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p75 Neurotrophin Receptor and Fenretinide-induced Signaling in Neuroblastoma

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Abstract

Purpose—Neuroblastoma is the most common extracranial solid tumor of childhood. The retinoic acid analogue, fenretinide (4-hydroxyphenyl retinamide; 4-HPR), induces apoptosis in neuroblastoma cells in vitro and is currently in clinical trials for children with refractory neuroblastoma. We have previously shown that expression of the p75 neurotrophin receptor (p75NTR) enhances apoptosis induction and mitochondrial accumulation of reactive oxygen species by 4-HPR in neuroblastoma cells. We now examine the signaling events that underlie this effect.

Methods—Systematic examination of pro- and anti-apoptotic signaling effectors was performed by Western blot. Specific inhibitors of JNK phosphorylation and scavengers of mitochondrial reactive oxygen species were used to demonstrate the roles of these phenomena in the enhancement of fenretinide efficacy.

Results—The present studies demonstrate that enhancement of 4-HPR-induced apoptosis by p75NTR is dependent upon p38MAPK phosphorylation, JNK phosphorylation, caspase 3 activation, Akt cleavage, and decreased Akt phosphorylation. In addition, treatment with 4-HPR results in upregulation of MKK4 and MEKK1, and phosphorylation of MKK3/6. Efforts to enhance the efficacy of 4-HPR and to identify those tumors most likely to respond to it might exploit these effectors of 4-HPR-induced apoptosis.

Conclusions—Pharmacological agents that enhance MKK4 or MEKK1 expression or JNK expression or phosphorylation may enhance efficacy of 4-HPR in neuroblastomas that do not express high levels of p75NTR.

Keywords

neuroblastoma; retinoids; signal transduction; apoptosis

Neuroblastoma is the most common extracranial tumor of childhood. Sixty-five percent of children diagnosed with neuroblastoma have bulky primary tumors or metastatic disease at the time of initial diagnosis. One theory of the pathogenesis of neuroblastoma posits that peripheral neural crest cells that fail to differentiate and thereby retain mitotic potential

develop into neuroblastomas. This theory has led to attempts to develop chemotherapeutic agents that induce neuroblastomas to differentiate. Retinoic acid and analogues thereof, termed “retinoids”, are among these agents.

Retinoic acid induces differentiation of neuroblastoma cells into mature neuronal cells and the 13-cis form has been used in treatment of residual stage 4 neuroblastoma following conventional chemotherapy [1,2]. Development of resistance to 13-cis retinoic acid led to studies of fenretinide (4-hydroxyphenyl retinamide; 4-HPR), which induces apoptosis, rather than differentiation, in neuroblastoma cells [3]. 4-HPR has been well tolerated in clinical trials and has shown synergistic responses with chemotherapeutic drugs in vitro [4]. The mechanisms through which 4-HPR induces apoptosis are not completely understood but have been shown to involve induction of oxidative stress in several cancer cell types. Previous studies have shown that mitochondrial accumulation of reactive oxygen species (ROS) plays a pivotal role in 4-HPR-induced apoptosis [3,5,6], and we have demonstrated that expression by neuroblastoma cells of the p75 neurotrophin receptor (p75NTR) enhances apoptosis induction by 4-HPR [7]. This finding is of therapeutic importance because p75NTR expression is especially high in Schwann cell-like (S-type) neuroblastoma cells, cells that are particularly resistant to conventional chemotherapy and, therefore, likely to constitute recurrent disease in children treated for metastatic neuroblastoma.

Our previous studies have suggested that p75NTR can be variably pro- or anti-apoptotic [8-11] and functions as a cellular redox modulator [7,12-15]. The effects of p75NTR signaling on the cell depend on the specific interacting proteins with which it is associated. The pro-apoptotic interacting proteins, TRAF-6, NRIF and NUAGE, induce JNK phosphorylation, an obligate event for initiation of developmental cell death [16,17]. Interestingly, apoptosis-inducing 4-HPR treatment causes activation of MAP-kinases, also followed by downstream phosphorylation of JNK and p38MAPK [18].

On the other hand, p75NTR can signal through activation of Ras, resulting in the induction of phosphatidyl inositol 3-kinase (PI3K)/Akt signaling, implicated in neural crest cell survival [14]. Potentiation by p75NTR of 4-HPR-induced cell death suggests that Akt signaling does not predominate in this system. Caspases 3, 6 and 9 are activated when cells undergo apoptosis in response to 4-HPR treatment. Akt is a physiological substrate of caspase 3 and cleavage of Akt could turn off the anti-apoptotic arm of the p75NTR signaling cascade [19].

In hopes of maximizing the efficacy and optimizing the choice of 4-HPR as chemotherapy for recurrent or refractory neuroblastoma, we sought to delineate the effects of p75NTR expression on 4-HPR-induced pro- and anti-apoptotic signaling. We further sought to demonstrate the roles of these signaling effectors in accumulation of mitochondrial ROS in S-type neuroblastoma cells treated with 4-HPR.

METHODS

Chemicals and Reagents

4-HPR, Hoechst dye #33342 and dehydroascorbic acid (DHA) were purchased from Sigma Chemical Corp. (St. Louis, MO). MitoSOX™ Red mitochondrial superoxide indicator was obtained from Molecular Probes, Inc. (Eugene, OR). Alamar blue and Trypan blue were purchased from Invitrogen (Carlsbad, CA). MTS Assay Reagent (CellTiter 96 Aqueous One Solution) was obtained from Promega (Madison, WI). SP600125 was purchased from Selleck Chemicals (Houston, TX).

Cell Lines and Cell Culture

The S-type human neuroblastoma cell line SH-EP1 was purchased from the American Tissue Culture Collection (ATCC, Rockville, MD) and maintained as recommended by the ATCC. Specifically, SH-EP1 cells were maintained in Dubecco's modified Eagle's medium supplemented with Ham's F-12 nutrient mixture with L-glutamine, 15 mM HEPES, fetal bovine serum (10%), and penicillin/streptomycin (1%). Cells were plated 24-48 h prior to transfection and 4-HPR treatment occurred 24 h after transfection.

Knockdown of p75NTR

p75NTR knockdown was performed as described in Ganeshan et al. [7] p75shRNA was purchased from Santa Cruz Biotechnology and transfected into the cells using an Amaxa Nucleofector II. SH-EP1 cells were transfected with Nucleofector Kit V as recommended by the manufacturer. Briefly, cells were plated at 70% confluency 24-48 h prior to transfection. Cells (approximately 2×10^6) were trypsinized and the supernatant was centrifuged and removed. The pellet was resuspended in a 100 ml mixture of Nucleofector solution (82%) and Supplement solution (18%) and added to the cuvette. This was followed by nucleofection using the corresponding program in the Nucleofector II machine (Amaxa). The transfected cells were plated in 6 cm diameter culture dishes and treated with the selection antibiotic (5 g/ml puromycin) to create stably transfected cells. In these p75shRNA cells, p75NTR was knocked down to 50% of its concentration in cells transfected with a scrambled construct ([7], Fig. 3).

Western Blotting

Native and p75NTR-deficient neuroblastoma cells were lysed in radioimmunoprecipitation buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 4 mg/ml aprotinin, 1 mM sodium orthovanadate) for at least 30 min on ice. Subsequently, the protein concentration was determined in triplicate samples using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard. Lysates containing equivalent amounts of protein were loaded onto each lane and electrophoresed in 7.5% SDS-polyacrylamide gel, followed by blotting onto a nitrocellulose membrane (Bio-Rad Laboratories). After blotting, non-specific binding was blocked with 5% non-fat dry milk in PBS for 1 h and the membrane was incubated for 2 h at room temperature with primary antibodies (anti-p75NTR antibody, 1:1000; Promega) diluted into 5% non-fat dry milk in PBS. Using the same technique, p-Akt was detected by Western blot using anti-p-Akt1/2/3 antibody from Santa Cruz (sc-16646-R), which detects p-Thr 308 of Akt1, p-Thr 309 of Akt2, and p-Thr 305 of Akt3. Akt phosphorylated at these sites signals downstream of PI kinases. Cleaved Akt was detected by Western blot using anti-Akt1/2 antibody from Santa Cruz (sc-1619), which detects both holo- and the N-terminal fragment of Akt. In all cases, the membrane was then washed with PBS and incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1000; Santa Cruz Biotechnology) for 1 h. The membrane was finally washed and developed with Western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology) following the manufacturer's instructions. The procedure was repeated staining for actin as a loading control. Densitometric analysis was done using Scion Image and the data were presented as the average ratio of p75NTR/actin from at least three independent experiments.

Assays of Mitochondrial ROS Accumulation

Mitochondrial superoxide was determined using the mitochondrial superoxide indicator, MitoSOX™ reagent. Neuroblastoma cells growing in 96 well plates were stained following the manufacturer's instructions. Briefly, cells were washed once with warm Hank's Balanced

Salt Solution/Ca/Mg and incubated in 5 μ M dihydroethidium or 2 μ M MitoSOX™ Red for 30 min at 37°C (protected from light) followed by three washes with warm buffer. The fluorescence was then measured with a fluorescence plate reader (SpectraMax M5) at the excitation wavelength of 505 nm and emission wavelength of 610 nm for MitoSox™. In addition, cells were viewed under a Nikon TE 2000 light microscope equipped for epifluorescent illumination to monitor the intracellular distribution of the fluorescence. Since all of the dyes used are light sensitive, low light conditions were maintained whenever possible. A SPOT digital camera with manual control was used to ensure the same exposure time for all pictures.

Mitochondrial Antioxidant Treatment

DHA (400 M) was used to scavenge mitochondrial ROS. Pretreatment with DHA for 24 h was followed by 4-HPR treatment (72 h; 0-20 M). The cells were collected following treatment and analyzed using Western blotting for protein expression.

RESULTS

Downregulation of p75NTR decreases phosphorylation of JNK and p38 in SH-EP1 cells treated with 4-HPR

SH-EP1 transfectants expressing native or downregulated p75NTR were treated with 6 μ M 4-HPR for 24 h and phosphorylation of JNK and p38 was measured. As is shown in Fig. 1A, downregulation of p75NTR expression decreases JNK phosphorylation in response to 4-HPR treatment. To test the hypothesis that JNK phosphorylation precedes apoptosis induction, we performed a time course experiment using SH-EP1 cells expressing control (SCR) and suppressed (p75shRNA) levels of p75NTR. Fig. 1B demonstrates that JNK phosphorylation increases within 5 min of 4-HPR treatment (6 μ M; 72 h). In contrast, our previous studies showed that apoptosis induction-associated caspase 3 activation occurs only at time points > 24 h after 4-HPR treatment [7]. Note that the magnitude of the increase in JNK phosphorylation in the SH-EP1 cells transfected with p75shRNA is significantly lower than the corresponding increase in SCR transfected control cells.

Osone et al. showed that MAPKKKs are activated with ROS production in 4-HPR treated neuroblastoma cells, leading to parallel phosphorylation of p38MAPK and JNK, and downstream activation of caspases [18]. SH-EP1 cells transfected with p75shRNA or control (SCR) shRNA constructs were treated with varying concentrations of 4-HPR for 72 h. Western blots for phosphorylated p38 show increased p38 phosphorylation with increasing concentrations of 4-HPR in SCR transfected cells but not in p75shRNA transfected cells (Fig. 1C).

JNK phosphorylation is necessary for 4-HPR-induced apoptosis in SH-EP1 neuroblastoma cells

As is shown in Fig. 2A, when SH-EP1 cells were pretreated with an inhibitor of JNK phosphorylation (SP600125), they were protected from the pro-apoptotic effects of 4-HPR (0-20 μ M; 72 h). Pre-treatment with SP600125 also inhibited caspase 9 cleavage (Fig. 2B), the final step in 4-HPR-induced apoptosis [20].

Mitochondrial superoxide production is a hallmark of the effects of 4-HPR on SH-EP1 neuroblastoma cells [7]. However, inhibition of mitochondrial superoxide production using DHA does not inhibit JNK phosphorylation (Fig 2C). On the other hand, inhibition of JNK phosphorylation in SH-EP1 neuroblastoma cells inhibits mitochondrial superoxide production, while scavenging of mitochondrial ROS with DHA does not inhibit JNK

phosphorylation, confirming that JNK phosphorylation is both upstream of and necessary for induction of accumulation of mitochondrial ROS by 4-HPR (Fig. 2D).

Activation of stress-activated MAP kinases establishes possible signaling pathways involved in 4-HPR-induced apoptosis

Since phosphorylation of JNK and p38 are key steps in initiation of 4-HPR-induced apoptosis in SH-EP1 neuroblastoma cells, the expression and activation of protein kinases involved in p38 and JNK phosphorylation were analyzed. 4-HPR treatment of SH-EP1 cells caused a significant increase in MKK4 and MEKK1 protein expression and MKK3/6 phosphorylation (Fig. 3).

Akt phosphorylation is increased in cells in which p75NTR is downregulated

SH-EP1 cells transfected with SCR control and p75shRNA constructs were treated with 8 μ M 4-HPR and analyzed at different time points using a Western blot. This time course study of Akt phosphorylation in response to 4-HPR treatment revealed a 30-50% increase in Akt phosphorylation in p75shRNA transfected compared to SCR transfected SH-EP1 cells (Fig. 4A). This implies that p75NTR expression suppresses Akt phosphorylation and thereby enhances susceptibility of SH-EP1 cells to 4-HPR-induced apoptosis. This is in concert with our previous demonstration that SH-EP1 cells that have a greater apoptotic response to 4-HPR treatment demonstrate suppression of the Akt signaling pathway [7].

4-HPR induces caspase 3 cleavage and downstream apoptosis in SH-EP1 neuroblastoma cells [7]. Fig. 4B shows that treatment of SH-EP1 cells with 4-HPR results in production of an Akt cleavage product. This is only seen at the later time points (24-72 h) in 4-HPR-treated SCR and p75shRNA transfected SH-EP1 cells, a finding that is in concert with the late activation of caspase 3 seen in this system [7]. However the amount of cleaved Akt is significantly higher in SCR than knockdown transfectants. In keeping with their resistance to 4-HPR-induced apoptosis, after transfection with p75shRNA, SH-EP1 cells treated with 4-HPR do not exhibit detectable cleavage of Akt (Fig. 4B).

DISCUSSION

As has been shown for other neuroblastoma cell lines [18], SH-EP1 transfectants expressing native and decreased amounts of p75NTR phosphorylate JNK when treated with 4-HPR. JNK phosphorylation is an early effect and is significantly higher in SH-EP1 cells with higher levels of p75NTR. 4-HPR treatment of neuroblastoma cells results in generation of mitochondrial ROS, activation of MAPKKs, and phosphorylation of p38MAPK and JNK with downstream activation caspases [18]. The present studies demonstrate an increase in p38MAPK phosphorylation with increasing 4-HPR concentration, and cleavage of Akt in 4-HPR-treated cells with native p75NTR content, but not in those with downregulated p75NTR.

Taken together our results confirm a pro-apoptotic role for JNK phosphorylation and demonstrate potentiation of JNK signaling by p75NTR in 4-HPR-treated neuroblastoma cells. Downregulation of p75NTR results in decreases in p38MAPK phosphorylation, JNK phosphorylation, caspase 3 and Akt cleavage, and mitochondrial superoxide accumulation in SH-EP1 human S-type neuroblastoma cells treated with 4-HPR. Downregulation of p75NTR also results in increased Akt phosphorylation, enhancing resistance of the p75shRNA-transfected cells to 4-HPR-induced apoptosis.

Finally, treatment with 4-HPR results in upregulation of MKK4 and MEKK1, and phosphorylation of MKK3/6. Efforts to enhance the efficacy of 4-HPR and to identify those tumors most likely to respond to it might exploit these effectors of 4-HPR-induced

apoptosis. For example, in neuroblastomas that do not express high levels of p75NTR, pharmacological agents that enhance MKK4 or MEKK1 expression or that enhance JNK expression or phosphorylation might be used adjunctively with 4-HPR.

The studies of others have indicated that SH-EP1 cells are not tumorigenic in murine xenograft models [21]. However, the correlation between xenograft tumorigenicity and human tumor recurrence from minimal residual disease is not clear. SH-EP1 cells were chosen for the present studies because they most closely resemble the cells that are most frequently evasive of and persistent in bone marrow despite chemotherapy.

Translational research to develop better agents and strategies with which to eradicate cells that are resistant to standard chemotherapeutic agents is essential to our ability to improve survival in this high risk disease. Identification of biomarkers that characterize those cells that are susceptible to particular agents and strategies will facilitate development of patient- and tumor-specific therapies for neuroblastoma.

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Figure 1.

Downregulation of p75NTR expression (50% relative to cells transfected with a scrambled construct (SCR; [7], Fig. 3) in SH-EP1 human S-type neuroblastoma cells (American Type Culture Collection, Rockville, MD) causes a decrease in 4-HPR-induced (Sigma Chemical Corp, St. Louis, MO) JNK and p38 phosphorylation. A. 4-HPR treatment of p75shRNA and scrambled control (SCR) transfected cells causes an increase in JNK phosphorylation which is inhibited by knockdown of p75NTR. A representative Western blot is shown. For this and all subsequent graphs, error bars signify the SEM of three or more independent values.

* $p < 0.05$; ** $p < 0.01$ relative to control; Student's t-test. B. Time course of 4-HPR treatment (6 μ M) in p75shRNA and SCR transfected cells shows a significant decrease in JNK phosphorylation when p75NTR is knocked down. A representative Western blot is shown and the associated graph depicts mean $\pm$ SEM for three independent determinations (Scion Image) of optical density of pJNK. * $p < 0.05$; ** $p < 0.01$ relative to control. C. Optical densitometric data from Western blots ($n=3$) showing no change in phosphorylation of p38 in p75shRNA transfected cells, but a significant increase in SCR transfected cells when treated for 72 h with different concentrations of 4-HPR. * $p < 0.05$; ** $p < 0.01$ relative to control; Student's t-test.

Figure 2.

JNK phosphorylation is a necessary intermediate step in 4-HPR-induced apoptosis in SH-EP1 neuroblastoma cells. A. Alamar blue assay (Invitrogen, Carlsbad, CA) absorbance demonstrating the protective effect of inhibiting JNK phosphorylation in 72 h treatment of 4-HPR cells. Differential results are observed with SP600125 (Selleck Chemicals, Houston, TX; white circles) and without SP600125 (black circles) pretreatment followed by 4-HPR treatment (0–20 μ M). At all concentrations of 4-HPR, SH-EP1 cell metabolic viability with JNK inhibitor pretreatment differs from that without pretreatment with $p < 0.01$. B. Caspase-3 cleavage (normalized to β -actin loading control) quantified from Western blots of SH-EP1 cells treated with 4-HPR (0–16 μ M). * $p < 0.05$; ** $p < 0.01$ relative to control; Student's t-test. C. Time course of JNK phosphorylation in p75shRNA and SCR transfected cells pretreated with the mitochondrial ROS scavenger, DHA (Sigma Chemical Corp., St. Louis, MO; 400 M; 24 h), then treated with 4-HPR (6 μ M) for 72 h. As is the case for Figure 1B, in the absence of DHA, quantified Western blots showing a significant decrease in JNK phosphorylation in p75shRNA transfected relative to SCR transfected SH-EP1 cells with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. However, DHA pretreatment does not alter JNK phosphorylation in either transfectant (Compare Figure 1B and Figure 2C. Note that the time course in Figure 1B is normalized actin at each time point and then normalized to $t=0$ for each transfectant, while that in Figure 2C is simply normalized to actin at each time point.). D. MitoSOX™ (Molecular Probes, Inc., Eugene, OR) staining of SH-EP1 cells treated with

4-HPR alone, SP600125 alone, and SP600125 pretreatment followed by 4-HPR treatment. A representative set of sister culture micrographs is shown of three independent sets performed. Fluorescence imaging of MitoSOX™ stain (seen as bright red dots on the otherwise black background) demonstrates accumulation of superoxide in mitochondria in 4-HPR treated SH-EP1 cells but significantly reduced MitoSOX™ staining with SP600125 treatment. The images shown are representative results of three experiments performed.

Figure 3.

MAPK signaling pathways activated in SH-EP1 neuroblastoma cells during 4-HPR-induced apoptosis. A. Time course of 4-HPR treatment of SCR and p75shRNA transfected SH-EP1 cells treated with 6 μ M 4-HPR. Western blots showing MEKK1, MEK3/6, MEK4, and β -actin protein expression for early and late time points following 4-HPR treatment. B. Quantification of Western blots shown in (A) demonstrating a significant increase in MEKK1 phosphorylation after 4-HPR treatment of SCR transfected cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ relative to control. Note that an approximately 2-fold increase in MEKK1 phosphorylation is seen in SCR relative to p75shRNA transfected cells. C. Quantification of Western blots shown in (A) demonstrating a significant increase in MEK4

phosphorylation after 4-HPR treatment of SCR transfected cells. $**p<0.01$ relative to control. Note that a 2-3 fold increase in MEK4 phosphorylation is seen in SCR relative to p75shRNA transfected cells. D. Quantification of Western blots shown in (A) demonstrating a significant increase in MEK3/6 phosphorylation after 4-HPR treatment of SCR transfected cells. $*p<0.05$; $**p<0.01$ relative to control. Note that an approximately 2-fold increase in MEK3/6 phosphorylation is seen in SCR relative to p75shRNA transfected cells.

Figure 4.

Akt phosphorylation protects SH-EP1 cells transfected with p75shRNA from and is cleaved during 4-HPR-induced apoptosis. A. Time course of Akt phosphorylation following 8 μ M 4-HPR treatment showing a significant and sustained increase in Akt phosphorylation in SH-EP1 cells transfected with p75shRNA with * $p < 0.05$; ** $p < 0.01$ relative to SCR transfected cells. A representative Western blot is shown and quantified across 3 repetitions. B. Akt cleavage was detected by Western blot using anti-Akt1/2 antibody from Santa Cruz (sc-1619), which detects both holo- and the N-terminal fragment of Akt. Akt cleavage at the later time points (24-72 h) following 4-HPR treatment of SH-EP1 cells was significantly higher in SCR transfected with ** $p < 0.01$; *** $p < 0.001$ relative to p75shRNA transfected cells.