Osmotic Swelling Induces p75 Neurotrophin Receptor (p75NTR) Expression via Nitric Oxide*†

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Brain injuries by physical trauma, epileptic seizures, or microbial infection upset the osmotic homeostasis resulting in cell swelling (cerebral edema), inflammation, and apoptosis. Expression of the neurotrophin receptor p75NTR is increased in the injured tissue and axon regeneration is repressed by the Nogo receptor using p75NTR as the signal transducer. Hence, p75NTR seems central to the injury response and we wished to determine the signals that regulate its expression. Here, we demonstrate that tonic mediated cell swelling rapidly activates transcription of the endogenous p75NTR gene and of a p75NTR promoter-reporter gene in various cell types. Transcription activation is independent of de novo protein synthesis and requires the activities of phospholipase C, protein kinase C, and nitric-oxide synthase. Hence, p75NTR is a nitric oxide effector gene regulated by osmotic swelling, thereby providing a strategy for therapeutic intervention to modulate p75NTR functions following injury.

Damage to the peripheral nervous system or the central nervous system represents a major medical problem, because of the lack of spontaneous tissue repair and the limited availability of tools for therapeutic intervention. Traumatic injury, epileptic seizures, microbial infections, ischemia, and others result in cerebral edema, inflammation, and ultimately death of neuronal and non-neuronal cells (1–3). Edema is described as an excess accumulation of water in the brain thereby inducing osmotic changes that lead to cell swelling (4–6). The cause of cell death is not entirely understood, but may be because of physical damage, release of cytokines by infiltrating inflammatory cells, and/or production of radicals, including nitric oxide (NO)³ (7). Injury-induced expression of p75NTR, the common receptor for neurotrophins (NT) may also contribute to apoptosis (8, 9). Neurite regeneration in the damaged central nervous system is muted by the Nogo receptor pathway, which requires the p75NTR receptor to transduce the repressing signal (10, 11). Thus, increased p75NTR expression is central to the injury response in nervous tissue.

The p75NTR receptor is a 75-kDa glycoprotein with an extracellular domain containing four cysteine-rich repeats required for NT binding; a single transmembrane domain, and an intracellular domain that lacks catalytic activity, yet contains a Type II death domain (8, 9). Binding of NTs to p75NTR can activate different signaling pathways, including NFκB, c-Jun NH₂-terminal kinase, and production of ceramide (12). Recently, p75NTR was shown to preferentially bind the uncleaved form of NTs (pro-NT) with high affinity, thereby inducing cell death (13). p75NTR also regulates RhoA activity, consistent with its role in neurite outgrowth, and the receptor plays a pivotal role in myelination in the peripheral nervous system (14, 15). Expression of p75NTR occurs in the developing central nervous system and peripheral nervous system, but is also found outside the nervous system and animals with complete p75NTR ablation have a high mortality rate because of severe defects in the vascular and nervous systems (16). The transcriptional control of p75NTR shows characteristics of a housekeeping gene and various agents either positively or negatively regulate its expression, yet no consensus mechanism of transcriptional control has emerged (17).

Given the importance of p75NTR in the injury response, we wished to determine the cellular signals that lead to its expression. We utilized an in vitro model that closely mimics injury conditions in vivo. Here, we demonstrate that cell swelling, a common response to tissue damage, ubiquitously activates p75NTR transcription in neuronal and non-neuronal cells. Transcription activation occurs independently of de novo protein synthesis and requires the enzymatic activities of phospholipase C (PLC), protein kinase C (PKC), and include nitric oxide synthase (NOS). Thus, p75NTR is an early effector gene of the NO signal pathway activated by volume stress, thereby implicating NO in p75NTR-regulated functions including neurite outgrowth and cell death.

MATERIALS AND METHODS

Reagents—1-Arginine, cycloheximide, actinomycin D, mannitol, EGTA, and thapsigargin were obtained from Sigma. BAPTA-AM and DAF-FM diacetate were purchased from Molecular Probes. Bisindolylmaleimide I (BIS), ionomycin, t-NAME, 7-NiNa, MGD, cPTIO, Rotterlin, U73122, and U73433 were obtained from Calbiochem.

Cell Culture—All cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (Irvine Scientific), supplemented with 10% heat inactivated fetal bovine serum (Omega Scientific), penicillin (100

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units/ml), streptomycin (100 units/ml), and 4 mM glutamine (all from Invitrogen). Hypoosmotic medium was prepared by dilution of complete growth medium with water to indicated osmolarities and confirmed with an osmometer (Advanced Instruments). Cell lines used included HTLA230 (18), GOTO (19), SK-N-BE(2), SK-N-SH, U-87 MG, Daoy, Y79, B104 (20), HEK293, NIH3T3, 10T1/2, HeLa, Neuro2a, brain capillary endothelial cells (21), and A875 (22). Cell lines were obtained from American Type Culture Collection, unless otherwise noted.

Cells were transiently transfected using LipofectAMINE 2000 (Invitrogen) in the presence of 10% serum for 4 h according to manufacturers instructions. Typically, 1 μg of DNA and 3 μl of LipofectAMINE were used per well of a 24-well plate and luciferase activity was determined from triplicates. Luciferase expression was assayed using the Luciferase Assay Kit (Tropix) according to the manufacturers instructions.

RT-PCR—Total RNA was harvested from cells using Trizol (Invitrogen) according to the manufacturers instructions. Equal amounts of each RNA sample (1–5 μg) were reverse transcribed using the Superscript Preamplification system for First-Strand cDNA synthesis (Invitrogen) followed by PCR amplification using the following protocol: 95 °C for 5 min, 5 cycles of 94 °C for 5 s, 72 °C for 4 min, 5 cycles of 94 °C for 5 s, 70 °C for 4 min, and 20 cycles of 94 °C for 5 s, 68 °C for 4 min. A 1.3-kb product was generated from the human p75NTR mRNA and RT-PCR amplification was done in the absence of RT as internal control. FasL was amplified as described (23). PCR amplification of the murine p75NTR was performed with primers from Promega. RT-PCR of β-actin expression was monitored using primers specific for human, mouse, and rat β-actin cDNA (Promega).

Flow Cytometry—p75NTR surface expression was analyzed as described (24). In brief, cultured cells were resuspended using PBS with 5 mM EDTA, washed in PBS, and incubated for 30 min in blocking solution (PBS, 5% bovine serum albumin, 1% goat serum) at 4 °C. Cells were then incubated with ME20.4 antibody (22) for 1 h followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Kirkgaard and Perry Laboratories) for 1 h. Cells were then washed 3 times with PBS and analyzed by FACS. Quantitation of FACS data was done based on mean fluorescence intensity as determined using CELLQuest software (BD Biosciences).

Cell Size Analysis—Cells were trypsinized, resuspended in medium with a defined osmolarity, and maximal cell size was determined from triplicate samples using a Coulter counter channelizer (Beckman Instruments). Relative cell volume is expressed as the median channel of the distribution determined by the channelizer.

PAGE Gels and Immunoblotting—Equal amounts of total cell protein were loaded onto 10% denaturing polyacrylamide gels (Invitrogen), followed by transfer to nitrocellulose membranes (Schleicher & Schull) as described (20). Membranes were immunoblotted with an anti-p75NTR antibody (Promega) at a 1:250 dilution using standard procedures. Blots were stripped and re-blotted with an anti-β-tubulin monoclonal antibody (Sigma) according to the manufacturers instructions to demonstrate equal loading. Quantitation of Western blot data was done using Labworks software (UVP).

125I-NGF Affinity Cross-linking Analysis—Cells were grown in isotonic or hypotonic medium for 24 h, resuspended in PBS supplemented with 5 mM EDTA. Cells (2 × 10^6) were incubated for 1 h at 4 °C in Krebs-Ringer solution supplemented with 1.25 nM [125I]-NGF (Amersham Biosciences, 1850 Ci/mmol) in the presence or absence of 200 nM unlabeled NGF. Cells were then incubated with BS3 (Pierce) (2 mM) for 15 min at room temperature, quenched with 80 mM glycine, washed twice with cold Tris-buffered saline, and boiled in 2 × SDS buffer. Complex formation was analyzed by PAGE gel electrophoresis and autoradiography.

Promoter Reporter Constructs—A 2.1-kb DNA fragment of the p75NTR promoter was amplified from normal human DNA using PCR primers located at −2000 (5′-ATCTTTAGAAGCGATCTCTGCGGAAATGACTC-3′) and at +100 (5′-CGGCGAAGATCGATATCC-3′), (transcription start site is considered 0). Amplification was done at 95 °C for 5 min, 5 cycles of 94 °C for 5 s and 72 °C for 5 min, 5 cycles of 94 °C for 5 s and 70 °C for 5 min, and 35 cycles of 94 °C for 5 s and 68 °C for 5 min. The PCR fragment was cloned in the pGL3 basic luciferase vector (Promega).

The NFXb-luciferase reporter construct was kindly provided by Dr. A. Baldwin (University of North Carolina, Chapel Hill, NC) and the dihydrofolate reductase (DHFR)-luciferase reporter construct was a gift from Dr. J. Horowitz (North Carolina State University, Durham, NC).

RESULTS

Stress-induced p75NTR Expression

We hypothesized that cell swelling, a common response to disruption of homeostasis by tissue damage, leads to p75NTR expression in vivo and decreasing the medium osmolarity would recapitulate these events in vitro. HTLA230 neuroblastoma cells that neither express the p75NTR nor the trkA genes were exposed to hypoosmotic (160 mosm/liter) medium and p75NTR expression was determined by semiquantitative RT-PCR. Total cellular RNA isolated from human melanoma (A875) cells known to express p75NTR was used as an internal control.
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Fig. 2. Analysis of the expressed p75NTR receptor. A, Western blot analysis of HTLA230 cells exposed for various time periods to hypoosmotic medium. The anti-p75NTR antibody recognized 2 proteins of 65 and 75 kDa in A875 cells. The p75NTR was undetectable in HTLA230 cells grown in isotonic medium, whereas both receptor proteins were present within 12 h upon exposure to hypotonic medium. β-Tubulin serves as internal loading control. B, HTLA230 cells were grown in either isotonic or hypoosmotic medium for 24 h and cells were stained with the anti-p75NTR antibody (ME20.4) and analyzed by FACS. The fluorescence histogram of p75NTR expression of A875 cells is shown for comparison. HTLA230 cells grown in isotonic medium, stained with the secondary fluorescein isothiocyanate-conjugated antibody only (row a), or stained with the anti-p75NTR antibody followed by the secondary antibody (row b) showed no specific staining. Similarly, cells grown in hypotonic medium but stained with the secondary antibody alone showed only background fluorescence (row c), whereas cells stained with both the primary and the secondary antibody (row d) showed a 22-fold increase in fluorescence intensity, when compared with untreated cells. C, HTLA230 and GOTO cells were grown in either isotonic or hypoosmotic medium for 24 h, followed by incubation with 125I-NGF in the presence or absence of 100-fold excess of unlabeled NGF. Receptor-ligand complexes were chemically cross-linked with BS3 and analyzed by PAGE and autoradiography. A875 cells were used as reference for identification of a 125I-NGF-p75NTR complex. A strong radioactive band with an approximate molecular weight of 100,000 was present in A875 cells, consistent with a 125I-NGF-p75NTR complex. This complex was completely abrogated by the presence of excess unlabeled NGF, demonstrating the specificity of the interaction. A similar 125I-NGF-p75NTR complex was present in both HTLA230 and GOTO cell lines exposed to hypoosmotic medium, but not in isotonic medium. Formation of this complex was abrogated by excess unlabeled NGF. Thus, the newly synthesized p75NTR is ligand binding competent.

Consistent with the hypothesis, hypoosmotic medium-induced p75NTR expression in HTLA230 cells, which remained at a high level as long as the toxicity stress was applied, but subsided within hours upon reversal of the medium to isotonicity (Fig. 1A). The DNA sequence of the RT-PCR fragment was consistent with that of the human p75NTR mRNA (data not shown). Interestingly, 30 min exposure to this toxicity stress was not sufficient to activate p75NTR expression (Fig. 1B), but mRNA was detectable following a 1-h treatment. Inhibition of protein synthesis with cycloheximide did not prevent stress-induced p75NTR transcription (Fig. 1C), thus suggesting that p75NTR is an early response gene. In contrast, actinomycin D abolished the increase in the p75NTR mRNA pool, demonstrating that p75NTR expression was due to de novo transcription, rather than stabilization of low abundant mRNA (Fig. 1D).

HTLA230 cells were grown in either control or hypoosmotic (160 mosm/liter) medium for various time periods and the newly synthesized p75NTR receptor was characterized using Western blots, FACS, and receptor/ligand cross-linking. On Western blots, the anti-p75NTR antibody recognized 2 protein bands with molecular weights of 65,000 and 75,000 in A875 cells, consistent with the described recognition profile for the antibody (25). In HTLA230 cells, p75NTR expression was undetectable at the onset of the experiment, whereas the 2 p75NTR immunoreactive proteins were present within 12 h of exposure to hypotonic medium (Fig. 2A). FACS analysis of A875 cells using the anti-p75NTR antibody (ME20.4) followed by incubation with a fluorescein isothiocyanate-conjugated secondary antibody demonstrated high p75NTR surface expression (Fig. 2B). Staining of HTLA230 cells with the secondary antibody alone (row a) or with ME20.4 followed by the secondary antibody (row b) showed no specific staining. Similarly, cells grown in hypotonic medium but stained with the secondary antibody alone showed only background fluorescence (row c), whereas cells stained with both the primary and the secondary antibodies (row d) showed a substantial shift in fluorescence intensity when stained with the primary and secondary antibodies (row d), consistent with p75NTR surface expression. This observation demonstrated that HTLA230 cells exposed to hypoosmotic medium showed a 22-fold increase in fluorescence intensity when compared with untreated cells. This fluorescence intensity is ~17 times lower than that seen in A875 cells. Chemical cross-linking of 125I-NGF (see “Materials and Methods”) was used to investigate the potential of the newly synthesized p75NTR to bind NGF. Incubation of A875 cells with 125I-NGF revealed a strong radioactive protein band with an approximate molecular weight of 100,000 consistent with the presence of a 125I-NGF-p75NTR complex (Fig. 2C). This complex was abrogated in the presence of 100-fold excess of unlabeled NGF, thus demonstrating the specificity of the 125I-NGF-p75NTR complex. HTLA230 cells did not reveal a radioactive complex when grown in control medium, whereas a labeled protein band with a similar molecular weight as that seen in A875 was present in cells exposed to hypoosmotic medium. Similar data were seen with GOTO cells, another human neuroblastoma, where an abundant 125I-NGF-p75NTR complex was present in osmotically stressed cells. This complex was abrogated in the presence of excess unlabeled NGF, thus indicating the presence of a 125I-NGF-p75NTR complex. Hence, surface-bound p75NTR receptors are ligand binding competent and thus represent the full-length receptor (16).

The observation that hypotonic medium induces p75NTR expression was not unique to HTLA230 cells. Analysis of a panel of human and murine cell lines using semiquantitative RT-PCR, Western blotting, or flow cytometry demonstrated that exposure to hypoosmotic medium activated p75NTR expression in almost all cell lines examined, albeit to various degrees (Table I). These cells included a series of neuronal,
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Analysis of p75NTR expression in various cell lines

*p75NTR expression was analyzed by flow cytometry, Western blotting, and/or semi-quantitative RT-PCR after culturing cells in isotonic and hypotonic medium for 24 hours. Quantitation of the -fold increase in p75NTR expression in hypotonic medium is given for cell lines in which analyses were done by flow cytometry or Western blotting.

| Cell type         | Cell line   | Increased p75NTR in hypotonic | -Fold increase |
|-------------------|-------------|--------------------------------|---------------|
| Human             | Neuroblastoma | HTLA230                        | Yes           | 22* |
|                   | Neuroblastoma | GOTO                           | Yes           | 30* |
|                   | Neuroblastoma | SK-N-BE(2)                     | Yes           | ND* |
|                   | Neuroblastoma | SK-N-SH                        | Yes           | ND  |
|                   | Glioblastoma | U-87 MG                        | Yes           | 9*  |
|                   | Medulloblastoma | Daoy                         | Yes           | ND  |
|                   | Melanoma      | A875                           | Yes           | 3*  |
|                   | Cervical carcinoma | HeLa                     | Yes           | ND  |
|                   | Retinoblastoma | Y79                            | Yes           | 24* |
|                   | Embryonic kidney | 293HEK                     | Yes           | 16* |
| Murine            | Brain endothelial cells | HBCEC                 | Yes           | 118* |
|                   | Central nervous system | B104              | Yes           | ND  |
|                   | Fibroblast    | NIH3T3                         | No            | ND  |
|                   | Fibroblast    | 10T1/2                         | No            | ND  |

* Analyzed by flow cytometry.
ND, no quantitative data available.
* Analyzed by Western blotting.

A 2.1-kb DNA fragment of the normal human p75NTR gene spanning from −2000 to +100 was PCR amplified and cloned into a luciferase reporter vector (see Materials and Methods) to facilitate a quantitative analysis of transcription activation. A875 melanoma cells, which are amenable to transfection, readily express the endogenous p75NTR gene, and demonstrate increased p75NTR expression upon cell swelling were transiently transfected with the p75NTR promoter-reporter gene, a CMV driven luciferase expression vector (CMV-luc), or a DHFR driven luciferase transgene. Cells were exposed 24 h later to either isotonic or hypotonic (160 mosm/liter) medium for 8 h, the relative light units (RLU) from triplicate cultures were determined, and the data represents the mean -fold increase in RLU/µg of protein from cells exposed to hypotonic medium over cells grown in isotonic medium (Fig. 3A). Constitutive expression of the CMV and the DHFR driven transgenes remained unchanged upon exposure of cells to hypotonic stress, whereas transcription of the p75NTR promoter-luciferase fusion gene was greater than 20-fold increased. Interestingly, both the DHFR and p75NTR promoter depend on the activities of members of the family of Sp transcription factors (26, 27), suggesting that toxicity mediated transcription is not just a hallmark of Sp driven promoters, but a unique property of the p75NTR gene. Various degrees of transcription activation of the p75NTR reporter gene occurred in other cell lines (Fig. 3B), re-enacting toxicity induced increase in expression of the endogenous p75NTR gene (see Table I).

The Role of Cell Volume in p75NTR Transcription Activation

Exposure of HTLA230 cells to medium of 160 mosm/liter rapidly induces a greater than 30% increase in cell volume (data not shown) and we hypothesized that processes accompanying cell swelling initiate cellular signals thereby triggering p75NTR expression. However, preparation of hypotonic medium reduces the concentration of all medium components (i.e. amino acids, vitamins, and salts), which could activate p75NTR expression. To test our hypothesis, we prepared hypotonic medium (160 mosm/liter) and added mannitol, a cell...
impermeable sugar, to restore isotonicity (320 mosm/liter). HTLA230 cells were then exposed to these culture conditions and expression of the endogenous \( p75NTR \) gene was determined (Fig. 4A). Cells grown in isotonic medium supplemented without or with 2.5% mannitol, and \( p75NTR \) expression was determined by RT-PCR. \( p75NTR \) expression is not activated by a mannitol-induced increase in the osmolarity of isotonic medium and restoration of isotonicity to hypotonic medium prevented \( p75NTR \) transcription activation. B, A875 cells were transfected with the \( p75NTR \) promoter-luciferase gene and promoter activity was determined after exposure of cells for 8 h to media with decreasing osmolarity. Maximal cell size increase induced by the different media was determined. \( p75NTR \) promoter activity was determined to be directly proportional to the increase in cell size. C, GOTO cells expressing low basal levels of \( p75NTR \) were exposed to media with decreasing osmolarity and \( p75NTR \) expression was determined by FACS analysis. \( p75NTR \) expression in A875 cells is shown for comparison. GOTO cells stained with the secondary antibody (2° only) showed only background fluorescence, whereas cells stained with the primary and secondary antibodies showed specific \( p75NTR \) surface expression. Exposure of cells to media with decreasing osmolarity resulted in an increase in fluorescence intensity, suggesting that \( p75NTR \) expression was proportional to cell volume increase mediated by osmotic swelling.

Cellular Signals Required for \( p75NTR \) Expression

**The Role of PLC Activation**—Hypotonicity induced cell swelling has been shown to activate PLC, which converts phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-triphosphate (28). As a consequence, diacylglycerol did not induce \( p75NTR \) expression. In contrast, hypotonic medium (160 mosm/liter) induced \( p75NTR \) expression, which was completely abrogated in hypotonic medium supplemented with 2.5% mannitol (320 mosm/liter). Thus, induction of \( p75NTR \) expression is the consequence of decreased medium osmolarity, rather than an effect of reduced levels of medium components.

To assess the relationship between osmolarity, cell volume, and transcription activity, A875 cells were transfected with the \( p75NTR \) reporter construct were exposed for 8 h to hypotonic medium in the absence (untreated) or presence of an active PLC inhibitor (U73122) or the less active analog (U73433). Data are expressed as % control RLU/µg of protein. Inhibition of PLC with U73122 essentially abrogated swelling induced reporter gene expression. B, GOTO cells were transfected with the \( p75NTR \)-luciferase reporter gene and exposed to hypotonic medium in the presence or absence of a broad spectrum (BIS) or a more selective (Rottlerin) PKC inhibitor. Both inhibitors effectively suppressed reporter gene expression, demonstrating the requirement for PKC activation.

![Fig. 5. Role of PLC and PKC.](image_url)
Swelling induced p75NTR transcription activation was abrogated in the presence of BAPTA-AM, demonstrating the need for intracellular Ca\(^{2+}\) signals. Similarly, depletion of the intracellular calcium stores with 10 \(\mu\)M thapsigargin significantly reduced swelling mediated p75NTR mRNA levels (Fig. 6B) consistent with the requirement for intracellular Ca\(^{2+}\) signals. Trypan blue exclusion analysis demonstrated that neither BAPTA-AM nor thapsigargin affected cell viability under hypotonic conditions (see Supplemental Materials Fig. 1B). However, extracellular Ca\(^{2+}\) ions seem not to be necessary, because the presence of EGTA (2.5 mM), a cell impermeable chelator, did not prevent p75NTR up-regulation (Fig. 6C). Nor were increases in [Ca\(^{2+}\)], sufficient as ionomycin (10 \(\mu\)M), a Ca\(^{2+}\) ionophore, activated transcription of the Fas ligand (FasL) gene as previously described (23), but did not induce p75NTR transcription (Fig. 6D). Hence, we concluded that increased [Ca\(^{2+}\)], is necessary, but not sufficient for swelling induced p75NTR expression.

Role of the NO/cGMP Pathway—Cellular stress as well as injury to the brain is associated with activation of NOS enzymes, which in the case of nNOS and eNOS requires Ca\(^{2+}\) ions (34). Hypotonicity activates NO synthesis in various cells (35) and we measured a tonicity mediated increase in NO in A875 cells using a fluorescent probe (DAF-FM diacetate) with high affinity for NO (see Supplemental Materials Fig. 2). To determine the requirement of NO for p75NTR up-regulation, we employed well characterized NO scavengers (i.e. cPTIO and MGD\(_2\)-Fe\(_{3}\)) (36) and inhibitors of NOS enzymes (\(\alpha\)-NAME and 7-NiNa) (34). Exposure of HTLA230 cells to hypoosmotic (160 mosm/liter) medium in the presence of cPTIO (100 and 250 \(\mu\)M) another NO scavenger. Altering NO synthesis with NOS enzyme inhibitors supported these observations (Fig. 7C). \(\alpha\)-NAME, an arginine-based broad spectrum

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**Fig. 6. Role of Ca\(^{2+}\) signals.** A, HTLA230 cells were preincubated with increasing concentrations of BAPTA-AM for 30 min, then grown for 8 h in hypoosmotic medium in the presence of BAPTA-AM, which prevented swelling induced expression of the endogenous p75NTR gene as determined by RT-PCR. B, HTLA230 cells were pretreated for 30 min with or without thapsigargin (TPG) in calcium-free or calcium containing medium to deplete internal calcium stores. Subsequent treatment of cells for 8 h with hypoosmotic medium resulted in a reduction of p75NTR mRNA levels in the presence of thapsigargin, independent of the presence of extracellular Ca\(^{2+}\) ions in the medium. C, HTLA230 cells were pretreated with or without EGTA for 30 min followed by exposure to either isotonic or hypotonic medium in the presence or absence of EGTA for 8 h. Extracellular Ca\(^{2+}\) ions are not required for toxicity induced p75NTR transcription, because osmotic swelling in the presence of EGTA activated p75NTR expression. D, cells were grown in isotonic Ca\(^{2+}\)-free medium or Ca\(^{2+}\)-free medium supplemented with 2.5 mM Ca\(_{2+}\) for 8 h. Ionomycin-mediated Ca\(^{2+}\) influx readily induced FasL expression, but was not sufficient to activate p75NTR transcription.
DISCUSSION

We have identified a novel mechanism by which various cell types ubiquitously up-regulate p75NTR expression. This discovery has potential clinical importance for treatment of patients with injury to the nervous system, because p75NTR functions include regulation of cell survival and axon regeneration. Swelling of the brain (cerebral edema) is a general and immediate injury response that often has a catastrophic outcome, despite therapeutic intervention. Accumulation of water in the brain tissue, partly because of a breakdown of the blood-brain barrier, results in an imbalanced osmotic homeostasis thereby inducing sustained cell swelling. Injuries that cause cerebral edema are associated with profound changes in gene expression including an increase in the expression of NOS and p75NTR genes (8, 38, 39). The signals that lead to increased NOS expression can be traced to local and infiltrating inflammatory cells, whereas the cues mediating p75NTR expression are poorly understood. The functional importance of p75NTR in the injured tissue justifies a search for these cues, however, identification of such signals is difficult to achieve in *vivo*. Hence, we employed osmotic cell swelling in tissue culture that mimics many aspects of the process of injury induced tissue swelling. Our data for the first time demonstrate that transcription activation of the endogenous p75NTR gene as well as a p75NTR promoter-driven reporter gene is directed connected to cell swelling. Moreover, the expression of surface bound p75NTR receptors is proportional to the maximal cell volume increase, suggesting that volume stress is a novel mechanism with which cells regulate p75NTR receptor density and ultimately p75NTR-mediated functions. Adjustment to osmotic stress is a universal property activating an immediate and coordinated cellular response. It is therefore not surprising that p75NTR transcription activation does not require *de novo* protein synthesis and occurs in a variety of different cell types with similar kinetics. Fluctuations of cell volume occur under many physiological circumstances, including cell cycle progression, cell differentiation, and cell migration, all of which have already been associated with increased p75NTR expression (9, 40, 41). Hence, p75NTR is an early volume stress response gene, although the detailed functions of p75NTR in volume stressed cells remain to be determined.

Cell swelling affects various cellular components, including membrane tension, the architecture of the cytoskeleton, actin depolymerization, activation of Na+/H+ exchanger, and various other channels (42, 43). Thus, multiple signal pathways are activated by cell swelling, all of which may modulate gene expression. We determined that PLC and its potential downstream effectors (*i.e.*, PKC and Ca^{2+} ions) are necessary to transduce the signal for tonicity mediated p75NTR expression. Yet, hypotonicity did not activate PLCγ in either GOTO or A875 cells (data not shown), implicating PLCβ or PLCδ, of which the latter enzyme is activated by environmental stresses, including hypotonicity (44). Various signal pathways can activate PLC including, but not limited to, G protein-coupled receptors and ion channels (28), and we have not yet identified the mechanism by which volume stress activates PLC. However, in agreement with the requirement for PLC activity is the necessity for PKC and Ca^{2+} mediated signals. Indeed, the PKC pathway is known to be activated by osmotic cell swelling (42, 45) and inhibition of PKC by a broad spectrum PKC inhibitor or a more selective PKCδ inhibitor abrogated swelling mediated p75NTR expression. Similarly, chelation of intracellular Ca^{2+} prevented toxicity induced p75NTR expression. However, ionomycin induced increases in [Ca^{2+}]i, activated *FasL* but not p75NTR expression, suggesting that Ca^{2+} ions are necessary but not sufficient for p75NTR up-regulation.

**Fig. 7. Role of NO.** A, the presence of increasing concentrations of the NO scavenger, cPTIO, abrogated swelling induced expression of the endogenous p75NTR gene in HTLA230 cells as determined by RT-PCR. β-Actin was used as internal control. B, toxicity induced reporter gene activity was determined in A875 cells. The presence of either cPTIO or MGD₂-Fe substantially reduced reporter gene expression. C, reporter gene activity was determined in the presence of 2 structurally dissimilar NOS inhibitors (L-NAME and 7-NiNa) both of which dramatically reduced transcription. Addition of L-arginine competes with the arginine-based NOS inhibitor L-NAME, thereby reversing the inhibition of reporter gene expression, demonstrating the specificity of the requirement for NOS activity.
Our data demonstrate that NO is required for the cell swelling mediated increase in p75NTR expression, because inhibitors of NOS and NO scavengers blocked the increase. This observation is consistent with findings of NO production following injury to nerve tissue, concomitant with increased p75NTR expression (46). The potential role of NO in the signals leading to p75NTR expression is intriguing given that NO, like p75NTR may exert a pro- or anti-apoptotic signal. The mechanisms by which NO regulates cell survival/cell death are diverse, yet our data imply a novel mechanism wherein NO may use p75NTR as an effector.

The molecular components that sense and determine cell volume are not completely elucidated, however, the tight regulation of p75NTR expression by cell volume suggests that the common NT receptor may be involved in this process. We envision that p75NTR may play a role in restoring volume homeostasis following cell swelling by its ability to modulate Rho activity (14, 47). Consistent with this idea, Rho GTPases have been shown to regulate cell size and inhibition of Rho kinase, a downstream effector of Rho increases cell volume (48). Alternatively, p75NTR may be involved in regulating cell survival following volume stress and we found that surface receptor density was proportional to the severity of the applied volume stress. As a consequence, levels of surface receptor expression may determine signal strength and ultimately p75NTR-mediated functions.

Treatment of patients with brain trauma aims at improving cell viability as well as functional regeneration of neuronal circuits. Although the precise function(s) of p75NTR in these disease states is not entirely clear, there is evidence that prevention of p75NTR functions may be beneficial. Death of neonatal Schwann cells following axotomy is significantly reduced in p75NTR null mutant mice, as is the death of oligodendrocytes upon spinal cord injury (13, 49). In addition, inactivation of the Nogo receptor pathway enabled limited neurite outgrowth following injury in mice, thereby providing a rational for therapeutic intervention in patients with central nervous system trauma (50), perhaps suggesting that similar or more pronounced effects could be achieved by preventing expression of p75NTR, an essential signaling component of the Nogo receptor pathway. Thus, identification of NO as a central regulator of p75NTR expression in volume stressed cells may provide a therapeutic strategy to modulate cell viability and neurite regeneration with clinically approved NO modifiers.

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