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Hypoxia-inducible Factor-1 and Neuroglobin Expression

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Abstract

Neuroglobin (Ngb) is a hypoxia-inducible protein with cytoprotective effects in animal models of stroke, Alzheimer's disease, and related disorders, but the molecular mechanisms involved in its induction are unknown. We tested the hypothesis that hypoxia-inducible factor-1 (HIF-1) regulates Ngb levels, using shRNA-mediated knockdown and lentiviral vector-mediated overexpression of the HIF-1 α subunit, in cultured neural (HN33) cells. HIF-1 α knockdown decreased and HIF-1 α overexpression increased Ngb levels, consistent with a connection between HIF-1 and Ngb induction. These findings may have implications for understanding the hypoxia-response repertoire of neural cells and devising therapeutic strategies for neurologic disorders.

Keywords

neuroglobin; hypoxia; hypoxia-inducible factor-1; stroke

Introduction

Expression of neuroglobin (Ngb), an oxygen-binding heme protein produced in neurons [2] and selected other cell types [16], is induced by hypoxia and related pathological states [18], against which it also confers protection [10, 19, 21]. The ability of cobalt and deferoxamine to also increase Ngb levels [18] is reminiscent of certain other hypoxia-responsive proteins, including vascular endothelial growth factor (VEGF) and erythropoietin (Epo), for which hypoxia is linked to increased expression through the transcription factor, hypoxia-inducible factor-1 (HIF-1), a heterodimer comprising inducible (HIF-1 α) and constitutive (HIF-1 β) subunits [14, 20]. However, a connection between HIF-1 and Ngb has not been demonstrated directly, and some evidence argues against it. First, the Ngb promoter region appears to lack consensus HIF-1-binding hypoxia-response elements [25]. Second, hypoxia [4], cobalt [6] and deferoxamine [5] can all induce protein expression independent of HIF-1. Third, even classic HIF-1-responsive proteins like VEGF are inducible via additional pathways [1]. In addition to these considerations, several transcription factors can be activated downstream of HIF-1 [11-13], so its effects on targets like Ngb could be indirect.

Interest in Ngb has been stimulated by its beneficial effects in a variety of animal models of human disease, including stroke [10], myocardial infarction [10], Alzheimer's disease [9], spinal cord injury [3], and glaucoma [23]. These findings suggest that increasing Ngb levels might have therapeutic value in such conditions. Direct administration of Ngb is impractical because mammalian Ngb, unlike that produced by zebrafish, does not penetrate cell

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membranes [22]. However, a few small molecule drugs have been identified which can enhance neuroglobin expression, although their mechanisms of action are unclear [8, 18, 26]. Additional insight into the molecular mechanisms that drive Ngb expression could reveal new pharmacological targets.

Accordingly, we investigated the connection between HIF and Ngb expression by increasing or decreasing HIF-1 α levels in cultured neural cells and determining the effect on Ngb. Our findings support such a connection, and may, therefore, have implications for understanding the hypoxia-response repertoire of neural cells and devising therapeutic strategies for hypoxic-ischemic disorders.

Materials and methods

CMV-HIF-1 α and CMV-GFP (Origene, Rockville, MD) and HIF-1 α shRNA (Open BioSystems, Huntsville, AL) vectors were grown overnight in Luria broth with ampicillin (100 μ g/mL). Plasmid was isolated using a Qiagen Maxi Prep Kit and resuspended in 10 mM Tris-Cl (pH 8.5). DNA concentration was determined by absorbance at 260 nm using a Nano-drop spectrophotometer (Thermo Scientific, Rockford, IL). Plasmid was sequenced by Sequetech (Mountain View, CA).

Mouse HN33 (hippocampal neuron \times N18TG2 neuroblastoma) cells were maintained at 37°C in humidified 5% CO₂/95% air, in DMEM supplemented with heat-inactivated FBS (10%) and 50 U/mL penicillin/streptomycin [26]. Cells were seeded for transfection at 80% confluence on 100-mm Nunclon Δ Surface plastic culture dishes in DMEM and FBS without antibiotics; 30 μ g of plasmid was incubated with 60 μ L of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in DMEM without FBS for 30 min to form liposomes. Medium containing liposomes was dropped onto 100-mm cell culture dishes and cells were incubated for 30 hr.

Lentivirus was generated as described [17]. Briefly, 293T cells were co-transfected with pHCMVG and HIF-1 α shRNA using Lipofectamine 2000. Virus was collected at 48 and 72 hr and added to 60% confluent HN33 cells in 10 μ g/mL of polybrene and 10 mM HEPES. Cells were split 2 days later at 1:5 and selected using 1 μ g/mL of puromycin. Colonies were expanded and western blots performed to identify clones showing knockdown of HIF-1 α .

For Ngb immunoblotting, medium was removed, and 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 Scientific). Lysates were sonicated for 10 sec and centrifuged at maximum speed for 20 min at 4°C. Supernatants were collected and protein concentration determined by BCA protein assay (Biorad, Hercules, CA); 120 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 onto a 0.2- μ m PVDF membrane (Thermo Scientific) at 70 V for 2 hr. Membranes were blocked in 5% dry milk and incubated in chicken anti-Ngb (1:1000; My Biosource, San Diego, CA) overnight. Membranes were washed three times with 1 \times PBS-Tween, incubated for 1 hr in goat anti-chicken HRP (1:10,000; Santa Cruz Biotech, Santa Cruz, CA), washed three times in PBS-Tween, incubated in Western Lightning Ultra (PerkinElmer, Waltham, MA) for 2 min, and blotted dry.

For HIF-1 α immunoblotting, cells were rinsed in PBS, trypsinized, centrifuged (700 \times g at 4°C), and rinsed with ice-cold PBS; cytosolic and nuclear fractions were isolated using a NEPER kit (Thermo Scientific). Nuclear protein (40 μ g) was run on 4-12% SDS-PAGE under denatured conditions and transferred at 30 V overnight at 4°C onto 0.2- μ m PVDF membranes. Membranes were blocked and probed overnight at 4°C with goat anti-HIF-1 α

(1:2000; R&D Systems, Minneapolis, MN), and later probed with rabbit-anti-goat-HRP (1:10,000; Santa Cruz Biotech).

Signal was detected by autoradiography. Protein levels were measured by densitometry with Quantity One (Biorad) and normalized using mouse anti- β -actin (Sigma, St. Louis, MO) or rabbit anti-TATA binding protein (Abcam, Cambridge, UK). Student's t-test was used to determine statistical significance ($p < 0.05$).

MatInspector (Genomatix, Munich, FRG) identified four candidate HIF binding sites within 10,000 (1993, 5012, 6725 and 7736) base pairs upstream of the neuroglobin ATG start site. No such sites were detected in the 500 base pair stretch immediately upstream of the start site, consistent with a previous report [25]. For chromatin immunoprecipitation (ChIP) analysis, PCR primers were designed between 150-200 base pairs upstream and downstream from these binding sites by VectorNTI (Life Technologies). HN33 cells were grown to 80% confluence and treated with 150 μ M CoCl₂ for 16 hrs. Protein and DNA were crosslinked with 1% formaldehyde (Sigma) for 15 min at room temperature. The crosslinking reaction was quenched with 125 mM glycine (Sigma) for 5 min at room temperature. Cells were pelleted at 500 \times g for 5 min at 4°C, resuspended in lysis buffer (R'D Systems) with protease inhibitors (Pierce), and sonicated twice for 20 sec at 25% power (McKinley Scientific). The sample was split and incubated overnight at 4°C with 5 μ g of goat-anti-HIF-1 α or goat IgG. Dynal magnetic beads crosslinked with protein A and G (Life Technologies) were added and the mixture was incubated for 5 hrs at 4°C. A high powered magnet rack (R&D Systems) was used to pull down the magnetic beads, which were washed progressively with buffers (R&D Systems) to remove unbound chromatin and protein. Crosslinking was reversed by resuspending the beads in reverse crosslinking buffer with proteinase K (Life Technologies) at 55°C for 15 min and later deactivated at 95°C for 15 min. Pellets were vortexed and centrifuged at maximum speed and supernatants were collected. Pellets were resuspended in 150 μ L of water, and recentrifuged, and the supernatants were removed. DNA was purified using a Gel Purification Kit (Qiagen) and eluted with 50 μ L of 10 mM Tris-Cl (pH 8.5). The PCR mix included Go-Taq Master Mix (Promega, Madison, WI), 1 μ M each of forward and reverse primers (Eurofins MWG Operon, Huntsville, AL), 2 μ L of template, and water. Denaturation was run at 95°C for 2 min, amplification was run at 30 cycles of 95°C for 30 sec, 56°C for 30 sec and 72°C for one minute, and final extension of 72°C for 5 min. PCR products were run on a 2% agarose gel (Sigma) in tris-acetate EDTA buffer (Biorad) and imaged under UV light (Biorad).

Results

HIF-1 α protein immunoreactivity detected by western blotting was decreased by ~50% in HN33 cells transfected with lentiviral HIF-1 α shRNA, compared to cells transfected with control vector (Figure 1A-B). Reduction of HIF-1 α immunoreactivity was accompanied by a decrease of ~60% in immunodetectable Ngb (Figure 1C-D). Conversely, transfection of HN33 cells with CMV HIF-1 α vector increased HIF-1 α expression ~8-fold; a similar effect was observed following treatment with the proline hydroxylase domain protein inhibitor, 2,2-dipyridyl (Figure 2A-B). Increased expression of HIF-1 α was associated with an ~2-fold increase in the expression of Ngb (Figure 2C-D).

ChIP assay showed binding of HIF-1 α to predicted sites 5012, 6725 and 7736, but not 1993, base pairs upstream of the neuroglobin ATG start site (Figure 3).

Discussion

The main finding of this study is that HIF-1 α regulates Ngb expression in neural cells. Thus, knockdown of HIF-1 α reduced, whereas forced overexpression of HIF-1 α enhanced, Ngb levels in HN33 (mouse hippocampal neuron \times N18TG2 neuroblastoma) cells. Hypoxia, ischemia, and related pathological states have been shown previously to induce Ngb expression [7], as have chemical activators of hypoxia sensing, including cobalt and the iron-chelating compound, deferoxamine [18]. The observation that hypoxia, cobalt and deferoxamine also stabilize HIF-1 α suggested that their induction of Ngb might be mediated through HIF-1 α , but this has not been demonstrated before.

How HIF-1 α modifies Ngb expression is unclear. Hypoxia raises HIF-1 α protein levels largely by interfering with hydroxylation of HIF-1 α and its subsequent ubiquitination and proteasomal degradation [15]. Upon binding to transcriptional coactivators, HIF-1 α can activate transcription of hypoxia-responsive genes such as VEGF, Epo, and glycolytic enzymes. These genes share a consensus HIF-1-binding hypoxia-response element in their promoter regions, which Ngb appears to lack [25], at least within about 5000 base pairs upstream of the ATG start site. However, HIF-1 can also enhance expression of hypoxia-responsive proteins indirectly, through the induction of other transcription factors, or by post-transcriptional regulation via microRNAs [15].

Transcription factors that are induced by HIF-1 and for which binding sites are present in the Ngb promoter region are candidates for linking hypoxic stabilization of HIF-1 α to increased Ngb expression. Numerous transcription factors are transcriptional targets for HIF-1 [11-13] and potential binding sites for Sp1, NF-1, AP-1 and NF κ B have been identified within the Ngb promoter [25]. Interestingly, Sp1 has recently been reported to be a target for HIF-1, and sequential activation of HIF-1 and Sp1 has been implicated in hypoxic induction of the sulfonyleurea receptor 1 following cerebral ischemia [24]. Future studies will be directed at determining if the same is true for induction of Ngb.

Acknowledgments

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Abbreviations

Ngb	neuroglobin
VEGF	vascular endothelial growth actor
Epo	erythropoietin
HIF-1	hypoxia-inducible factor-1

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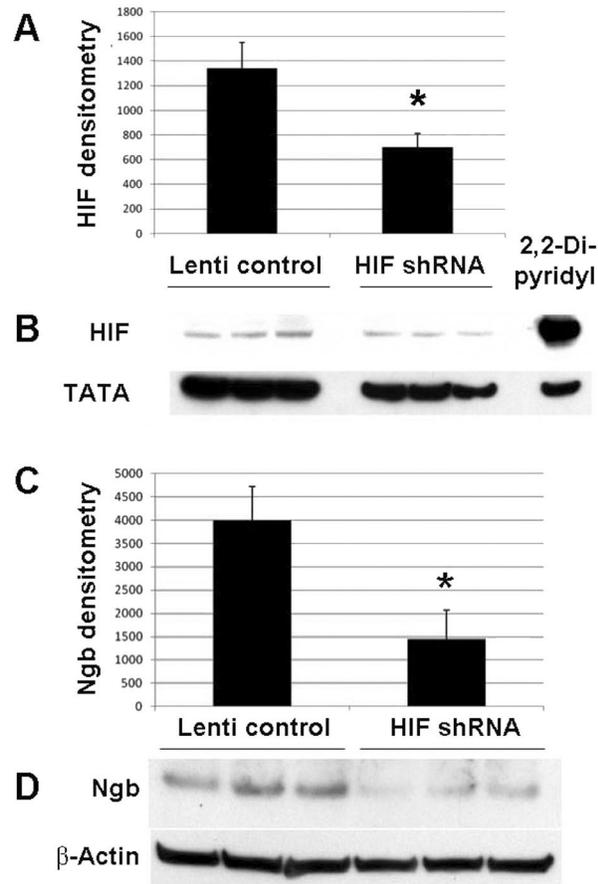


Figure 1. shRNA-mediated knockdown of HIF-1 α decreases Ngb protein levels

(A) Normalized densitometric analysis and (B) western blots of nuclear HIF-1 α protein in HN33 cells infected with control (left) or HIF-1 α shRNA-expressing (right) lentivirus (*, $p < 0.02$). Band at far right is from cells treated for 4 h with the prolyl hydroxylase domain-containing protein inhibitor, 2,2-dipyridyl (50 μ M). (C) Normalized densitometric analysis and (D) western blots of cytoplasmic Ngb protein in HN33 cells infected with control (left) or HIF-1 α shRNA-expressing (right) lentivirus (*, $p < 0.01$).

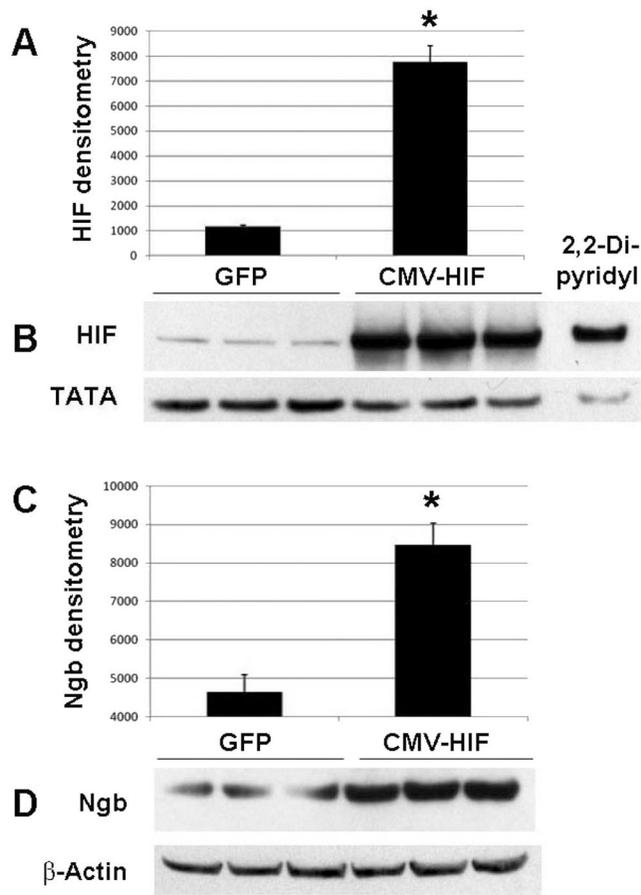


Figure 2. CMV-mediated HIF-1 α overexpression increases Ngf protein levels

(A) Normalized densitometric analysis and (B) western blots of nuclear HIF-1 α protein in HN33 cells infected with CMV-GFP control (left) or CMV-HIF-1 α (right) vector (*, $p < 0.001$). Band at far right is from cells treated for 4 h with the prolyl hydroxylase domain-containing protein inhibitor, 2,2-dipyridyl (50 μ M). (C) Normalized densitometric analysis and (D) western blots of cytoplasmic Ngf protein in HN33 cells infected with CMV-GFP control (left) or CMV-HIF-1 α (right) vector (*, $p < 0.001$).

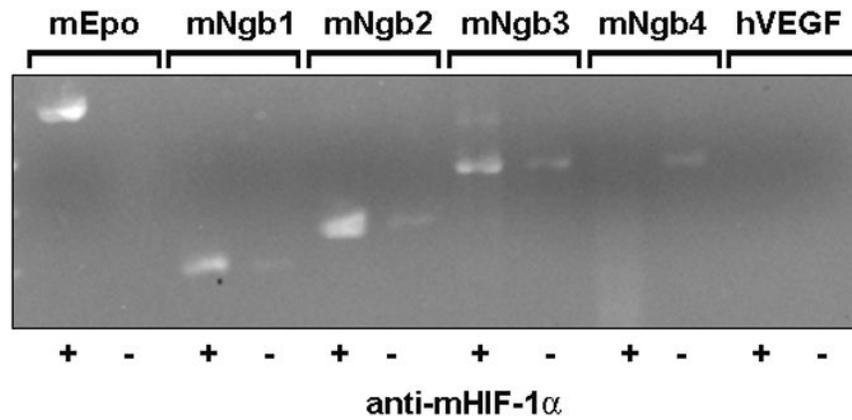


Figure 3. ChIP analysis of predicted HIF-1 α binding sites upstream of the Ngb ATG start site Anti-HIF-1 α was used to immunoprecipitate HIF-1 α -crosslinked DNA sequences amplified with primers for candidate HIF-1 α binding sites located 7736 (mNgb1), 6725 (mNgb2), 5012 (mNgb3), and 1993 (mNgb4) base pairs upstream of the neuroglobin ATG start site. All except mNgb4 yielded bands. mEpo served as a positive control for the ability to detect HIF-1 α binding sites, hVEGF as a negative control for DNA amplification, and goat IgG (lanes labeled "-") as a negative control for immunoprecipitation.