Human Neuroglobin Functions as an Oxidative Stress-responsive Sensor for Neuroprotection*

Seiji Watanabe1, Nozomu Takahashi, Hiroyuki Uchida, and Keisuke Wakasugi2
From the Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

Background: Mammalian neuroglobin (Ngb) is involved in neuroprotection under oxidative stress conditions. The ferrous oxygen-bound form of Ngb, which exists under normoxia, is converted to the ferric bis-His conformation during oxidative stress, inducing large tertiary structural changes. We clarified that ferric bis-His Ngb, but not ferrous ligand-bound Ngb, specifically binds to flotillin-1, a lipid raft microdomain-associated protein, as well as to α-subunits of heterotrimeric G proteins (Gαi). Moreover, we found that human ferric bis-His Ngb acts as a guanine nucleotide dissociation inhibitor for Gαi, that has been modified by oxidative stress. In addition, our data shows that Ngb inhibits the decrease in cAMP concentration that occurs under oxidative stress, leading to protection against cell death. Furthermore, by using a mutated Ngb protein that cannot form the bis-His conformation, we demonstrate that the oxidative stress-induced structural changes of human Ngb are essential for its neuroprotective activity.

Mammalian neuroglobin (Ngb) protects neuronal cells under conditions of oxidative stress. The mechanism underlying this function is only partly understood. Here, we report that human Ngb exists in lipid rafts only during oxidative stress and that lipid rafts are crucial for neuroprotection by Ngb. The ferrous oxygen-bound form of Ngb, which exists under normoxia, is converted to the ferric bis-His conformation during oxidative stress, inducing large tertiary structural changes. We clarified that ferric bis-His Ngb, but not ferrous ligand-bound Ngb, specifically binds to flotillin-1, a lipid raft microdomain-associated protein, as well as to α-subunits of heterotrimeric G proteins (Gαi). Moreover, we found that human ferric bis-His Ngb acts as a guanine nucleotide dissociation inhibitor for Gαi, that has been modified by oxidative stress. In addition, our data shows that Ngb inhibits the decrease in cAMP concentration that occurs under oxidative stress, leading to protection against cell death. Furthermore, by using a mutated Ngb protein that cannot form the bis-His conformation, we demonstrate that the oxidative stress-induced structural changes of human Ngb are essential for its neuroprotective activity.

Mammalian neuroglobin (Ngb) is an oxygen (O2)-binding heme protein that has a classical globin fold and is widely expressed in the cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum, and retina (1–3). Previous studies have shown that inhibiting Ngb expression with an antisense oligodeoxynucleotide decreases, while Ngb overexpression increases neuronal survival after oxidative stress, supporting the notion that mammalian Ngb protects neurons from hypoxic-ischemic insults (4–7). Mammalian Ngb has been reported to protect the brain from experimentally induced stroke in vivo (8, 9). Moreover, overexpression of Ngb in the hearts of transgenic mice reduced ischemic injury to myocardial cells, which contain vastly greater amounts of myoglobin (Mb) (8), suggesting that mammalian Ngb has a novel unique function, not shared by Mb, to protect against oxidative stress-induced cell death.

Mb is an intracellular globin that stores O2 in muscle tissue and facilitates its diffusion from the periphery of the cell to the mitochondria. Although Ngb shares only 20–25% sequence identity with Mb, the key amino acid residues required for Mb function are conserved (1). The iron atom in the heme prosthetic group of each globin normally exists in either the ferrous (Fe2+) or ferric (Fe3+) redox state. In the absence of exogenous ligands, Mb is normally pentacoordinated in the ferrous form, leaving the sixth position available to bind exogenous ligands such as O2 or carbon monoxide (CO), or hexacoordinated in the ferric state, with a water molecule coordinated to the ferric iron. In contrast, as shown in Fig. 1, both the ferric and ferrous forms of Ngb are hexacoordinated to endogenous protein ligands, namely proximal and distal histidine (His) residues, and O2 or CO displaces the distal His residue of ferrous Ngb to produce ferrous O2− or CO-bound Ngb (10).

We previously found that the ferric form of human Ngb (HNgb) binds exclusively to the GDP-bound form of the α subunit of heterotrimeric G proteins (Gα) and competes with Gβγ for binding to Goα (11, 12). Heterotrimeric G proteins consist of Goα with GTPase activity and a βγ dimer (Gβγ) and belong to a family of proteins, whose signal transduction functions depend on the binding of guanine nucleotides (13–15). Ligand- or signal-activated G protein–coupled receptors (GPCRs) induce the release of GDP from Goα, allowing Goα to bind to GTP. Binding of GTP to Goα “turns on” the system and causes conformational changes that result in dissociation of the GTP-bound Goα from both the receptor and Gβγ. The GTP-bound Goα and Gβγ can then regulate the activity of different effector molecules, such as...
Neuroprotective Mechanism of Human Neuroglobin

The distal histidine residue of HNgb is His-64.

adenyl cyclase, and ion channels. Signal transduction is “turned off” by the intrinsic GTPase activity of the Gα protein, which hydrolyzes bound GTP to GDP, inducing the reassociation of GDP-bound Gα to Gβγ. Gα proteins are grouped into four distinct families: Gαq/11, Gαo, Gα12/13 (14, 15). We previously showed that ferric HNgb binds exclusively to the GDP-bound form of Gαi/o and acts as a guanine nucleotide dissociation inhibitor (GDI) for Gαi/o, whereas human Mb cannot interact with Gαo (12). In contrast, ferrous ligand-bound HNgb under normoxia does not interact with Gαi/o, nor has GDI activity (11, 12). Together, these findings indicated that HNgb may be a novel oxidative stress-responsive sensor for signal transduction in the brain (12, 16).

Although Ngb was originally identified in mammalian species, it is also present in non-mammalian vertebrates (17, 18). Mammalian and fish Ngb proteins share about 50% amino acid sequence identity. Fish Ngb proteins are also hexacoordinated globins with similar oxygen-binding kinetics (18). Previously, we showed that zebrafish Ngb (ZNgb) has a cell membrane-penetrating activity (19–22), but does not exhibit GDI activity (23). To identify residues in HNgb that are crucial for its GDI activity, we previously generated HNgb mutants by focusing on those residues that differ between HNgb and ZNgb and on residues with positive or negative charges on the protein surface (23). R47A, K102N, K119N, and D149A HNgb mutants, which retained GDI activity, protected PC12 cells against cell death caused by hypoxia/reoxygenation as did wild-type (WT) HNgb (7). In contrast, E53Q, R97Q, E118Q, and E151N HNgb mutants, which did not function as GDI proteins, did not rescue cell death under oxidative stress conditions (7). These results clearly showed that the GDI activity of HNgb is tightly correlated with its neuroprotective activity.

Furthermore, we previously performed yeast two hybrid screening using HNgb as bait and identified flotillin-1 as a HNgb-binding protein (24). Flotillin-1 exists within lipid rafts, which are detergent-resistant, cholesterol- and sphingolipid-rich membrane domains that are involved in important cellular processes such as signal transduction and intracellular trafficking (25–29). Within the lipid rafts of the rat brain exist heterotrimeric G proteins (Gα, Gβγ, and Gδ), Src family protein kinases, and some glycosylphosphatidyl inositol (GPI)-anchored proteins (30–33). Flotillin-1 has been shown to recruit signaling proteins to lipid rafts that mediate the compartmentalization of crucial signal transduction pathways (27, 28). Because HNgb interacts with Gαi/o, which also exists within lipid rafts, the association between HNgb and flotillin-1 may position it within lipid rafts containing heterotrimeric G proteins as a means of preventing neuronal death.

In the present study, we aimed to clarify the roles of lipid rafts in HNgb-mediated neuroprotection. Because Gαi/o proteins have been reported to be targets of reactive oxygen species (ROS) (34, 35), we investigated whether ferric HNgb interacts with the Gαi/o that is modified by ROS, thereby acting as a GDI for modified Gαi/o. Moreover, we substituted the distal His residue with Val to create an HNgb mutant (H64V HNgb) that cannot form a bis-His conformation and investigated the significance of the structural changes of HNgb induced by oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Rat myristoylated Gαi1 was purchased from Calbiochem. The short splice variant of rat Gαi protein was obtained from Jena Bioscience (Jena, Germany). Horse heart Mb, and methyl-β-cyclodextrin (MβCD) were obtained from Sigma-Aldrich (St. Louis, MO). [8,5-3H]GDP (20–50 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS) and adenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPS) were from Biolog (Bremen, Germany). Cholesterol was from Wako chemicals (Osaka, Japan).

**Cell Culture**—Rat pheochromocytoma PC12 cell line (RCB009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). PC12 cells were maintained in culture in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose 4.5 g/liters, 10% (v/v) fetal bovine serum (FBS), 10% (v/v) heat-inactivated horse serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (all from Invitrogen (Carlsbad, CA)) in a humidified atmosphere containing 5% CO2 at 37 °C. The medium was changed twice weekly and the cultures were split at a 1:8 ratio once a week.

PC12 cells (CRL-1721.1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in F-12 nutrient mixture (Ham’s F-12), supplemented with 15% (v/v) horse serum, 2.5% (v/v) FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen) in a humidified atmosphere containing 5% CO2 at 37 °C. The
medium was changed every 3 days, and the cultures were split at a 1:8 ratio once a week.

SH-SY5Y human neuroblastoma cells (CRL-2266) were obtained from ATCC and maintained in a 1:1 mixture of DMEM and Ham's F-12 containing 2.5 mM glutamine, supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen) in a humidified atmosphere containing 5% CO₂ at 37 °C. The medium was changed every 4 days, and the cultures were split at a 1:20 ratio once a week. Cultured cells were induced to differentiate into a neuronal phenotype by treatment with 10 μM retinoic acid (Sigma-Aldrich) over a period of 6 days (media were exchanged every 3 days during sub-culture). Differentiation was verified by monitoring macroscopic changes to the cells.

**Detergent Solubilization and Sucrose Density Gradient Fractionation**—HNgb cDNA was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen). The construct was confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services). The PC12 cells (CRL-1721.1) were grown on 100-mm culture plates at a density of 5.0 × 10⁵ cells/ml for 24 h. The diluted sample was then concentrated and incubated with either GST alone or GST-Ngb immobilized on glutathione-Sepharose 4B beads (GE Healthcare Biosciences) equilibrated with 50 mM Tris-HCl, pH 8.0, and eluted with a linear NaCl gradient from 0 to 0.7 M.

**Preparation and Purification of Human Truncated Flotillrin-1** (a.a. 137–427)—Human truncated flotillin-1 was expressed in Escherichia coli and purified as described previously (24).

**Gust** Pull-down Assays of Truncated Flotillrin-1—Human truncated flotillin-1, solubilized in buffer (8 M urea, 100 mM sodium phosphate buffer, and 10 mM Tris-HCl, pH 4.5), was diluted 16 times with buffer B (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, pH 7.4 or pH 8.0) containing EDTA-free complete protease inhibitor mixture (Roche Diagnostics). The diluted sample was then concentrated and incubated with either GST alone or GST-Ngb immobilized on glutathione-Sepharose 4B beads (GE Healthcare Biosciences) for 1 h at 4 °C. The beads were washed extensively three times with buffer B. The samples were then resuspended in Laemmlli sample buffer, heated for 5 min at 95 °C, and separated by 12% SDS/PAGE. For Western blot analyses, the proteins were transferred onto Hybond-P PVDF membranes (GE Healthcare Biosciences), which were then blocked with phosphate-buffered saline (PBS) and 5% skim milk (Wako chemicals) and incubated with primary antibody. The blots were then incubated with the secondary antibody conjugated to horseradish peroxidase (HRP) and visualized using Enhanced Chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare Biosciences) according to the manufacturer's instructions.
with rabbit anti-flofllin-1 (H-104) polyclonal antibody (Santa Cruz Biotechnology). The membranes were washed and then incubated with an HRP-linked F(ab)2 fragment of donkey anti-rabbit IgG (GE Healthcare Biosciences).

**GST Pull-down Assays using a Rat Brain Extract**—Freshly excised SD rat brains purchased from Japan SLC (Shizuoka, Japan) were homogenized on ice in Tris-buffered saline buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT, pH 8.0) containing 1% n-octyl-β-glucoside and protease inhibitor mixture. The homogenates were incubated on ice, and insoluble fractions were removed by centrifugation. The supernatants were used in the GST pull-down assays. The experimental conditions of the GST pull-down assays have been described above for recombinant truncated flofllin-1.

**Construction, Expression, and Purification of Recombinant Human Gαi1 Protein**—The DNA fragment containing the human Gαi1 subunit (residues 1–354) was amplified by PCR and cloned into the pET15/D-TOPO® vector (Invitrogen) to be expressed as human wild-type Gαi1 protein fused to a TEV protease recognition site directly after an N-terminal tag of six histidine residues (His-tag). The construct was confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services). The resulting Gαi1 was expressed in the Escherichia coli strain BL21 (DE3) by induction with 30 μM IPTG for 18 h at 25 °C. Cells were extracted in buffer C (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol, 0.4% (v/v) phenylmethylsulfonylfluoride (PMSF)) supplemented with complete, EDTA-free; protease inhibitor mixture (Roche Diagnostics). After centrifuging at 10,000 rpm for 30 min at 4 °C, the supernatant including human Gαi1 protein with His-tag was applied to a nickel affinity column (His-Bind® resin; Novagen) equilibrated with buffer C. The column was washed with 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 20 mM β-mercaptoethanol and 0.4% (v/v) PMSF. Gαi1 was then eluted with 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 250 mM imidazole, 20 mM β-mercaptoethanol, and 0.4% (v/v) PMSF. The buffer was immediately replaced with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 50 μM GDP, 20 mM β-mercaptoethanol and 0.4% (v/v) PMSF. To eliminate the N-terminal His-tag from Gαi1, the sample was incubated for 24 h at 4 °C with His-tagged TEV protease (MoBiTec GmbH, Göttingen, Germany) added in a ratio of 1:200 (w/w). The sample was then loaded onto a His-Bind® column equilibrated with buffer C to separate the cleaved Gαi1 from the cleaved His-tag, any uncleaved protein, and His-tagged TEV protease. The purified Gαi1 (flow-through fraction) was concentrated in 50 mM HEPES (pH 8.0), 1 mM EDTA, 2 mM DTT, and 200 μM GDP and stored at −80 °C.

**GST Pull-down Assays using Gαi1 or Gαi1—Gαi1 or Gαi1** was incubated with either GST alone or GST-Ngb immobilized on glutathione-Sepharose 4B beads (GE Healthcare Biosciences) in HEPES buffer (10 mM HEPES, 150 mM NaCl, 10 mM MgCl2, 10 mM GDP, 0.1% Tween 20, pH 7.4) in the absence or presence of 10 mM NaF and 30 mM AlCl3 for 1 h at 4 °C. The beads were washed extensively three times with the buffer, and the samples were then resuspended in Laemmli sample buffer, heated for 5 min at 95 °C, and separated by 12.0% SDS/PAGE. For Western blot analyses, the proteins were transferred onto Hybond-P PVDF membranes (GE Healthcare Biosciences), which were then blocked with PBS and 5% skim milk (Wako chemicals) and incubated with mouse anti-Gαi1 (Ab-3; clone R4.5) monoclonal antibody (Thermo Fisher Scientific, Fremont, CA) or mouse anti-Gαi1 (12) monoclonal antibody (Santa Cruz Biotechnology). After washing, the membranes were incubated with an HRP-linked whole antibody of sheep anti-mouse IgG (GE Healthcare Biosciences).

**Preparation of Ngb Proteins**—Plasmids for HNgb or ZNgb were prepared as described previously (12, 23). A Quick-Change® site-directed mutagenesis system (Stratagene, La Jolla, CA) was used to introduce substitutions at specific sites according to the manufacturer’s instructions. The constructs were confirmed by DNA sequencing (FASMAC Co., Ltd., DNA sequencing services, Atsugi, Japan). Overexpression of each Ngb was induced in the E. coli strain BL 21 (DE 3) by treatment with IPTG, and each Ngb protein was purified as previously described (7, 20, 23). Briefly, soluble cell extracts were loaded onto DEAE Sepharose anion-exchange columns equilibrated with buffer D (20 mM Tris-HCl, pH 8.0). Ngb proteins were eluted from the columns with buffer D containing 75 mM NaCl and further purified by passage through Sephacryl S-200 HR gel filtration columns. Ngb proteins were then applied to a HiTrap Q HP column (GE Healthcare Bio-Sciences) and eluted with a 0–500 mM linear NaCl gradient in buffer D. Purified Ngb was dialyzed overnight against PBS. Endotoxin was removed from the protein solutions by phase separation using Triton X-114 (Sigma–Aldrich) (36, 37). Trace amounts of Triton X-114 were removed by passage through Sephadex G25 gel (GE Healthcare Bio-Sciences) equilibrated with PBS. The protein concentration of human ferric Ngb was determined spectrophotometrically using an extinction coefficient of 122 mM−1 cm−1 at the Soret peak.

Purified H64V HNgb was in the ferrous O2-bound form. Ferric H64V HNgb was obtained by treating the protein with a 20% excess of K2Fe(CN)6 and quickly passing the resulting solution over a Sephadex G25 gel filtration column equilibrated with PBS (pH 7.4).

**[3H]GDP Dissociation Assays**—Gαi1, complexed with [3H]GDP (0.3 μM) was prepared by incubating 0.3 μM Gαi1 with 2 μM [3H]GDP in buffer E (20 mM Tris-HCl, 100 mM NaCl and 10 mM MgSO4 at pH 8.0) with or without 2 mM DTT for 1.5 h at 25 °C. Excess unlabeled GTP (2 μM) was added to monitor dissociation of [3H]GDP from Gαi1 in the absence or presence of Ngb (10 μM). Aliquots were withdrawn at 0, 5, and 10 min and were passed through nitrocellulose filters (0.45 μM) (Advantec Toyo). The filters were then washed three times with 1 ml of
ice-cold buffer E and were counted in a liquid scintillation counter (LS6500; Beckman Coulter).

Treatment of SH-SY5Y Cells with cAMP Analog or Antagonist and Hydrogen Peroxide—Differentiated SH-SY5Y cells were plated on poly-D-lysine coated 96-well plates at a density of $5.0 \times 10^5$ cells/ml for 24 h. The pcDNA3.1-HNgb expression vector or control vector (pcDNA3.1 empty vector) was transfected by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h of transfection, cells were treated with Sp-cAMPS or Rp-cAMPS at a concentration of $300 \mu M$ for 1 h. The hydrogen peroxide was then added at $100 \mu M$, and cells were incubated for 24 h.

cAMP Immunoassay—Differentiated SH-SY5Y cells were seeded at $5.0 \times 10^5$/ml in 12-well cell culture plates the day before experiments. The control vector (pcDNA3.1 empty vector) or pcDNA3.1-HNgb expression vector was transfected by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h of transfection, the cells were treated with 100 $\mu M$ hydrogen peroxide for 24 h at 37 °C. Then, the concentrations of intracellular cAMP were determined using cAMP Biotrak™ competitive enzymeimmunoassay system (GE Healthcare Biosciences) according to the manufacturer’s instructions.

Protein Transduction by Chariot—Protein transduction was performed by using Chariot™ (Active Motif, Carlsbad, CA) as described previously (7, 20). Each purified globin protein (3 $\mu g$ per well) was incubated in the presence of diluted Chariot for 30 min at room temperature. Next, the mixture was added to cells that had been washed in DMEM without serum. DMEM without serum was added and the cells were incubated at 37 °C for 1 h; FBS was then added to a final concentration of 2%. The cells were incubated at 37 °C for another 2 h to allow Ngb internalization.

Protein transduction was confirmed by Western blot analyses using rabbit anti-HNgb (FL-151) polyclonal antibody (Santa Cruz Biotechnology), mouse anti-β-actin monoclonal antibody (Sigma-Aldrich), or rabbit anti-Mb polyclonal antibody (Spring Bioscience, Pleasanton, CA).

Hypoxia/Reoxygenation—PC12 cells (RCB009) were plated on a poly-D-lysine-coated 96-well tissue culture plate at a density of $1.0 \times 10^5$ cells/ml in DMEM containing 2.0 g/liter glucose, 2% (v/v) FBS, and 2 mM glutamine for 24 h. Each Ngb was transduced with or without Chariot™. Hypoxia was induced in a multigas incubator (Astec, Fukuoka, Japan; set to 1% O$_2$, with 5% CO$_2$ and 94% N$_2$) at 37 °C for 24 h. After hypoxia, the culture medium was replaced with fresh DMEM containing 2.0 g/L glucose, 2% (v/v) FBS, and 2 mm glutamine, and the cells were incubated at 37 °C for 24 h under normoxia (95% air/5% CO$_2$).

Statistics—Data were analyzed by one-way ANOVA followed by Tukey-Kramer post hoc tests.

RESULTS

Ngb Levels Increase in Lipid Rafts under Oxidative Stress, and Lipid Rafts Are Essential for the Neuroprotective Activity of HNgb—First, we examined whether oxidative stress affects the intracellular localization of Ngb in PC12 cells. Lipid rafts were isolated conventionally by treatment with Brij-58. The 12 gradient fractions were then analyzed by immunoblotting with anti-flotillin-1 or anti-Ngb antibody. Flotillin-1, a marker for lipid rafts, was found intensively in fractions 3–6 (Fig. 2A), indi-
cating that lipid rafts float into these fractions during centrifuga-
tion. Ngb was detected not only in fractions 10–12 at the
bottom of the gradient but also in fraction 5 of lipid rafts under
hydrogen peroxide-induced oxidative stress. Thus, these
results indicate that part of Ngb is recruited to lipid rafts in
PC12 cells only during oxidative stress.

Because raft domains are enriched in cholesterol, a com-
 pound that interacts with and sequesters cholesterol may be a
useful tool for investigating the structural and functional signif-
icance of plasma membrane compartments. MβCD, known as a
lipid raft-disrupting agent, extracts cholesterol from the plasma
membrane (29, 38). To investigate the role of lipid rafts in the
signaling process, SH-SY5Y cells were pretreated with MβCD
to ablate raft structures and were exposed to hydrogen perox-
ide. Addition of MβCD attenuated the neuroprotective activity
of HNgb (Fig. 2B) as shown by cell viability assays. To restore
cholesterol-rich raft domains, cholesterol-depleted cell cul-
tures were incubated with cholesterol-loaded MβCD.

Addition of MβCD attenuated the neuroprotective activity
of HNgb (Fig. 2B) as shown by cell viability assays. To restore
cholesterol-rich raft domains, cholesterol-depleted cell cul-
tures were incubated with cholesterol-loaded MβCD. After raft
constitution, cells were exposed to hydrogen peroxide. Reconsti-
tution of the lipid raft domains restored the neuroprotective
activity of HNgb (Fig. 2B). Taken together, these findings sug-
gest that plasma membrane compartments rich in cholesterol
participate in HNgb-mediated signal transduction pathways
during oxidative stress.

Ferric, but Not Ferrous Ligand-bound, HNgb Binds to
Flotillin-1—In the present study, we carried out GST pull-
down assays in which GST-fused HNgb or ZNgb was used to
characterize the interaction of Ngb and proteins in vitro.

Previously, we showed that ferric HNgb, which is generated
spontaneously as a result of rapid autoxidation, interacts with a
truncated human flotillin-1 protein (a.a. 137–427) and with
WT rat flotillin-1 (24). In the present study, we investigated
whether ZNgb or ferrous ligand-bound HNgb interacts with
flotillin-1. As shown in Fig. 3B, the ferric form, but not the
ferrous CO-bound form of HNgb or ZNgb interacts with trun-
cated human flotillin-1 (Fig. 3B). Moreover, we demonstrated

FIGURE 3. Structural and flotillin-1-binding properties of GST-fused HNgb or ZNgb. A, electronic absorption spectra of the ferric (bold line), ferrous deoxy
(fine line), and ferrous-CO (dotted line) forms of GST-fused HNgb or GST-fused ZNgb. The concentration of each recombinant protein was ~5 μM on the basis
of heme content. The Q bands from 500 to 600 nm are enlarged by a factor of 3 on the perpendicular axis. B, GST pull-down assays with Ngb and flotillin-1.
Truncated or full-length flotillin-1 was incubated with GST-HNgb, GST-ZNgb, or GST in buffer (pH 8.0), washed, and analyzed by Western blotting with rabbit
anti-flotillin-1 polyclonal antibody.
that ferric, but not ferrous CO-bound HNgb binds to full-length WT flotillin-1 (Fig. 3B).

**Ferric HNgb Acts as a GDI for G\(\alpha_{i} / i/o\) Modified by ROS and Inhibits the Oxidative Stress-mediated Decrease in cAMP to Protect against Cell Death**—Using surface plasmon resonance, we previously showed that ferric HNgb binds exclusively to GDP-bound G\(\alpha_{i} / i/o\), whereas ferrous CO-bound HNgb does not interact with G\(\alpha_{i} / i/o\) (12). Moreover, GST pull-down assays using GST-fused HNgb have demonstrated that GST-ferric, but not GST-ferrous CO-bound HNgb interacts with G\(\alpha_{i}\) (11, 16). These data are consistent with those obtained by surface plasmon resonance of untagged Ngb (12), suggesting that the GST tag has no effect on protein-protein interactions between HNgb and G\(\alpha_{i}/i/o\).

In the present study, GST-HNgb, GST-ZNgb, or GST was incubated with rat myristoylated G\(\alpha_{i}/i/o\), and Western blot analyses were performed with anti-G\(\alpha_{i}\) mouse monoclonal antibody. B. comparison of HNgb binding between different forms of recombinant human G\(\alpha_{i}/i/o\). GDP-bound G\(\alpha_{i} / i/o\) in the absence or presence of AlF\(_4^{-}\) was incubated with GST-HNgb or GST. C. GST pull-down assays with HNgb and G\(\alpha_{i}/i/o\). GDP-bound G\(\alpha_{i}/i/o\) was incubated with GST-HNgb or GST, washed, and analyzed by Western blotting with anti-G\(\alpha_{i}\) antibody. D. GST pull-down assays with HNgb and ROS-treated G\(\alpha_{i}/i/o\). GST-HNgb or GST was incubated with untreated or ROS-treated human G\(\alpha_{i}/i/o\). The same concentration and volume of untreated and ROS-treated human G\(\alpha_{i}/i/o\) were used. E. effects of HNgb on dissociation of GDP from untreated or ROS-treated rat G\(\alpha_{i}/i/o\). The amount of [\(^3\)H]GDP bound to untreated G\(\alpha_{i}/i/o\) in the absence of HNgb at 0 min was defined as 100%. Data are expressed as means ± S.E. from four independent experiments. *, p < 0.05; **, p < 0.01. F. protection by HNgb is mediated by cAMP. Differentiated SH-SY5Y cells were pretreated with Sp-cAMPS or Rp-cAMPS, and then incubated with hydrogen peroxide. Cell viability was measured by MTS and LDH assays. Each graph represents data from three independent experiments each carried out in triplicate. Data are expressed as means ± S.E. **, p < 0.01. G. WT HNgb increases intracellular cAMP concentration under oxidative stress. Differentiated SH-SY5Y cells were treated with hydrogen peroxide, and intracellular cAMP concentrations were determined by cAMP enzyme immunoassay kit. Data are expressed as means ± S.E. from five experiments. *, p < 0.05.
bound to the GDP-bound form of nonmyristoylated Ga\textsubscript{11}, expressed in \textit{E. coli} (Fig. 4B). Aluminum tetrafluoride (AlF\textsubscript{4}^-) can interact with Ga\textsubscript{11}-bound GDP and mimic GTP, thereby activating Ga\textsubscript{11} (14). In the presence of GDP and AlF\textsubscript{4}^-, ferric HNgb did not bind to activated Ga\textsubscript{11} (Fig. 4B). Therefore, ferric HNgb clearly interacts with the inactive (GDP-bound) form of Ga\textsubscript{11}. Moreover, we clarified that HNgb does not bind to Ga\textsubscript{11} (Fig. 4C).

Ga\textsubscript{11/o} proteins are targets of ROS, which directly activate Ga\textsubscript{11/o} without receptor activation by modification of specific cysteine residues that exist only in Ga\textsubscript{11/o} but not in other Ga families, leading to the selective activation of Ga\textsubscript{11/o} under conditions of oxidative stress (34, 35). ROS, such as hydroxyl radical, were generated in the presence of hydrogen peroxide and Fe\textsuperscript{2+}. GST pull-down assays showed that WT HNgb interacts comparably with ROS-modified and non-modified Ga\textsubscript{11} (Fig. 4D). Two cysteine residues of Ga\textsubscript{11/o} have been reported to be oxidized by ROS, leading to activation of Ga\textsubscript{11/o} and dissociation into Ga\textsubscript{11} and Gβγ (35). Fig. 4E shows that modification of Ga\textsubscript{11} by ROS decreased the percentage of GDP-bound Ga\textsubscript{11} at 0 min as compared with non-modified Ga\textsubscript{11}, suggesting that modified Ga\textsubscript{11} has decreased affinity for GDP. This is consistent with previous data that Ga\textsubscript{11/o} mutated on a Cys residue that is modified by ROS showed decreased affinity for GDP under normal conditions (40). As shown in Fig. 4E, WT HNgb functioned as a GDI for modified Ga\textsubscript{11}, whereas the E53Q HNgb mutant did not. Treatment with DTT significantly increased the percentage of GDP-bound form of Ga\textsubscript{11} (Fig. 4E), suggesting that cysteine residues of Ga\textsubscript{11} are modified by ROS.

Next, we considered whether HNgb may protect neuronal cells by increasing intracellular levels of cAMP. Sp-cAMPS and Rp-cAMPS are an activator and an inhibitor, respectively, of cAMP-dependent protein kinases. Incubation of cells with the stable cAMP analog Sp-cAMPS led to protection during oxidative stress induced by hydrogen peroxide (Fig. 4F). In contrast, the cAMP antagonist Rp-cAMPS did not rescue cell death. To verify this relationship between protection by HNgb and cAMP levels, we inhibited cAMP signaling by adding Rp-cAMPS prior to hydrogen peroxide treatment. This led to a significant reduction in HNgb-mediated protection (Fig. 4F). Next, we measured the intracellular concentration of cAMP in SH-SY5Y cells transfected with the control vector or WT HNgb expression vector under hydrogen peroxide-induced oxidative stress. As shown in Fig. 4G, WT HNgb significantly increased the amount of intracellular cAMP under oxidative stress. Taken together, these data suggest that neuroprotective activity by HNgb is correlated with increasing cAMP concentration.

**A Structural Change in HNgb Induced by Oxidative Stress Is Crucial for Its Neuroprotective Activity**—To investigate the effects of structural changes in HNgb on its neuroprotective activity, we prepared an H64V HNgb mutant, in which the distal His residue was substituted with Val, as a model of HNgb that cannot form a bis-His conformation. The UV-visible spectra recorded immediately after purification of H64V HNgb showed features typical of ferrous O\textsubscript{2}-bound globins. Ferrous O\textsubscript{2}-bound H64V HNgb was converted to its ferric form by the addition of ferricyanide. The Soret peak of ferric H64V HNgb was 406 nm, corresponding to previous observations (41, 42).

These data confirm that ferric H64V HNgb forms a mono-His conformation as does ferric Mb.

As shown in Fig. 5A, the GST-fused H64V HNgb mutant did not interact with human Ga\textsubscript{11} or flotillin-1, suggesting that H64V HNgb can be used as a model of ferrous ligand-bound HNgb, which does not form the bis-His conformation. To examine the effect of H64V mutation of HNgb on the release of GDP from Ga\textsubscript{11}, we measured the rates of GDP dissociation in the absence or presence of HNgb. In the presence of an excess amount of unlabeled GTP, [\textsuperscript{3}H]GDP release from [\textsuperscript{3}H]GDP-bound Ga\textsubscript{11} was inhibited by ferric WT HNgb (Fig. 5B). In contrast, ferric H64V HNgb did not act as a GDI for Ga\textsubscript{11} (Fig. 5B). Next, we tested whether H64V HNgb can protect cells against hypoxia/reoxygenation, which induces oxidative stress by generating ROS (43). Protein transduction was achieved by using the protein delivery reagent Chariot, which can efficiently deliver a variety of proteins into several cell lines in a fully biologically active form (7, 20, 44, 45), and was confirmed by Western blot analyses (Fig. 5C). MTS assays showed that cell survival was significantly enhanced by the transduction of WT HNgb into PC12 cells (Fig. 5D). TBE assays also showed that protein transduction of WT HNgb with Chariot resulted in a significant increase in cell viability (Fig. 5D). These results suggest that WT HNgb is effective in rescuing PC12 cell death induced by hypoxia/reoxygenation. By contrast, H64V HNgb, which cannot form the bis-His conformation, did not significantly rescue cell death during oxidative stress (Fig. 5D). These results indicate that oxidative stress-induced structural change in HNgb is crucial for its neuroprotective activity. As shown in Fig. 5D, Mb, which does not interact with Ga\textsubscript{11} (12), did not protect PC12 cells against cell death caused by hypoxia/reoxygenation and did not induce PC12 cell death, similar to the PBS control.

**DISCUSSION**

In the present study, we found that Ngb exists in lipid rafts only during oxidative stress. In addition, our data shows that addition of a lipid raft disruptor, M\textsubscript{13}, attenuates the neuroprotective activity of HNgb, whereas reconstruction of lipid raft domains restores it, suggesting that lipid rafts are crucial for HNgb-mediated neuroprotection. Because HNgb binds to flotillin-1, a lipid raft microdomain-associated protein (24), and Ga\textsubscript{11/o} also exists in lipid rafts (30–33), flotillin-1 recruits HNgb to lipid rafts, where HNgb then binds to Ga\textsubscript{11/o} and acts as a GDI for Ga\textsubscript{11/o}, as a means of preventing neuronal death (Fig. 6A).

We clarified that ferric bis-His, but not ferrous ligand-bound HNgb interacts with both flotillin-1 and Ga\textsubscript{11}. Moreover, we showed that the H64V HNgb mutant, which cannot form a bis-His conformation, did not interact with Ga\textsubscript{11} or flotillin-1. Molecular dynamics simulations of mouse ferrous deoxy and CO-bound Ngb have documented that alteration of the His configuration upon CO binding changes the dynamic behavior of the CD corner, which comprises α-helices C and D and the CD loop (46). Glu-53, which we identified as crucial for the HNgb-Ga\textsubscript{11} interaction (23), is located in the CD corner of HNgb. Thus, we propose that HNgb undergoes structural changes during oxidative stress and functions as a non-receptor-mediated oxidative stress-responsive sensor for neuroprotection.
Furthermore, we showed that ferric HNgb, but not ferric ZNgb bound to human G/H9251i1. Previously, we generated HNgb mutants in the amino acids that differ between HNgb and ZNgb and clarified that Glu-53, Arg-97, Glu-118, and Glu-151 of HNgb are crucial for its GDI and neuroprotective activities (7, 23). By contrast, the present study showed that human flotillin-1 bound to both ferric ZNgb and ferric HNgb, suggesting that residues conserved between HNgb and ZNgb proteins are crucial for the interaction with human flotillin-1. Further studies to identify these crucial residues are now in progress.

The G/H9251i/o family is specifically activated by ROS produced during conditions of oxidative stress because the cysteine residues that are modified during oxidative stress are conserved only among G/H9251i/o, and not among other G/H9251 families such as

**FIGURE 5. Functional analyses of the H64V HNgb mutant.** A, GST pull-down assays with H64V HNgb and G/H9251i1 or truncated flotillin-1. GST, GST-fused WT or H64V HNgb mutant was incubated with human GDP-bound G/H9251i1 or truncated flotillin-1 in a buffer (pH 7.4). Western blot analyses were performed with anti-G/H9251i1 mouse monoclonal antibody or with anti-flotillin-1 rabbit polyclonal antibody. B, effect of the H64V mutation on dissociation of GDP from recombinant human GDP-bound G/H9251i1. The amount of [3H]GDP bound to G/H9251i1 in the absence of HNgb at 0 min was defined as 100%. All data are expressed as means ± S.E. from four independent experiments. **p < 0.01. C, Western blot analyses of PC12 cell lysates after protein transduction. PBS, WT HNgb, H64V HNgb or Mb was applied to PC12 cells with Chariot. The cells were then incubated for 3 h. Cell lysates were analyzed on 15.0% SDS/PAGE and by Western blot analyses using rabbit anti-HNgb polyclonal antibody, mouse anti-β-actin monoclonal antibody or rabbit anti-Mb polyclonal antibody. D, protective effect of WT or H64V HNgb or WT Mb on PC12 cell death induced by hypoxia/reoxygenation. WT or H64V HNgb or Mb was applied to PC12 cells with Chariot, and cell viabilities were measured by MTS and TBE assays. Data are expressed as means ± S.E. from three independent experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01.

**FIGURE 6. Schematic representation of the neuroprotective mechanism of HNgb.** A, recruitment of HNgb to lipid rafts by specific interaction with flotillin-1 under oxidative stress conditions. B, regulation of enzymatic activity of ROS-modified G/H9251i1 by ferric HNgb under oxidative stress conditions. Furthermore, we showed that ferric HNgb, but not ferric ZNgb bound to human G/H9251i1. Previously, we generated HNgb mutants in the amino acids that differ between HNgb and ZNgb and clarified that Glu-53, Arg-97, Glu-118, and Glu-151 of HNgb are crucial for its GDI and neuroprotective activities (7, 23). By contrast, the present study showed that human flotillin-1 bound to both ferric ZNgb and ferric HNgb, suggesting that residues conserved between HNgb and ZNgb proteins are crucial for the interaction with human flotillin-1. Further studies to identify these crucial residues are now in progress.

The G/H9251i1 family is specifically activated by ROS produced during conditions of oxidative stress because the cysteine residues that are modified during oxidative stress are conserved only among G/H9251i1 and not among other Gα families such as
ROS modifies Gαs, which leads to activation of Gαi/o and dissociation into Gαi/o and Gβγ (Fig. 6B). Activation of Gαi/o decreases the intracellular cAMP concentration, leading to cell death (Fig. 6B). In the presence of HNgb, however, ferric bis-His HNgb is recruited to lipid rafts by interacting with flotillin-1, where it acts as a GDI for modified Gαi/o, resulting in inhibition of the decrease in intracellular cAMP concentration (Fig. 6B). Thus, HNgb can protect against cell death.

The functional roles of Gαs and Gαi in cell survival or death are completely opposite (47–49). Gαs stimulates adenylate cyclases to activate the cAMP signaling pathway, whereas Gαi/o inhibits adenylate cyclases. The activation of Gs has been shown to increase glutathione and to protect neuronal cells against oxidative stress (49). Recently, it has been reported that Gαi/o inhibits hydrogen peroxide-induced apoptosis of SH-SY5Y cells (48); overexpression of a constitutively active Gαi/o mutant protected, while Gαi/o siRNA augmented hydrogen peroxide-induced apoptosis in SH-SY5Y cells (48). In addition, the protective effect of Gαi/o was abolished by co-expressing a constitutively active Gαi/o mutant, which antagonizes Gαi/o by inhibiting adenylate cyclase (48). Moreover, hydrogen peroxide-induced apoptosis was reduced by treating cells with prostaglandin E2, which activates Gαs, but was augmented by CCPA, which activates Gαi/o, causing a decrease in cAMP levels (48). Furthermore, it should be also noted that a GDI and a guanine nucleotide dissociation inhibitor activity of human Ngb have been reported (50, 51). Initially, Ngb was suggested to be an O2 storage protein similar to Mb (1). However, the low concentration (in the micromolar range) of Ngb in brain tissues except for the retina perhaps argues against a role for Ngb in storing and carrying significant amounts of O2. Alternatively, Ngb may act as an intracellular scavenger of ROS and/or nitric oxide (NO) (5, 52–54). It has been reported that both H64V HNgb and Mb generate very reactive cytotoxic ferryl (Fe4+) species upon treatment of the ferric form with peroxide (42). In the present study, we showed that neither Mb nor H64V HNgb enhanced cell death. Moreover, our previous studies demonstrated that ZNgb and the E53Q, R97Q, E118Q, and E151N HNgb mutants, which can act as ROS scavengers by forming the bis-His conformation, do not inhibit cell death (7). Therefore, we conclude that the neuroprotective effect of human Ngb is due to its GDI activity and not to its scavenging activity against ROS.

HNgb is the first reported sensor that consists of a single globin domain alone, although some globin-coupled sensors in which globin domains are fused to methyl-accepting chemotaxis protein domains or domains for second-messenger regulation have been reported (55). It should be also noted that the function of Ngb proteins has been changing dynamically throughout the evolution of life. For example, fish Ngb has a very different function, i.e., a cell-membrane-penetrating activity, without the fusion of other domains (19). Further studies to investigate the function of Ngb from several different species will provide clues to not only its molecular evolutionary process but also its physiological significance.

REFERENCES

1. Burmester, T., Weich, B., Reinhardt, S., and Hankeln, T. (2000) A vertebrate globin expressed in the brain. Nature 407, 520–523
2. Reuss, S., Saaler-Reinhardt, S., Weich, B., Wystub, S., Reuss, M. H., Burmester, T., and Hankeln, T. (2002) Expression analysis of neuroglobin mRNA in rodent tissues. Neuroscience 115, 645–656
3. Schmidt, M., Giessl, A., Lauß, T., Hankeln, T., Wolfrum, U., and Burmester, T. (2003) How does the eye breathe? Evidence for neuroglobin-mediated oxygen supply in the mammalian retina. J. Biol. Chem. 278, 1932–1935
4. Li, R. C., Morris, M. W., Lee, S. K., Pouranfar, F., Wang, Y., and Gozal, D. (2008) Neuroglobin protects PC12 cells against oxidative stress. Brain Res. 1190, 159–166
5. Li, R. C., Pouranfar, F., Lee, S. K., Morris, M. W., Wang, Y., and Gozal, D. (2008) Neuroglobin protects PC12 cells against β-amyloid-induced cell injury. Neurobiol Aging 29, 1815–1822
6. Sun, Y., Jin, K., Mao, X. O., Zhu, Y., and Greenberg, D. A. (2001) Neuroglobin is up-regulated and protects neurons from hypoxic-ischemic injury. Proc. Natl. Acad. Sci. U.S.A. 98, 15306–15311
7. Watanabe, S., and Wakasugi, K. (2006) Neuroprotective function of human neuroglobin is correlated with its guanine nucleotide dissociation inhibitor activity. Biochem. Biophys. Res. Commun. 369, 695–700
8. Khan, A. A., Wang, Y., Sun, Y., Mao, X. O., Xie, L., Miles, E., Graboski, J., Chen, S., Ellerby, L. M., Jin, K., and Greenberg, D. A. (2006) Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. Proc. Natl. Acad. Sci. U.S.A. 103, 17944–17948
9. Sun, Y., Jin, K., Peel, A., Mao, X. O., Xie, L., and Greenberg, D. A. (2003) Neuroglobin protects the brain from experimental stroke in vivo. Proc. Natl. Acad. Sci. U.S.A. 100, 3497–3500
10. Dewilde, S., Kiger, L., Burmester, T., Hankeln, T., Baudin-Creuzat, V., Aerts, T., Marden, M. C., Caubergs, R., and Moens, L. (2001) Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. J. Biol. Chem. 276, 38949–38955
11. Kitatsui, C., Kurogochi, M., Nishimura, S., Ishimori, K., and Wakasugi, K. (2007) Molecular basis of guanine nucleotide dissociation inhibitor activity of human neuroglobin by chemical cross-linking and mass spectrometry. J. Mol. Biol. 368, 150–160
12. Wakasugi, K., Nakano, T., and Morishima, I. (2003) Oxidized human neuroglobin as a heterotrimetric Gα protein guanine nucleotide dissociation inhibitor. J. Biol. Chem. 278, 36505–36512
13. Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56, 615–649
14. Hepler, J. R., and Gilman, A. G. (1992) G Proteins. Trends Biol. Sci. 17, 383–387
15. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Diversity of G proteins in signal transduction. Science 252, 802–808
16. Wakasugi, K., Kitatsui, C., and Morishima, I. (2005) Possible neuroprotective mechanism of human neuroglobin. Ann. N.Y. Acad. Sci. 1053, 220–230
17. Awenius, C., Hankeln, T., and Burmester, T. (2001) Neuroglobins from the zebrafish Danio rerio and the pufferfish Tetraodon nigroviridis. Biochem. Biophys. Res. Commun. 287, 418–421
18. Fuchs, C., Heib, V., Kiger, L., Haberkamp, M., Roesner, A., Schmidt, M., Hamdane, D., Marden, M. C., Hankeln, T., and Burmester, T. (2004) Zebrafish reveals different and conserved features of vertebrate neuroglobin gene structure, expression pattern, and ligand binding. J. Biol. Chem. 279, 24116–24122
19. Wakasugi, K., Takahashi, N., Uchida, H., and Watanabe, S. (2011) Species-specific functional evolution of neuroglobin. Mar. Genomics 4, 137–142
20. Watanabe, S., and Wakasugi, K. (2008) Zebrafish neuroglobin is a cell-membrane-penetrating globin. Biochemistry 47, 5266–5270
21. Watanabe, S., and Wakasugi, K. (2010) Identification of residues critical...
Neuroprotective Mechanism of Human Neuroglobin

for the cell-membrane-penetrating activity of zebrafish neuroglobin. FEBS Lett. 584, 2467–2472.
22. Watanabe, S., and Wakasugi, K. (2011) Module M1 of zebrafish neuroglobin acts as a structural and functional protein building block for a cell-membrane-penetrating activity. PLoS ONE 6, e16808.
23. Wakasugi, K., and Morishima, I. (2005) Identification of residues in human neuroglobin crucial for guanine nucleotide dissociation inhibitor activity. Biochemistry 44, 2943–2948.
24. Wakasugi, K., Nakano, T., Kitatsui, C., and Morishima, I. (2004) Human neuroglobin interacts with flotillin-1, a lipid raft microdomain-associated protein. Biochem. Biophys. Res. Commun. 318, 453–460.
25. Bickel, P. E., Scherer, P. E., Schnitzer, J. E., Oh, P., Lantis, M. P., and Lodish, H. F. (1997) Flotillin and epidermal surface antigen define a new family of caveolea-associated integral membrane proteins. J. Biol. Chem. 272, 13793–13802.
26. Brownman, D. T., Hoegg, M. B., and Robbins, S. M. (2007) The SPFH domain-containing proteins: more than lipid raft markers. Trends Cell Biol. 17, 394–402.
27. Steurmer, C. A. (2009) The reggie/flotillin connection to growth. Trends Cell Biol. 20, 6–13.
28. Zhao, F., Zhang, J., Liu, Y. S., Li, L., and He, Y. L. (2011) Research advances on flotillins. Virol. J. 8, 479.
29. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. 1, 31–39.
30. Moffett, S., Brown, D. A., and Linder, M. E. (2000) Lipid-dependent targeting of G proteins into rafts. J. Biol. Chem. 275, 2191–2198.
31. Oh, P., and Schnitzer, J. E. (2001) Segregation of heterotrimetric G proteins in cell surface microdomains. Gα binds caveolin to concentrate in caveolae, wheeas Gβ and Gδ target lipid rafts by default. Mol. Biol. Cell 12, 685–698.
32. Quinton, T. M., Kim, S., Jin, J., and Kunapuli, S. P. (2005) Lipid rafts are required in Goα signaling downstream of the P2Y12 receptor during ADP-mediated platelet activation. J. Thromb. Haemost. 3, 1036–1041.
33. Yuyama, K., Sekino-Suzuki, N., Sanai, Y., and Kasahara, K. (2007) Translocation of activated heterotrimetric G protein Goα to ganglioside-enriched detergent-resistant membrane rafts in developing cerebellum. J. Biol. Chem. 282, 26392–26400.
34. Nishida, M., Maruyama, Y., Tanaka, R., Kontani, K., Nagao, T., and Kurose, H. (2000) Goα, Goβ, and Goγ are target proteins of reactive oxygen species. Nature 408, 492–495.
35. Nishida, M., Schey, K. L., Takagahara, S., Kontani, K., Katada, T., Urano, Y., Nagano, T., Nagao, T., and Kurose, H. (2002) Activation mechanism of Gα and Gβ, by reactive oxygen species. J. Biol. Chem. 277, 9036–9042.
36. Aida, Y., and Pabst, M. J. (1990) Removal of endotoxin from protein solutions by phase separation using Triton X-114. J. Immunol. Methods 132, 191–195.
37. Liu, S., Tobias, R., McClure, S., Styba, G., Shi, Q., and Jackowski, G. (1997) Removal of endotoxin from recombinant protein preparations. Clin. Biochem. 30, 455–463.
38. Yang, B., Oo, T. N., and Rizzo, V. (2006) Lipid rafts mediate H2O2 prosurvival effects in cultured endothelial cells. FASEB J. 20, E688–E697.
39. Wakasugi, K., and Morishima, I. (2005) Preparation and characterization of a chimeric zebrafish-human neuroglobin engineered by module substitution. Biochem. Biophys. Res. Commun. 330, 591–597.
40. Thomas, T. C., Schmidt, C. I., and Neer, E. J. (1993) G-protein α, subunit: Mutation of conserved cysteine identifies a subunit contact surface and alters GDP affinity. Proc. Natl. Acad. Sci. U.S.A. 90, 10295–10299.
41. Uno, T., Ryu, D., Tsutsuhi, H., Tomisugi, Y., Ishikawa, Y., Wilkinson, A. J., Sato, H., and Hayashi, T. (2004) Residues in the distal heme pocket of neuroglobin. Implications for the multiple ligand binding steps. J. Biol. Chem. 279, 5886–5893.
42. Lardinois, O. M., Tomer, K. B., Mason, R. P., and Deterding, L. J. (2008) Identification of protein radicals formed in the human neuroglobin–H2O2 reaction using immuno-spin trapping and mass spectrometry. Biochemistry 47, 10440–10448.
43. Abramov, A. Y., Scorziello, A., and Duchen, M. R. (2007) Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. J. Neurosci. 27, 1129–1138.
44. Morris, M. C., Depollier, J., Mery, J., Heitz, F., and Divita, G. (2001) A peptide carrier for the delivery of biologically active proteins into mamalian cells. Nat. Biotechnol. 19, 1173–1176.
45. Wakasugi, K., Takahashi, N., and Watanabe, S. (2011) Chimeric ZHHH neuroglobin is a novel cell membrane-penetrating, neuroprotective agent. Am. J. Neurorpept. Neuroregen. 3, 42–47.
46. Anselmi, M., Brunori, M., Vallone, B., and Di Nola, A. (2007) Molecular dynamics simulation of deoxy and carboxy murine neuroglobin in water. Biochem. Biophys. Res. Commun. 358, 591–597.
47. Garcia-Marcos, M., Ear, J., Farquhar, M. G., and Ghosh, P. (2011) A GDI (AGS3) and a GEF (GIV) regulate autophagy by balancing G protein activity and growth factor signals. Mol. Biol. Cell 22, 673–686.
48. Kim, S. Y., Seo, M., Kim, Y., Lee, Y. I., Oh, J. M., Cho, E. A., Kang, J. S., and Juhnn, Y. S. (2008) Stimulatory heterotrimetric GTP-binding protein inhibits hydrogen peroxide-induced apoptosis by repressing BAK induction in SH-SYSY human neuroblastoma cells. J. Biol. Chem. 283, 1350–1361.
49. Lewerenz, J., Letz, J., and Methner, A. (2003) Activation of stimulatory heterotrimetric G proteins increases glutathione and protects neuronal cells against oxidative stress. J. Neurochem. 87, 522–531.
50. Brunori, M., and Vallone, B. (2006) A globin for the brain. FASEB J. 20, 2192–2197.
51. Nienhaus, K., and Nienhaus, G. U. (2007) Searching for neuroglobin’s role in the brain. IUBMB Life 59, 490–497.
52. Fordel, E., Thijs, L., Martinet, W., Lenjou, M., Laufs, T., Van Bockstaele, D., Moens, L., and Dewilde, S. (2006) Neuroglobin and cytoglobin overexpression protects human SH-SYSY neuroblastoma cells against oxidative stress-induced cell death. Neurosci. Lett. 410, 146–151.
53. Fordel, E., Thijs, L., Martinet, W., Schrijvers, D., Moens, L., and Dewilde, S. (2007) Anoxia or oxygen and glucose deprivation in SH-SYSY cells: A step closer to the unraveling of neuroglobin and cytoglobin functions. Gene 398, 114–122.
54. Herold, S., Fago, A., Weber, R. E., De Vries, A., and Moens, L. (2004) Reactivity studies of the Fe(III) and Fe(II)NO forms of human neuroglobin reveal a potential role against oxidative stress. J. Biol. Chem. 279, 22841–22847.
55. Gilles-Gonzalez, M. A., and Gonzalez, G. (2005) Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. J. Inorg. Biochem. 99, 1–22.