect of HNE on neuronal survival in our culture system, rat sympathetic neurons were treated with a range of concentrations of HNE and scored for apoptosis on the basis of nuclear morphology. HNE dose-dependently induced death of sympathetic neurons (Fig. 1A). We confirmed that the cell death

In Vivo Assessment of 6-OHDA-induced Neurite Degeneration—Adult, age-matched p75NTR(exonIII)−/− or +/+ mice were administered 100 mg/kg 6-OHDA-hydrochloride (Sigma, freshly prepared in phosphate-buffered saline (pH 7.3) supplemented with 0.02% ascorbate) or vehicle solution by intraperitoneal injection once daily for 2 days. Animals were sacrificed 1 week later, and the spleens were collected for determination of norepinephrine concentrations or used for immunohistochemical localization of tyrosine hydroxylase (TH)-immunoreactive axons. For the latter studies, animals were perfused transcardially with heparinized saline, followed by 4% paraformaldehyde. The spleens were then collected, post-fixed, cryosectioned at 12 μm, and collected onto slides. Noradrenergic axons were detected by immunofluorescent localization of TH-immunoreactivity using a mouse anti-TH antibody (1:750, Abcam, Cambridge, MA). Splenic norepinephrine concentrations were determined by HPLC with electrochemical detection, following our method described previously (46).

FIGURE 1. HNE triggered apoptosis of sympathetic neurons. A, quantification of neuronal death elicited by HNE (n = 3). After exposing rat sympathetic neurons to various concentrations of HNE for 20 h, the cells were fixed, immunostained with the neuron-specific marker TUJ1, and labeled with the nuclear stain DAPI. TUJ1-positive neurons were scored blindly as apoptotic or non-apoptotic on the basis of the appearance of the nuclei. B, representative Western blot analysis of cleaved caspase 3 (CC3) from the lysate of rat sympathetic neurons treated for 12 h with 25 μM HNE (n = 3). C, carbonylation of proteins, detected by the Oxyblot™ protein oxidation detection kit, in lysates of sympathetic neurons exposed for 30 min or 2 h to 12 or 25 μM HNE (n = 3). DR, derivatization reaction; NC, negative control.
induced by HNE was apoptotic, indicated by a marked increase in the levels of cleaved caspase 3 (Fig. 1B). HNE is known to promote apoptosis through its ability to form protein adducts and modify cell signaling (32). Additionally, HNE can propagate oxidative stress through mitochondrial impairment or depletion of antioxidants (36–39). Because amino acid side chains are abundant targets of oxidation by reactive oxygen species and lipid-derived \( \alpha,\beta \)-unsaturated aldehydes, increased protein carbonylation is commonly used as a biomarker of oxidative stress (48). Treatment of sympathetic neurons with HNE caused a rapid increase in protein carbonylation, observed within 30 min of HNE treatment (Fig. 1C). These results indicate that exposure of sympathetic neurons to HNE models oxidative stress-induced apoptosis.

The p75NTR has been implicated as a mediator of apoptosis in many pathological conditions involving oxidative stress (16, 20–24). Therefore, we studied sympathetic neurons exposed to HNE to evaluate whether p75NTR contributes to oxidative stress-induced neuronal apoptosis. Sympathetic neurons were cultured from p75NTR knockout or wild-type mice and assessed for survival following exposure to various concentrations of HNE. Compared with neurons from wild-type mice, sympathetic neurons lacking p75NTR were protected significantly from HNE-induced apoptosis (Fig. 2, A and B). These findings indicate that p75NTR contributes to neuronal apoptosis induced by HNE.

HNE Stimulates p75NTR-dependent Neurite Degeneration—During survival analysis of sympathetic neurons exposed to 12 \( \mu \text{M} \) HNE, we observed extensive fragmentation of neuronal processes throughout the culture despite less than maximal cell death. Although the ability to induce neuronal apoptosis has been the most studied function of p75NTR, recent investigations have also demonstrated a function for the receptor in promoting axonal degeneration (13, 14, 24). Because of our observations and because numerous pathological conditions related to oxidative stress have also been associated with axonal degeneration (49, 50), we hypothesized that p75NTR mediates the degeneration of axons caused by HNE. Therefore, sympathetic neurons were treated with 12 \( \mu \text{M} \) HNE, and axonal degeneration was quantified from phase-contrast images. Using an automated method of image analysis, we measured the degeneration index, the ratio of the fragmented neurite area over the total neurite area (43–45, 51). Remarkably, although HNE-treated neurons from wild-type animals had substantial neurite fragmentation, the processes from cells lacking p75NTR were healthy and intact (Fig. 3A). Indeed, on the basis of the degeneration index, the p75NTR \(-/-\) neurons were protected significantly (Fig. 3B). These results reveal that p75NTR is necessary for HNE-induced neurite degeneration and suggest that oxidative stress invokes p75NTR signaling to promote axon fragmentation.

Induction of p75NTR-mediated Neurite Degeneration and Apoptosis by HNE Occurs through a Ligand-independent Mechanism—Because of the effects of p75NTR on HNE-induced neurite degeneration and apoptosis, we speculated that oxidative stress promotes neurotrophin or proneurotrophin release,
thereby leading to autocrine or paracrine activation of p75NTR. We considered BDNF the most likely candidate because BDNF can be produced by sympathetic neurons (52, 53) and can promote their apoptosis through activation of p75NTR (5, 6, 11). Therefore, we collected lysates from neurons treated with 25 μM HNE, the maximally effective dose, and measured BDNF by Western blotting. Surprisingly, however, no BDNF was detected, even after treatment with HNE (Fig. 4A). We next analyzed other neurotrophins. The precursor form of NGF, proNGF, is a known proapoptotic ligand for p75NTR (17, 19, 54), whereas mature NGF is a well defined prosurvival factor for sympathetic neurons (55–57). We detected no proNGF in the neurons and found only low levels of mature NGF, likely because of its internalization from the media, which were unchanged in sympathetic neurons treated with vehicle or HNE (Fig. 4A). Similar analyses revealed that sympathetic neurons also do not produce NT-3 or NT-4 in response to HNE (Fig. 4A). Although substantial levels of proapoptotic neurotrophins would need to be present to induce neuronal death in the presence of NGF, which was in the media, it is theoretically possible that neurotrophins remaining in the neurons were below our detection limit. Therefore, we next used an antibody to the extracellular domain of p75NTR that blocks neurotphin-mediated activation of the receptor to further explore whether HNE-induced axon degeneration and apoptosis requires activation of p75NTR by neurotrophins. As observed in previous studies (42), blockade of the extracellular domain with the p75NTR antibody prevented BDNF-induced death of sym-
Oxidative Stress Activates p75NTR-mediated Neurodegeneration

pathic neurons. However, the antibody failed to prevent HNE-induced neurite degeneration and apoptosis (Fig. 4, B and C). Together, these data suggest that oxidative stress promotes p75NTR-mediated axonal degeneration and apoptosis through a ligand-independent mechanism.

**HNE Stimulates Proteolytic Cleavage of p75NTR**—Because our results indicated that the effects of HNE did not require ligand binding to p75NTR, we hypothesized that oxidative stress triggers intracellular receptor signaling. We demonstrated previously that p75NTR-mediated apoptosis in sympathetic neurons requires proteolytic cleavage of the receptor, first by the metalloprotease TACE/ADAM17 and then by γ-secretase (5, 6). Therefore, we investigated whether HNE stimulates p75NTR proteolysis. Sympathetic neurons were treated with various concentrations of HNE and subjected to Western blot analysis using an antibody that recognizes the intracellular domain of p75NTR. Compared with neurons treated with vehicle, HNE-treated neurons had a robust and dose-dependent increase in the 25- and 20-kDa fragments of p75NTR corresponding to the p75NTR C-terminal fragment and p75NTR ICD, respectively (Fig. 5A). Cleavage of p75NTR in response to HNE was observed even after just 6 h of treatment (Fig. 5B), which was before apoptosis was visually apparent (data not shown), suggesting that proteolysis of the receptor precedes cell death. No change in the total expression level of p75NTR was observed in response to HNE (Fig. 4D), indicating that cleavage of p75NTR occurs through regulated activation of proteases rather than because of up-regulation of the full-length receptor. Because TACE and γ-secretase have been shown to mediate cleavage of p75NTR in response to neurotrophins, we hypothesized that similar enzymatic activities may be induced by oxidative stress. Treatment of sympathetic neurons with the TACE inhibitor TAPI-1 or with the γ-secretase inhibitor DAPT blocked HNE-induced cleavage of p75NTR (Fig. 5, C and D), thus indicating that HNE stimulates proteolytic cleavage of p75NTR by TACE and γ-secretase.

**Cleavage of p75NTR Is Required for HNE-induced Neurite Degeneration and Apoptosis**—To determine whether proteolysis of p75NTR is required for HNE-induced axon degeneration and apoptosis, we next blocked cleavage of p75NTR by pretreating sympathetic neurons with the TACE inhibitor TAPI-1 and then assessed neurite integrity and neuronal death following exposure to HNE. Compared with neurons pretreated with vehicle, HNE-induced neurite fragmentation was reduced dramatically in sympathetic neurons pretreated with TAPI-1 (Fig. 6, A and B). Similarly, HNE-induced apoptosis was decreased significantly in neurons pretreated with TAPI-1 (Fig. 6C). Hence, receptor proteolysis is required for p75NTR-mediated axon degeneration and apoptosis induced by HNE.

**Oxidative Stress-associated Axonal Degeneration Requires p75NTR in Vivo**—We next sought to evaluate the effects of p75NTR in axonal degeneration induced by oxidative stress in vivo. Because our aim was to promote oxidative stress specifically in neurons, we chose to use 6-OHDA, which is selectively taken up in cells expressing catecholaminergic transporters (38), rather than HNE, which reacts with a wide variety of cell types (35). 6-OHDA is a neurotoxin that has long been used systematically to selectively induce degeneration of sympathetic axons.
that express the norepinephrine transporter (59, 60). It is thought to promote the degeneration of catecholaminergic neurons primarily by increasing intracellular levels of reactive oxygen species, partially because of its tendency to undergo auto-oxidation to generate the hydroxyl radical, quinones, and other reactive species (58, 61, 62). Before utilizing 6-OHDA to model oxidative stress in vivo, we first tested whether 6-OHDA activates p75NTR signaling in cultured sympathetic neurons. As we observed following treatment with HNE, exposure of sympathetic neurons to 15 μM 6-OHDA caused cleavage of p75NTR, promoting a robust increase in the p75NTR C-terminal and p75NTR ICD fragments (Fig. 7A).

We next administered 6-OHDA to adult wild-type or p75NTR knockout mice to promote degeneration of sympathetic axons in vivo. One week after 6-OHDA treatment, a marked loss of TH-immunoreactive axons in the spleen was observed in wild-type mice (Fig. 7B). Interestingly in p75NTR−/− mice treated with vehicle, the density of TH-immunoreactive axons in the spleen appeared lower than in vehicle-treated wild-type control animals. However, there also appeared to be partial protection from 6-OHDA-induced degeneration in the p75NTR−/− null mice (Fig. 7B). To quantitatively assess the level of the sympathetic innervation, we determined the splenic norepinephrine content by HPLC. Administration of 6-OHDA caused a significantly greater loss of splenic norepinephrine in wild-type mice than in p75NTR−/− knockout mice (Fig. 7C), indicating that p75NTR contributes to 6-OHDA-induced axonal degeneration in vivo.

Additionally, expression of the norepinephrine transporter was similar in sympathetic ganglia of wild-type and p75NTR−/− mice (Fig. 7D), and, therefore, protection of the null animals from 6-OHDA-induced degeneration was likely not the result of altered 6-OHDA transport. These findings, together with the results obtained from cultured sympathetic neurons, suggest that oxidative stress promotes ligand-independent cleavage of p75NTR by TACE and γ-secretase, thereby leading to axonal degeneration and neuronal apoptosis.

**DISCUSSION**

The p75NTR is up-regulated in response to a variety of conditions involving oxidative stress [16, 20–23, 63], suggesting that the receptor may contribute to the associated cell death. However, a direct role for p75NTR as an apoptotic mediator in response to oxidative stress had not been established. Here, we demonstrate that HNE, an endogenous product of oxidative stress, activates p75NTR signaling by initiating proteolysis of the receptor, which results in axonal degeneration and neuronal apoptosis.

Oxidative stress can promote death through a variety of cell signaling mechanisms. Therefore, blocking an individual pathway may not be sufficient to confer significant protection. That neurons lacking p75NTR were markedly protected from axonal degeneration and apoptosis induced by HNE or 6-OHDA is, therefore, quite remarkable because it indicates that p75NTR is a critical regulator of neuronal responses to oxidative stress. Nev-
Oxidative Stress Activates p75NTR-mediated Neurodegeneration

A. Vehicle HNE HNE+TAPI

B. Degeneration Index

C. % Apoptosis

FIGURE 6. Cleavage of p75NTR is required for HNE-induced neurite degeneration and apoptosis. A, phase-contrast microscopy images of C57Bl6 sympathetic neurons treated with vehicle or 12 μM HNE for 20 h following 1 h of pretreatment with DMSO or 10 μM TAPI-1. Scale bar = 12.5 μm. B, measurement of neurite degeneration after 20 h exposure of sympathetic neurons to vehicle or 12 μM HNE following pretreatment with DMSO or 10 μM TAPI-1 (n = 3). Data are mean ± S.E. ***, p < 0.001; ANOVA with Bonferroni post hoc analysis. C, quantification of apoptosis of sympathetic neurons treated for 20 h with 12 μM HNE after 1 h of pretreatment with DMSO or 10 μM TAPI-1 (n = 3). Data are mean ± S.E. *, p < 0.05; ANOVA with Bonferroni post hoc analysis.

Nevertheless, other signaling pathways also likely contribute to HNE-induced apoptosis because some apoptosis, although reduced, was still detected in cultures of p75NTR−/− neurons exposed to the highest tested concentrations of HNE. Results from these studies were obtained from both cultured sympathetic neurons and from sympathetic target tissues in vivo. Sympathetic neurons are susceptible to a variety of neurodegenerative conditions. For example, they develop neurofibrillary tangles in association with tauopathies or myotonic dystrophy (64, 65). Additionally, apart from the lower brainstem and olfactory bulb, peripheral autonomic nuclei are among the earliest cell populations affected by Parkinson disease, and sympathetic neurons of Parkinson disease patients are susceptible to Lewy pathology and progressive neurodegeneration (66–68). The oxidant 6-OHDA has long been used to mimic Parkinson disease in the CNS and was initially characterized for its ability to selectively induce degeneration of sympathetic nerve terminals (58, 68). Our findings suggest that the activation of p75NTR by this oxidant plays a key role in promoting the breakdown of these axons. Interestingly, p75NTR has also been detected in neurons of the substantia nigra (69), and the receptor has been reported to be up-regulated in a mouse model with Parkinsonian-like neuronal loss and motor deficits (63, 70). Oxidative stress is widely regarded as a contributing factor to the pathogenesis of Parkinson disease (29), and, therefore, induction of p75NTR signaling by reactive oxygen species may contribute to neurodegeneration caused by the disorder.

Interestingly, we did not observe an up-regulation of the p75NTR in response to HNE. These findings suggest that the reported increases in p75NTR expression observed under conditions associated with oxidative stress are likely not due to oxidants acting directly on neurons but, instead, are the result of neighboring glial cell activation, leading to the production of cytokines. Choi and Friedman (73, 74) have shown that proinflammatory cytokines such as TNFα and IL-1β, which can be released by microglia and astrocytes (71, 72), up-regulate the expression of p75NTR.

Although most investigations of p75NTR-mediated cell death have focused on neurotrophin- or proneurotrophin-induced apoptosis, studies over expressing recombinant p75NTR or its cleavage fragments have revealed the potential for ligand-independent apoptotic signaling by the receptor (75–79). Other non-apoptotic functions of p75NTR have also been reported to occur independently of neurotrophin binding, such as inhibition of fibrinolysis through down-regulation of the serine protease tissue plasminogen activator (80). We did not observe any induction of NGF, BDNF, NT-3, or NT-4 expression in response to HNE, and use of a ligand-blocking antibody failed to prevent HNE-induced neurite degeneration and apoptosis, suggesting that initiation of these functions by p75NTR occurs through a ligand-independent mechanism.

Numerous studies have demonstrated that ROS activate TACE. For example, H2O2 has been found to activate TACE through a mechanism suggested to involve oxidative disruption of inhibitory interactions between the TACE prodomain and the Zn2+-containing catalytic site (81). More recently, a study by Walcheck and co-workers (82) revealed that the activity of purified TACE lacking its prodomain and intracellular region is enhanced by H2O2. Their results indicate that oxidation of conserved CXXX motifs within the extracellular domain of TACE promotes its activation (82). These and other studies (83, 84) demonstrate that ROS can activate TACE through multiple mechanisms, and, therefore, we hypothesized that similar mechanisms could link oxidative stress to cleavage of p75NTR. Fitting with this hypothesis, treatment of sympathetic neurons with HNE promoted the robust cleavage of p75NTR, indicating that oxidative stress promotes the activation of the regulatory proteases of the receptor. Pretreatment with the matrix metalloprotease and TACE inhibitor TAPI-1 or the γ-secretase inhibitor DAPT blocked HNE-induced p75NTR cleavage. Although cleavage of the p75NTR extracellular domain by metalloproteases other than TACE is also feasible, previous work has demonstrated that TACE is required for p75NTR cleavage in sympathetic neurons (6). Induction of p75NTR cleavage was observed not only after exposure of sympathetic neurons to HNE but also after treatment with 6-OHDA, indicating that different oxidants are capable of initiating p75NTR signaling. Therefore, our results support a model in which oxidative stress promotes ligand-independent cleavage of p75NTR by TACE and γ-secretase, leading to axonal degeneration and programmed cell death.
Although our findings demonstrate that oxidants can trigger activation of p75NTR-mediated apoptotic signaling in neurons, a previous report using PC12 cells found the intracellular domain of the receptor to have antioxidant capability, thereby conferring resistance to ROS (85). Because p75NTR has been shown to have cell-specific effects on survival, cleavage of the receptor in response to oxidative stress may confer death in specific populations of postmitotic neurons, but similar signaling mechanisms may lead to cell survival in other non-mitotic cell types. Fitting with this hypothesis is the fact that p75NTR cleavage has been reported to promote cell survival by enhancing Trk receptor signaling in PC12 cells (8, 86), but, in sympathetic neurons, cleavage of p75NTR induces programmed cell death (5, 6). Thus, p75NTR may regulate cell survival in different cell populations through similar proteolytic signaling mechanisms that lead to cell-specific physiological responses.

Although previous studies have demonstrated that p75NTR mediates axonal degeneration as part of developmental pruning (14), our findings indicate that this function of the receptor is also engaged in the response to oxidative stress. Blocking cleavage of p75NTR with the metalloprotease inhibitor TAPI-1 significantly protected sympathetic neurons from HNE-induced neurite degeneration as well as apoptosis, indicating that proteolysis of the receptor is required for oxidative stress-induced neurodegeneration. These results provide the first evidence that p75NTR-mediated axonal degeneration requires receptor proteolysis, similar to p75NTR-mediated inhibition of axon outgrowth and neuronal apoptosis. Although neuronal death and axonal degeneration were correlated in our in vitro studies, administration of 6-OHDA in vivo caused axonal loss without leading to apoptosis of sympathetic neurons (data not shown). These findings are in agreement with earlier studies of 6-OHDA administration in which axonal degeneration was detected without sympathetic neuron loss (59, 60, 87). Therefore, these functions of the receptor appear to have similar upstream components but, in particular situations, produce different functional outcomes. Further studies are needed to understand how the degenerative signaling of p75NTR can be confined so that axonal regression occurs without neuronal apoptosis.

Acknowledgments—We thank members of the Carter laboratory and Dr. Phil Barker for comments and suggestions. We also thank Regeneron for the BDNF and Lauren Herrera for assistance with tyrosine hydroxylase immunostaining.

REFERENCES

1. Kraemer, B. R., Yoon, S. O., and Carter, B. D. (2014) The biological functions and signaling mechanisms of the p75 neurotrophin receptor. Handb. Exp. Pharmacol. 220, 121–164

2. Jung, K. M., Tan, S., Landman, N., Petrova, K., Murray, S., Lewis, R., Kim, P. K., Kim, D. S., Ryu, S. H., Chao, M. V., and Kim, T. W. (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. J. Biol. Chem. 278, 42161–42169
Oxidative Stress Activates p75NTR-mediated Neurodegeneration

3. Kanning, K. C., Hudson, M., Amieux, P. S., Wiley, J. C., Bothwell, M., and Schecter, L. C. (2003) Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. J. Neurosci. 23, 5425–5436

4. Weskamp, G., Schlöndorff, J., Lum, L., Becherer, J. D., Kim, T. W., Saftig, P., Hartmann, D., Murphy, G., and Blobel, C. P. (2004) Evidence for a critical role of the tumor necrosis factor α convertase (TACE) in ectodomain shedding of the p75 neurotrophin receptor (p75NTR). J. Biol. Chem. 279, 4241–4249

5. Kenchappa, R. S., Zampieri, N., Chao, M. V., Barker, P. A., Teng, H. K., Hempstead, B. L., and Carter, B. D. (2006) Ligand-dependent cleavage of the p75 neurotrophin receptor is necessary for NRF nuclear translocation and apoptosis in sympathetic neurons. Neuroreport 50, 219–232

6. Kenchappa, R. S., Tep, C., Korade, Z., Urra, S., Bronfman, F. C., Yoon, S. O., and Carter, B. D. (2010) p75 neurotrophin receptor-mediated apoptosis in sympathetic neurons involves a biphasic activation of JNK and up-regulation of tumor necrosis factor-α-converting enzyme/ADAM17. J. Biol. Chem. 285, 20358–20366

7. Domenici, M., Zampieri, N., Spencer, T., Hilaire, M., Mellado, W., Chao, M. V., and Filbin, M. T. (2005) MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. Neuron 46, 849–855

8. Kommadadi, R. P., Thomas, R. Ceni, C., Daugnault, K., and Barker, P. A. (2011) Trk-dependent ADAM17 activation facilitates neurotrophin survival signaling. FASEB J. 25, 2061–2070

9. Frade, J. M., Rodriguez-Tébar, A., and Barde, Y. A. (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383, 166–168

10. Naumann, T., Casademunt, E., Hollerbach, E., Hofmann, J., Dechant, G., and Meyer, M. (2001) Endogenous brain-derived neurotrophic factor and prion neurotrophins and activation of p75NTR-mediated apoptosis via neurotrophin receptor-interacting factor in hippocampal neurons after seizure. Med. Res. Rev. 21, 2234–2242

11. Turner, B. J., Cheah, I. K., Macfarlane, K. J., Lopes, E. C., Petratos, S., Langford, S. I., and Cheema, S. S. (2003) Antisense peptide nucleic acid-mediated knockdown of the p75 neurotrophin receptor delays motor neuron disease in mutant SOD1 transgenic mice. J. Neurochem. 87, 752–763

12. Kokaia, Z., Andsberg, G., Martinez-Serrano, A., and Lindvall, O. (1998) Focal cerebral ischemia in rats induces expression of p75 neurotrophin receptor in resistant striatal cholinergic neurons. Neuroscience 84, 1113–1125

13. Manzano, S., Santoro, T., and Arumugam, T. V. (2013) Neuronal oxidative stress in acute ischemic stroke: sources and contribution to cell injury. Neurochem. Int. 62, 712–718

14. Zuo, L., and Motherwell, M. S. (2013) The impact of reactive oxygen species and genetic mitochondrial mutations in Parkinson’s disease. Gene 532, 18–23

15. Mazzera, A. T., Beat, A., Singh, A., and Bullock, M. R. (2009) The role of mitochondrial transition pore and its modulation, in traumatic brain injury and delayed neurodegeneration after TBI. Exp. Neurol. 218, 363–370

16. Caldeira, G. L., Ferreira, I. L., and Rego, A. C. (2013) Impaired transcription in Alzheimer’s disease: key role in mitochondrial dysfunction and oxidative stress. J. Alzheimers Dis. 34, 115–131

17. Dalleau, S., Baradat, M., Guérard, F., and Huc, L. (2013) Cell death and diseases related to oxidative stress: 4-hydroxyxenon-1-HNE in the balance. Cell Death Differ. 20, 1615–1630

18. Perluigi, M., Coccia, R., and Butterfield, D. A. (2012) 4-Hydroxy-2-nonenal, a reactive product of lipid peroxidation, and neurodegenerative diseases: a toxic combination illuminated by redox proteomics studies. Antioxid. Redox Signal. 17, 1590–1609

19. Krumn, I., Bruce-Keller, A. J., Breiden, W., Hauw, J. G., and Mattson, M. P. (1997) Evidence that 4-hydroxyxenon-1-HNE mediates oxidative stress-induced neuronal apoptosis. J. Neurosci. 17, 5089–5100

20. Poli, G., Schaur, R. J., Siems, W. G., and Leonarduzzi, G. (2008) 4-Hydroxyxenon-1-HNE: a membrane lipid oxidation product of medicinal interest. Med. Res. Rev. 28, 569–631

21. Uchida, K., Shiraishi, M., Naito, Y., Torii, Y., and Osawa, T. (2009) Activation of stress signaling pathways by the end product of lipid peroxidation: 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. J. Biol. Chem. 274, 2234–2242

22. Kutuk, O., Adli, M., Poli, G., and Basaga, H. (2004) Resveratrol protects against 4-HNE induced oxidative stress and apoptosis in Swiss 3T3 fibroblasts. Biofactors 20, 1–10

23. Liu, W., Kato, M., Akhand, A. A., Hayakawa, A., Suzuki, H., Miyata, T., Kurokawa, K., Hotta, Y., Ishikawa, N., and Nakashima, I. (2000) 4-Hydroxyxenon-1-HNE induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. J. Cell Sci. 113, 635–641

24. Lee, J. Y., Jung, G. Y., Heo, H. J., Yun, M. R., Park, J. Y., Bae, S. S., Hong, K. W., Lee, W. S., and Kim, C. D. (2006) 4-Hydroxyxenon-1-HNE induces vascular smooth muscle cell apoptosis through mitochondrial generation of...
reactive oxygen species. *Toxicol. Lett.* 166, 212–221
40. Grau, C. M., and Greene, L. A. (2012) Use of PC12 cells and rat superior cervical ganglion sympathetic neurons as models for neuroprotective agents relevant to Parkinson’s disease. *Methods Mol. Biol.* 846, 201–211
41. Kurangi, R. F., Tilve, S. G., and Blair, I. A. (2006) Convenient and efficient syntheses of 4-hydroxy-2(E)-nonenal and 4-oxo-2(E)-nonenal. *Lipids* 41, 877–880
42. Palmada, M., Kanwal, S., Rutkoski, M. J., Gustafson-Brown, C., Johnson, R. S., Wisdom, R., and Bartlett, P. F. (2002) C-jun is essential for sympathetic neuronal death induced by NGF withdrawal but not by p75 activation. *J. Cell Biol.* 158, 453–461
43. Sasaki, Y., Vohra, B. P., Lund, F. E., and Milbrandt, J. (2009) Nicotinamide mononucleotide adenyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. *J. Neurosci.* 29, 5525–5535
44. Shin, J. E., Miller, B. R., Babetto, E., Cho, Y., Vohra, B., Qayum, S., Russler, E. V., Cavalli, V., Milbrandt, J., and DiAntonio, A. (2012) SCG10 is a jnk target in the axonal degeneration pathway. *Proc. Natl. Acad. Sci. U.S.A.* 109, E3696–E3705
45. Gerdts, J., Sasaki, Y., Vohra, B., Marasa, J., and Milbrandt, J. (2011) Image-based screening identifies novel roles for iakb kinase and glycosyn synthase kinase 3 in axonal degeneration. *J. Biol. Chem.* 286, 28011–28018
46. Deutch, A. Y., and Cameron, D. S. (1992) Pharmacological characterization of dopamine systems in the nucleus accumbens core and shell. *Neuroscience* 46, 49–56
47. Bruckner, S. R., and Estus, S. (2002) JNK3 contributes to c-jun induction and apoptosis in 4-hydroxynonenal-treated sympathetic neurons. *J. Neurosci. Res.* 70, 665–670
48. Chevion, M., Bersonstein, E., and Stadtman, E. R. (2000) Human studies related to protein oxidation: protein carbonyl content as a marker of damage. *Free Radic. Res.* 33, 599–108
49. Fischer-Hayes, L. R., Brotherton, T., and Glass, J. D. (2013) Axonal degeneration in the peripheral nervous system: implications for the pathogenesis of amyotrophic lateral sclerosis. *Exp. Neurol.* 246, 6–13
50. Overk, C. R., and Maslia, E. (2014) Pathogenesis of synaptic degeneration in Alzheimer’s disease and Lewy body disease. *Biochem. Pharmacol.* 88, 508–516
51. Babetto, E., Beirowski, B., Russler, E. V., Milbrandt, J., and DiAntonio, A. (2013) The Pfl ubiquitin ligase promotes injury-induced axon self-destruction. *Cell Rep.* 3, 1422–1429
52. Causing, C. G., G lobster, A., Aloyz, R., Banji, S. X., Chang, E., Fawcett, J., Kuchel, G., and Miller, F. D. (1997) Synaptic innervation density is regulated by neuron-derived BDNF. *Neuron* 18, 257–267
53. Deppmann, C. D., Mihalas, S., Sharma, N., Lonze, B. E., Niebur, E., and Ginty, D. D. (2008) A model for neuronal competition during development. *Science* 320, 369–373
54. Lee, R., Kermani, P., Teng, K. K., and Hempstead, B. L. (2001) Regulation of cell survival by secreted proenocrinotrophins. *Science* 294, 1945–1948
55. Levi-Montalcini, R., and Hamburger, V. (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool.* 116, 321–361
56. Cohen, S. (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antisemur. *Proc. Natl. Acad. Sci. U.S.A.* 46, 302–311
57. Bothwell, M. (2014) NGF, BDNF, NT3, and NT4. *Handb. Exp. Pharmacol.* 220, 3–15
58. Bové, J., and Perier, C. (2012) Neurotoxin-based models of Parkinson’s disease. *Neuroscience* 211, 51–76
59. Tranzer, J. P., and Thoenen, H. (1968) An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. *Experientia* 24, 155–156
60. Angelotti, P. U., and Levi-Montalcini, R. (1970) Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. Natl. Acad. Sci. U.S.A.* 65, 114–121
61. Saner, A., and Thoenen, H. (1971) Model experiments on the molecular mechanism of action of 6-hydroxydopamine. *Mol. Pharmacol.* 7, 147–154
62. Cohen, G., and Heikkila, R. E. (1974) The generation of hydrogen perox-
Oxidative Stress Activates p75NTR-mediated Neurodegeneration

81. Zhang, Z., Oliver, P., Lancaster, J. R., Jr., Schwarzenberger, P. O., Joshi, M. S., Cork, J., and Kolls, J. K. (2001) Reactive oxygen species mediate tumor necrosis factor α-converting, enzyme-dependent ectodomain shedding induced by phorbol myristate acetate. *FASEB J.* **15**, 303–305
82. Wang, Y., Herrera, A. H., Li, Y., Belani, K. K., and Walcheck, B. (2009) Regulation of mature ADAM17 by redox agents for L-selectin shedding. *J. Immunol.* **182**, 2449–2457
83. Myers, T. J., Brennaman, L. H., Stevenson, M., Higashiyama, S., Russell, W. E., Lee, D. C., and Sunnarborg, S. W. (2009) Mitochondrial reactive oxygen species mediate GPCR-induced TACE/ADAM17-dependent transforming growth factor-α shedding. *Mol. Biol. Cell* **20**, 5236–5249
84. Brill, A., Chauhan, A. K., Canault, M., Walsh, M. T., Bergmeier, W., and Wagner, D. D. (2009) Oxidative stress activates ADAM17/TACE and induces its target receptor shedding in platelets in a p38-dependent fashion. *Cardiovasc. Res.* **84**, 137–144
85. Tyurina, Y. Y., Nylander, K. D., Mirnics, Z. K., Portugal, C., Yan, C., Zac-caro, C., Saragovi, H. U., Kagan, V. E., and Schor, N. F. (2005) The intracellular domain of p75NTR as a determinant of cellular reducing potential and response to oxidant stress. *Aging Cell* **4**, 187–196
86. Ceni, C., Kommaddi, R. P., Thomas, R., Vereker, E., Liu, X., McPherson, P. S., Ritter, B., and Barker, P. A. (2010) The p75NTR intracellular domain generated by neurotrophin-induced receptor cleavage potentiates Trk signaling. *J. Cell Sci.* **123**, 2299–2307
87. Thoenen, H., and Tranzer, J. P. (1973) The pharmacology of 6-hydroxydopamine. *Annu. Rev. Pharmacol.* **13**, 169–180