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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 may contribute to Ngb's neuroprotection.

Key words: neuroglobin, mitochondria, oxygen–glucose deprivation, permeability transition pore.

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 retinal tissues (Schmidt et al., 2003). Like other globins such as hemoglobin and myoglobin, Ngb binds to various gaseous ligands including O_2, CO and NO (Dewilde et al., 2001). The specific expression of Ngb in brain neurons and its high O_2-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 laboratory 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,

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Abbreviations: BK, bongkrekic acid; CsA, cyclosporine A; Cyc1, cytochrome c1; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; LDH, lactate dehydrogenase; mPTP, mitochondria permeability transition pore; MTS, mitochondrial-targeting sequence; Ngb, neuroglobin; OGD, oxygen–glucose deprivation; PBS, phosphate-buffered saline; VDAC, Voltage-Dependent Anion Channel.

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but not all, of its functions (Burmester and Hankeln, 2009). Our laboratory has demonstrated that Ngb overexpression 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 Voltage-Dependent Anion Channel (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.

EXPERIMENTAL PROCEDURES

Animals

Wild-type female C57BL/6 mice at 15 days of pregnancy 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-h OGD plus 4-h reoxygenation. For mitochondria permeability transition pore (mPTP) blockage, neurons were pre-treated with cyclosporine A (10 μM) or bonkrekic acid (5 μM) for 1 h before OGD treatment (Liu et al., 2008). VDAC inhibitors, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (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 mitochondrial isolation. Mitochondria were isolated using differential centrifugation with the Mitochondria Isolation Kit (ThermoScientific, Rockford, IL, USA) following the manufacturer's instructions.

Western blot

To test the total intracellular Ngb level, cortical neurons at day 9 of culture were subjected to OGD and collected for protein extraction. Total intracellular Ngb levels were examined by Western blot following a previously described protocol (Wang et al., 2008). To test Ngb levels in mitochondria, isolated mitochondria were subject to SDS–PAGE and Ngb levels were examined by Western blot. VDAC, 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 Ngb to total Ngb level in neurons

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

Immunocytochemistry

After treatment, neurons were washed with cold phosphate-buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde (PFA) for 30 min. After washing with PBS containing 0.1% Tween, cells were incubated with 5% fetal bovine serum (FBS) for 1 h, and then incubated with primary antibodies against Ngb (mouse poly Ab, 1:100; Abnova, Walnut, CA, USA) or mitochondria marker, CoxIV (rabbit poly Ab, 1:200; Abcam, Cambridge, MA, USA) at 4 °C overnight. After PBS washing, they were incubated with donkey anti mouse IgG conjugated with Tritc (1:150; Jackson ImmunoResearch, West Grove, PA, USA) and goat anti rabbit IgG conjugated with FITC (1:150; Jackson ImmunoResearch, West Grove, PA, USA), respectively, for 1 h 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 min at 3000 rpm. The cells were fixed with 4% PFA for 2 h followed by washing with PBS. Prior to freezing in liquid nitrogen the cell pellets were infiltrated with 2.3 M sucrose in PBS for 15 min. 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 μl drops of primary antibody (mouse anti-Ngb, Abnova, 1:100) and incubated for 30 min. The grids were then washed in four drops of PBS for 15 min, transferred to 5 μl drops of Protein-Q gold for 20 min, washed in four drops of PBS for 15 min and five 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 Ngb cDNA was cloned into an AAV-based vector, pACP, with a 6x-His tag added at the C terminus of Ngb 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).