Review

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Neuroglobin – recent developments

Abstract: Neuroglobin (Ngb), a monomeric hexacoordinated heme protein of 17 kDa, was identified in 2000 in the nervous system. Accumulative evidence has proved that Ngb is an endogenous neuroprotective molecule against ischemic/hypoxic insults and oxidative stresses, and in most ischemic conditions, Ngb is up-regulated. The underlying mechanisms, however, are not fully clarified. Here we review the recent experimental findings, mainly focusing on the mechanisms of Ngb's protection and induction during ischemic/hypoxic conditions, the roles of Ngb in astrocytes and tumors, as well as Ngb's function in neurite outgrowth.

Keywords: astrocyte; cancer; hypoxia-inducible factor-1α; neuron; neuroprotection.

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List of abbreviations: ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, cAMP response element binding protein; E2, ^{17}β-estradiol; EMSA, electrophoretic mobility shift assay; EPO, erythropoietin; GDI, guanine nucleotide dissociation inhibitor; GST, glutathione S-transferase; HIF-1, hypoxia-inducible factor 1; MCAO, middle cerebral artery occlusion; mPTP, mitochondrial perrmeability transition pore; Ngb, neuroglobin; NO, nitric oxide; OGD, oxygen-glucose deprivation; PHD, prolyl hydroxylase; PHD1, prolyl hydroxylase-1; ROS, reactive oxygen species; RNS, reactive nitrogen species; Sp1, specificity protein; Sp3, specificity protein 3; TBI, traumatic brain injury; VEGF, vascular endothelial growth factor; VDAC, voltage-dependent anion channel.

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Introduction

Neuroglobin (Ngb), a monomeric globin of 17 kDa, was first identified in mammalian brains in the year 2000 (1). Although Ngb is a member of the globin superfamily, it seems to be impossible for Ngb to be a normal oxygen transporter like hemoglobin and myoglobin, because of Ngb's low content in the brain and its high affinity for oxygen (1–3). It is also distinctive for its hexacoordinate structure (2). In the absence of exogenous ligands, the iron in the heme of ferric and ferrous forms of Ngb is hexacoordinated with distal (His-64) and proximal histidine (His-96) (2, 4). Exogenous ligands (e.g., O_2 and CO) can displace the distal histidine of ferrous (Fe^{2+}) Ngb and form Fe^{2+}-O_2-Ngb or Fe^{2+}-CO-Ngb (2). However, Fe^{2+}-O_2-Ngb is unstable and can be converted to ferric Ngb (Fe^{3+}) very rapidly because of its autoxidation (2). These Ngb forms in the 'Ngb system' have differential biological functions. For instance, ferrous Ngb functions as nitrite reductase (5) and cytochrome c reductase (6), whereas ferric Ngb acts as guanine nucleotide dissociation inhibitor (GDI) for Gα (7). Early studies have shown that hypoxia could up-regulate Ngb expression (8) and the overexpression of Ngb confirmed its neuroprotection role against ischemia/hypoxia/oxidative stresses, although the underlying mechanisms were not fully understood (8, 9). These findings strongly suggest that Ngb is a promising protective molecule against stroke. In this review, we propose a possible pathway for Ngb's induction by hypoxia and discuss possible mechanisms underlying the neuroprotection of Ngb through its GDI and nitrite reductase activities. Finally, we summarize the role of Ngb in neurite development, astrocytes and tumors.

Induction of Ngb expression

Ischemia/hypoxia can up-regulate Ngb

Ngb expression can be induced by ischemia/hypoxia *in vitro*. Both hypoxia alone (95% N_2/5% CO_2) for up to 8–16 h (8) or 4 h OGD (oxygen-glucose deprivation) followed by 4 h reoxygenation (10) in primary cultured mouse cortical neurons, up-regulated Ngb expressions. Up-regulation of Ngb was also observed in cultured neuronal cell lines (PC12 and HN33) after exposure to hypoxia (0.3% O_2 for PC12, 0.3% and 1% O_2 for HN33) for 24 h (11).

Studies have also proved the induction of Ngb by ischemia/hypoxia *in vivo*, but the situation more complex. An early study reported that immunostaining sections from cerebral cortex of mice subjected to focal cerebral ischemia by middle cerebral artery occlusion (MCAO) for 90 min, followed by reperfusion for 4–24 h, showed increased Ngb immunoreactivity in the cytoplasm of cortical neurons from the ischemic hemisphere compared to the nonischemic hemisphere (8). Another experiment in rat brain cortex also demonstrated that both Ngb mRNA (RT-PCR) and Ngb protein (Western blot) increased after sustained exposure to hypoxia of 10% O_2 for 1, 3, 7 and 14 days (12). Compared with the above reports in cerebral cortex, the opposite results are often found in hippocampus. Li et al. demonstrated down-regulation of Ngb mRNA and protein in CA1 hippocampus of rats 3 h and 6 h after global brain ischemia (four-vessel occlusion for 8 min) (13). Shang et al. further confirmed a different response of Ngb between hippocampal neurons and cortical neurons to transient global ischemia in Mongolian gerbils: the number of Ngb-positive neurons by immunocytochemistry was increased in the cerebral cortex but lowered in the hippocampus after 16, 24 and 48 h of reperfusion following 20 min of bilateral common carotid arteries occlusion (14). The seemingly contradictory findings might be a result of the diverse ischemic/hypoxic model used and different reactions of cerebral cortex and hippocampus neurons in response to ischemia/hypoxia. Another experiment reported that in the CA1 and CA3 regions of hippocampus, decreased numbers of Ngb-positive neurons (immunocytochemistry) were also reported in rats exposed to chronic hypobaric hypoxia for 7 days (by an animal decompression chamber) compared to normoxic controls (15). Western blot results confirmed the decrease of Ngb in rat hippocampus after 7 days of hypoxia but also detected an increase of Ngb after 1 day of hypoxia (15). It indicated that Ngb could also be up-regulated in hippocampus, but the response might be much sooner and the duration might be much shorter in hippocampus compared with that in cortex. Unknown differences may exist in the regulatory mechanisms of Ngb expression between cortical and hippocampal neurons in response to ischemia, and it possibly contributes to their differential tolerance to hypoxic or ischemic injury.

Notably, the duration of ischemic/hypoxic injury may be another important factor affecting the expression of Ngb. The expression of Ngb (immunocytochemistry) in the cerebral cortex of Mongolian gerbils was increased after 16, 24 and 48 h of reperfusion (peak at 24 h) following 20 min of bilateral common carotid arteries occlusion but reduced to normal level at 72 h (14). *In vitro* studies further confirmed this phenomenon. An early study by Sun et al. (8) had also revealed a temporal effect of Ngb expression (Western blot) in HN33 cell line upon hypoxia (95% N_2/5% CO_2): increased first at 4 h of hypoxia, peaked at 8 h, and then decreased to control level at 24 h. Similar effects were also reported in N2a cells exposed to hypoxia (90% N_2/5% CO_2/5% H_2), showing that 8 h or 16 h of hypoxia significantly increased Ngb protein levels, but prolonged 24 h of hypoxia detected no significant increase (16). Present evidence indicates that ischemia/hypoxia-induced Ngb expression in neurons is a transient effect of ischemic/hypoxic responses and the mechanisms underlying this phenomenon remain unclear. Recently studies of Ngb have extended from metabolic brain injury to traumatic brain injury (TBI), which is also common in brain diseases. Similar fluctuation of Ngb expression was observed in various TBI models (17–20).

In summary, the responses of Ngb expression to brain injuries are complicated and depend on many factors, such as the duration of injury, the regions of brain as well as the ischemic/hypoxic model used.

Mechanisms of Ngb up-regulation

Introduction

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that responds to hypoxia. It consists of two subunits – HIF-1α and HIF-1β. HIF-1β is expressed constitutively in all cells and does not respond to changes of oxygen tension. However, it is required for hypoxia-induced transcriptional changes mediated by the HIF-1 heterodimer. HIF-1α level is regulated by various conditions. It is already clarified that in presence of O_2 (normoxia), iron and 2-oxoglutarate, HIF-1α is hydroxylated by prolyl hydroxylase (PHD), thereby targeting it for proteasomal degradation. Under hypoxic conditions, PHD activity is inhibited because of the lack of

O_2, leading to accumulation of HIF-1α and activating the downstream transcriptions (21). The correlation between HIF-1α and the up-regulation of Ngb has been reported by different research groups.

Hypoxia up-regulates Ngb expression via stabilization of HIF-1α

A significant up-regulation of both Ngb (mRNA and protein) and HIF-dependent target genes such as ADM and EPO (mRNA) was observed in neonatal mice at postnatal day 0 (P0) upon acute systemic hypoxia (8% O_2, 6 h), but none of above genes was altered in mice exposed to chronic hypoxia (10% O_2, 7 days), implying a close relationship between Ngb and HIF-1 expressions upon hypoxia (22). Furthermore, the DNA binding ability of HIF-1α (electrophoretic mobility shift assay; EMSA) in rat hippocampus was increased after 1 day of hypoxic exposure but progressively decreased afterwards along the hypoxic time up to 7 days, consistent with the changes of Ngb expression in the same hypoxic model (15). In lung cancer tissues from patients, results of RT-PCR also revealed that both HIF-1α and Ngb were overexpressed in tumor samples compared with its adjacent normal tissues (23). These studies both suggested the same tendency between Ngb and HIF-1 levels.

More importantly, the stabilization of HIF-1α was proved to be an induction of Ngb expression by *in vitro* studies. Induction of Ngb was detected in cultured cortical neurons treated with HIF-1α stabilizer, i.e., 300 μM of $CoCl_2$ or 100 μM of deferoxamine (8). Stabilization of HIF-1α via deletion of the von Hippel-Lindau – a component of the E3 ubiquitin ligase complex that can facilitate oxygen-dependent polyubiquitination and proteasomal degradation of HIF-1α subunits – also up-regulated Ngb expression from renal glomerular podocytes (24). Haines et al. reported that HIF-1α knock-down by lentiviral HIF-1α-shRNA decreased Ngb levels while HIF-1α overexpression increased Ngb expression in HN33 cells (25). Similarly, siRNA knock-down of HIF-1α in N2a cells significantly attenuated the hypoxia-induced Ngb protein expression (16). Another *in vitro* study showed that the transcriptional activity of Ngb was increased in mouse embryonic neuronal cultures after incubating with FG-4497 (PHD inhibitor, a HIF-1α stablizer) for 6 h under both normoxic and hypoxic (1% O_2/5% CO_2/94% N_2) conditions; without the synergistic effects of hypoxia and FG-4497 treatment on Ngb expression (22), suggesting a center role of PHD activity inhibition in the hypoxia-upregulated Ngb. However, in contrary to the *in vitro* observations, cerebral Ngb expression was not altered under normoxic conditions when different dosages (30, 60 and 100 mg/kg, i.p.) of FG-4497 were administrated in rats (22), suggesting other important regulatory mechanisms of Ngb expression in the brain.

How HIF-1 up-regulates Ngb expression is further investigated. Although there is no conserved hypoxia-response element in mouse Ngb promoter (26), the 5′-untranslated region of Ngb contains several copies of consensus HIF-1-binding sequence 5′-RCGTG-3′, located at 2073, 1977, 1445, 1041, 985, 627, 522, and 64 nucleotides upstream of the transcription initiation site (8). Chromatin immunoprecipitation (ChIP) assay verified the binding of HIF-1α to the sites 5012, 6725 and 7736 base pairs upstream of the Ngb ATG start site (25). Liu et al. (16) further confirmed the effects of HIF-1α on the Ngb promoter activity upon hypoxic stimulation by luciferase assay. Co-transfection of pGL3-Basic reporter vector containing the promoter -2027/+6 of Ngb and HIF-1α-siRNA for 24 h followed by hypoxia for 16 h showed that HIF-1α-siRNA dramatically attenuated hypoxia-induced Ngb promoter activity (16). Taken together, present evidence supports that hypoxia up-regulates Ngb expression through stabilizing HIF-1α, which initiates Ngb transcription by binding to its promoter region.

Other transcription factors involving in Ngb expression

Zhang et al. (27) analyzed the 5′-flanking region of human Ngb gene and found two GC-boxes located at -16 and +30 bp relative to the transcription start site. Luciferase assay demonstrated that mutation of either GC-box in SH-SY5Y cells led to a significant reduction in Ngb promoter activity, indicating that the two GC-boxes are positive regulatory elements of Ngb. ChIP assay verified that Sp1 (specificity protein 1) and Sp3 (specificity protein 3) bound to the two GC-boxes. Further luciferase assay verified that Sp1 and Sp3 were transactivators of Ngb promoter in SH-SY5Y cells. It is likely that Sp1 and Sp3 might promote Ngb transcription through interacting GC-boxes.

Analysis of the Ngb promoter sequence further revealed that Ngb contains one Egr1-binding site (overlapping with a Sp1-binding site) and two NF-κB (p65) binding sites around -549/+6 relative to the start codon ATG (16). Overexpression of p65 or Egr1 significantly enhanced the Ngb promoter activities, while knock-down of p65, Egr1 or Sp1 significantly reduced Ngb promoter activity in N2a cells (16). Correspondingly, knock-down of p65, Egr1 or Sp1 by siRNA technique decreased Ngb mRNA and protein levels in N2a cells (16). In hypoxic (16 h) N2a cells,

knock-down of transcription factors p65 or Sp1 by RNA interference technique significantly reduced hypoxia-enhanced Ngb promoter activity as measured by an Ngb promoter-driven luciferase assay. These results suggest that NF-κB and Sp1 might be also responsible for hypoxia-induced Ngb up-regulation (16).

Recently, another transcription factor binding site, cAMP response element (CRE), was identified around -854 bp in the promoter region of mouse Ngb gene (28). ChIP assays demonstrated that cAMP response element binding protein (CREB) bound to the Ngb promoter region spanning from -1016 to -793 upstream of the starting codon of Ngb (28). CREB knock-down decreased Ngb gene expression while overexpression of the wild-type CREB, but not the mutant CREB, significantly increased Ngb gene expression in N2a cells (28). CREB might be an additional important transactivator involving in Ngb gene expression upon hypoxia.

Possible hypoxia-induced Ngb up-regulation mechanisms

As listed above, HIF-1α, NF-κB, Sp1, Egr1 and CREB are potential transactivators of Ngb gene, and HIF-1α, NF-κB and Sp1 were involved in the hypoxia-induced Ngb up-regulation. The role of HIF-1α in hypoxia-induced Ngb up-regulation is partly clarified as stated, whereas how NF-κB and Sp1 play their roles in the process is little understood.

It was reported that NF-κB and Sp1 could be up-regulated by hypoxia, although it is still not clear whether their up-regulation is directly correlated to Ngb or not. IκB kinase-β (IKKβ) was negatively regulated by prolyl hydroxylase-1 (PHD1) whose activity can be inhibited by hypoxia (29). Therefore, hypoxia might activate NFκB through a pathway involving IKKβ activation leading to phosphorylation-dependent degradation of IκBα and liberation of NFκB (29). In terms of Sp1, sequential activation of HIF-1 and Sp1 existed in and was required for hypoxic induction of the sulfonylurea receptor 1 following cerebral ischemia in rats (30). The fact that HIF-1 can bind to HIF-binding sites on the Sp1 promoter to stimulate transcription of the Sp1 gene in rats after cerebral ischemia (30) suggests that Sp1 expression could be altered during hypoxia.

CREB is also reported to be involved in ischemic process. The binding activity for CREB was increased in ischemic hippocampus and cortex and the phosphorylation of CREB and CRE-mediated gene expression in neurons was robustly triggered by cerebral ischemia, as reviewed by Kitagawa (31). However, whether hypoxia can up-regulate Ngb expression through the activation of CREB remains not clear, which requires further investigations.

The possible mechanisms of hypoxia-induced Ngb up-regulation are summarized in Figure 1. Hypoxia, as a result of lack of oxygen, inhibits PHD activity, leading to the stabilization and accumulation of HIF-1α. HIF-1α may elicit Ngb gene transcription either by itself binding to Ngb promoter or by promoting Sp1 expression. Meanwhile, hypoxia can increase NF-κB level by inhibiting PHD1 activity, thus NF-κB may enhance Ngb transcription by directly binding to its promoter. Further, hypoxia may induce CREB to enhance Ngb expression.

In addition to inducing Ngb-related transcriptional factors, hypoxia may also enhance Ngb expression through other mechanisms such as induction of erythropoietin (EPO) and vascular endothelial growth factor (VEGF). EPO and VEGF are HIF-1 target genes and are up-regulated by hypoxia (21). Stabilization of HIF-1α by deletion of von Hippel-Lindau gene from renal glomerular podocytes up-regulated both VEGF and Ngb expressions (24). In cortical neuron cultures, administration of VEGF increased Ngb protein expression in a VEGF receptor 2 (VEGFR2/Flk1) dependent manner (32). In primary cultures from Ngb-overexpressing transgenic mice, VEGF expression was found to be decreased, suggesting that Ngb overexpression in turn suppressed VEGF expression (32). Several studies have demonstrated the induction of Ngb by EPO *in vivo*. Ngb expression in the brain was up-regulated by EPO

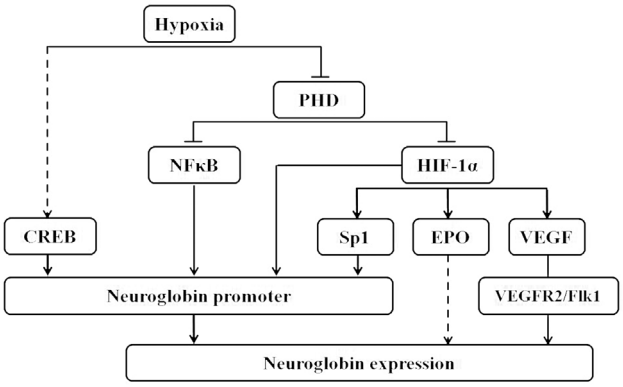

Figure 1 Possible mechanisms for Ngb up-regulation in response to hypoxia.
Hypoxia inhibits PHD activity, leading to stabilization and accumulation of HIF-1α and increased level of free NF-κB. HIF-1α binds directly to Ngb promoter or via inducing Sp1, EPO or VEGF. In addition, hypoxia can trigger the activation of CREB. Both NF-κB, CREB and Sp1 can enhance Ngb transcription by directly binding to its promoter. VEGF up-regulates Ngb expression in a VEGFR2/Flk1-dependent manner, whereas how EPO enhances Ngb expression is little understood. CREB, cAMP response element binding protein; EPO, erythropoietin; HIF-1, hypoxia-inducible factor 1; Ngb, neuroglobin; PHD, prolyl hydroxylase; Sp1, specificity protein; VEGF, vascular endothelial growth factor.

administration after brain ischemia (33–35). In the cerebral cortex of gerbil brain, Ngb (immunohistochemistry) was up-regulated by intranasal administration of recombinant human neuronal EPO for 1 or 5 weeks immediately after unilateral permanent ischemia (36). Therefore, it is reasonable to propose that VEGF and EPO contribute to hypoxia-induced Ngb up-regulation although the detailed mechanisms remain largely unclear (Figure 1).

Similar to Ngb and VEGF, Ngb and HIF-1α expressions are regulated by each other. In N2a cells upon 48 h of hypoxia (1% O_2), Ngb overexpression enhanced the DNA binding ability of HIF-1α (EMSA) while silencing the DNA binding ability of Ngb reduced HIF-1α (15). Contrary to these *in vitro* results, HIF-1α mRNA was increased in Ngb-null mice upon acute hypoxia compared to that in wild-type mice (37). This evidence suggests complicated Ngb-HIF1α feedback regulatory mechanisms in hypoxic conditions.

With progress, other regulatory mechanisms of Ngb were also revealed, e.g., the methylation of Ngb promoter. Upon 5-aza-2′-deoxycytidine treatment, Ngb promoter methylation was decreased while Ngb expression was remarkably increased in normal primary epithelial cell line (NHBE), non-small cell lung carcinoma cells (HTB182 and H2073), and other cancer cells (HeLa and BEAS-2B) (23, 27). It is likely that DNA methylation might be also a mechanism regulating Ngb expression in neurons or in the brain.

Chronic/prolonged hypoxia does not increase Ngb

As mentioned above, Ngb level after chronic or prolonged hypoxia exposure appears to be decreased. Correspondingly, HIF-1α levels showed a similar trend. After 7 days of hypobaric hypoxic exposure, Ngb protein (Western blot) as well as the DNA binding ability of HIF-1α (EMSA), decreased progressively from its peak around 1 day of hypoxia (15). These results suggested that the decrease of Ngb expression during prolonged hypoxia might be caused by the decline of HIF-1α level. In addition, Ngb oxidation and ubiquitination in the hippocampus of rats were significantly increased after 3 or 7 days of hypoxia (15), whereas oral administration of anti-oxidant N-acetylcysteine (150 mg/kg body weight) significantly attenuated the oxidation and ubiquitination of Ngb (15). This evidence indicates that disturbance of redox homeostasis during chronic/prolonged hypoxia might decrease Ngb via Ngb oxidation or ubiquitination. However, until now, the exact mechanism regulating Ngb expression during chronic/prolonged hypoxia remains largely unclear.

Induction of Ngb by chemical compounds

In addition to pathological inducers, some compounds can be also used to induce Ngb expression. These compounds include cinnamic acid (38), valproic acid (38), hemin (39) and ^{17}β-estradiol (E_2, the most efficient within estrogens) (40).

E_2 is a strong inducer of Ngb expression and Ngb participates in the protection of E_2 against H_2O_2-induced neurotoxicity (40, 41). Ngb is also involved in the E_2-mediated anti-inflammatory effects in primary cortical astrocytes (42). The effects of E_2 on Ngb induction and the biological functions of E_2-induced Ngb have been reviewed in details by Fiocchetti (43).

Like hemoglobin and myoglobin, Ngb expression can also be induced by hemin (39). RT-PCR and Northern blot analyses demonstrated a dosage- and time-dependent effect of Ngb mRNA induction by hemin in HN33 cells (39). Western blot showed that Ngb protein was increased evidently after 2 h of 50 μM hemin exposure in HN33 cells and lasted for at least 3 days with a maximal 4-fold increase of the basal Ngb level (39). *In vivo* studies further confirmed the effect of hemin on Ngb induction. Intraperitoneal injection of hemin at 15, 30, and 60 μM/kg/day prior to global brain ischemia (four-vessel-occlusion for 8 min) showed a dose-dependent up-regulation of Ngb mRNA (3 h after ischemia) and protein (6 h after ischemia), which might prevent the delayed neuronal death in CA1 hippocampus induced by ischemia (13). Protein kinase G and soluble guanylate cyclase were considered to be responsible for the hemin-induced Ngb as their inhibitors or mimics blocked or promoted the effects of hemin on Ngb expression (39). E_2 and hemin might be promising molecules for the treatment of ischemia in clinic.

Physiological and pathological role of Ngb in nervous system

Effects of Ngb overexpression and downexpression *in vitro* and *in vivo*

Incubation of TAT PTD-Ngb (transactivator of transcription protein-transduction domain-Ngb fusion protein) in primary neurons for 2 h before hypoxia showed increased cell viability and survival (44). Ngb overexpression by adeno-associated virus also significantly reduced neuronal cell death after 4 h OGD followed by 20 h reoxygenation (10). Transfection of pcDNA-Ngb into HN33

cells increased the viability of hypoxic HN33 cells (8). It is known that ischemia/hypoxia injury is usually linked to enhanced oxidative stress. The insult purely induced by oxidative stress (H_2O_2) could also be attenuated by Ngb overexpression. Antao et al. (45) reported that overexpressing Ngb increased cell tolerance to H_2O_2 insult with a decrease of necrosis exposed to 50–200 μM H_2O_2 for 2 h. Consistently, knocking down Ngb by stable transfection of Ngb-shRNA plasmid decreased cell viability, increased cell death and increased apoptotic cells in N2a cells following 150–300 μM H_2O_2 treatment for 12 h (35). Further, Ngb knock-down decreased cell survival and led to degeneration in primary retinal ganglion cells without any extra insult treatment (46), suggesting the essential role of Ngb for the primary retinal ganglion cells. In addition, Ngb overexpression could confer protection against mechanical injury in B104 cells (17). These *in vitro* studies together support a protective role of Ngb upon both metabolic and mechanical injuries.

The protective role of Ngb has also been proved *in vivo* by many studies. Intracerebral administration of the Ngb-expressing adeno-associated virus 3 weeks before MCAO (occlusion for 90 min followed by reperfusion for 24 h) reduced infarct size and improved functional outcomes in rat (9). Administration of Ngb-expressing canine adenovirus-2 into the cortex of rats 5 days before transient MCAO (occlusion for 45 min) also reduced infarct volume and increased neurologic outcome (47). Consistently, TAT-Ngb, a fusion protein that can cross the blood-brain barrier, enhanced neuron survival in the striatum when intravenously administrated immediately after MCAO (30 min), and reduced brain infarct volume and improved neurologic outcomes by intravenous injection of TAT-Ngb 2 h before MCAO (occlusion 2 h followed by reperfusion 24 h) in mice (48). The protective effects of Ngb was further confirmed in Ngb-overexpressing transgenic mice, in which neuronal injury or death in CA1 region was markedly reduced in response to ischemia-reperfusion injury induced by a 10-min bilateral occlusion of the common carotid arteries (34). Correspondingly, reducing Ngb by intracerebroventricular infusion for 72 h of an Ngb anti-sense oligodeoxynucleotide 24 h before MCAO (90 min followed by 24 h reperfusion) increased infarct volume and aggravated functional neurological outcome (9). The protective role of Ngb after mechanical injury has also been reported. In Ngb-overexpressing transgenic mice, the brain lesion volume was significantly reduced in mice 3 weeks after TBI as compared to wide type mice (19). Ngb overexpression by GFP-rNgb-carrying adenovirus injecting at the injury sites after TBI in rats (18) or by Ngb-lentiviral injection at the injury sites 24 h after spinal cord injury in rabbits (49), both demonstrated that apoptotic cells decreased at the region of injury as compared to their corresponding vehicle controls. These *in vivo* data certainly support the protective role of Ngb in the central nervous system.

Although most studies have verified the protective role of Ngb, opposing results are also reported. In Ngb-null mice, ischemic infarct volume was significantly smaller than that in wild-type mice 24 h after permanent MCAO (50). Further investigations in Ngb-null mice reported that the number of neurons was not altered after 48 h of hypoxia as compared to that in wide type mice (37), which seemed to be contradictory to Lechauve's report in primary retinal ganglion cells mentioned above (46). Moreover, HIF-1α mRNA, c-FOS protein and the glycolytic pathway genes were increased in Ngb-null mice compared with that in wild-type mice upon acute hypoxia (37) and Ngb-null mice exhibited an increased behavioral response to light concomitant with increased Per1 gene expression in suprachiasmatic nucleus upon light stimulation at the early subjective night in relation to wild-type mice (51), indicating that systemic compensatory reactions for total deletion of Ngb existed. We may speculate that endogenous Ngb plays a pivotal role in maintaining brain homeostasis as numerous reactions were elicited to compensate for the unbalance caused by Ngb knockout. What is more, the compensatory reactions may overwhelm the protective effects of Ngb upon injury, resulting in decreased infarct volume. As there are only few studies against the protective roles of Ngb, it is still too early to make a conclusion.

Protective mechanisms of Ngb

Anti-apoptotic role of Ngb

Mitochondria play a central role in apoptosis. Accumulating evidences showed that the neuroprotection role of Ngb is closely related to its mitochondrial function, e.g., preservation of ATP level, scavenging of ROS (reactive oxygen species)/RNS (reactive nitrogen species), reduction of ferric cytochrome c to ferrous cytochrome c by ferrous Ngb, and suppression of calcium release into the cytoplasm (52). The existence of Ngb in mitochondria was proved by various studies (10, 46, and 53). Ngb distribution in mitochondria could be further increased by OGD in primary mouse cortical neurons and was involved in maintaining mitochondrial permeability transition pore (mPTP) and voltage-dependent anion channel (VDAC) (10, 54). In mitochondria, Ngb interacted with cytochrome c-1 (a subunit of mitochondria complex III) and cytochrome c (54), and Ngb knock-down reduced respiratory chain

complexes III and cytochrome c activities (46), strongly suggesting a role of Ngb in mitochondrial respiration.

Upon OGD (4 h OGD and 4 h reoxygenation), the interaction of Ngb and VDAC in primary cultured mouse cortical neurons was increased (55). Ngb overexpression inhibited OGD-induced mPTP opening, cytochrome c release from mitochondria, and decreased OGD-induced neuronal cell death (55). Ngb overexpression alone showed similar neuroprotection effects as inhibiting mPTP opening by administration of CsA, and the combination of Ngb overexpression and CsA did not further reduce OGD-induced neuron death (55). This evidence suggests that Ngb might play a key role in preventing mPTP opening during ischemia. The OGD-induced translocation of Ngb in mitochondria may be a complementary protective mechanism as a result of mitochondrial injury.

Different protection mechanisms were reported in neuronal cells against oxidative stress. Ngb overexpression protected SH-SY5Y cells upon H_2O_2 insult through increasing ATP level by activating mito-K (ATP) channel and Akt (45). Moreover, silencing Ngb in N2a cells enhanced neuronal vulnerability to H_2O_2 by down-regulating 14-3-3γ, indicating that 14-3-3γ was also involved in the anti-apoptotic mechanism of Ngb (35). As 14-3-3 proteins are signaling modulating proteins and are essential for cell survival, Ngb might be heavily involved in neuronal signaling including apoptotic signaling pathways.

Fe^{2+}-Ngb functions as a nitrite reductase

Under physiological conditions, the majority of circulating and storing nitrite is derived from the oxidation of nitric oxide (NO), and nitrite can be reversely reduced to NO by reducing proteins such as hemoglobin and myoglobin, which confers protective effects against hypoxia (56). Ngb has similar effects on reducing nitrite. Ferrous deoxy-Ngb (Fe^{2+}-deoxy-Ngb) could reduce nitrite to NO, generating ferric Ngb (Fe^{3+}-Ngb) (5, 57). The reducing rate of nitrite was faster when Ngb is penta-coordinated (5), although penta-coordinated Ngb accounted smaller fraction of total Ngb (0.1–5%) at equilibrium (58). It is proposed that the penta/hexa-coordinated Ngb equilibrium was regulated by redox-sensitive surface thiols, i.e., cysteine 46 and 55 (5, 59). Under oxidative stress conditions, cysteine 46 and 55 were oxidized and formed an intramolecular disulfide bond. This change increased the formation of penta-coordinated Ngb, facilitated the opening of the heme pocket, allowed nitrite binding, and thus improved the reductase activity of Ngb (5). Moreover, penta-coordinated conformation of Ngb could be stabilized and protected by post-translational modifications such as phosphorylation by Erk 1/2 (60). 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) (60). In neuroglobin-GFP expressing SH-SY5Y cells, 3 h of hypoxic treatment (1% O_2 and 5% CO_2) increased cellular Erk activity without affecting total Erk1/2 expression compared to the normoxic control, and 3 h of hypoxia together with glucose and serum deprivation heavily enhanced Ngb phosphorylation (60). Moreover, cell-permeable Erk inhibitor FR180204 partly inhibited Ngb phosphorylation in Ngb-expressing cells upon 3 h hypoxia (1% O_2 and 5% CO_2) (60), supporting the involvement of Erk in Ngb's phosphorylation upon hypoxia. In addition, immunoprecipitation showed increased 14-3-3 binding to neuroglobin in Ngb-GFP expressing cells cultured under hypoxic conditions over 3 h, which could increase the half-life of the phosphorylation and further stabilize penta-coordinated Ngb (60).

The biological significance of Ngb's nitrite reductase activity is further investigated. Tiso et al. (5) reported that under hypoxic conditions, Ngb inhibited mitochondrial respiration in the presence of nitrite *in vitro*. Further evidences suggested that the produced NO might bind to cytochrome c oxidase to suppress cellular mitochondrial respiration, oxygen consumption and ROS production (5). NO is also known as a soluble guanylyl cyclase activator, promoting the formation of cGMP (61). Whether this property of NO contributed to Ngb's biological function is unknown. In summary, Ngb may serve as a sensor to hypoxic stress through the post-translational modifications and play a role in nirite-dependent-NO signaling to protect cells under hypoxia conditions (Figure 2).

Fe^{3+}-Ngb functions as a guanine nucleotide dissociation inhibitor

Heterotrimeric G proteins (G proteins) consist of an α subunit (Gα) with GTPase activity and a βγ dimer (Gβγ), propagating incoming messages from receptors to effectors. Wakasugi et al. (7) first reported that ferric Ngb but not ferrous ligand-bound Ngb behaved as a GDI for Gαi/o, by binding exclusively to the GDP-bound form of Gα, thus liberating Gβγ leading to protection against neuronal death. The selective Ngb conformational change with cellular oxidative states indicates that Ngb may be an oxidative stress-responsive sensor for signal transduction in the brain (7). Further study revealed that the GDI activity of human Ngb was highly correlated to its neuroprotective

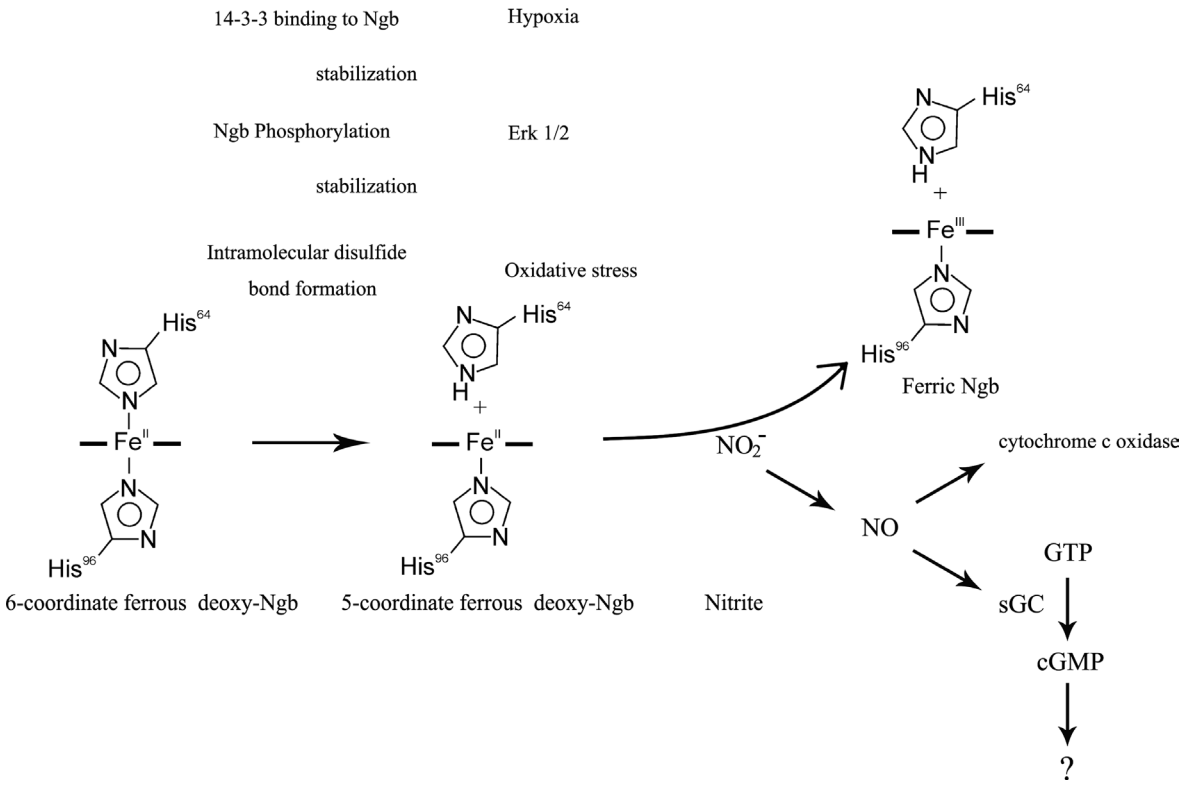

Figure 2 Possible role of Ngb serving as a hypoxic stress sensor.
Upon oxidative stress, Ngb cysteine 46 and 55 are oxidized forming an intramolecular disulfide bond, which promotes the conformational change of Ngb from its hexa-coordiante to penta-coordinate status. Erk 1/2 is activated under hypoxia conditions and phosphorylates Ngb, which stabilizes the intramolecular disulfide bond. Meanwhile, hypoxia enhances the binding of 14-3-3 to Ngb, which prolongs the half-time of phosphorylation. The two together facilitate the transformation from hexa- to penta-coordinated Ngb. The penta-coordinated ferrous deoxy-Ngb reacts with nitrite to produce NO and ferric Ngb. NO acts as a cytochrome c oxidase inhibitor to suppress cellular mitochondrial respiration, oxygen consumption and ROS production. It may also activate sGC, promoting the formation of cGMP that may initiate downstream reactions. sGC, soluble guanylyl cyclase; ROS, reactive oxygen species.

activity, because abolishing GDI activity of Ngb by single mutation (E53Q, R97Q, E118Q, or E151N) did not confer a protection in PC12 cells under hypoxic conditions while other single Ngb mutations (R47A, K102N, K119N, and D149A) without affecting the GDI activity preserved their protective roles in PC12 cells upon hypoxia (62).

Flotillin-1, a lipid raft microdomain-associated protein, was identified as a Ngb binding partner by yeast two-hybrid screening and glutathione S-transferase (GST) pull-down (63). A recent study further showed that it was ferric but not ferrous ligand-bound Ngb that bound to flotillin-1 (64), consistent with the Ngb conformation required for its GDI activity. By using flotillin-1 as a marker for lipid rafts, Ngb were found to be recruited to lipid rafts in PC12 cells only during oxidative stress (64). Disruption of lipid rafts by MβCD attenuated the neuroprotective activity by Ngb against H_2O_2, whereas reconstruction of the lipid raft domains by cholesterol restored Ngb's protection in H_2O_2-treated PC12 cells, suggesting that the lipid rafts participates in Ngb-mediated signal transduction during oxidative stresses (64). These evidences suggest that flotillin-1 might recruit Ngb to lipid rafts, where Ngb acts as a GDI for Gαi to inhibit oxidative stress-mediated cAMP decrease and liberate Gβγ, exerting a protection role (64). The downstream molecules of Gβγ, e.g., PI3K/Akt and Raf/Erk, may also be responsible for the protective effects of Ngb (65). Akt and Erk are key enzymes known to phosphorylate Bad, which binds to 14-3-3 and prevents apoptotic cell death (66, 67).

Role of Ngb in hypoxic preconditioning

Hypoxic preconditioning is hypothesized to be caused, at least in part, by hypoxic induction of HIF-1α, which forms heterodimer with HIF-1β and binds to hypoxia-response element of a number of HIF target genes, including VEGF and EPO (21). As mentioned, HIF-1 is a major

transactivator of Ngb expression and VEGF/EPO can also up-regulate Ngb level. Recent studies demonstrated that Ngb was up-regulated by both hypoxic preconditioning (68) and limb ischemic preconditioning (13). Administration of Ngb anti-sense oligodeoxynucleotides was effective in blocking the protective effect of limb ischemic preconditioning, supporting that Ngb contributes to the ischemic tolerance induced by preconditioning (13). These results also imply that Ngb may be a downstream executor of HIF/VEGF/EPO signaling, which still needs further investigation.

Role of Ngb in neurite development

Very recently, we discovered a novel function of Ngb in neuronal development. In N2a cells and primary cultured rat cerebral cortical neurons, Ngb knock-down exhibited significantly shorter neurite length, whereas Ngb overexpression promoted neurite outgrowth (69). In primary cultured rat cerebral cortical neurons, Ngb was preferentially distributed in the neurites during neurite outgrowth.

The underlying mechanism of the neurite outgrowth is further investigated. Bimolecular fluorescence complementation and GST pull-down assays revealed that Ngb and its mutants (E53Q, E118Q, K119N, H64A, H64L, and Y44D) bound differentially with Akt and phosphatase and tensin homolog (PTEN). Western blot demonstrated that Ngb overexpression suppressed PTEN but increased Akt phosphorylation during induction of N2a neurite outgrowth by serum deprivation. Interestingly, single mutation of Ngb (E53Q) reduced its PTEN binding prominently but increased its Akt binding, decreased p-PTEN but increased p-Akt correspondingly, and increased neurite length. Therefore, we proposed that Ngb may promote neurite outgrowth via differential binding to PTEN and Akt (69).

Role of Ngb in astrocytes

Astrocytes are the most abundant cells in the brain. Whether Ngb is also expressed in glial cells or not is debatable (70). We first detected Ngb mRNA in primary cultured mouse cerebral cortical astrocytes by quantitative RT–PCR and Ngb proteins by cytoimmunostaining (71). Consistently, Avivi et al. (72) reported that Ngb (cytoimmunostaining) was expressed in astrocytes isolated from the corpus callosum of subterranean mole rat Spalax. Very recently, Lechauve et al. (73) reported that Ngb (histoimmunostaining) was detected in optic nerve astrocyte processes in physiological conditions *in vivo*. Under pathological conditions, Ngb expression was detected in reactive actrocytes. For example, Ngb level was elevated in Müller cells during reactive gliosis (73); the expression of Ngb was up-regulated in reactive astrocytes located in the proximity of a penetrating cortical injury *in vivo* (42) or located in regions associated with most severe pathology and the astroglial scar in murine models of TBI, cerebral malaria and experimental autoimmune encephalitis (74). Moreover, Ngb was detected in astrocytic tumors, e.g., rat astrocytoma cells (C6), human astrocytoma cells (U251) (75), human astrocytoma tissues (75, 76). Taken together, these data support that Ngb is expressed in glial cells in the brain.

In many studies, however, Ngb was not detected in astrocytes by conventional immunohistochemistry or fluorescent immunostaining in normal mice brains (74), in hypoxic mice brain (51) and in stroke patient brain tissues (77). These results reflect that Ngb expression in astrocytes in most areas is much lower than that of neurons in the brain. A major concern for these negative results is that all present Ngb antibodies usually detect multiple bands, with a minor band around 17 kDa (the predicted size of endogenous Ngb) in Western blot although Ngb antibodies detect exogenous overexpressed Ngb well. Therefore, how much the signal of immunostaining of Ngb in the brain represents that of endogenous Ngb is unknown. If the immunosignals of Ngb antibodies do represent endogenous Ngb, then the positive stain of Ngb in primary cultured astrocytes (71) and freshly dissociated astrocytes from the brains (unpublished data) already make the conclusion. The protective role of Ngb in neuronal cells has been well documented; however, the function of Ngb in astrocytes is less well studied. We had previously reported that transfecting anti-sense Ngb oligonucleotides significantly exacerbated cell death in primary cultures of cerebral cortical astrocytes upon OGD as compared to the untransfected controls and sense Ngb oligonucleotides controlse (71), consistent to the results of anti-Ngb oligonucleotides in cultured neurons (8). Therefore, Ngb might play a similar protective role in both neurons and astrocytes upon ischemia. 14-3-3γ is a brain-specific isoform of 14-3-3 protein family and was prominently up-regulated in primary cortical astrocytes after ischemia (78). In astrocytes, 14-3-3γ bound to more p-Bad and reduced apoptosis of astrocytes upon OGD (67). In N2a cells, we found that knocking down of Ngb enhanced neuronal vulnerability to H_2O_2 by down-regulating 14-3-3γ (35). Therefore, it is likely that Ngb may play its protective role in astrocyts through modulating 14-3-3γ level or directly binding to it (60).

Role of Ngb in tumors

Ngb has been detected in both brain and non-brain tumors. Ngb mRNA and protein were reported to be expressed in brain tumor cell lines, including rat astrocytoma cells (C6), human astrocytoma cells (U251) and many other non-brain cancer cells, including non-small cell lung cancer (NSCLC) cells (CRL5935, H2073, HTB182 and LUDLU1), SCLC cells (DMS53 and CORL88) and human hepatocellular carcinoma cells (HepG2) (23, 75, 79–81). In addition, Ngb was detected in various human cancer samples, including tumors of breast, bladder, thyroid, lung, liver, and brain (astrocytomas, ependymoblastomas, gangliogliomas and oligodendrogliomas) (23, 75, 80, 81).

As mentioned above, most studies demonstrated that Ngb was up-regulated by hypoxia or ischemia. In tumor tissues such as breast/liver/bladder/thyroid tumors (81) and lung cancer (23), Ngb was found to be increased together with hypoxic markers such as carbonic anhydrase IX. In human hepatocellular carcinoma tissues, we found that Ngb protein and mRNA were significantly down-regulated as compared to its adjacent non-tumor tissues and normal liver tissues (80). The expression of Ngb might be complicated in cancers, as hypoxic or oxidative conditions in cancers last a long time and may vary during the development of cancers. In HepG2 cells, we first demonstrated a functional role of Ngb in cancers (80). Knock-down of Ngb by RNA interference promoted while the overexpression of Ngb suppressed HepG2 cells growth and proliferation via Raf/MEK/Erk axis *in vitro* and *in vivo* (80). The interaction between wild-type Ngb and Raf-1 was demonstrated by GST pull-down, which was attenuated by Ngb mutation at its oxygen-binding site (H64L) (80). These results indicate the involvement of oxygen/ROS signals by Ngb in cancers. Whether Ngb plays similar tumor suppressor function in other tumors remains to be investigated.

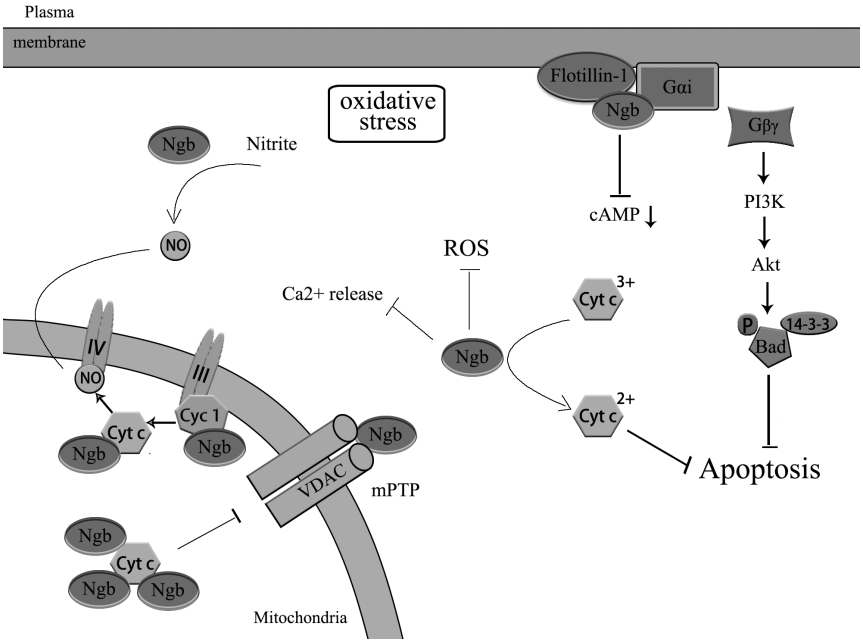

Figure 3 Possible mechanisms underlying the neuroprotective function of Ngb against hypoxia/oxidative stress.
In response to hypoxia, calcium is released into cytoplasm to induce ROS and apoptosis. However, NGB can attenuate these changes via various mechanisms. Firstly, Ngb scavenges ROS and suppresses calcium release into the cytoplasm. Secondly, hypoxia and oxidative stress facilitate the transformation of NGB into 5-coordination ferrous deoxy-NGB, which reduces nitrite to NO that diffuses into mitochondria and binds to cytochrome c oxidase. Also, Ngb can bind to cytochrome c-1 and cytochrome c to reduce oxygen exhaust and ROS production. Thirdly, the ferrous NGB can react with ferric cytochrome c in cytoplasm to generate ferrous cytochrome c to prevent apoptosis. Ngb also binds to VDAC on mitochondria membrane to prevent cytochrome c release. Ngb may also enter into the mitochondria to bind Cyt c and then prevent its outflow upon injury stimulation. Fourthly, the ferric Ngb produced from autoxidation and Ngb-cytochrome c reaction might be recruited to lipid raft by flotillin-1 and interacts with Gαi, thereby inhibiting oxidative stress-mediated cAMP decrease and liberating Gβγ that activates downstream PI3K/Akt. Akt phosphorylates Bad, which binds to 14-3-3 and prevents apoptosis. Cyt c, cytochrome c; Cyc 1, cytochrome c-1; III, mitochondria complex III; IV, mitochondria complex IV; mPTP, mitochondrias permeability transition pore. ROS, reactive oxygen species.

Summary

Ngb expression is low and may be at a 'rest state' under normoxia. Upon hypoxia, Ngb is up-regulated by HIF-1, NFκB, SP1 or CREB transcriptional factors or their downstream targets such as VEGF and EPO. However, the elevation of Ngb level does not persist when exposed to prolonged hypoxia/ischemia, indicating that Ngb induction is mainly an acute response of hypoxia or ischemia. Moreover, prolonged hypoxia or ischemia may promote Ngb degradation through its oxidation and ubiquitination.

Upon hypoxia or oxidative stress, Ngb tends to transfer to its penta-coordinated ferrous status, which facilitates NO production. NO binds to cytochrome c oxidase to inhibit cellular mitochondrial respiration, oxygen consumption and ROS production. Ferrous Ngb can also reduce ferric cytochrome c to ferrous cytochrome c to prevent apoptosis, with itself oxidized to ferric Ngb. The accumulated ferric Ngb can be recruited to lipid raft by flotillin-1 and binds to Gα, which inhibits oxidative stress-mediated cAMP decrease and liberates Gβγ to activate downstream anti-apoptotic signaling pathways. In mitochondria, Ngb can directly bind to VDAC to prevent cytochrome c release. Furthermore, Ngb can bind to cytochrome c-1, which compromises mitochondrial respiration to reduce oxygen consumption and ROS production (Figure 3).

Apart from the protective effects of Ngb in response to hypoxia/ischemia/oxidative stress, Ngb plays a physiological role in neuron or brain development through promoting neurite outgrowth by directly binding to PTEN and Akt.

Moreover, investigations in astrocytes demonstrated the expression of Ngb in astrocytes and its protective role in astrocytes from ischemic death. It is worth noting that detection of Ngb in astrocytes *in vivo* depends on specific pathological conditions.

In terms of tumors, the expression of Ngb in cancer cells varies among cancers. Ngb binds to Raf-1, suppresses Raf/Erk signaling and functions as a tumor suppressor in hepatocellular cancer. Further studies are required for understanding the role of Ngb in other kinds of tumors.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors declare that there is no conflict of interest regarding the publication of this article.

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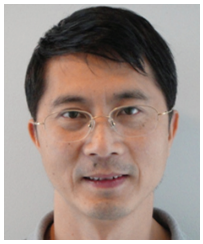

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