14-3-3 Binding and Phosphorylation of Neuroglobin during Hypoxia Modulate Six-to-Five Heme Pocket Coordination and Rate of Nitrite Reduction to Nitric Oxide

Received for publication, June 14, 2011, and in revised form, September 21, 2011. Published, JBC Papers in Press, September 30, 2011, DOI 10.1074/jbc.M111.271973

Thottala Jayaraman‡§¹, Jesús Tejero‡§¹, Bill B. Chen‡, Arlin B. Blood‡, Sheila Frizzell¹, Cali Shapiro¹, Mauro Tiso¹, Brian L. Hood¹, Xunde Wang**, Xuejun Zhao¹, Thomas P. Conrads¹, Rama K. Mallampalli†‡∥‡, and Mark T. Gladwin†§³

From the †Vascular Medicine Institute and ||Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, the ‡||Medical Specialty Service Line, Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, Pennsylvania 15240, the ¶Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, **NHBLI, National Institutes of Health, Bethesda, Maryland 20892, and the ¶¶Division of Neumatology, Department of Pediatrics, School of Medicine, Loma Linda University, Loma Linda, California 92354

Background: Neuroglobin protects neurons from hypoxia; however, the underlying mechanisms for this effect remain poorly understood.

Results: Hypoxia increases neuroglobin phosphorylation, binding to 14-3-3, and nitrite reduction to form nitric oxide.

Conclusion: Hypoxia-dependent post-translational modifications to neuroglobin regulate the six-to-five heme pocket equilibrium and heme access to ligands.

Significance: Hypoxia-regulated neuroglobin may contribute to the cellular adaptation to hypoxia.

Neuroglobin protects neurons from hypoxia in vitro and in vivo; however, the underlying mechanisms for this effect remain poorly understood. Most of the neuroglobin is present in a hexa-coordinate state with proximal and distal histidines in the heme pocket directly bound to the heme iron. At equilibrium, the concentration of the five-coordinate neuroglobin remains very low (0.1–5%). Recent studies have shown that post-translational redox regulation of neuroglobin surface thiol disulfide formation increases the open probability of the heme pocket and allows nitrite binding and reaction to form NO. We hypothesized that the equilibrium between the six- and five-coordinate states and secondary reactions with nitrite to form NO could be regulated by other hypoxia-dependent post-translational modification(s). Protein sequence models identified candidate sites for both 14-3-3 binding and phosphorylation. In both in vitro experiments and human SH-SY5Y neuronal cells exposed to hypoxia and glucose deprivation, we observed that 1) neuroglobin phosphorylation and protein-protein interactions with 14-3-3 increase during hypoxic and metabolic stress; 2) neuroglobin binding to 14-3-3 stabilizes and increases the half-life of phosphorylation; and 3) phosphorylation increases the open probability of the heme pocket, which increases ligand binding (CO and nitrite) and accelerates the rate of anaerobic nitrite reduction to form NO. These data reveal a series of hypoxia-dependent post-translational modifications to neuroglobin that regulate the six-to-five heme pocket equilibrium and heme access to ligands. Hypoxia-regulated reactions of nitrite and neuroglobin may contribute to the cellular adaptation to hypoxia.

Neurons require a constant supply of oxygen to maintain adequate energy production to support normal cellular survival. These cells have an ability to adapt to sudden deprivation of O₂ by activating specific cellular machinery through transcriptional and post-translational regulation. This is an area of intensive research, as understanding the regulation of endogenous respiratory proteins that protect neurons from ischemic and hypoxic insults is required to develop effective therapeutic strategies for common human diseases such as stroke and epilepsy. The 16.9-kDa protein neuroglobin is one such endogenous protein, as it is highly expressed in the neuronal structures of brain and retina (1–4) and shares ~16 and 23% sequence similarity with the O₂ carrier proteins myoglobin (in muscle) and hemoglobin (in blood) (1, 5), respectively. Neuroglobin expression is up-regulated during hypoxia (6–9), and neuroglobin protects neurons from hypoxic/ischemic injury and cytotoxicity in vitro and in vivo (10–14). However, the cellular mechanism(s) that regulate neuroglobin expression and activity during hypoxia remain incompletely understood.

A notable property of neuroglobin is that it possesses the bishistidine six-coordinate heme geometry with proximal and distal histidines in the heme pocket directly bound to the heme iron (both Fe²⁺ or Fe³⁺ oxidation states) (15). At equilibrium, the six-coordinate configuration is favored (16). The binding of oxygen or other gas ligands such as NO or carbon monoxide to the heme iron requires displacement of the sixth coordination...
bond with the distal His64 residue (17–20). Interestingly, the low tissue concentration of neuroglobin and the rapid autoxidation of the oxygen-bound species suggest that neuroglobin may not have evolved to store and supply oxygen, leading to a number of different hypotheses about its molecular functionality (2–4). These include roles in the scavenging of reactive oxygen and nitrogen species and cytochrome c reduction (10, 21).

We have recently characterized its activity as a nitrite reductase that reduces nitrite to NO based on studies of other related heme globins, myoglobin and hemoglobin, which function as nitrite reductases and NO signaling molecules (22–24). Although the signaling function of neuroglobin remains the subject of active speculation and research, the protein’s six-to-five heme coordination appears to be post-translationally regulated. One example is that oxidation induces a disulfide bridge formation between two surface cysteines (Cys46 and Cys55) in neuroglobin (25) and decreases the distal histidine binding affinity for heme iron ($K_{d_{hi}}$ value has been shown to decrease from ~3000 to 280 and is calculated as $k_{on}/k_{off}$) (25). This is accompanied by a five-coordinate neuroglobin subpopulation increase with enhanced affinity for endogenous ligands such as oxygen ($P_{so}$ shift from ~9 to 1 mm Hg) (26). In addition, oxidized disulfide-bridged neuroglobin also exhibits a higher affinity for nitrite than the thiol-reduced form (27) and a higher nitrite reductase activity (28). We have recently found that mutation of the distal His64 residue locks neuroglobin in a pentacoordinate state, increasing its catalytic nitrite binding and reductase activity compared with hexacoordinate neuroglobin (28). Therefore, any cellular mechanism that displaces His64 and stabilizes a five-coordinate conformation would be expected to increase its ligand binding affinity and potential nitrite reductase activity.

To identify potential post-translational modifications, especially those potentially regulated during hypoxia or ischemia, we analyzed the primary amino acid sequence of neuroglobin and identified the presence of several putative phosphorylation motifs, an ERK docking domain proximal to His64, and two potential 14–3–3-binding sites. Because ERK1/2 and PKA pathways are activated by hypoxic/ischemic insults (29, 30), we hypothesized that neurons could utilize the ERK1/2 and PKA pathways to modulate neuroglobin catalytic activity through phosphorylation. Similarly, 14–3–3 protein–protein interactions could modulate kinase binding and phosphorylation (31), stability of phosphorylation (32–34), or protein tertiary structure. Both modifications could alter catalytic activity through conformational changes affecting the heme pocket coordination. We therefore sought to determine whether neuroglobin is a target for these kinase pathways and 14–3–3 binding, how these post-translational modifications are influenced by hypoxia, and what effects they have on the heme pocket coordination and rates of nitrite reduction.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animal protocols were preapproved by the Loma Linda University Institutional Animal Care and Use Committee. Non-pregnant and pregnant ewes (135–140 days gestation, term 145) were killed by intravenous injection of sodium pentobarbital (100 mg/kg). Fetal and adult brains were immediately removed, and samples from various brain regions were isolated, snap-frozen in liquid nitrogen, and stored at −70 °C until further analysis.

**Reagents**—Polyclonal anti-phospho-ERK (sc-101760), polyclonal anti-ERK (sc-93), polyclonal anti-neuroglobin (sc-30144), monoclonal anti-GFP (sc-9996), monoclonal anti-β-actin (sc-47778), polyclonal anti-14-3-3 (sc-629), and monoclonal anti-14-3-3 (sc-133232) antibodies and protein A/G-coupled agarose beads were purchased from Santa Cruz Biotechnology, Inc. ERK1/2 and PKA kinase inhibitors (U0126 and PKA synthetic peptide inhibitor) were obtained from EMD Biosciences. The cell-permeable ERK1/2 inhibitor FR180204 was purchased from GenWay Biotech, Inc. Radioactive inorganic orthophosphate and [γ-32P]ATP were obtained from PerkinElmer Life Sciences.

**Generation of Cell Lines**—Stable neuronal cell lines were developed by transducing GFP-tagged wild-type and mutant (H64L) neuroglobin into neuroblastoma SH-SY5Y cells. High intensity GFP-positive cells were further sorted by flow cytometry and cloned by limiting dilution. The clonal lines that expressed comparable levels of neuroglobin were chosen for this study.

**Cell Culture and Treatments**—Human neuroblastoma cell lines (SH-SY5Y) expressing GFP, GFP-tagged neuroglobin, and GFP-tagged mutant neuroglobin (H64L) were maintained in regular growth DMEM containing 10% FBS, 100 units/ml penicillin/streptomycin, and 5 μg/ml puromycin (Sigma). The cells were split every 2 days and maintained in regular growth DMEM. Unless indicated otherwise, cells were cultured for 4 days in regular growth medium and changed to glucose- and serum-deprived DMEM before placing cells in a hypoxic chamber (1% O2 and 5% CO2). For normoxic controls, cells were placed in a CO2 incubator in regular growth DMEM (10% FBS and glucose) for the indicated times.

**cDNA Constructs**—Human neuroglobin cDNA containing clone SC122910 (NCBI accession number NM_021257) was purchased from OriGene (Rockville, MD) and used as a template for generating mutants.

**siRNA Transfections**—siRNA transfections in neuroglobin-GFP-expressing cells were carried out according to the manufacturer’s instructions (Santa Cruz Biotechnology, Inc.). Briefly, $2 \times 10^5$ neuroglobin-GFP-expressing cells were seeded into 6-well plates in 2 ml of antibiotic-free DMEM supplemented with 10% FBS for 24 h. Cells were rinsed with transfection medium, followed by the addition of 60 pmol of 14–3–3ζ and 14–3–3ζ siRNA duplex mixture in a total volume of 1 ml of transfection medium. The cells were incubated for 6 h at 37 °C in a CO2 incubator. Cells were cultured for an additional 21 h with the addition of 1 ml of 2× regular growth DMEM. 2 ml of 1× regular growth medium was exchanged, and the cells were cultured for 3 days. Two sets of cells were placed in the hypoxic chamber for 3 h after replacing the medium with glucose- and serum-deprived DMEM. One set of cells was used for confirming 14–3–3 silencing by immunoblot analysis using anti-pan-14-
3-3 antibody, and the duplicate set of cells was metabolically labeled with $^{32}$P to assess in vivo neuroglobin phosphorylation.

**Generation and Purification of Recombinant Neuroglobin**—

Human wild-type neuroglobin protein was overexpressed in *Escherichia coli* strain BL21(DE3)pLysS. A His$_6$ tag was included in the N terminus of the protein to aid purification on a nickel resin affinity column. Briefly, human neuroglobin cDNA was used as a template. PCR was performed for 23 cycles, each cycle at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. The 466-bp PCR product was digested with EcoRI and HindIII and cloned into pET28a at EcoRI and HindIII sites (EMD Chemicals, Gibbstown, NJ), resulting in fusion of the human neuroglobin with the His tag. Both strands of the cloned neuroglobin gene were sequenced. This neuroglobin construct for neuroglobin containing 36 additional amino acids at its N terminus, including six histidines. BL21(DE3)pLysS cells expressing pET28a-neuroglobin were exponentially grown in LB broth (1.1% dextrose, 0.5% tryptone, 0.5% yeast extract), and stored in aliquots at −80 °C.

**In Vitro Phosphorylation Assays**—In vitro neuroglobin phosphorylation assays were performed using either recombinant neuroglobin as described (36). In vitro kinase reactions were performed in the presence and absence of purified ERK1, ERK2, and PKA (200 ng; Millipore), and contained 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP. The reactions were performed at 30 °C, and the reaction products were visualized by SDS-PAGE and autoradiography.

**In Vivo Phosphorylation of Neuroglobin in SH-SY5Y Neuronal Cells**—2 × 10$^5$ neuroglobin-GFP-expressing cells were cultured for 4 days in 6-well plates and then subjected to either normoxia in regular or glucose- and serum-deprived DMEM or hypoxia in glucose- and serum-deprived DMEM for 3 h. The medium was then aspirated, washed with phosphate-free DMEM, and incubated with 0.1 mCi of inorganic orthophosphate for an additional 2 h. The cells were gently washed with prewarmed phosphate-free DMEM, lysed, and immunoprecipitated with polyclonal anti-neuroglobin antibody; immune complexes were resolved on 4–15% SDS-polyacrylamide gels; and the phosphorylation signals were visualized by autoradiography.

To examine if increased ERK1/2 activity during hypoxia resulted in neuroglobin phosphorylation in cells, neuroglobin-GFP-expressing cells were subjected to normoxia and hypoxia in the presence and absence of 0.5 $\mu$m FR180204 for 3 h. The phosphorylation state of neuroglobin was assessed by Western blotting using anti-phosphoserine antibody.

**Co-immunoprecipitation and Western Blotting**—To determine 14-3-3 interaction with neuroglobin, neuroglobin-GFP-expressing cells were subjected to normoxia or hypoxia for the indicated times and lysed in mammalian extraction buffer (Thermo Scientific) containing a mixture of protease and phosphatase inhibitors (Roche Applied Science) on ice for 30 min, and the lysates were cleared of debris by centrifugation at 7700 × g for 15 min at 4 °C. 4 $\mu$g of either polyclonal anti-14-3-3 or polyclonal anti-neuroglobin antibody was added to pre-cleared cell lysates for 2 h, followed by mixing with 75 $\mu$l of protein A/G-agarose beads in a rotator for 2 h. The beads containing the immune complex were washed five times with lysis buffer, resuspended in 50 $\mu$l of 1× SDS sample buffer, boiled for 5 min, and resolved on 4–15% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membrane for 3 h and probed with monoclonal anti-14-3-3, monoclonal anti-GFP, or polyclonal anti-neuroglobin antibody as indicated (37). The immunoreactive bands were analyzed using an Odyssey infrared imager (LI-COR).

To determine whether endogenous neuroglobin interacts with 14-3-3, fetal and adult brain segments were homogenized in mammalian lysis buffer containing a mixture of protease and phosphatase inhibitors. Co-immunoprecipitations were carried out with anti-neuroglobin and anti-14-3-3 antibodies, followed by Western blotting with the indicated antibodies as described above.

**FRET**—FRET analysis was essentially carried out as described (38). A 455-bp neuroglobin fragment was cloned and ligated into pAMCyto-CI (Clontech) to generate cyan fluorescent protein (CFP)-neuroglobin. To obtain YFP-14-3-3, a 741-bp fragment encoding 14-3-3$\zeta$, was ligated into the YFP vector at Sall and BglII. To analyze the interaction between 14-3-3 and neuroglobin by FRET, cells were cotransfected with YFP-14-3-3$\zeta$ and CFP-neuroglobin with FuGENE 6 transfection reagent for 24 h and then subjected to normoxia or hypoxia for 3 h. The interaction between neuroglobin and 14-3-3$\zeta$ was detected using a combination laser scanning microscope system (Zeiss LSM 510 META-ConfoCovor 2) as described (38). FRET was quantified by acceptor photobleaching. To achieve excitation, the 458-nm line of an argon ion laser was focused through the Zeiss ×63 oil differential interference contrast objective lens onto the cell. Emissions of YFP (the FRET acceptor) and CFP were collected through 535–595- and 470–500-nm barrier filters, respectively. Photobleaching was performed with 50 iterations and 100% intensity of a 514-nm laser. FRET quantification of fluorescence images was generated using Zeiss Rel 3.2 image software, and the average fluorescence intensities/pixel was calculated following background subtraction. FRET efficiency was calculated as followed: $E_{FRET} = (1 - CFP_{before}/CFP_{after}) \times 100$. $n$ = three separate experiments and >12 randomly selected cells for each condition were analyzed.

To map out neuroglobin interaction site(s) with 14-3-3, FRET analysis was performed after replacing the serine residues (Ser$^{17}$ and Ser$^{50}$) in each of the two 14-3-3-binding sites on neuroglobin with alanine using a QuikChange site-directed mutagenesis kit (Agilent Technologies).

**Effect of Neuroglobin Phosphorylation on CO Binding**—CO binding to ferrous neuroglobin was studied by stopped-flow spectroscopy (39). All experiments were carried out in an Applied Photophysics SX-20 instrument equipped with a diode array detector. Reactions were carried out in 100 mM potassium phosphate buffer (pH 7.4) at 25 °C. A solution of ~5 $\mu$m neuroglobin (phosphorylated or non-phosphorylated) and 5 mm...
sodium dithionite was mixed with a CO-saturated solution ([CO] ≈ 1 mM, CO concentration after mixing ≈500 μM), which also contained 5 mM sodium dithionite. The rates of decay of the deoxyneuroglobin and concomitant formation of neuroglobin-CO were calculated from the absorbance changes at 425.3 nm. The decay was best fit in all cases to a double exponential equation. Data fitting was done with Origin 8.0 software (OriginLab, Northampton, MA).

**Nitrite Reductase Activity of Neuroglobin**—The nitrite reductase activity of neuroglobin was studied in the presence of sodium dithionite (28). Assays were carried out at 25 °C in an Agilent HP 8453 spectrophotometer using 500-μl quartz cuvettes sealed by rubber septa. Assay mixtures (500 μM) contained 5–10 μM neuroglobin (phosphorylated or non-phosphorylated) in 100 mM potassium phosphate buffer (pH 7.4). Dithionite was added to a final concentration of 2.5 mM to form quantitatively the ferrous form of neuroglobin and to ensure anaerobic conditions. A final concentration of 5 mM anaerobic sodium nitrate was used to start the reaction. Upon addition of nitrite, the ferrous protein spectra shift to the ferrous nitrosyl spectra according to Reactions 1–4.

\[
\begin{align*}
\text{Ngb-Fe}^{2+} + \text{NO}_2^- & \rightarrow \text{Ngb-Fe}^{3+} + \text{NO} \\
\text{Ngb-Fe}^{3+} & \rightarrow \text{Ngb-Fe}^{2+} (\text{heme reduction by dithionite}) \\
\text{Ngb-Fe}^{2+} + \text{NO} & \rightarrow \text{Ngb-Fe}^{2+}\text{-NO}
\end{align*}
\]

Reactions 1–4

In the presence of excess dithionite, the rates of the Reactions 2 and 3 vastly exceed the rate of the Reaction 1, which is therefore the rate-limiting step. The observed rates of ferrous nitrosyl formation and ferrous protein decay are similar and correspond to the rate of the nitrite reductase reaction (Reaction 1). The spectrum shifts from ferrous neuroglobin to ferrous nitrosyl neuroglobin with clear isosbestic points, indicating the absence of noticeable intermediate species. The absorbance at 559 nm minus the absorbance at 579 nm was plotted versus the time and fitted to a single exponential equation in the form \( A_{559\text{ nm}} - A_{579\text{ nm}} = A_0 e^{-k_{\text{obs}} t} + C \). In the pseudo-first-order conditions of the assay, the observed rate corresponds to \( k_{\text{obs}} = k_{\text{BRC}} [\text{NO}_2^-] \), where \( k_{\text{BRC}} \) is the bimolecular rate constant of the reaction between ferrous neuroglobin and nitrite and the instantaneous reaction rate equal to \( d[Ngb-Fe^{2+/}] / dt = k_{\text{BRC}} [\text{Ngb-Fe}^{2+}] [\text{NO}_2^-] \), where Ngb is neuroglobin. The observed rate \( k_{\text{obs}} \) was divided by the concentration of nitrite to calculate the bimolecular reaction rate constant \( k_{\text{BRC}} \).

**RESULTS**

Neuroglobin Is Phosphorylated in Vitro and in Neuronal Cells after Hypoxia—Ischemic and hypoxic insults activate many signaling pathways in neurons, including the ERK1, ERK2, and PKA pathways. ERK1/2 plays an important role in transducing extracellular signals to the nucleus through phosphorylation of many signaling proteins at (S/T)P sites, whereas PKA phosphorylates target proteins at RXS consensus motifs. To investigate whether neuroglobin has the potential to serve as a substrate for these kinases, we analyzed the primary amino acid sequences for putative phosphorylation and docking motifs on neuroglobin. Phosphorylation motif analysis of neuroglobin revealed the presence of phosphorylation motifs for ERK1/2 and PKA and two putative binding sites for the scaffold protein 14-3-3 (amino acids 14–17 and 47–50) (Fig. 1A). Interestingly, the phosphorylation sites in neuroglobin are highly conserved from rodents to human. To determine whether neuroglobin is phosphorylated by these kinases, we performed in vitro kinase reactions using bacterially purified neuroglobin and purified recombinant ERK1, ERK2, and PKA. The results show that both ERK1/2 and PKA phosphorylated neuroglobin (Fig. 1, B and C) in vitro. The addition of specific inhibitors to ERK1/2 and PKA significantly inhibited neuroglobin phosphorylation (Fig. 1, B–D). To further examine neuroglobin phosphorylation during ischemic and hypoxic insults in live neuronal cells, which express minimal neuroglobin in culture, we developed stable cell lines by transducing GFP-tagged wild-type (GFP-neuroglobin) and H64L mutant neuroglobin into neuroblastoma SH-SY5Y cells. High intensity GFP cells were further sorted by flow cytometry and cloned by limiting dilution. The clones that expressed comparable levels of neuroglobin were chosen for this study (Fig. 1F). To explore if ERK1 and ERK2 are activated during hypoxia, we subjected neuroglobin-GFP cells to either normoxia or hypoxia for 3 h. After this, cells were lysed and immunoblotted with antibodies that recognize total ERK1/2 and phospho-ERK, as expression of phospho-ERK is an indicator of ERK1/2 activity.3ho f hypoxic treatment increased cellular ERK activity without affecting total ERK1/2 expression (Fig. 1F). With these observations, we next examined hypoxia-dependent neuroglobin phosphorylation in neuronal cells by metabolic labeling with inorganic orthophosphate in cells incubated without serum or glucose. Increased neuroglobin phosphorylation occurred when cells were subjected to both glucose and serum deprivation and hypoxia (Fig. 1, G and H), suggesting that neuroglobin phosphorylation increases with the severity of cellular metabolic stress.

Cell-permeable ERK Inhibitor FR180204 Inhibits Neuroglobin Phosphorylation in Neuroglobin-expressing Cells—To directly examine if increased ERK1/2 activity after hypoxia results in neuroglobin phosphorylation, we exposed neuroglobin-GFP-expressing cells to normoxia or hypoxia in the presence and absence of 0.5 μM FR180204 for 3 h. After 3 h, cells were lysed, and the phosphorylation status of neuroglobin was determined by immunoblotting with anti-phosphoserine antibody. The results show that hypoxia increased neuroglobin phosphorylation in neuronal cells and that FR180204 significantly inhibited this modification (Fig. 2A). Equal amounts of protein were loaded from hypoxic cells with and without FR180204 as evidenced by equivalent β-actin expression (Fig. 2B).

Neuroglobin Associates with 14-3-3 during Hypoxia—14-3-3 proteins are a group of scaffold proteins that modulate target protein activity after binding. To explore if 14-3-3 is associated with neuroglobin during hypoxia, we subjected neuroglobin-GFP-expressing cells to either normoxia or hypoxia for 3 h, followed by immunoprecipitation with anti-14-3-3 antibody.
The neuroglobin-14-3-3 immune complex was resolved by 4–12% SDS-PAGE and probed with anti-pan-14-3-3 (Fig. 3A) and anti-GFP (Fig. 3B) antibodies. The quantitative results show increased 14-3-3 binding to neuroglobin after 3 h of hypoxia (Fig. 3C). To determine the time-dependent increase in the neuroglobin-14-3-3 interaction, we cultured these cells under hypoxic conditions from 3 to 24 h, followed by immunoprecipitation with anti-neuroglobin antibody. The neuroglobin complex was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-GFP (Fig. 3D) and anti-14-3-3 (Fig. 3E) antibodies. Again, quantitative analysis showed a time-dependent increase in 14-3-3 binding to neuroglobin in cells cultured under hypoxic conditions over 3 h (Fig. 3F). To further examine if 14-3-3 binding to neuroglobin occurs in live cells during hypoxia, cells were either singly transfected or cotransfected with YFP-14-3-3 and CFP-neuroglobin before subjecting them to normoxic and hypoxic conditions for 3 h. In cells singly transfected with the CFP or YFP protein, fluorescent profiles in the FRET channel were documented and set for subtraction as a control for the background (Fig. 3G). In Cotransfected cells expressing YFP-14-3-3 and CFP-neuroglobin before subjecting them to normoxic and hypoxic conditions for 3 h. In cells singly transfected with the CFP or YFP protein, fluorescent profiles in the FRET channel were documented and set for subtraction as a control for the background (Fig. 3G). In Cotransfected cells expressing YFP-14-3-3 and CFP-neuroglobin before subjecting them to normoxic and hypoxic conditions for 3 h. In cells singly transfected with the CFP or YFP protein, fluorescent profiles in the FRET channel were documented and set for subtraction as a control for the background (Fig. 3G). In Cotransfected cells expressing YFP-14-3-3 and CFP-neuroglobin before subjecting them to normoxic and hypoxic conditions for 3 h. In cells singly transfected with the CFP or YFP protein, fluorescent profiles in the FRET channel were documented and set for subtraction as a control for the background (Fig. 3G). In
formed FRET analysis using a photobleaching approach (Fig. 4). In FRET, energy is transferred from the donor fluorophore (CFP-neuroglobin) to an acceptor fluorophore (YFP-14-3-3). If FRET is observed using the acceptor photobleaching method, indicative of protein interaction between binding partners, the donor emission (CFP) signal increases after a nearby acceptor fluorophore (YFP) is inactivated by irreversible photobleaching. Upon bleaching, there was decreased acceptor fluorescence (YFP-14-3-3) coupled with increased donor emission fluorescence (CFP-WT neuroglobin),

**FIGURE 3. Neuroglobin association with 14-3-3 under hypoxia.** A and B, GFP- and neuroglobin (Ngb)-expressing cells were subjected to either normoxia or 1% hypoxia, lysed, and immunoprecipitated (IP) with anti-14-3-3 antibody, followed by probing with anti-pan-14-3-3 and anti-GFP antibodies. C, quantitative analysis revealed that neuroglobin binding to 14-3-3 was increased in neuronal cells during hypoxia. D and E, time-dependent increase in 14-3-3 binding to neuroglobin after hypoxia. Neuroglobin-GFP-expressing cells were subjected to either normoxia or 1% hypoxia at the indicated times, lysed, immunoprecipitated with anti-neuroglobin antibody, and immunoblotted with anti-GFP and anti-14-3-3 antibodies, respectively. F, quantitation of the time-dependent increase in 14-3-3 binding to neuroglobin. G, neuroglobin association with 14-3-3 in intact live cells as determined by FRET analysis. Cells were exposed to normoxia or hypoxia for 3 h prior to analysis for protein-protein interaction by FRET. Cells were either singly transfected (upper panels) or cotransfected with YFP-14-3-3 and CFP-neuroglobin (Ngb; middle and lower panels). The panels show CFP, YFP, merge, or FRET (pseudo-color, far right panels); the latter represents intensity of the FRET signal.
consistent with wild-type neuroglobin interaction with 14-3-3 during hypoxia compared with normoxia (Fig. 4, B and C). In a negative control, we did not observe increased donor emission fluorescence (CFP) upon photobleaching the acceptor YFP in cells transfected with CFP/YFP alone (Fig. 4A).

Phosphorylation Regulates Neuroglobin Activity

To obtain additional insight into neuroglobin interaction with 14-3-3, we generated neuroglobin mutants S17A and S50A and examined their interaction with 14-3-3 after hypoxia also using FRET photobleaching analysis. The emission fluorescence of both the donor CFP-neuroglobin and acceptor YFP-14-3-3 before and after acceptor photobleaching in the region of interest (red arrows) is shown (Fig. 4, D and E, upper and lower panels). The data from >12 randomly selected cells for each condition were analyzed and are shown graphically. As shown in Fig. 4D, cotransfection of YFP-14-3-3 with CFP-S17A neuroglobin showed that, after bleaching, there was decreased acceptor fluorescence (YFP) coupled with increased donor emission fluorescence (CFP) because the acceptor cannot take in energy after its photobleaching. Thus, the data show that neuroglobin and 14-3-3 interaction occurred in cells expressing mutant S17A, although slightly reduced, compared with cells expressing wild-type neuroglobin (Fig. 4, C, D, and F). In contrast, experiments revealed that 14-3-3 binding to S50A neuroglobin was significantly reduced, as we observed no increase in donor emission fluorescence (CFP) (Fig. 4, E and F). To quantify our results, FRET efficiency was calculated based on the following: FRET efficiency (%) = 100 × (CFP_{after}/CFP_{before} − 1) (Fig. 4F). Taken together, these data suggest that 14-3-3 associates with neuroglobin after an ischemic hypoxic insult, with binding occurring predominately at a critical molecular site (Ser50).

Endogenous Neuroglobin Associates with 14-3-3 in Vivo in Brain—Because we observed neuroglobin association with 14-3-3 in neuroglobin-overexpressing cells, we next examined whether endogenous neuroglobin in brain associates with 14-3-3. For this, we first examined neuroglobin expression in various regions of sheep brain by Western blotting using neuroglobin-specific antibody. Our results show that neuroglobin is highly expressed in the brainstem and thalamic regions of fetal and adult brain samples (Fig. 5A). β-actin expression is shown in the same sample for comparison (Fig. 5A). To determine whether neuroglobin is associated with 14-3-3, we utilized brainstem and thalamic brain samples from fetal and adult sheep to pull down neuroglobin or 14-3-3 by immunoprecipitation first prior to either neuroglobin or 14-3-3 immunoblotting. In companion studies, the antibodies were reversed. The results indicate that neuroglobin associates with 14-3-3 in both fetal and adult brain samples in vivo (Fig. 5, B and C).

14-3-3 Binding Stabilizes Neuroglobin Phosphorylation—To further explore the significance of 14-3-3 binding to neuroglobin function, we first silenced 14-3-3 protein expression in neuronal cells using a siRNA duplex mixture containing 14-3-3ε and 14-3-3ζ. The siRNAs significantly reduced 14-3-3 expression (~50–60%) without affecting β-action expression compared with control siRNA-treated cells (Fig. 6, A–C). We next subjected these cells to hypoxia for 3 h and examined neuroglobin phosphorylation in cells as described above by metabolic labeling with inorganic orthophosphate. Our results show that 14-3-3 silencing significantly reduced neuroglobin phosphorylation during hypoxia (Fig. 6, D and E), suggesting that 14-3-3 binding protects and stabilizes the neuroglobin phosphorylation state in neuronal cells.
Neuroglobin Phosphorylation Increases Nitrite Reductase Activity—Previous work has shown that neuroglobin possesses nitrite reductase activity and generates NO after nitrite addition (28, 40). To determine whether the nitrite reductase activity is influenced by the phosphorylation state of neuroglobin, we assessed nitrite reductase activity using neuroglobin phosphorylated by ERK2. Our results show that ERK2 strongly phosphorylated neuroglobin (Fig. 7A) with increased nitrite reductase activity by 3–4-fold compared with non-phosphorylated neuroglobin at 25 °C and pH 7.4 (Fig. 7, B–E).

Phosphorylation Modifies Protein Structure as Indicated by Changes in CO Binding—To confirm that phosphorylation modulates the six-to-five heme pocket coordination equilibrium of neuroglobin, we also examined CO binding kinetics. The effect of phosphorylation on the dynamics and kinetic behavior of CO binding to the heme group was assessed by CO binding to ferrous neuroglobin using stopped-flow spectroscopy. 5 μM phosphorylated and non-phosphorylated neuroglobin was mixed with CO-saturated solution containing 5 mM sodium dithionite. We calculated the rates of decay of the deoxyneuroglobin and concomitant formation of neuroglobin-CO from the absorbance changes at 425.3 nm. The decay was best fit in all cases to a double exponential equation. In both cases, two phases were observed. The rates were similar for phosphorylated and non-phosphorylated neuroglobin (phosphorylated, fast phase = 4.15 ± 0.21 s⁻¹ and slow phase = 0.68 ± 0.03 s⁻¹; non-phosphorylated, fast phase = 3.46 ± 0.43 s⁻¹ and slow phase = 0.67 ± 0.04 s⁻¹), but the amplitudes of each phase were reversed, with the phosphorylated protein showing 67% amplitude in the fast phase with only 40.8% in non-phosphorylated neuroglobin (Fig. 7F).

Neuroglobin Phosphorylation Increases Nitrite Reductase Activity—Previous work has shown that neuroglobin possesses nitrite reductase activity and generates NO after nitrite addition (28, 40). To determine whether the nitrite reductase activity is influenced by the phosphorylation state of neuroglobin, we assessed nitrite reductase activity using neuroglobin phosphorylated by ERK2. Our results show that ERK2 strongly phosphorylated neuroglobin (Fig. 7A) with increased nitrite reductase activity by 3–4-fold compared with non-phosphorylated neuroglobin at 25 °C and pH 7.4 (Fig. 7, B–E).

Phosphorylation Modifies Protein Structure as Indicated by Changes in CO Binding—To confirm that phosphorylation modulates the six-to-five heme pocket coordination equilibrium of neuroglobin, we also examined CO binding kinetics. The effect of phosphorylation on the dynamics and kinetic behavior of CO binding to the heme group was assessed by CO binding to ferrous neuroglobin using stopped-flow spectroscopy. 5 μM phosphorylated and non-phosphorylated neuroglobin was mixed with CO-saturated solution containing 5 mM sodium dithionite. We calculated the rates of decay of the deoxyneuroglobin and concomitant formation of neuroglobin-CO from the absorbance changes at 425.3 nm. The decay was best fit in all cases to a double exponential equation. In both cases, two phases were observed. The rates were similar for phosphorylated and non-phosphorylated neuroglobin (phosphorylated, fast phase = 4.15 ± 0.21 s⁻¹ and slow phase = 0.68 ± 0.03 s⁻¹; non-phosphorylated, fast phase = 3.46 ± 0.43 s⁻¹ and slow phase = 0.67 ± 0.04 s⁻¹), but the amplitudes of each phase were reversed, with the phosphorylated protein showing 67% amplitude in the fast phase with only 40.8% in non-phosphorylated neuroglobin (Fig. 7F).
The observed rates from the biexponential fittings seem independent of CO concentrations and thus may correspond to the histidine dissociation rates of each population.

**Effect of Neuroglobin Phosphorylation and 14-3-3 Binding on Nitrite Reductase Activity**—We next examined the effect of 14-3-3 binding on the nitrite reductase activity. We performed kinase reactions on recombinant neuroglobin in the presence and absence of ERK2. Purified 14-3-3 was added to both non-phosphorylated and phosphorylated neuroglobin. Phosphorylation was seen only in the presence of a kinase (Fig. 8A), although equal amounts of neuroglobin were seen in both non-phosphorylated and phosphorylated neuroglobin samples by Coomassie Blue staining (Fig. 8B). Despite very strong neuroglobin phosphorylation (Fig. 8A), 14-3-3 additions had no significant effect beyond the phosphorylation effect (Fig. 8C).

**DISCUSSION**

In this study, we have shown that neuroglobin activity is regulated by post-translational phosphorylation and protein-protein interactions in response to hypoxic stress. We found that neuroglobin phosphorylation and 14-3-3 interaction increase in neuronal cells after hypoxia, that 14-3-3 appears to stabilize protein phosphorylation, and that phosphorylation increases the heme pocket reactivity with nitrite. Our *in vitro* kinase reactions suggest that neuroglobin is phosphorylated by ERK1/2 and PKA and could be a target for these pathways during hypoxia. Inhibition of hypoxia-induced neuroglobin phosphorylation in neuronal cells by the cell-permeable ERK inhibitor FR180204 further supports that ERK1 and ERK2 phosphorylate neuroglobin *in vivo* (35). We have shown here that neuroglobin interacts with 14-3-3 by both co-immunoprecipitation and...
Phosphorylation Regulates Neuroglobin Activity

FRET analyses. Furthermore, examination of neuroglobin mutants determined that, although Ser\textsuperscript{17} and Ser\textsuperscript{50} are involved in 14-3-3 interactions, Ser\textsuperscript{50} is more critical than Ser\textsuperscript{17} in 14-3-3 binding. It is also possible that different 14-3-3-binding sites could be utilized under different stress conditions depending on the phosphorylated residues as well as the kinases that are involved in phosphorylation. Our co-immunoprecipitation studies in sheep brain segments (brainstem and thalamus) suggest that endogenous neuroglobin interacts with 14-3-3 in vivo in brain.

Our results further support that hypoxia-dependent phosphorylation is greatly reduced in 14-3-3-depleted cells, suggesting that 14-3-3 binding protects neuroglobin phosphorylation from phosphatases activity. A recent study has shown that neuroglobin itself directly regulates 14-3-3\textgamma expression, although the mechanism is not known (41). Further studies are required to understand the dynamic nature of neuroglobin-14-3-3 interaction as well as isoform-specific differences and the necessity in shaping these signaling networks in cells that are under stress.

We examined the effect on nitrite reduction, as recent studies suggest that nitrite serves as a reservoir of NO that can be reduced by hemoglobin or myoglobin to regulate hypoxic NO signaling, including hypoxic vasodilation and mitochondrial respiration (42). As with other heme proteins, neuroglobin is able to catalyze the formation of NO from nitrite. Phosphorylation of neuroglobin increases the reaction rate by \(~3\)-fold. The binding of CO to deoxyneuroglobin is also affected by phosphorylation, indicating effects on the heme coordination. The biphasic behavior observed here for CO binding has been previously observed, with slightly different rates (19, 25, 42, 43). We did not observe a noticeable increase in CO binding in the dead time of the instrument, and the binding of CO to the five-coordinate species is several orders of magnitude faster than the rates observed here (43); therefore, a significant increase in the initial five-coordinate population seems an unlikely cause for the biphasic behavior. Previous work suggested that there are two subpopulations of six-coordinate neuroglobin that can revert to five-coordinate neuroglobin with different histidine binding constants (K\textsubscript{d}) (19, 20). Moreover, thiol-reducing agents have been shown to change the rates of CO binding (25, 42), indicating that the internal Cys\textsuperscript{46} - Cys\textsuperscript{55} disulfide bond formation can alter CO binding rates. Our data indicate that the heme coordination environment in neuroglobin is also altered by phosphorylation, either directly or through changes in disulfide bonds.

Consistent with our present studies showing that phosphorylation of neuroglobin modulates this reaction rate, we have recently reported that neuroglobin surface thiol oxidation increases the five-coordinate heme pocket equilibrium, increases nitrite binding, and increases nitrite reaction rates to form NO (28). Mutagenesis studies confirmed that heme pocket mutations of neuroglobin His\textsuperscript{64} “lock” neuroglobin in the five-coordinate state. These five-coordinate mutant neuroglobins (H64L and H64Q) reduce nitrite to NO at rates \(~2000\) times faster than wild-type neuroglobin (28). These studies strongly suggest that post-translational modifications that increase the five-coordinate heme pocket equilibrium will increase the nitrite reaction rate, as observed in this study with neuroglobin phosphorylation. At this time, the physiological reaction catalyzed by neuroglobin is unknown, but the activities studied serve as surrogates and suggest that phosphorylation will have the potential to alter the kinetics of other neuroglobin-catalyzed reactions as well.

On the basis of our study, we suggest that neuroglobin phosphorylation serves as a sensor to hypoxic stress, potentially generating an adaptive response. It is also tempting to suggest that neuroglobin phosphorylation may serve as a signal to promote cellular adaptive responses to other stress conditions through protein-protein interactions. Several stress conditions, including hypoxia, induce protein kinase C and prostaglandin E\textsubscript{2}, which are known to stabilize hypoxia-inducible factor 1\textalpha (44 – 46). Because neuroglobin transcription is regulated in part by the ERK/MAPK pathway (8, 47), which is known to activate hypoxia-inducible factor 1\textalpha by recruitment of the co-activating p300/CREB (cAMP-responsive element-binding protein)-binding protein (48), we predict that neuroglobin expression may be increased by phorbol esters through protein kinase C activation and prostaglandin E\textsubscript{2} induction. Given that multiple kinases are activated during stress conditions, we suggest that characterization of phosphorylation by these kinases involved in hypoxia and other stress conditions may lead to better identification of its role in these pathological processes.

In conclusion, our data suggest that neuroglobin is phosphorylated during hypoxia and physically interacts with 14-3-3, which further stabilizes the phosphorylation state in vivo. Given that neuroglobin phosphorylation modulates CO binding kinetics and increases the nitrite reductase activity of neuroglobin, these data suggest that the heme environment in neuroglobin is altered by phosphorylation and protein-protein interactions. Characterization of neuroglobin offers great potential to markedly increase our understanding of the function of six-coordinate cellular heme globins and their role in nitrite and NO signaling and the cellular resistance to hypoxia.
