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Mitochondrial Distribution of Neuroglobin and Its Response to Oxygen-Glucose Deprivation in Primary Cultured Mouse Cortical Neurons

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Abstract

Neuroglobin (Ngb) is a new member of the globin family and a novel endogenous neuroprotective molecule, but its neuroprotective mechanisms remain largely undefined. Previous studies suggest Ngb is both physically and functionally related to mitochondria, however without direct evidence. Our recent discovery has shown that Ngb can physically interact with a number of mitochondrial proteins. In this study we aimed to define the physical interaction between Ngb and mitochondria by determining whether there is a mitochondrial distribution of Ngb under both physiological resting and pathological oxygen-glucose deprivation (OGD) conditions. Western blot for the first time revealed a small portion of Ngb was physically localized in mitochondria, and the relative mitochondrial Ngb level was significantly increased after OGD in primary cultured mouse cortical neurons, indicating a translocation of Ngb into mitochondria. Complementary approaches including confocal imaging and immuno-electron microscopy confirmed Ngb distribution in mitochondria under both basal resting condition and OGD. Inhibitors of mitochondria permeability transition pore (mPTP) and Voltage Dependent Anion Channel (VDAC) blocked OGD-induced increase of mitochondrial Ngb level, demonstrating a possible role of mPTP in Ngb's mitochondrial translocation. We further found that Ngb overexpression-conferred neuroprotection was correlated with increased mitochondrial Ngb level, suggesting the mitochondria distribution of Ngb is clearly associated with and even might contribute to Ngb's neuroprotection.

Introduction

Neuroglobin (Ngb) is a new member of the globin family identified in 2000 (Burmester et al., 2000) and is highly expressed in brain neurons, some endocrine and retina tissues

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(Schmidt et al., 2003). Like other globins such as hemoglobin and myoglobin, Ngb binds to various gaseous ligands including O₂, CO and NO (Dewilde et al., 2001). The specific expression of Ngb in brain neurons and its high O₂ binding affinity suggest an important role of Ngb in neuron function and hypoxic/ischemic injuries. In the past decade, a large volume of evidence have proven Ngb is an endogenous protective molecule for neurons against hypoxic/ischemic insults (Greenberg et al., 2008, Burmester and Hankeln, 2009, Yu et al., 2009a). Enhanced Ngb gene expression inversely correlates with the severity of histological and functional deficits after ischemic stroke in a manner that is consistent with its neuroprotective effect (Sun et al., 2003, Peroni et al., 2007, Wang et al., 2008). Moreover, emerging evidences from our laboratory and others have demonstrated that Ngb's broad neuroprotection property may have translational importance in other neurological disorders such as beta-amyloid neurotoxicity and Alzheimer's phenotype (Khan et al., 2007), and glaucomatous retinal ganglion cell damage (Wei et al., 2011).

Despite Ngb's generally recognized roles in neuroprotection, the molecular modes of its action remain not fully understood, although a number of hypotheses have been proposed. Ngb may play a role in scavenging reactive oxygen species (ROS) (Fordel et al., 2007), modulating nitric oxide homeostasis (Brunori et al., 2005), serving as a hypoxia sensor and initiating a signal cascade (Wakasugi and Morishima, 2005) in neurons. Our lab has found that Ngb over-expression alters the expression of a group of hypoxia-response genes in primary cortical neurons after oxygen-glucose deprivation (OGD) (Yu et al., 2009b). Furthermore, Ngb has been found to be closely related to mitochondria (Schmidt et al., 2003) and reversibly binds oxygen, suggesting that storing and supplying oxygen to neurons might be one, but not all, of its functions (Burmester and Hankeln, 2009). Our lab has demonstrated that Ngb over-expression preserved mitochondrial function in primary cortical neurons after hypoxia (Liu et al., 2009) and in an experimental model of glaucoma in mice (Wei et al., 2011). Our recent study using yeast two-hybrid screening further identified a group of Ngb-interacting proteins, some of which are mitochondrial proteins including cytochrome c1 (Cyc1), which is a subunit of mitochondria complex III; and VDAC, a component of mitochondria permeability transition pore (Yu et al., 2012). However, no direct evidence of Ngb's physical interaction with mitochondria has been obtained. In this study, we aimed to investigate whether Ngb is physically localized in mitochondria and its changes after OGD in primary cultured mouse cortical neurons.

Methods

Animals

Wild type female C57BL/6 mice at the pregnancy of 15 days were used for primary cortical neuron culture. All animal procedures were following protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Primary mouse cortical neuron culture and OGD treatment

Primary mouse cortical neuron culture and OGD treatment were performed as previously described (Yu et al., 2009b). Unless otherwise specified, the "OGD" treatment in this study refers to 4 hr OGD plus 4 hr reoxygenation. For mPTP blockage, neurons were pre-treated with Cyclosporine A (10 μ M) or bongkrekic acid (5 μ M) for 1 hr before OGD treatment (Liu et al., 2008). VDAC inhibitors, DIDS (0.4 mM) and dextran sulfate (0.2 mM), were used to treat neurons during reoxygenation (Madesh and Hajnoczky, 2001).

Mitochondria isolation

At 9 days of culture, primary cortical neurons were subjected to OGD and then collected for mitochondria isolation. Mitochondria were isolated using differential centrifugation with the Mitochondria Isolation Kit (ThermoScientific) following the manufacturer's instructions.

Western blot

To test the total intracellular Ngf level, cortical neurons at day 9 of culture were subjected to OGD and collected for protein extraction. Total intracellular Ngf levels were examined by Western blot following a previously described protocol (Wang et al., 2008). To test Ngf levels in mitochondria, isolated mitochondria were subject to SDS-PAGE and Ngf levels were examined by Western blot. VDAC (Voltage Dependent Anion Channel), an outer membrane protein of mitochondria, was used as mitochondrial protein marker (Monick et al., 2008); GAPDH was used as cytosol protein marker to verify the purity of isolated mitochondria.

Quantitative analysis of the ratio of mitochondrial Ngf to total Ngf level in neurons

Based on Figure 1A, the intensity of Ngf and VDAC protein bands in the gel were measured with image analysis software-Image J, and the ratio of mitochondrial Ngf to total intracellular Ngf level was calculated as: (mitochondrial Ngf band intensity/mitochondrial VDAC band intensity)/(total Ngf band intensity/total VDAC band intensity).

Immunocytochemistry

After treatment, neurons were washed with cold PBS (pH 7.4), followed by 4% paraformaldehyde for 30 min. After washing with PBS containing 0.1% Tween, cells were incubated with 5% FBS for 1 h, and then incubated with primary antibodies against Ngf (mouse poly Ab, 1:100; Abnova) or mitochondria marker, CoxIV (rabbit poly Ab, 1:200; Abcam) at 4°C overnight. After PBS washing, they were incubated with donkey anti mouse IgG conjugated with Tritc (1:150; Jackson ImmunoResearch) and goat anti rabbit IgG conjugated with FITC (1:150; Jackson ImmunoResearch), respectively, for 1 hr at room temperature. Immunostaining was analyzed using a fluorescence microscope (Olympus BX51) interfaced with a digital camera and an image analysis system.

Electron Microscopy

For preparation of cryosections, neurons were rinsed once with PBS and removed from the dish with 0.5 mM EDTA in PBS, and then pelleted for 3 minutes at 3000 rpm. The cells were fixed with 4% PFA for 2 hr followed by washing with PBS. Prior to freezing in liquid nitrogen the cell pellets were infiltrated with 2.3M sucrose in PBS for 15 minutes. Frozen samples were sectioned at -120°C, and the sections were transferred to fromvar-carbon coated copper grids. Grids were floated on drops of 1% BSA for 10 min to block unspecific labeling, transferred to 5 ul drops of primary antibody (mouse anti-Ngf, Abnova, 1:100) and incubated for 30 minutes. The grids were then washed in 4 drops of PBS for 15 min, transferred to 5 ul drops of Protein-Q gold for 20 min, washed in 4 drops of PBS for 15 min and 5 drops of double distilled water. Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 min. Grids were then examined in a JEOL 1200EX Trans electron microscope and images were recorded with an AMP 2k CCD camera.

AAV construction and packaging

Construction and packaging of AAV was conducted as previously described (Xiao et al., 1998; Xu et al., 2005). Mouse Ngf cDNA was cloned into an AAV-based vector, pACP, with a 6× His tag added at the C terminus of Ngf sequence. Vectors were packaged in a 3

plasmid system by co-complementation of the AAV vector plasmid with another two plasmids, pXX2 and pXX6.

Neurotoxicity

Lactate dehydrogenase (LDH) release assay was used to measure neurotoxicity using LDH Assay Kit (Roche Applied Science). LDH release is an indicator of plasma membrane damage and is commonly used for determination of neurotoxicity as we previously described (Wang et al., 2002).

Statistical Analysis

Results were expressed as mean $\pm$ SEM. Each experiment was repeated three-five times. Multiple comparisons were evaluated by ANOVA followed by Tukey–Kramer’s tests between all groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Total intracellular Ngb expression in primary cultured mouse cortical neurons after OGD

To test the total intracellular Ngb protein levels, primary cultured mouse cortical neurons under physiological resting condition or after OGD (4 hr OGD followed by 4 hr reoxygenation) were collected for protein extraction. Western blot showed that Ngb protein level was significantly increased about 1.8 fold in OGD-insulted neurons compared with normal control neurons (Figure 1, A and B).

Ngb localization in mitochondria of primary cultured mouse cortical neurons after OGD

To test whether Ngb is physically localized in mitochondria, we isolated mitochondria from primary cultured mouse cortical neurons and examined Ngb protein level by Western blot. The results showed, for the first time, that Ngb was detectable in mitochondria protein extraction, and mitochondrial Ngb level was significantly increased about 2.1 fold after OGD (Figure 1, A and C), indicating that Ngb is localized not solely in cytosol, but also in mitochondria, and that this mitochondrial distribution is increased after OGD.

We further analyzed the ratio of mitochondrial Ngb to total intracellular Ngb level in primary cultured neurons based on the data in Figure 1A. The results showed this ratio was about 7.8% for normal neurons, and slightly increased to about 9.9% after OGD (Figure 1, A and D). These results indicate that mitochondrial Ngb is only a small portion of total intracellular Ngb, but increases in response to OGD as well.

To further define the mitochondrial localization of Ngb and its changes after OGD, confocal fluorescent imaging was performed following immuno-cytochemistry. We show that Ngb and mitochondria marker Cox IV partially co-localize in primary cortical neurons both under resting conditions and after OGD (Figure 2A).

To ensure the specificity of anti-Ngb antibody used in this study, we conducted immunocytochemistry using secondary antibody alone (Figure 2B, left column) or anti-Ngb antibody pre-adsorbed with recombinant Ngb protein (Figure 2B, middle column) in primary cultured mouse cortical neurons, and anti-Ngb antibody in endothelial cells (Fig 2B, right column) as a negative control since Ngb protein was undetectable in endothelial cells (Burmester et al., 2000). No Ngb signal was detected in these experiments (Figure 2B, bottom row), indicating the anti-Ngb antibody used in our study is specific.

To further confirm the mitochondrial localization of Ngb, we performed immuno-gold labeling and electron-microscopical (EM) imaging for Ngb in primary neurons under basal

condition and after OGD. The results clearly showed the localization of Ngb in neuronal cytosol and inside mitochondria under both basal resting condition and OGD (Figure 2C). Stereological quantitation of Ngb's mitochondria distribution is needed in future study. Endothelial cell served as a negative control.

Effects of AAV-His-Ngb transduction in Ngb's mitochondria distribution and neuroprotection against OGD

To explore the correlation of Ngb's mitochondrial distribution and neuroprotection, we over-expressed Ngb in primary cultured mouse cortical neurons by transduction with AAV-His-Ngb. In control neurons, the mitochondrial Ngb level was increased about 2 fold by 4 hr OGD followed by 4 hr reoxygenation compared to normal basal condition; while in AAV-His-Ngb transduced neurons, Ngb level in mitochondria was significantly increased about 3.5 fold by OGD (Figure 3A). Cytosol Ngb level was also increased by OGD in non-transduced and GFP-transduced neurons. AAV-His-Ngb transduction significantly increased cytosol Ngb under basal condition compared to non-transduced or GFP-transduced neurons, but OGD did not significantly further increase the cytosol Ngb level in AAV-His-Ngb transduced neurons (Figure 3A). At the later time point, 4 hr OGD followed by 20 hr reoxygenation, Ngb over-expression significantly reduced OGD-induced neuronal cell death (Figure 3B).

Translocation of Ngb from cytosol to mitochondria after OGD

To explore the potential paths of Ngb translocation from cytosol to mitochondria, we tracked the localization and translocation of exogenous His-Ngb produced by AAV-His-Ngb transduction. Western blot with anti-His tag antibody was used to detect His-Ngb in cytosol and mitochondria fractions. A weak signal of His-Ngb was detected in mitochondria, which significantly increased about 1.6 fold after OGD (Figure 3A) in AAV-His-Ngb transduced neurons, suggesting that increased Ngb was translocated to mitochondria after OGD. No positive signal of His-Ngb was detected in AAV-GFP transduced neurons, indicating the specificity of the anti-His tag antibody.

Effects of mPTP inhibitors in Ngb's mitochondrial translocation in neurons after OGD

We used two mPTP inhibitors, cyclosporine A (CsA) and bongkreikic acid (BK), to test the effect of mPTP blockage in Ngb's mitochondrial distribution after OGD. While neither CsA nor BK significantly changed the cytosol or mitochondrial Ngb levels under basal normal condition (Figure 4A), both of them significantly diminished OGD-induced increases of mitochondrial Ngb distribution (Figure 4B), suggesting that mPTP opening at least partially contributes to OGD-induced increases of Ngb mitochondrial distribution.

Effects of VDCA inhibitors on Ngb's mitochondrial translocation after OGD

We further tested the role of VDAC in mitochondrial translocation of Ngb. Two VDAC inhibitors, DIDS and dextran sulfate (Han et al., 2003), were used to treat neurons after OGD, and Ngb level in mitochondria was measured. We showed that DIDS and dextran sulfate did not significantly change the mitochondrial Ngb levels under basal normal condition (Figure 4C), however both of them significantly decreased OGD-induced Ngb translocation into mitochondria, which suggests that VDAC plays an important role in this process (Figure 4D).

DISCUSSION

Since Ngb was first identified in 2000, a large array of studies have demonstrated that Ngb is neuroprotective against stroke and related neurodegenerative disorders, but the underlying

mechanisms remain elusive (Khan et al., 2006, Wang et al., 2008, Burmester and Hankeln, 2009, Yu et al., 2009a, Brittain et al., 2010). Among the hypotheses to explain Ngb's neuroprotection mechanisms, the physical and functional association between Ngb and mitochondria has been considered important, but remains largely unexplored (Schmidt et al., 2003, Rajendram and Rao, 2007, Yu et al., 2009a).

In this study for the first time, we aimed to seek direct evidence of Ngb's physical interaction with mitochondria by determining whether there is a mitochondria distribution of Ngb and its response to OGD. A number of important findings were achieved. First, we clearly demonstrated that Ngb was physically localized in mitochondria under physiological resting condition, and the mitochondrial localization was increased after OGD/reoxygenation. Second, we found that Ngb overexpression-conferred neuroprotection was correlated with increased mitochondrial distribution of Ngb, suggesting a potential role of Ngb's mitochondrial localization in neuroprotection. Third, we found inhibitors of mPTP and VDAC significantly reduced the OGD-induced increase of Ngb translocation to mitochondria, indicating this translocation may be at least partially through mPTP opening and associated with VDAC. These findings imply that the increased mitochondrial localization of Ngb in response to hypoxia/OGD might lead to a new direction in understanding Ngb's function and neuroprotection mechanisms.

Previous studies showed an association of higher Ngb expression with highly metabolically active and mitochondria-rich cell types in brain (Fuchs et al., 2004), and Ngb was localized close to and even possibly physically within mitochondria (Hundahl et al., 2010). However, these observations were not experimentally well characterized and defined. In this short study, we clearly demonstrated the existence of Ngb inside mitochondria and the translocation of Ngb into mitochondria in response to OGD by complementary approaches, including the evidence that His-tagged Ngb was detectable in mitochondria and increased after OGD. The detectable Ngb in mitochondria of cortical neurons under normal resting condition indicates a basal level of Ngb translocation into mitochondria because Ngb gene is encoded by nuclear genomic DNA, but not mitochondrial DNA (Burmester et al., 2000). Indeed, we have recently identified a group of Ngb-interacting proteins using yeast two-hybrid screening and confirmed their interaction by Co-IP (Yu et al., 2012), some of which are mitochondrial proteins such as VDAC, cytochrome c1 (Cyc1) - a subunit of mitochondria complex III (Zhu et al., 2011), and electron-transfer flavoprotein (Etf). These findings provided additional evidence in support of the mitochondrial distribution of Ngb, although the detailed actions of Ngb in mitochondria warrant further investigations.

In this study, we also explored the possible paths of Ngb's translocation into mitochondria. Previous studies have shown that mitochondria permeability transition pore (mPTP) is responsible for certain protein translocation into mitochondria, such as p53, in response to death stimuli (Liu et al., 2008). More interestingly, our recent study using yeast two-hybrid has identified VDAC, a key component of mPTP that resides in mitochondria outer membrane, as an Ngb-interacting protein (Yu et al., 2012). VDAC is also reportedly involved in metabolite and protein trafficking across mitochondria (Shoshan-Barmatz et al., 2006, Scharstuhl et al., 2009), thus implying a possible role of mPTP and VDAC in Ngb's mitochondrial translocation. Our data showed that mPTP and VDAC inhibitors decreased Ngb translocation into mitochondria in response to OGD, suggesting that OGD-induced mPTP opening may be involved in this process.

It should be noted that mPTP may not be the only pathway for mitochondria translocation of Ngb, since Ngb can be translocated into mitochondria at resting condition, without mPTP opening. Most mitochondrial proteins are imported into mitochondria through recognition of the mitochondria targeting sequence (MTS) by TOM complex (translocase of the outer

membrane). This targeting sequence normally resides at the N-terminus of the preprotein, and in some cases in the mature protein as internal signals (Pfanner and Geissler, 2001, Truscott et al., 2003, Chacinska et al., 2009). However, no mitochondrial targeting signal in Ngb protein sequence has been reported, nor could it be found by prediction using bioinformatics tools such as MitoProt or Predotar (data not shown). One explanation is that the mitochondrial targeting signals are not very highly conserved, but rather consist of a handful of subtle distinguishing properties (Truscott et al., 2003), and Ngb may have a very unique mitochondria targeting signal. Site-directed mutagenesis of Ngb can be applied to identify the required sequences for Ngb's mitochondrial translocation. Another possible mechanism may be through the recognition of specific mRNA by mitochondria and subsequent targeting of ribosomes that synthesize the protein (Marc et al., 2002, Riemer et al., 2011), facilitating a co-translational transportation of Ngb into mitochondria. These hypotheses for the mechanisms of Ngb's mitochondrial translocation should be further investigated.

We are aware there are a few limitations of this study. First, the quantification of mitochondrial Ngb to total intracellular Ngb ratio might not be accurate, likely being down-scored using our Western blot assessment and data analysis since we were unable to differentiate whether Ngb is bound to mitochondria membrane or inside mitochondria with this method, although immuno-electron microscopic (EM) images clearly showed the presence of Ngb inside mitochondria. Thus both the Ngb bound to mitochondria outer membrane and those localized inside mitochondria are considered "mitochondria translocation". More detailed study on Ngb localization in sub-structures of mitochondria will be valuable to further define Ngb's action in mitochondria by stereological EM histological analysis. Another limitation is that we were unable to fully dissect the biological role and mechanism of Ngb mitochondrial distribution and translocation. It was mainly due to the technical limitation of lacking the means to specifically block the translocation of Ngb into mitochondria. For future studies, one approach may be applied to address this issue by specifically enhancing Ngb's mitochondrial distribution using a typical mitochondria targeting sequence (MTS) (Lu et al., 2010).

We strongly believe the discovery of Ngb's mitochondrial distribution is translationally important, as Ngb is increasingly recognized as a novel endogenous neuroprotection molecule against hypoxic/ischemic insults, neurodegenerative disorders and glaucomatous retinal ganglion cell damage (Burmester and Hankeln, 2009, Jin et al., 2010, Raychaudhuri et al., 2010, Wei et al., 2011). In addition, disruption of mitochondria function has been regarded as one critical pathological mechanism and therapeutic target (Christophe and Nicolas, 2006, Vosler et al., 2009), thus findings from this study open a new direction for further investigation of Ngb's neuroprotective mechanisms and for the development of strategies for treating stroke and related neurological disorders.

In summary, this study clearly demonstrated the localization of Ngb in mitochondria of primary cultured mouse cortical neurons and its increase after OGD in accordance to the increased total intracellular Ngb levels. The mitochondrial translocation of Ngb may be partially through mPTP opening, and the mPTP component VDAC may also contribute to this process. Our findings established a direct link between Ngb and mitochondria, but the roles and mechanism of this physiological phenomenon need to be elucidated by further investigating the molecular interactions between Ngb and mitochondrial proteins, which may lead to better dissection of Ngb's biological function and neuroprotection mechanisms, and to the development of new therapeutic strategies for neuroprotection.

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Highlights

1. We showed that neuroglobin (Ngb) is localized in mitochondria in primary neurons.
2. Ngb's mitochondrial localization is increased after OGD.
3. Ngb neuroprotection is correlated with increased mitochondria distribution of Ngb.
4. Mitochondria permeability transition pore is involved in Ngb's mito translocation.

Figure 1. Quantitative analysis of NgB distribution in mitochondria of neurons after OGD
 (A) Total intracellular NgB level and NgB in mitochondria measured by Western blot. Mitochondria was isolated from primary cortical neurons after OGD (4 hr OGD followed by 4 hr reoxygenation) and subjected to Western blot. VDAC was used as a mitochondria protein marker, and GAPDH as a cytosol protein marker. (B) Quantitation of relative total intracellular NgB level in OGD-treated versus non-treated neurons; (C) Quantitation of relative mitochondrial NgB level in OGD-treated versus non-treated neurons; (D) Quantitation of the ratio of mitochondrial NgB to total NgB level in OGD-treated versus non-treated neurons. (n=5, *P<0.05)

2A

2B

2C

Figure 2. Ngb localization in mitochondria of neurons after OGD

(A) Double immunostaining of Ngb and Cox IV in normal or OGD-treated neurons. (Scale bar = 10 μ m). (B) Primary cultured mouse cortical neurons stained with secondary antibody alone (left column), with anti-Ngb antibody pre-adsorbed with recombinant Ngb protein (middle column), and endothelial cells stained with anti-Ngb antibody (right column). Cell morphology was shown on the top row and fluorescent imaging at the bottom. (C) Ngb localization in the subcellular structure of normal or OGD-treated neurons detected by immuno-electron microscopy. (Scale bar = 200 nm).

Figure 3. Ngf distribution in mitochondria of neurons with Ngf-overexpression

(A) Ngf protein level in mitochondria and cytosol from non-transduced, AAV-GFP or AAV-His-Ngf transduced neurons at normal condition or after OGD. Anti-Ngf antibody was used to measure total Ngf level in mitochondria. Anti-His tag antibody was used to measure His-Ngf level in mitochondria. The graph shows quantification of mitochondrial Ngf level (n=4, * P<0.05 versus non-transduced neurons at normal condition; # P<0.05 versus OGD-treated AAV-GFP neurons). (B) OGD-induced neuron death for non-transduced, AAV-GFP or AAV-His-Ngf transduced neurons. After 4 hr OGD plus 20 hr reoxygenation, cell death was measured by LDH release assay (n=4, * P<0.05 versus AAV-GFP transduced neurons, and non-transduced neurons).

Figure 4. Effects of mPTP and VDAC inhibitors on OGD-induced NgB translocation into mitochondria

(A) Mitochondrial NgB levels in primary cortical neurons under basal condition and treated with mPTP inhibitors, Cyclosporine A (10 μ M) and bongkrekic acid (5 μ M). (B) Mitochondrial NgB levels in primary cortical neurons after OGD and treated with mPTP inhibitors, Cyclosporine A (10 μ M) and bongkrekic acid (5 μ M). (C) Mitochondrial NgB levels in primary mouse cortical neurons under basal condition and treated with VDAC inhibitors, DIDS (0.4 mM) and dextran sulfate (0.2 mM). (D) Mitochondrial NgB levels in primary mouse cortical neurons after OGD and treated with VDAC inhibitors, DIDS (0.4

mM) and dextran sulfate (0.2 mM). (n=3, * $P < 0.05$ versus neurons at normal condition; # $P < 0.05$ versus OGD-treated neurons).