Role of tyrosine phosphorylation in the antioxidant effects of the p75 neurotrophin receptor

Tong Zhang, Zhiping Mi and Nina F. Schor*

Department of Pediatrics and the Center for Neural Development and Disease; University of Rochester Medical Center; Rochester, NY USA

Key words: neurotrophins, neurotrophin receptors, reactive oxygen species, oxidative stress, apoptosis, tyrosine phosphorylation

Introduction

The p75 neurotrophin receptor (p75NTR) has been shown to mediate antioxidant function in PC12 rat pheochromocytoma cells.1 Physiological signaling through p75NTR includes ligand binding and receptor dimerization, cleavage of p75NTR by α- and γ-secretases,2 phosphorylation of the intracellular domain (p75ICD) thereby liberated, binding of interactor proteins to phosphorylated p75ICD (with phosphorylation site dependence of the specific interactor that binds), and several interactor-dependent signaling pathways that determine whether p75NTR signaling leads to the life or death of the cell. In circumstances in which “life” signaling is enacted, p75ICD is as effective an antioxidant as full-length p75NTR.1 Thus, under physiological circumstances, the nature of the activity of p75NTR depends critically upon its phosphorylation; that is, the specific function of p75NTR in a given milieu is, in part, dictated by the site(s) at which phosphorylation occurs.3,4

The importance of cleavage of p75NTR, liberation of p75ICD, and the antioxidant signaling this triggers is underscored by the effects of mutation of the γ-secretase, presenilin-1, in familial Alzheimer’s disease. While p75NTR is ubiquitously expressed in embryonic brain, its expression is successively more and more restricted as the fetus matures. In the adult central nervous system, p75NTR is preferentially expressed in the cholinergic neurons of the nucleus basalis of Meynert, the hippocampus, and the cerebellum, loci implicated in the pathology of Alzheimer’s disease. Unlike its wildtype counterpart, familial Alzheimer’s disease mutant presenilin-1 is unable to cleave p75NTR and p75ICD is not liberated. It is hypothesized that p75NTR antioxidant activity is therefore not present in the neurons of patients with familial Alzheimer’s disease. Consistent with this hypothesis, reactive oxygen species have been hypothesized to play a role in the loss of cholinergic neurons in the brains of patients with Alzheimer’s disease.1,5,6

Coordinate regulation of phosphorylation occurs between Y366 and Y337. Under baseline conditions, blocking phosphorylation by replacing Y366 with F results in increased spontaneous phosphorylation of Y337, and vice versa. However, neither mono-phosphorylated molecule is as heavily phosphorylated after NGF treatment as wildtype p75NTR.3 Furthermore, phosphorylation at...
Y337 is necessary (although not sufficient) for c-Cbl-dependent ubiquitination and consequent internalization and degradation of p75NTR. In addition, p75NTR-mediated inactivation of the inhibitor of neurite outgrowth, Rho, requires phosphorylation of Y366 within the death domain of p75NTR. This residue lies between helices 2 and 3 and it is hypothesized that phosphorylation at this locus controls spacing between helices 2 and 3 and helices 4 and 5. Thus, phosphorylation of p75NTR is one of the arbiters of the function of this receptor.

We sought to examine the effects of phosphorylation of Y337 and Y366 on the antioxidant effects of p75ICD in PC12 cells. Using site-directed mutagenesis, we transfected p75NTR-negative PC12 cells with wildtype and each of three mutant (Y337F, Y366F, and Y337F/Y366F, respectively) p75ICD expression constructs. Interfering with phosphorylation at Y337 impairs antioxidant function, while interfering with phosphorylation at Y366 enhances neuroprotection. These findings suggest potential therapeutic targets for enhancing (as one might want to do in combating neural tumors) or diminishing (as one might want to do in preventing Alzheimer’s disease) the cytotoxic effects of reactive oxygen species in the nervous system.

Results

Expression and phosphorylation of p75ICD in transfected p75NTR-deficient cells. Western blot analysis demonstrates that p75ICD is expressed in all of the transfectants except the mock transfectants (Fig. 1A and B). Although the relative intensity of the bands varied from blot to blot, on repeated assessment, the p75ICD protein content (n = 3 independent blots) does not differ significantly from transfectant to transfectant (p > 0.05 for all paired comparisons; Student’s t-test, Bonferroni correction). Maintenance of p75ICD-transfected PC12 cells in the presence of the tyrosine phosphorylase inhibitor, sodium orthovanadate results in detection of phosphorylated Y (pY) in each of the immunoprecipitated p75ICD mutants (Fig. 1C).

Cell survival after treatment with 6-OHDA. As we have previously reported, transfection of p75NTR-deficient PC12 cells with wildtype p75ICD protects them from 6-OHDA-induced cell death (Fig. 2A; p < 0.04, Mann-Whitney U test, aggregate of all points for wild type p75ICD vs. aggregate of all points for mock transfectants). Cells transfected with the Y366F mutant or Y337F/Y366F double mutant, respectively, are more sensitive to 6-OHDA than are wildtype or Y366F mutant transfectants (Fig. 2B; p < 0.05, Mann-Whitney U test, aggregate of all points for Y337F + Y337F/Y366F vs. aggregate of all points for wild type + Y366F transfectants). Cells transfected with the Y366F mutant are less susceptible to 6-OHDA-induced cell death than are Y337F transfectants (p < 0.03, Mann-Whitney U test, all points for Y366F vs. all points for Y337F transfectants). Double mutants behave more like Y337F than like Y366F mutants. Results obtained using the Alamar blue assay were confirmed using LDH release into the medium (Fig. 2C).

Assessment of nuclear morphology (20–24 h; Fig. 3A and B) and caspase-3 cleavage (0 and 6 h; Fig. 4) after 6-OHDA (200 μM) treatment suggests the apoptotic nature of 6-OHDA-induced cell death in this model and confirms the importance of phosphorylation of Y337 for the anti-oxidant protective effects of p75ICD. Interestingly, fractional poly-ADP-ribose polymerase (PARP) cleavage at 6 and 9 h after addition of 6-OHDA to the medium of mutant p75ICD transfectants (Fig. 5) did not differ significantly from that of wild type p75ICD transfectants, except in the case of the 9 h determination for the double mutant (p < 0.01 compared to wild type, Student’s t-test). Similarly, by 6 h after treatment with 6-OHDA, the PARP cleavage product is most abundant in double mutant-transfected cells.

Activation of signal transductants by 6-OHDA. Cellular content of activated p38 and ERK1,2, MAP kinases (MAPKs)
Phosphorylation of p75NTR and neuroprotection

Discussion

The neurotrophin receptor, p75NTR, protects PC12 pheochromocytoma cells from oxidant stress. Implications of p75NTR signaling, and of the activation product of NF-κB, IκB-α, were examined in untreated wildtype and mutant p75NTR cells grown in serum- (i.e., NGF-) containing medium. Total ERK1,2 content is similar for all four cell lines. Resting p-ERK1,2 content is highest in wildtype cells and lowest in Y366F cells (Fig. 6). The time course of ERK1,2 activation differs among the transfectants. Mock- and Y337F-transfected cells exhibit biphasic enhancement of p-ERK/Total ERK in the 4 h following 6-OHDA treatment, with return to baseline levels between phases. By the 4 h and 1 h time points, respectively, wildtype and double mutant-transfected cells exhibit a monophasic decrease in p-ERK/Total ERK, while Y366F cells exhibit a monophasic increase in p-ERK/Total ERK. Resting p-p38 is lowest in double mutant- and Y366F-transfected cells. Resting p-IκB-α content is highest in Y366F cells (Fig. 7).

Figure 2. Concentration-response curves (Alamar blue and LDH release determinations of relative cell death; see Experimental Procedure) for p75NTR-deficient PC12 cell p75ICD transfectants treated with 6-OHDA. (A) Mock-transfected cells are compared by Alamar blue assay with cells transfected with wildtype (WT) p75ICD; (B) Cells transfected with WT p75ICD are compared by Alamar blue assay with cells transfected, respectively, with each of the mutant p75ICD constructs. DM = Y337F/Y366F p75ICD. Each point represents the mean ± SEM of nine independent determinations. (C) Cells transfected with WT p75ICD are compared by LDH release assay with cells transfected, respectively, with each of the mutant p75ICD constructs. DM = Y337F/Y366F p75ICD. Each point represents the mean ± SEM of 9 independent determinations. For (A, B and C), individual time point results differ from that obtained for WT p75ICD cells with p < 0.05 (*); p < 0.01 (**); or p < 0.001 (***) as determined by Student’s t test.
Phosphorylation of p75NTR and neuroprotection

A

WT

Y337F

Y366F

DM

Y337F

WT

DM

Y366F

B

** p<0.01 compared to WT
*** p<0.001 compared to WT

% Apoptotic Cells

[6-OHDA] (μM)

WT

Y337F

Y366F

Y337FY366F
Phosphorylation of p75NTR and neuroprotection

We have used site-directed mutagenesis to generate single and double mutants of p75ICD at Y337 and Y366. The mutants involve substitution of F for Y and therefore cannot be phosphorylated at the mutated position. These studies demonstrate that p75NTR-negative PC12 cells transfected with wildtype or Y366F p75ICD are more resistant to oxidative stress than cells that are transfected with Y337F or Y337F/Y366F double mutant p75ICD. As both wildtype and Y366F p75ICD mutant can be phosphorylated on Y337 and both Y337F and Y337F/Y366F cannot, our results suggest that phosphorylation of Y337 of p75ICD plays an essential role in the p75ICD-mediated protection of PC12 cells from cell death due to oxidant stress.

Interestingly, as phosphorylation of Y337 is essential for ubiquitination of p75NTR prior to its degradation,4 protein levels of the p75ICD Y366F mutant might be expected to be lower than that of the other mutants. On the other hand, in the Y337F mutant, none of Y337 is phosphorylated and thus the p75ICD protein level might be predicted to be highest compared to the other three mutants. However, on average, there were no significant differences in p75ICD content among the cells expressing the four different p75ICD sequences (Fig. 1).

Phosphorylation of p75NTR has been hypothesized to involve transfer of a phosphate from MAPK p38 to p75NTR; there is evidence that p38 can interact directly with the fifth and sixth alpha helices of p75NTR in the C-terminal death domain.8 Interestingly, Y366 is located between helix 2 and 3 of p75ICD in the open pocket for p38. However, our studies demonstrate no correlation between protective activity of phosphorylation site mutants of p75ICD and cellular content of phosphorylated p38 (Fig. 7). Of interest in this regard, members of the MAPK family, including p38 and ERK1,2, have been shown, like p75NTR, to be pro- or anti-apoptotic in neuronal systems and to be critical in oxidant-induced apoptosis in hepatocytes in culture.9,10 Furthermore, p38 activation is critical for 6-OHDA-induced
Phosphorylation of p75NTR and neuroprotection

The time course of ERK activation is of particular interest in light of the observation of Luo and DeFranco\textsuperscript{12} that activation of ERK in response to oxidative stress is biphasic and consists of an early, brief, compensatory, protective phase followed by a late, prolonged, cell-injurious phase. Mock- and Y337F-transfected cells demonstrate both of these phases of ERK activation and are most susceptible to 6-OHDA-induced death. Cells transfected with wildtype p75NTR have high levels of activated ERK at baseline and demonstrate a paradoxical decrease in activated ERK at the usual time of the injurious phase of ERK activation. This is perhaps responsible for the protection of these cells from 6-OHDA-induced death. Y366F- and double mutant-transfected cells exhibit ERK activation time courses that do not fit a conventional pattern. Y366F-transfected cells demonstrate early increased ERK activation that increases in magnitude with time, without return to baseline. Given the resistance of these cells to 6-OHDA-induced cell death, it is tempting to speculate that the return to baseline between ERK activation phases is necessary for the transition of p-ERK from protective to injurious. Double mutant-transfected cells demonstrate high baseline levels of ERK activation and early-onset progressively decreasing ERK activation.

Phosphorylation of p75NTR has been shown to enhance NF-kB activation.\textsuperscript{8,13,14} Our studies demonstrate that activation of NF-kB is greatest in the Y366F mutant (Fig. 7), implying that phosphorylation of Y366 may inhibit and phosphorylation of Y337 may enhance activation of NF-kB.

Several markers of apoptosis were examined in the present study. Exposure to 6-OHDA results in nuclear margination and fragmentation (Fig. 3A and B) and cleavage of caspase-3 (Fig. 4) and PARP (Fig. 5) in all transfectants. It is interesting that the fractional caspase-3 activation and fractional PARP cleavage for each transfectant do not correlate absolutely and quantitatively with resistance to oxidant stress. There are two possible, non-mutually exclusive explanations for this. First, it likely that cell death in this model, as in others,\textsuperscript{15} occurs by a combination of apoptosis and necrosis and the relative incidence of these two mechanisms of cell death differ among the transfectants. Second, while useful for the binary decision of whether cells in a given model die by apoptosis or necrosis, Western blotting does not allow for precise kinetic or quantitative comparisons of PARP cleavage from cell line to cell line or tissue to tissue.\textsuperscript{16}

The significance of the differing roles in the anti-oxidant effects of p75ICD of phosphorylation of p75ICD at Y337 and Y366,
respectively, for Alzheimer's disease is not clear. Oxidative injury to cholinergic central neurons plays a role in this disorder. In familial Alzheimer's disease, mutation of presenilin, a γ-secretase for which p75NTR is a substrate, prevents release of p75ICD. However, sporadic Alzheimer's disease, the more common of the two, does not involve mutation of presenilin. Our studies suggest that altered phosphorylation of p75ICD could also result in the enhanced likelihood of apoptotic death of neurons subjected to oxidative stress.

**Experimental Procedure**

**Chemicals.** Ascorbic acid and 6-OHDA were obtained from Sigma Aldrich, Inc. (St. Louis, MO). To prevent extracellular oxidation of 6-OHDA, solutions for use in cell culture experiments were made up in iced saline containing 250 μM ascorbic acid with a minimum of agitation immediately before use. Ascorbic acid does not penetrate the cell membrane, and therefore does not alter the intracellular effects of 6-OHDA. Catecholamine transporter-mediated uptake of 6-OHDA by and intracellular generation of reactive oxygen species in PC12 cells have been previously demonstrated.17,18

**Cell cultures.** Polyclonal p75NTR-deficient PC12 cells have been described in our previous publications.5,7 Briefly, as a population, native PC12 cells are p75NTR- and TrkA-positive (~40,000 p75NTR receptors per cell and ~400 TrkA receptors per cell). p75NTR-deficient (0 p75NTR receptors per cell and ~400 TrkA receptors per cell) PC12 cells were selected and assayed for receptor content initially and again every several passages as follows. Subclones of PC12 cells were selected on the basis of absent cell-surface p75NTR receptors as determined by FACScan analysis using the monoclonal antibody mAbMC192, a ligand of the extracellular domain of p75NTR.19,20 Subclones were then grown and examined by RT-PCR for mRNA for the p75NTR receptor. Studies were performed on pooled subclones, comprising a polyclonal, p75NTR-negative population of PC12 cells.

The p75NTR-deficient PC12 cells were maintained in Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) supplemented with 10% horse serum and 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 1.1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). The cells were fed twice weekly.

**Site-directed mutagenesis.** Site-directed mutagenesis was carried out following the protocol from Stratagene (La Jolla, CA) for the QuickChange Site-Directed Mutagenesis Kit. The template for PCR was the p75ICD gene sequence cloned into the pcDNA3.1 TOPO V5 His plasmid.1 Primers were designed to change Y to F.
by changing the A of the Y codon (TAC) to T giving the F codon (TTF) for both Y337 and Y366. Primers were constructed so that, in each case, the changed nucleotide was the fifth nucleotide from the 5' end. We used forward CTCTTCACTAGCTGCCCTGC-CCTGACC and reverse ACTGAAGAGGTTCATCACC-CCGGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTG GG
added to the supernatant and incubated overnight in the cold room. The next day, Protein A agarose beads were added again and tubes were incubated in a rotor for at least 3 h in the cold room. The beads were then spun down and washed with RIPA buffer at least four times and then dissolved into 50 μl SDS sample loading buffer for SDS electrophoresis.

Acknowledgements
The authors thank Dr. Marc Halterman for many helpful discussions and technical advice. The studies were funded through grants from the National Institutes of Health (R01-CA074289 and R01-NS038569) and the William H. Eilinger endowment of the Golisano Children's Hospital at the University of Rochester Medical Center.

References
Submit your manuscripts at
http://www.hindawi.com