Brain-Derived Neurotrophic Factor Activation of TrkB Induces Vascular Endothelial Growth Factor Expression via Hypoxia-Inducible Factor-1α in Neuroblastoma Cells

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Abstract

The extent of angiogenesis and/or vascular endothelial growth factor (VEGF) expression in neuroblastoma tumors correlates with metastases, N-myc amplification, and poor clinical outcome. Recently, we have shown that insulin-like growth factor-I and serum-derived growth factors stimulate VEGF expression in neuroblastoma cells via induction of hypoxia-inducible factor-1α (HIF-1α). Because another marker of poor prognosis in neuroblastoma tumors is high expression of brain-derived neurotrophic factor (BDNF) and its tyrosine kinase receptor, TrkB, we sought to evaluate the involvement of BDNF and TrkB in the regulation of VEGF expression. VEGF mRNA levels in neuroblastoma cells cultured in serum-free media increased after 8 to 16 hours in BDNF. BDNF induced increases in VEGF and HIF-1α protein, whereas HIF-1β levels were unaffected. BDNF induced a 2- to 4-fold increase in VEGF promoter activity, which could be abrogated if the hypoxia response element in the VEGF promoter was mutated. Transfection of HIF-1α small interfering RNA blocked BDNF-stimulated increases in VEGF promoter activity and VEGF protein expression. The BDNF-stimulated increases in HIF-1α and VEGF expression required TrkB tyrosine kinase activity and were completely blocked by inhibitors of phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways. These data indicate that BDNF plays a role in regulating VEGF levels in neuroblastoma cells and that targeted therapies to BDNF/TrkB, PI3K, mTOR signal transduction pathways, and/or HIF-1α have the potential to inhibit VEGF expression and limit neuroblastoma tumor growth. (Cancer Res 2006; 66(8): 4249-55)

Introduction

Patients with neuroblastoma whose tumors express high levels of brain-derived neurotrophic factor (BDNF) and its tyrosine kinase receptor, TrkB, have a poor prognosis (1, 2). Recent studies indicate that BDNF activation of the TrkB pathway increases neuroblastoma cell survival (2–4), neurite extension (2, 5), and cell invasion (3) and protects cells from chemotherapy (6–9). These studies indicate that BDNF activation of the TrkB pathway contributes to the biology of neuroblastoma tumors aside from being just markers of poor prognosis. The extent of angiogenesis and elevated levels of vascular endothelial growth factor (VEGF) and other proangiogenic factors also correlate with poor clinical outcome in neuroblastoma (10, 11). Recently we found that insulin-like growth factor-I (IGF-I) activation of the IGF receptor tyrosine kinase was important in regulating hypoxia-inducible factor-1α (HIF-1α) and VEGF levels under normoxic conditions in neuroblastoma cells (12). Based on this study, we hypothesize that BDNF activation of the TrkB pathway may regulate VEGF levels in neuroblastoma cells.

A role for neurotrophins during angiogeneisis has recently emerged. Neurotrophic factors are structurally and functionally related growth factors, including nerve growth factor (NGF), BDNF, NT-3, and NT-4/5, that stimulate the survival, differentiation, and function of neural cells via selective activation of tyrosine kinase receptors (TrkA, TrkB, and TrkC) in the absence or presence of p75NTR (13). NGF increases the levels of VEGF in normal neural cells (14), induces proliferation of TrkA and p75-expressing endothelial cells (15), and stimulates angiogenesis in animal models of ischemia (16). BDNF is an endothelial survival factor (17), stimulates angiogenesis (18), and can increase levels of VEGFR in TrkB-expressing endothelial cells (19).

Angiogenesis is essential for tumor development and metastasis. To initiate angiogenesis, tumor cells make an angiogenic switch by perturbing the local balance of proangiogenic and antiangiogenic factors (20). VEGF is a strong proangiogenic factor and an attractive target for antiangiogenic therapies. A major regulator of VEGF is HIF-1α. The importance of this factor in the regulation of VEGF levels was revealed with the finding that the kidney cancer von-Hippel-Lindau tumor suppressor gene (*VHL*) encoded a protein that targets HIF-1α protein to the proteasome for degradation (21). Mutations in VHL lead to increased levels of HIF-1α and, in turn, constitutively high VEGF levels. Recently, we have shown that the constitutive expression of VEGF by neuroblastoma cells under normoxic conditions is due not to mutations in VHL but rather to serum-derived growth factors, including IGF-I, that stimulate increases in HIF-1α and lead to increases in VEGF mRNA and protein expression (12).

In this study, we examine the ability of BDNF activation of the TrkB signal transduction pathway to regulate VEGF levels in neuroblastoma cells. We find that BDNF increases VEGF expression and secretion in neuroblastoma cells. The BDNF/TrkB activation of the phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways leads to an increase in HIF-1α that stimulates VEGF promoter activity and transcription. Targeted decreases in HIF-1α expression using a HIF-1α small interfering RNA (siRNA) block BDNF's ability to stimulate VEGF transcription and expression in neuroblastoma cells.

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Materials and Methods

Cell lines. The tetracycline-regulated, TrkB-expressing SY5Y cells (TB3 and TB8) have been previously described (8, 9, 22). SY5Y cells were transfected with the tetracycline-regulated vector, pBPSTR1, as a control (E2). SMS-KCNR neuroblastoma cells were transfected with pBPSTR1-TrkB (22) by electroporation, and puromycin-resistant clones were isolated (KCNR-TrkB).

Cell culture and reagents. One million neuroblastoma cells (E2, TB3, TB8, and KCNR-TrkB cells) per well were cultured into six-well plates in RPMI 1640 containing 10% fetal bovine serum for 16 hours. Neuroblastoma cells were shifted into serum-free RPMI for 6 hours and then treated with control media, BDNF (100 ng/mL; PeproTech, Inc., Rocky Hill, NJ), or NGF (100 ng/mL; Upstate, Lake Placid, NY) for 8 hours. Cells and media were collected and stored as previously described (12). Cells were treated with 100 ng/mL BDNF for the indicated times with or without a 1-hour pretreatment with the pharmacologic inhibitors. TrkB inhibitor K252a (Calbiochem, San Diego, CA), PI3K inhibitor LY294002 (Sigma, St. Louis, MO), MAPK kinase inhibitor PD98059 (Sigma), and mTOR inhibitor rapamycin (Cell Signaling Technology, Beverly, MA) were obtained and reconstituted according to manufacturer's specifications.

Protein assays. Western blotting and immunoprecipitation were done as described previously (8, 12). Antibodies used were anti-phospho-tyrosine antibody (PY-99; Santa Cruz Biotechnology, Santa Cruz, CA), anti-pan-Trk antibody (C-14; Santa Cruz Biotechnology), anti-TrkB antibody (H-181; Santa Cruz Biotechnology), anti-HIF-1α antibody (BD Transduction laboratories, San Jose, CA), anti-HIF-2α antibody (Novus Biologicals, Littleton, CO), anti-HIF-1β antibody (Novus Biologicals). The concentration of VEGF protein was measured using an ELISA kit with a mouse monoclonal antibody against VEGF (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Plasmids. The series of VEGF reporter constructs, including pVEGF-*Kpn*I (from −2274 to +379; ref. 23), P11w (from −985 to −939: CCACAGTGCA*TACGTGGG*CTCCAACAGGTCCTCTTCCCTCCCATGCA; ref. 23), and P11m (from −985 to −939: CCACAGTGCA*TAAAAGGG*CTCCAACAGGTCCTCTTCCCTCCCATGCA; ref. 23), were purchased from American Type Culture Collection (Manassas, VA). The pGL2TKHRE plasmid containing three copies of the hypoxia response element (HRE; 5′-GTGAC*TACGTGCT*GCCTAG-3′) was a generous gift from Dr. Giovanni Melillo (Science Applications International Corporation-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD; ref. 24).

Transient transfection. DNA plasmids were prepared using a commercially available kit (Endofree Maxi-Prep; Qiagen, Valencia CA). Transfections were done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 16 hours, the protein lysates were made, and luciferase assays were done in 96-well optiplates (Packard Instrument, Inc., Meriden, CT) as previously described (25).

RNA interference. The HIF-1α siRNA (AAAGGACAAGTCACCACAGGA) to target HIF-1α and a nonspecific siRNA control (AATTCTCCGAACGTGTCACGT; Qiagen) were used. One million TB8 cells were cotransfected with *Kpn*I, P11w, P11m, or pGL2TKHRE and HIF-1α siRNA using LipotectAMINE 2000 (Invitrogen) according to manufacturer's the instructions. After 24 hours, the cell supernatants were collected and evaluated for VEGF protein expression by ELISA; the cells were lysed with 1% NP-40 lysis buffer, and protein expression was evaluated by Western blot analysis. Luciferase assays were done in 96-well optiplates (Packard Instrument).

Results

BDNF induces VEGF protein expression. To assess whether BDNF regulates VEGF expression, SMS-KCNR cells were incubated for 8 or 16 hours in serum-free media followed by the addition of 10% FCS or BDNF (100 ng/mL). Such treatment results in an increase in VEGF mRNA expression at 16 hours as determined by Northern blot (Fig. 1*A*). To study BDNF regulation of VEGF in a more defined system, we used the well-characterized TB3 and TB8 cell lines in which SY5Y neuroblastoma cells stably express a tetracycline (TET)–regulated TrkB expression plasmid (pBPSTR1-TrkB). E2 cells in which SY5Y neuroblastoma cells have been transfected with the control empty vector plasmid (pBPSTR1) were also used (8, 9, 22). In E2 cells, the basal level of TrkB does not change in either the TET^+ or TET^- conditions. In contrast, both TB3 and TB8 cells express a 4- to 5-fold increase in TrkB levels when cultured in the absence of TET, and BDNF induced an almost 4-fold increase in phospho-Trk levels under TrkB high-expressing condition (TET^-) in both TB3 and TB8 cells (Fig. 1*B*). Conversely, there was no detectable phosphorylation of Trk in E2 cells. To examine whether BDNF stimulates VEGF expression, we evaluated the levels of VEGF protein secretion in E2, TB3, and TB8 cells. BDNF stimulates 1.2- and 2-fold increases in VEGF secretion protein in TB3 cells expressing low (TET^+) and high (TET^-) levels of TrkB, respectively, after both 8 hours (Fig. 1*C*) and 16 hours (data not shown). BDNF stimulates an almost 2- and 2.5-fold increase in VEGF secretion protein in TB8 cells expressing low (TET^+) and high (TET^-) levels of TrkB, respectively, after both 8 hours (Fig. 1*C*) and

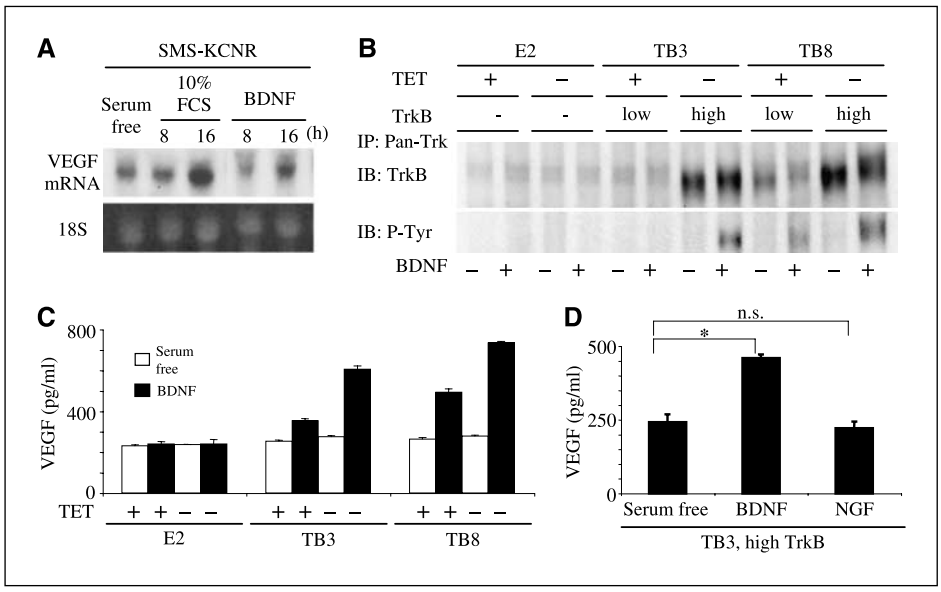

Figure 1. BDNF regulates VEGF levels in TrkB-expressing NB cells. *A,* Northern analysis of 25 μg of total RNA from SMS-KCNR cells after stimulation of serum-deprived cells (6 hours) with 10% FCS or BDNF for an additional 8 or 16 hours. 18S ribosomal RNA is shown as a loading control. *B,* TrkB pathway. Protein lysates (500 μg) from E2, TB3, and TB8 cells treated with or without TET or BDNF were immunoprecipitated with rabbit polyclonal anti-pan Trk antibody (C-14) and subjected to Western analysis (as described in Materials and Methods) for evaluation of phosphorylation of TrkB and total TrkB levels. *C,* conditioned medium from E2, TB3, and TB8 cells (1×10^6) treated with or without TET or BDNF (100 ng/mL) for 8 hours was analyzed by ELISA for VEGF expression. *Columns,* mean of triplicate values for each condition; *bars,* SD. *D,* conditioned medium from TB3 cells (1×10^6) expressing high TrkB (TET^-) treated with or without BDNF (100 ng/mL) or NGF (100 ng/mL) for 8 hours was analyzed by ELISA for VEGF expression. *Columns,* mean of triplicate values for each condition; *bars,* SD.
*, $P < 0.05$.

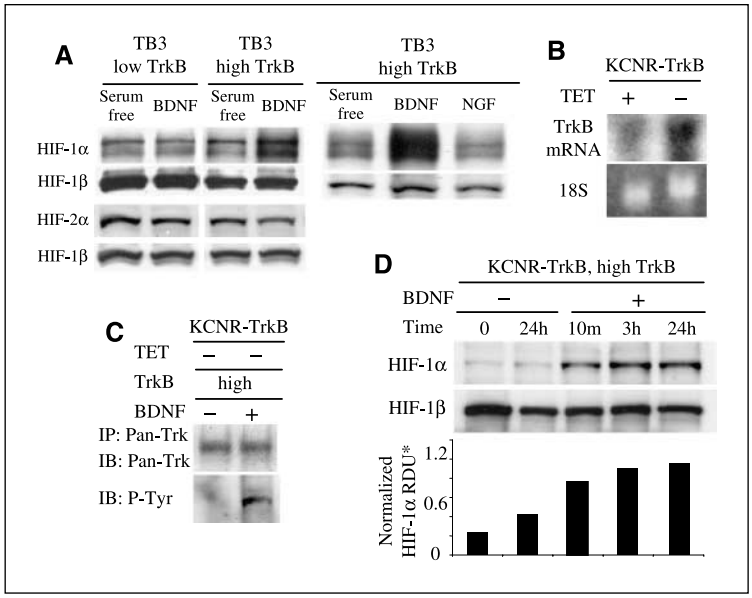

Figure 2. BDNF regulates HIF-1α levels in TrkB-expressing NB cells. *A,* Western analysis of total protein lysates (30 μg) from indicated TB3 cells treated for 8 hours with or without TET or BDNF (100 ng/mL) for HIF-1α, HIF-2α, and HIF-1β levels. Total protein lysates from indicated TB3 cells expressing high TrkB (TET^-) treated for 8 hours with or without BDNF (100 ng/mL) or NGF (100 ng/mL) for HIF-1α and HIF-1β levels. *B,* Northern analysis of 25 μg of total RNA from KCNR-TrkB cells with or without TET for 48 hours. 18S ribosomal RNA is shown as a loading control. *C,* TrkB pathway. Protein lysates (500 μg) from KCNR-TrkB treated with or without BDNF were immunoprecipitated with rabbit polyclonal anti-pan Trk antibody (C-14) and subjected to Western analysis (as described in Materials and Methods) for evaluation of TrkB autophosphorylation and total TrkB levels. *D,* Western analysis of 30 μg of HIF-1α levels in protein lysates from indicated KCNR-TrkB cells treated for 0 to 24 hours with or without BDNF (100 ng/mL) for HIF-1α and HIF-1β levels. Normalized HIF-1α protein expression. **RDU,* relative densitometric unit.

16 hours (data not shown). However, BDNF does not stimulate VEGF protein secretion in E2 cells (Fig. 1*C*). These data indicate that the levels of endogenous TrkB are not sufficiently high to stimulate VEGF protein secretion in the E2 cells or are below the levels detectable in our system. Moreover, when cells are treated with retinoic acid to induce endogenous TrkB (5), BDNF stimulates an induction of VEGF protein secretion in the parental SY5Y and KCNR cells (data not shown). BDNF acts as a ligand of TrkB and the p75 neurotrophin receptor, a member of the death receptor family that binds all NGF family neurotrophins. To examine whether activation of p75 alone stimulates an increase in VEGF, we treated TB3 cells expressing high levels of TrkB (TET^-) with NGF (100 ng/mL), which is also a ligand of p75. BDNF increases VEGF protein expression (*, $P < 0.05$), but NGF does not cause a significant change in VEGF levels (Fig. 1*D*). These data indicate that activation of p75 alone is not sufficient to induce VEGF secretion.

BDNF induces HIF-1α protein expression. Previously, we have shown that activation of the IGF-I/IGF-I–receptor pathway stimulated VEGF expression in neuroblastoma cells, and that this increase was mediated by an IGF-I-stimulated increase in HIF-1α expression (12). Recently it was reported that HIF-2α is expressed in human fetal paraganglia and neuroblastoma (26). To determine whether BDNF stimulates HIF-1α and HIF-2α expression, we treated TB3 cells cultured in the absence or presence of TET with BDNF. BDNF does not stimulate HIF-1α protein levels in TB3 cells expressing low (TET^+) levels of TrkB, and BDNF stimulates a 2-fold increase in HIF-1α protein in TB3 cells expressing high (TET^-) levels of TrkB (Fig. 2*A*). However, under these conditions, the levels of HIF-2α and HIF-1β are unchanged (Fig. 2*A*). These data indicate that BDNF does not induce HIF-2α expression in this system. We treated the high TrkB-expressing TB3 cells (TET^-) with BDNF or NGF and found that BDNF, but not NGF, induces HIF-1α protein (Fig. 2*A*). To extend these findings to an additional neuroblastoma cell line expressing TrkB, we developed a TrkB-regulated neuroblastoma cell line in the N-myc-amplified KCNR cell line using the pBPSTR1-TrkB expression vector (22). KCNR-TrkB cells incubated for 48 hours in the absence of TET showed an increase in TrkB mRNA expression compared with cells cultured in the presence of TET as determined by Northern blot (Fig. 2*B*). BDNF stimulation of KCNR-TrkB (TET^-) causes a 2-fold increase in autophosphorylation of the TrkB receptor compared with media-treated cells (Fig. 2*C*). BDNF treatment of KCNR-TrkB cells expressing high levels of TrkB (TET^-) stimulated an increase in HIF-1α expression within 10 minutes of treatment that further increased up to 3-fold by 3 hours and remained at these levels 24 hours after treatment (Fig. 2*D*). Thus, in two distinct types of neuroblastoma cell lines expressing TrkB, BDNF stimulates HIF-1α expression.

HIF-1α induced by BDNF mediates VEGF protein expression. To directly evaluate whether the BDNF induction of HIF-1α plays a role in stimulating VEGF protein expression, we transfected an artificial HRE promoter containing three copies of HRE (pGL2TKHRE) into the high TrkB-expressing TB3 cells (TET^-) and cultured for 16 hours in the absence of serum (serum-free), with BDNF (100 ng/mL), or with NGF (100 ng/mL). BDNF stimulated a 2.5-fold increase in HIF-1α transcriptional activity in TB3 cells expressing high levels of TrkB, but NGF did not (Fig. 3*A*). The increase in VEGF transcriptional activity is also regulated by the level of TrkB expression, because BDNF stimulated a 3.5-fold increase in TB8 cells expressing high levels of TrkB (TET^-) compared with a 2.8-fold increase in TB8 cells expressing low levels of TrkB (TET^+; Fig. 3*B*).

To evaluate BDNF regulation of the natural VEGF promoter, we transfected TB3 and TB8 cells expressing high levels of TrkB with a luciferase reporter plasmid containing sequence −2272 to +379 of the VEGF promoter (*Kpn*I; ref. 23). BDNF induced a 3- to 4-fold increase in transcriptional activity in TB3 cells expressing high levels of TrkB (TET^-) that were transfected with *Kpn*I or with P11w, a luciferase reporter construct containing the HRE of the natural VEGF promoter (Fig. 3*C*). BDNF did not stimulate transcriptional activity in P11m, a luciferase reporter construct in which the HRE was mutated and nonfunctional (Fig. 3*C*). Similar data have been generated using TB8 cells expressing high levels of TrkB (TET^-). BDNF induced 1.5- to 4-fold increases in transcriptional activity transfected with *Kpn*I, P11w, and pGL2TKHRE into the high TrkB expressing TB8 cells (TET^-; Fig. 3*D*). However, BDNF failed to stimulate an increase in transcriptional activity in P11m, which contained the mutated HRE (Fig. 3*D*). These data indicate

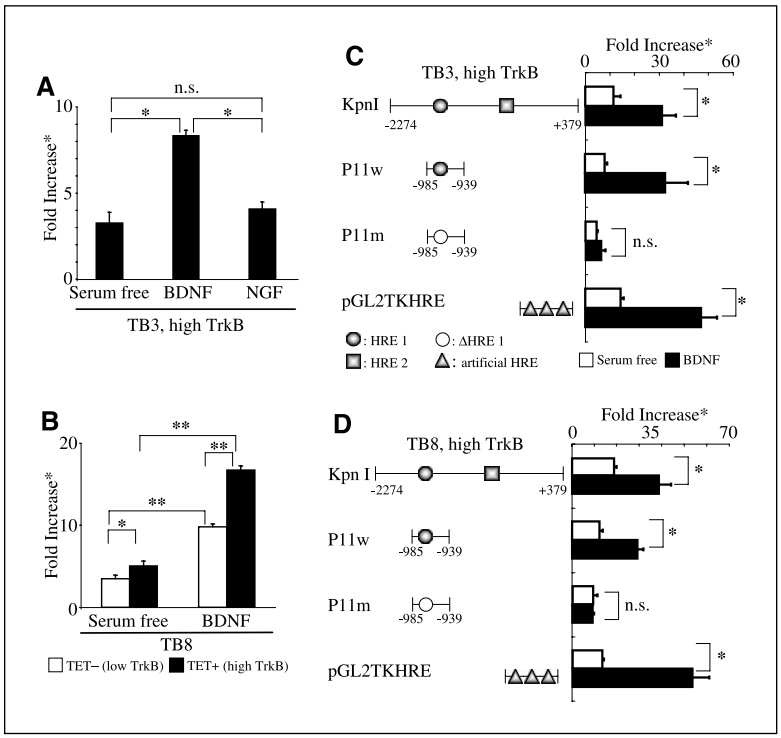

Figure 3. HIF-1α induced by BDNF mediates VEGF protein expression. *A,* luciferase activity from TB3 cells (1×10^6) at TrkB high expressing conditions (TET^-) treated with or without BDNF (100 ng/mL) or NGF (100 ng/mL) for 16 hours was analyzed by luciferase assay. *Columns,* mean of triplicate values for each condition; *bars,* SD. *, $P < 0.01$. **Fold increase,* relative fold increase of normalized luciferase activity. *B,* luciferase activity from TB8 cells (1×10^6) at both high and low TrkB (TET^- and TET^+) treated with or without BDNF (100 ng/mL) for 16 hours was analyzed by luciferase assay. *Columns,* mean of triplicate values for each condition; *bars,* SD. *, $P < 0.05$; **, $P < 0.001$. *C,* luciferase activity from the high TrkB-expressing TB3 cells (TET^-; 1×10^6) transfected with *Kpn*I, P11w, P11m, or pGL2TKHRE treated with or without BDNF (100 ng/mL) for 16 hours was analyzed by luciferase assay. *Columns,* mean of triplicate values for each condition; *bars,* SD. *, $P < 0.01$. *D,* luciferase activity from the high TrkB-expressing TB8 cells (TET^-; 1×10^6) transfected with *Kpn*I, P11w, P11m, or pGL2TKHRE treated with or without BDNF (100 ng/mL) for 16 hours was analyzed by luciferase assay. *Columns,* mean of triplicate values for each condition; *bars,* SD. *, $P < 0.01$.

that the BDNF/TrkB signal transduction pathway via HIF-1α drives VEGF transcription.

HIF-1α mediates activity stimulating VEGF protein expression. To directly evaluate whether HIF-1α plays a role in BDNF/TrkB–stimulated VEGF protein expression, we transfected a HIF-1α siRNA or a control siRNA into the high TrkB-expressing TB8 cells (TET^-) and cultured for 24 hours after BDNF (100 ng/mL) stimulation. According to Western blot analysis, HIF-1α protein expression dramatically decreased in cells transfected with the HIF-1α siRNA compared with cells transfected with the control siRNA treated with or without BDNF (Fig. 4*A*). Furthermore, HIF-1α siRNA knockdown caused a 65%, 74%, and 58% decrease in BDNF-induced transcriptional activity in the high TrkB expressing TB8 cells (TET^-) transfected with *Kpn*I, P11w, and pGL2TKHRE, respectively (Fig. 4*B*). When VEGF protein levels were measured in the supernatants from these same samples, the BDNF-stimulated VEGF protein levels decreased ~50% in the HIF-1α siRNA-transfected samples compared with the control siRNA-transfected samples (Fig. 4*C*). These results indicate that a large portion of the BDNF-induced VEGF expression is regulated by HIF-1α.

BDNF/TrkB signaling transductional pathway is essential in BDNF-induced HIF-1α and VEGF expression. To determine the role that the level of TrkB expression plays in modulating downstream BDNF-stimulated increases in VEGF expression, we evaluated HIF-1α levels in BDNF-stimulated TB3 cells cultured in the absence or presence of BDNF. BDNF stimulates a 4- to 6.5-fold

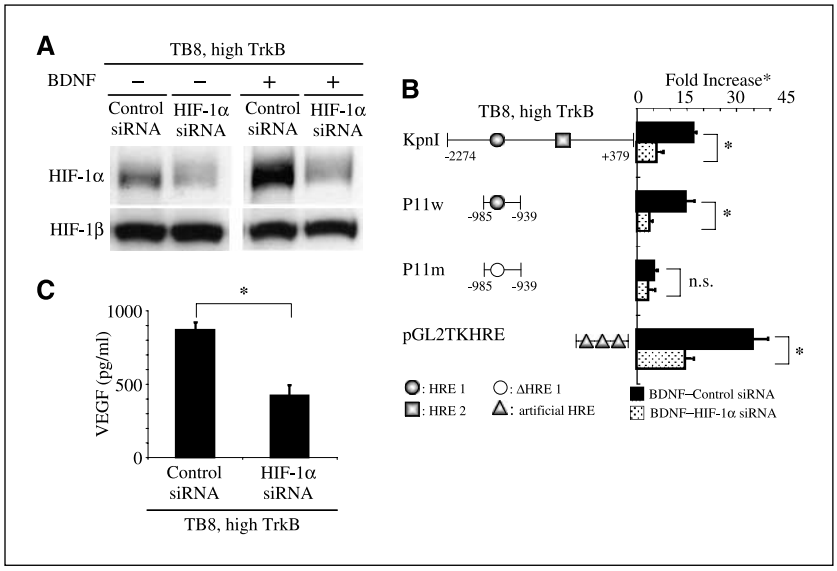

Figure 4. HIF-1α siRNA knockdown of VEGF protein expression. *A,* Western analysis of total protein lysates (30 μg) from indicated TB8 cells expressing high TrkB (TET^-) for 6 hours in serum-free RPMI transfected HIF-1α siRNA with or without BDNF after 24 h for HIF-1α and HIF-1β levels. *B,* luciferase activity from the high TrkB-expressing TB8 cells (TET^-; 1×10^6) cotransfected with *Kpn*I, P11w, P11m, or pGL2TKHRE with control siRNA or HIF-1α siRNA treated with BDNF (100 ng/mL) for 16 hours was analyzed by luciferase assay. *Columns,* mean of triplicate values for each condition; *bars,* SD. *, $P < 0.01$. *C,* VEGF protein levels from the same samples in (*B*) were analyzed by ELISA. *Columns,* mean of triplicate values for each condition; *bars,* SD. *, $P < 0.01$. **Fold increase,* relative fold increase of normalized luciferase activity.

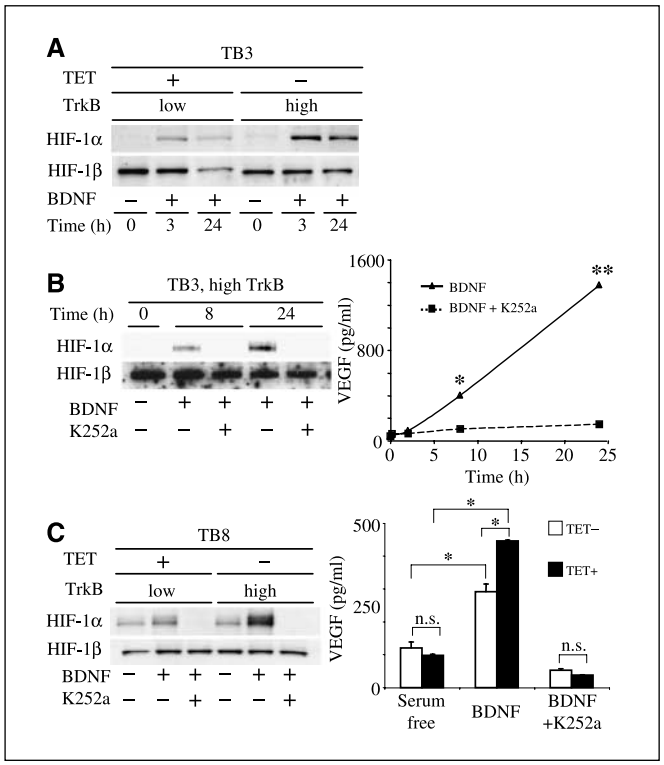

Figure 5. BDNF/TrkB pathway is essential in BDNF-induced HIF-1α and VEGF expression. *A,* Western analysis of 30 μg of HIF-1α levels in protein lysates from indicated TB3 cells treated for 0, 3, or 24 hours with or without TET or BDNF (100 ng/mL) for HIF-1α and HIF-1β levels. *B,* Western analysis of 30 μg of HIF-1α and HIF-1β levels in protein lysates from indicated TB3 cells under high TrkB (TET^-) treated for 0, 8, or 24 hours with or without BDNF (100 ng/mL) or K252a (0.5 μmol/L). VEGF protein expression by ELISA (*solid line*, BDNF; *dashed line*, BDNF + K252a) *, $P < 0.05$; **, $P < 0.01$. *C,* Western analysis of 30 μg of HIF-1α and HIF-1β levels in protein lysates from indicated TB8 cells under both high and low TrkB (TET^-, TET^+) treated for 8 hours with or without BDNF (100 ng/mL) or K252a (0.5 μmol/L). VEGF protein expression by ELISA (*white columns*, TET^+; *black columns*, TET^-) *, $P < 0.01$.

increase of HIF-1α expression in cells expressing high levels of TrkB (TET^-) compared with cells expressing low levels of TrkB (TET^+) at 3 to 24 hours (2- to 2.5-fold increase; Fig. 5*A*). Furthermore, we measured the expression of HIF-1α and VEGF induced by BDNF in the presence of K252a, which selectively inhibits Trk receptors. K252a completely abrogates both HIF-1α and VEGF protein expression at 8 to 24 hours (Fig. 5*B*). We also evaluated the effects of the BDNF/TrkB signal transduction pathway in TB8 cells. BDNF stimulates a 3-fold increase in HIF-1α and VEGF in high TrkB-expressing cells (TET^-) compared with serum-free conditions, whereas BDNF stimulates up to a 2-fold increase in HIF-1α and VEGF in low TrkB-expressing cells (TET^+) compared with the serum-free conditions (Fig. 5*C*). Furthermore, K252a completely inhibits expression of HIF-1α and VEGF (Fig. 5*C*). However, it should be noted that pretreatment with K252a leads to a lower level of HIF-1α than that detected at the baseline condition, which may be due to off target effects of K252a.

Both PI3K/mTOR pathway and mitogen-activated protein kinase pathway are involved in BDNF-induced HIF-1α and VEGF expression. To evaluate the signaling paths that contribute to the BDNF-induced HIF-1α and VEGF expression in TrkB expressing neuroblastoma cells, we used pharmacologic inhibitors of the mitogen-activated protein kinase (MAPK) pathway (PD98059), PI3K (LY294002), and mTOR (rapamycin). When TB8 cells were treated with BDNF following pretreatment with LY294002 or rapamycin, BDNF-induced HIF-1α expression was markedly inhibited, and HIF-1β and gel stain were shown as loading control (Fig. 6*A*). In addition, pretreatment with PD98059, the MAPK inhibitor, partially inhibited the BDNF-induced HIF-1α expression (Fig. 6*A*). Consistent with inhibition of HIF-1α protein, LY294002 and rapamycin blocked the BDNF-induced VEGF protein secretion from TB8 cells. PD98059 partially inhibited the VEGF secretion (*, $P = 0.0001$; **, $P < 0.05$; ***, $P < 0.01$; Fig. 6*B*). Although treatment with the mTOR and PI3K inhibitors significantly blocked VEGF secretion, the levels were slightly higher than the levels at baseline. This may be due to the contribution of MAPK to stimulate a small but significant change in VEGF levels (27). These findings indicate that the PI3K/mTOR pathway markedly decreases expression of HIF-1α, which leads to decreases in VEGF expression.

Discussion

Poor prognosis neuroblastoma tumors are marked by expression of BDNF, its tyrosine kinase receptor TrkB (1, 2), proangiogenic cytokines (11), and a high degree of vascularization (10). Previously,

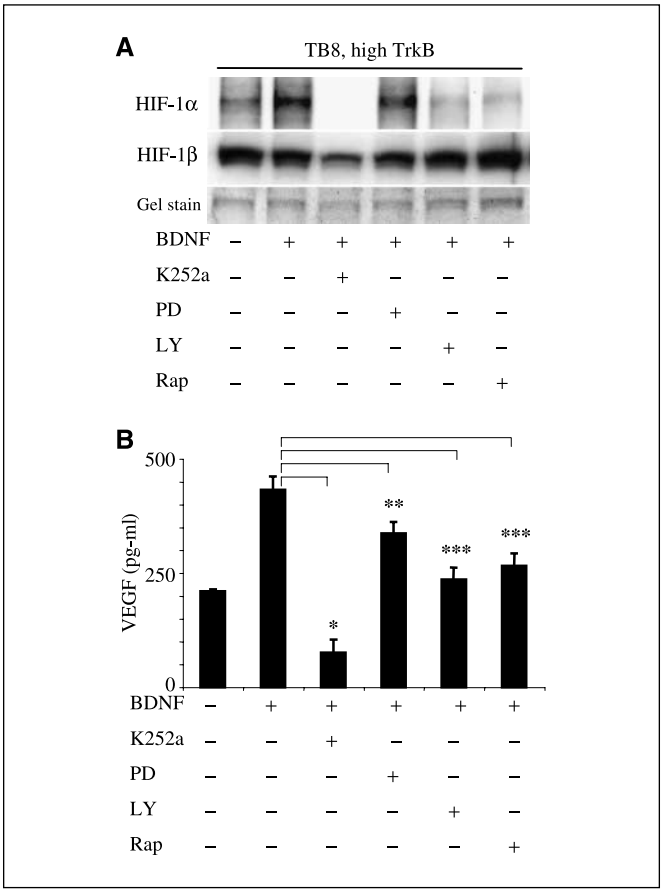

Figure 6. BDNF regulates HIF-1α and VEGF levels via PI3K/mTOR and MAPK pathways. *A,* Western analysis of 30 μg of HIF-1α levels in protein lysates from the serum-deprived (6 hours) high TrkB-expressing TB8 cells (TET^-) pretreated with control solvent or indicated inhibitor for 1 hour followed by stimulation with BDNF for 8 hours. HIF-1β and gel stain are shown as loading control. *B,* conditioned media from the high TrkB-expressing TB8 cells (TET^-; 1×10^6) incubated with serum-free media for 6 hours following a 1-hour preincubation with control solvent or indicated inhibitor was analyzed by ELISA for VEGF expression. *Columns,* mean of triplicate values for each condition; *bars,* SD. *, $P = 0.0001$; **, $P < 0.05$; ***, $P < 0.01$.

we identified that serum-derived growth factors increased VEGF levels in neuroblastoma cells (12). In this study, we sought to evaluate whether the neurotrophic factor BDNF affects the regulation of VEGF expression in neuroblastoma cells. We find that BDNF activation of TrkB stimulates VEGF mRNA transcription via induction of HIF-1α. This increase in VEGF mRNA leads to an increase in VEGF secreted by neuroblastoma cells. Moreover, we find that BDNF induces VEGF expression via the TrkB/PI3K/mTOR pathway. BDNF induction of HIF-1α is a major mediator of the increased expression of VEGF in TrkB-expressing neuroblastoma cells. Thus, in addition to its previously described effects on neuroblastoma cell survival, chemosensitivity, and metastasis, activation of the BDNF/TrkB signal transduction pathway stimulates VEGF levels in neuroblastoma. Because VEGF is a proangiogenic cytokine whose high expression in neuroblastoma tumors is a marker of poor prognosis, the activation of TrkB may contribute to the proangiogenic phenotype seen in neuroblastoma tumors.

Our evidence that the TrkB receptor is key to the BDNF-mediated increase in HIIF-1α and VEGF expression is primarily based on our findings that (*a*) TrkB-selective ligands mediate the effect; (*b*) the TET-regulatable expression vector shows that with increasing TrkB expression, there is increasing expression of HIF-1α and VEGF; (*c*) cells transfected with the control vector fail to induce HIF-1α and VEGF when treated with BDNF; and (*d*) K252a can inhibit activation of the Trk tyrosine kinase (8) and in this study also diminishes HIF-1α and VEGF. The latter finding with K252a is consistent with regulation through Trk, although limited by recent findings that K252a also blocks expression of other proteins such as c-*jun* and activator protein that are involved in regulation of HIF-1α (28).

Neurotrophins (NGF, BDNF, NT-3, and NT-4) through their activation of Trks (TrkA, TrkB, and TrkC) are major effectors of the survival, axonal growth, and differentiation of neurons (29, 30). Recent studies also indicate that there is a complex interaction among neurotrophins, endothelial, and neural cells during development and during ischemic alterations in the brain. NGF promotes endothelial vessel growth (16, 31–33) and increases in arteriole length density (16). NGF also stimulates VEGF expression in normal neurons (14). BDNF activation of TrkB promotes angiogenesis in the developing embryonic myocardium (17), recruits brain-derived (19) and bone marrow-derived TrkB-expressing endothelial precursor cells from the bone marrow (18) and increases VEGFR on endothelial cells (19). Thus, in normal tissues, neurotrophins act as proangiogenic stimuli.

Tumors from neuroblastoma patients with a favorable prognosis express relatively high levels of TrkA (34), whereas those from poor prognosis patients express BDNF and TrkB (1, 2). The differential expression of Trk receptors has been proposed to affect the biology of these tumors and affect the survival of patients. Our data unequivocally show that BDNF stimulates an increase in HIF-1α expression that increases VEGF transcription leading to an increase in VEGF secretion by neuroblastoma cells. This is similar to the effects of BDNF on normal neural tissues. Our finding that NGF does not alter VEGF levels indicates only that the effects of neurotrophic factors are not solely mediated by the pan-neurotrophic factor receptor p75 and do not address whether NGF activation of TrkA affects VEGF levels. However, in another study, overexpression of TrkA under either normoxic or hypoxic conditions inhibits angiogenesis in SY5Y neuroblastoma cells by down-regulation of angiogenic factors, including VEGF. In this study, the addition of NGF did not dramatically alter the effect of increased TrkA on VEGF levels (35). A more detailed study is needed to assess whether this finding is applicable to other neuroblastoma tumors cells and what role NGF activation of TrkA may play in the regulation of HIF-1α and VEGF transcription in normal and tumor cells.

This study identifies a potential mechanism for neurotrophin regulation of VEGF expression in neural-derived cells by finding that BDNF induction of HIF-1α mediates the increase in VEGF transcription. Recent studies show that hypoxia induces BDNF (36), which has a protective effect on normal neural tissue that may be related to vascular adjustments (37). Whether the increase in BDNF under hypoxic conditions requires induction of HIF-1α or whether BDNF-stimulated increases in HIF-1α are required for neuroprotection against hypoxia in normal tissues remains to be elucidated.

Our finding that BDNF activation of TrkB stimulates VEGF expression may also be relevant to a number of other human tumors that express BDNF and TrkB. The BDNF/TrkB pathway is expressed in hepatocellular carcinoma (38), prostate cancer (39, 40), medulloblastoma (41), lung cancer (42), and pancreatic carcinoma (43). In hepatocellular carcinoma patients, the serum levels of BDNF correlated with the status of microsatellites and tumor recurrences (38). It is possible that these findings may be related to the ability of BDNF activation of TrkB to affect survival and angiogenesis.

In conclusion, our study has identified an additional role for BDNF/TrkB in neuroblastoma tumorgenesis as a potential stimulator of angiogenesis. Furthermore, it has identified a number of potential target sites in the TrkB, PI3K, and mTOR pathways and HIF-1α, which have the potential to inhibit VEGF expression and limit neuroblastoma tumor growth. Our further studies will be aimed at blocking BDNF effects on tumor growth and angiogenesis *in vivo* in neuroblastoma tumors.

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