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Interactions between Vascular Endothelial Growth Factor and Neuroglobin

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Abstract

Vascular endothelial growth factor (VEGF) and neuroglobin (Ngb) participate in neuronal responses to hypoxia and ischemia, but the relationship between their effects, if any, is unknown. To address this issue, we measured Ngb levels in VEGF-treated mouse cerebrocortical cultures and VEGF levels in cerebrocortical cultures from Ngb-overexpressing transgenic mice. VEGF stimulated Ngb expression in a VEGFR2/Flk1 receptor-dependent manner, whereas Ngb overexpression suppressed expression of VEGF. These findings provide further insight into hypoxia-stimulated neuronal signaling pathways.

Keywords

Vascular endothelial growth factor; neuroglobin; hypoxia

Introduction

Neuronal adaptation to hypoxic or ischemic injury is mediated partly through the increased expression of neuroprotective proteins, such as vascular endothelial growth factor (VEGF) [8] and neuroglobin (Ngb) [9]. VEGF, which stimulates angiogenesis and neurogenesis in addition to affording neuroprotection, is transcriptionally activated by hypoxia-inducible factor-1 (HIF-1) [5]. Ngb protein levels are also enhanced by HIF-1 [10], although apparently indirectly, as the Ngb promoter lacks HIF-1-binding hypoxia-response elements [31].

In addition to hypoxia and HIF-1, Ngb expression can be enhanced experimentally by cobalt [28], deferoxamine [28], hemin [35], and certain short chain fatty acids [15], but how Ngb is regulated under physiological or pathological conditions in vivo is poorly understood. Hormones—in particular 17 β -estradiol [3] and erythropoietin [7]—can stimulate Ngb protein expression in cultured neural cells and gerbil brain respectively, and the neuroprotective effect of 17 β -estradiol against H₂O₂ toxicity is Ngb-dependent. Other globins are similarly hormone-inducible, as in upregulation of neuronal hemoglobin by Epo

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[25]. Growth factors have also been implicated in regulating globin expression, as exemplified by the ability of platelet-derived growth factor and transforming growth factor- β 1 to augment cytoglobin levels in cultured hepatic stellate cells [24].

Although growth factors, like Ngb, are induced by cerebral ischemia [2,20], the effect of growth factors on Ngb expression and signaling has received little attention. In a single study [26], conditional deletion of the von Hippel-Lindau gene from renal glomerular podocytes, resulting in stabilization of HIF-1, upregulated both VEGF and Ngb; however, the relationship between these effects, if any, was not examined. Accordingly, we investigated possible interrelationships between VEGF and Ngb expression in cultured mouse cortical neurons. The results provide new insight into signaling pathways involving hypoxia-inducible neuroprotective proteins.

Materials and methods

Neuronal cultures were prepared from cerebral cortex of 16 day old wild type or NGB-overexpressing transgenic [17,18] mouse embryos (Jackson Laboratory, Sacramento, CA). Cells were grown in neurobasal medium containing B27 supplement, 2 mM glutamate, and 1% penicillin and streptomycin. After 4 days, one-half of the medium was replaced with neurobasal medium, and experiments were conducted at 8 days in vitro [16]. The cellular composition of cultures at this time, based on counting of cells immunostained for cell type-specific protein markers, was 98% neuronal and 2% astrocytic. Cultures were treated for 24 h with VEGF (VEGF-A; R&D Systems, Minneapolis, MN) with or without the VEGF receptor tyrosine kinase (VEGFR2/Flk1) inhibitors SU1498 ((E)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-n-propyl)amino-carbonyl]acrylonitrile; EMD4Biosciences, Gibbstown, NJ) [27] and VEGFR2 kinase inhibitor IV (3-(3-thienyl)-6-(4-methoxyphenyl)pyrazolo[1,5-a]pyrimidine; EMD4Biosciences) [6], or the mitogen-activated protein kinase (MAPK) inhibitor PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; Calbiochem, La Jolla, CA).

For immunoblotting, medium was removed. Cells were scraped from culture dishes, briefly centrifuged to remove remaining medium, and resuspended in RIPA protein extraction buffer with 50 μ M EDTA and Pierce protease inhibitor (Thermo Fisher Scientific, Waltham, MA). Lysates were sonicated for 10 sec and centrifuged at maximal speed for 20 min at 4°C. Supernatants were collected and protein concentration determined by BCA protein assay (Biorad, Hercules, CA); 50 μ g of protein was placed in SDS running buffer with dithiothreitol, incubated for 10 min at 70°C, run on 12% SDS-PAGE for 35 min at 200 V, and transferred to a 0.2- μ m PVDF membrane (Thermo Fisher Scientific) at 70 V for 2 h. Membranes were blocked in 5% dry milk and incubated with rabbit anti-VEGF-A (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or chicken anti-Ngb (1:1000; My Biosource, San Diego, CA) overnight. Membranes were washed three times with 1 $\times$ PBS-Tween, incubated for 1 h in goat anti-chicken HRP (1:10,000; Santa Cruz), washed three times in PBS-Tween, incubated in Western Lightning Ultra (PerkinElmer, Waltham, MA) for 2 min, and blotted dry. Signal on blots was detected by autoradiography. Protein levels were measured by densitometry with Quantity One (Biorad) and normalized using mouse anti- β -actin (1:10,000; Sigma, St. Louis, MO). Student's paired *t*-test was used to determine statistical significance (*p*<0.05).

Results

Treatment with VEGF increased Ngb protein expression in cortical neuron cultures, with maximal (about threefold) enhancement at 20 ng/ml of VEGF (Figure 1), which is similar to

the concentration achieved with hypoxia in vitro [12] and at which other VEGF effects have been observed in these cultures [14].

To investigate the site through which VEGF acts, we first probed cultures for expression of the two principal tyrosine kinase receptors that mediate its effects—VEGFR1/Flt1 and VEGFR2/Flk1 [19]. As shown in Figure 2, both receptors were expressed in our cultures; in principle, therefore, either could be responsible for the effect of VEGF on Ngf levels.

Next we examined the effect of selective VEGFR2/Flk1 inhibitors on induction of Ngf by VEGF. SU1498 was tested at 10 μ M, which is about ten times its half-maximally effective concentration (IC_{50}) for inhibiting VEGFR2/Flk1 [27], but has no effect on VEGFR1/Flt1 [1]. VEGFR kinase inhibitor IV was tested at 20 nM, which approximates its IC_{50} for VEGFR2/Flk1 and is about tenfold lower than its IC_{50} for VEGFR1/Flt1 [6]. Each inhibitor reversed VEGF-induced elevation of Ngf levels, consistent with a VEGFR2/Flk1-mediated effect (Figure 3).

Because we found previously that hypoxic induction of Ngf may involve MAPK [35], we also tested the effect of PD98059, a MAPK inhibitor, on induction of Ngf by VEGF. Figure 4 shows that in the presence of 1–100 μ M PD98059, VEGF failed to increase Ngf levels.

Finally, we investigated whether Ngf exerts a reciprocal influence on VEGF levels, by comparing levels in cultures from wild type and Ngf-overexpressing transgenic mice. Figure 5 illustrates that VEGF levels were reduced by about 50% in transgenic compared to wild type mice, suggesting that Ngf downregulates VEGF.

Discussion

The main findings of this study are that VEGF upregulates Ngf expression and Ngf downregulates VEGF expression in neuron-enriched murine cerebrocortical cultures. Accordingly, Ngf levels were increased by VEGF treatment, through an effect mediated largely via VEGFR2/Flk1 receptors, whereas overexpression of Ngf reduced VEGF levels.

The detailed mechanisms that underlie the reciprocal effects of VEGF and Ngf are uncertain. In endothelial cells, VEGF can induce the expression of several proteins, including transforming growth factor- β 1 (TGF- β 1) [22], connective tissue growth factor (CTGF) [29], and profibrotic and extracellular matrix genes [21], and exerts a positive feedback effect on HIF-1 α expression [4]. VEGF-induced HIF-1 α expression is thus one mechanism that could account for induction of Ngf. Alternatively, the induction by VEGF of TGF- β 1 and CTGF has been attributed to MAPK and phosphatidylinositol 3'-kinase (PI3K) signaling, which VEGF also activates in neural cells [13,34]. Moreover, MAPK has been implicated in the induction of Ngf by hypoxia [35] and 17 β -estradiol [3], so MAPK might mediate the effect of VEGF on Ngf as well. Our finding that the VEGF-induced increase in Ngf expression was blocked by a MAPK inhibitor is consistent with this possibility. Other potential mediators include reactive oxidative species, which have been proposed to be responsible for the induction of HIF-1 α by VEGF in endothelial cells [4] and of Ngf by arsenite in cerebellum [30].

How Ngf decreases VEGF levels is also unclear, but altered Ngf gene expression produces changes in the neuronal expression of several genes that regulate VEGF. These include HIF-1 α [5], which is suppressed in Ngf-overexpressing transgenic [33] and enhanced in Ngf-knockout [11] mice, and endothelial PAS domain protein 1 [32], which is also suppressed by Ngf overexpression [33]. Therefore, Ngf might reduce VEGF levels through its effect on one or more of these genes.

The detailed mechanisms involved in Ngb induction and Ngb-mediated neuroprotection remain to be defined. However, the findings reported here add to current understanding of these interactions. As schematized in Figure 6, elements include HIF-dependent [5] and HIF-independent [23] induction of VEGF by hypoxia, positive feedback of VEGF on HIF expression [4], induction of Ngb through hypoxia [28] and HIF [10], and induction of Ngb by VEGF and suppression of VEGF by Ngb (this report).

Acknowledgments

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Abbreviations

VEGF	vascular endothelial growth factor
Ngb	neuroglobin
HIF-1	hypoxia-inducible factor-1
SU1498	(E)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-n-propyl)amino-carbonyl]acrylonitrile
VEGFR2 kinase inhibitor IV	3-(3-thienyl)-6-(4-methoxyphenyl)pyrazolo[1,5-a]pyrimidine
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
IC₅₀	half-maximally effective inhibitory concentration
TGF-β1	transforming growth factor- β 1
CTGF	connective tissue growth factor
MAPK	mitogen-activated protein kinase
PI3K	phosphatidyl inositol 3'-kinase

References

1. Buharalioglu CK, Song CY, Yaghini FA, Ghafoor HU, Motiwala M, Adris T, Estes AM, Malik KU. Angiotensin II-induced process of angiogenesis is mediated by spleen tyrosine kinase via VEGF receptor-1 phosphorylation. *Am J Physiol Heart Circ Physiol.* 2011; 301:H1043–1055. [PubMed: 21642504]
2. Croll SD, Wiegand SJ. Vascular growth factors in cerebral ischemia. *Mol Neurobiol.* 2001; 23:121–135. [PubMed: 11817215]
3. De Marinis E, Ascenzi P, Pellegrini M, Galluzzo P, Bulzomi P, Arevalo MA, Garcia-Segura LM, Marino M. 17 β -Estradiol--a new modulator of neuroglobin levels in neurons: role in neuroprotection against H₂O₂-induced toxicity. *Neurosignals.* 2010; 18:223–235. [PubMed: 21335947]
4. Deudero JJ, Caramelo C, Castellanos MC, Neria F, Fernandez-Sanchez R, Calabia O, Penate S, Gonzalez-Pacheco FR. Induction of hypoxia-inducible factor 1 α gene expression by vascular endothelial growth factor. *J Biol Chem.* 2008; 283:11435–11444. [PubMed: 18305118]
5. Forsythe JA, Jiang B-H, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol.* 1996; 16:4604–4613. [PubMed: 8756616]
6. Fraley ME, Hoffman WF, Rubino RS, Hungate RW, Tebben AJ, Rutledge RZ, McFall RC, Huckle WR, Kendall RL, Coll KE, Thomas KA. Synthesis and initial SAR studies of 3,6-disubstituted pyrazolo[1,5-a]pyrimidines: a new class of KDR kinase inhibitors. *Bioorg Med Chem Lett.* 2002; 12:2767–2770. [PubMed: 12217372]

7. Gao Y, Mengana Y, Cruz YR, Munoz A, Teste IS, Garcia JD, Wu Y, Rodriguez JC, Zhang C. Different expression patterns of Ngb and EPOR in the cerebral cortex and hippocampus revealed distinctive therapeutic effects of intranasal delivery of Neuro-EPO for ischemic insults to the gerbil brain. *J Histochem Cytochem*. 2011; 59:214–227. [PubMed: 21339183]
8. Greenberg DA, Jin K. From angiogenesis to neuropathology. *Nature*. 2005; 438:954–959. [PubMed: 16355213]
9. Greenberg D, Jin K, Khan A. Neuroglobin: an endogenous neuroprotectant. *Curr Opin Pharmacol*. 2008; 8:20–24. [PubMed: 17942367]
10. Haines B, Demaria M, Mao X, Xie L, Campisi J, Jin K, Greenberg DA. Hypoxia-inducible factor-1 and neuroglobin expression. *Neurosci Lett*. 2012; 514:137–140. [PubMed: 22342914]
11. Hundahl CA, Luuk H, Ilmarj S, Falktoft B, Raida Z, Vikesaa J, Friis-Hansen L, Hay-Schmidt A. Neuroglobin-deficiency exacerbates Hif1A and c-FOS response, but does not affect neuronal survival during severe hypoxia in vivo. *PLoS One*. 2011; 6:e28160. [PubMed: 22164238]
12. Ishikawa A, Yoshida H, Metoki N, Toki T, Imaizumi T, Matsumiya T, Yamashita K, Taima K, Satoh K. Edaravone inhibits the expression of vascular endothelial growth factor in human astrocytes exposed to hypoxia. *Neurosci Res*. 2007; 59:406–412. [PubMed: 17889387]
13. Jin KL, Mao XO, Greenberg DA. Vascular endothelial growth factor rescues HN33 neural cells from death induced by serum withdrawal. *J Molec Neurosci*. 2000; 14:197–203. [PubMed: 10984196]
14. Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:11946–11950. [PubMed: 12181492]
15. Jin K, Mao XO, Xie L, John V, Greenberg DA. Pharmacological induction of neuroglobin Expression. *Pharmacology*. 2011; 87:81–84. [PubMed: 21228614]
16. Khan AA, Mao XO, Banwait S, Dermardirossian CM, Bokoch GM, Jin K, Greenberg DA. Regulation of hypoxic neuronal death signaling by neuroglobin. *The FASEB Journal*. 2008; 22:1737–1747.
17. Khan AA, Sun Y, Jin K, Mao XO, Chen S, Ellerby LM, Greenberg DA. A neuroglobin-overexpressing transgenic mouse. *Gene*. 2007; 398:172–176. [PubMed: 17537594]
18. Khan AA, Wang Y, Sun Y, Mao XO, Xie L, Miles E, Graboski J, Chen S, Ellerby LM, Jin K, Greenberg DA. Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:17944–17948. [PubMed: 17098866]
19. Koch S, Tugues S, Li X, Gualandi L, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. *Biochem J*. 2011; 437:169–183. [PubMed: 21711246]
20. Kogure K, Kato H. Altered gene expression in cerebral ischemia. *Stroke*. 1993; 24:2121–2127. [PubMed: 8248999]
21. Kuiper EJ, Hughes JM, Van Geest RJ, Vogels IM, Goldschmeding R, Van Noorden CJ, Schlingemann RO, Klaassen I. Effect of VEGF-A on expression of profibrotic growth factor and extracellular matrix genes in the retina. *Invest Ophthalmol Vis Sci*. 2007; 48:4267–4276. [PubMed: 17724216]
22. Li ZD, Bork JP, Krueger B, Patsenker E, Schulze-Krebs A, Hahn EG, Schuppan D. VEGF induces proliferation, migration, and TGF- β 1 expression in mouse glomerular endothelial cells via mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Biochem Biophys Res Commun*. 2005; 334:1049–1060. [PubMed: 16039615]
23. Nakatani K, Okuyama H, Shimahara Y, Saeki S, Kim DH, Nakajima Y, Seki S, Kawada N, Yoshizato K. Cytoglobin/STAP, its unique localization in splanchnic fibroblast-like cells and function in organ fibrogenesis. *Lab Invest*. 2004; 84:91–101. [PubMed: 14647402]
24. Mizukami Y, Li J, Zhang X, Zimmer MA, Iliopoulos O, Chung DC. Hypoxia-inducible factor-1-independent regulation of vascular endothelial growth factor by hypoxia in colon cancer. *Cancer Res*. 2004; 64:1765–1772. [PubMed: 14996738]
25. Schelshorn DW, Schneider A, Kuschinsky W, Weber D, Kruger C, Dittgen T, Burgers HF, Sabouri F, Gassler N, Bach A, Maurer MH. Expression of hemoglobin in rodent neurons. *J Cereb Blood Flow Metab*. 2009; 29:585–595. [PubMed: 19116637]

26. Steenhard BM, Isom K, Stroganova L, St John PL, Zelenchuk A, Freeburg PB, Holzman LB, Abrahamson DR. Deletion of von Hippel-Lindau in glomerular podocytes results in glomerular basement membrane thickening, ectopic subepithelial deposition of collagen $\alpha 1\alpha 2\alpha 1$ (IV), expression of neuroglobin and proteinuria. *Am J Pathol.* 2010; 177:84–96. [PubMed: 20522651]
27. Strawn LM, McMahon G, App H, Schreck R, Kuchler WR, Longhi MP, Hui TH, Tang C, Levitzki A, Gazit A, Chen I, Keri G, Orfi L, Risau W, Flamme I, Ullrich A, Hirth KP, Shawver LK. Flk-1 as a target for tumor growth inhibition. *Cancer Res.* 1996; 56:3540–3545. [PubMed: 8758924]
28. Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proceedings of the National Academy of Sciences of the United States of America.* 2001; 98:15306–15311. [PubMed: 11742077]
29. Suzuma K, Naruse K, Suzuma I, Takahara N, Ueki K, Aiello LP, King GL. Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-Akt-dependent pathways in retinal vascular cells. *J Biol Chem.* 2000; 275:40725–40731. [PubMed: 11018037]
30. Wang J, Zhang W, Sun D, Song L, Li Y, Xu C. Analysis of neuroglobin mRNA expression in rat brain due to arsenite-induced oxidative stress. *Environ Toxicol.* 2010 Nov 29. [Epub ahead of print].
31. Wystub S, Ebner B, Fuchs C, Weich B, Burmester T, Hankeln T. Interspecies comparison of neuroglobin, cytoglobin and myoglobin: sequence evolution and candidate regulatory elements. *Cytogenet Genome Res.* 2004; 105:65–78. [PubMed: 15218260]
32. Xia G, Kageyama Y, Hayashi T, Kawakami S, Yoshida M, Kihara K. Regulation of vascular endothelial growth factor transcription by endothelial PAS domain protein 1 (EPAS1) and possible involvement of EPAS1 in the angiogenesis of renal cell carcinoma. *Cancer.* 2001; 91:1429–1436. [PubMed: 11301389]
33. Yu Z, Liu J, Guo S, Xing C, Fan X, Ning M, Yuan JC, Lo EH, Wang X. Neuroglobin-overexpression alters hypoxic response gene expression in primary neuron culture following oxygen glucose deprivation. *Neuroscience.* 2009; 162:396–403. [PubMed: 19401220]
34. Zhu Y, Jin K, Mao X, Greenberg D. Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. *Faseb J.* 2003; 17:186–193. [PubMed: 12554697]
35. Zhu Y, Sun Y, Jin K, Greenberg DA. Hemin induces neuroglobin expression in neural cells. *Blood.* 2002; 100:2494–2498. [PubMed: 12239161]

Highlights

- Vascular endothelial growth factor (VEGF) increased neuroglobin (Ngb) levels in mouse cerebrocortical cultures.
- This effect was reduced by VEGF receptor 2 (VEGFR2/Flk1) inhibitors.
- Transgenic overexpression of Ngb reduced VEGF levels in mouse cerebrocortical cultures.

Figure 1. VEGF-induced Ngf protein expression

Mouse cerebrocortical neuron cultures were treated for 24 h with the indicated concentrations of VEGF, Ngf ($M_r \sim 17$ kDa) levels were measured by immunoblotting (top), and the results were analyzed by densitometry, normalized to β -actin, and plotted as mean $\pm$ SEM (bottom). * $p < 0.05$, ** $p < 0.002$ compared to 0 ng/ml VEGF (N=4).

Figure 2. VEGF receptor expression

Triplicate mouse cerebrocortical neuron cultures were probed for expression of VEGFR1/Flt1 ($M_r \sim 180$ kDa, top) and VEGFR2/Flk1 ($M_r \sim 150$ kDa, bottom), with mouse hippocampal neuron $\times$ N18TG2 neuroblastoma (HN33) cells (right lane) used as a positive control.

Figure 3. Effect of VEGFR2/Flk1 receptor kinase inhibitors on VEGF-induced Ngf protein expression

Mouse cerebrocortical neuron cultures were treated for 24 h with 20 ng/ml of VEGF, with or without 10 μ M SU1498 (SU) or 20 nM VEGFR kinase inhibitor IV (Inh IV). Ngf levels were measured by immunoblotting (top), and the results were analyzed by densitometry, normalized to β -actin, and plotted as mean $\pm$ SEM (bottom). * p <0.05 (N=3), ** p <0.01 (N=4) compared to control.

Figure 4. Effect of MAPK inhibition on VEGF-induced Ngn expression

Mouse cerebrocortical neuron cultures were treated for 24 h with 20 ng/ml of VEGF, with or without 1–100 μ M PD90859 (PD), or with 100 μ M PD90859 alone. Ngn levels were measured by immunoblotting (top), and the results were analyzed by densitometry, normalized to β -actin, and plotted as mean $\pm$ SEM (bottom). *p<0.05 (N=3) compared to control.

Figure 5. VEGF expression in wild type and Ngb-overexpressing transgenic cultures
VEGF levels in cerebrocortical neuron cultures from wild type and Ngb-overexpressing transgenic mice were measured by immunoblotting (top), and the results were analyzed by densitometry, normalized to β -actin, and plotted as mean $\pm$ SEM (bottom). * $p < 0.02$ compared to wild type (N=4).

Figure 6. Overview of interactions between neuronal hypoxia-response mediators

Previously described, positive interactions are indicated by thin arrows. Positive and negative interactions described in this report are indicated by the thick pointed arrow and flat-tipped arrow, respectively.