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METHYLENE BLUE-INDUCED NEURONAL PROTECTIVE MECHANISM AGAINST HYPOXIA-REOXYGENATION STRESS

M.-G. RYOU,[a,b*] G. R. CHOUDHURY,[a] W. LI,[a] A. WINTERS,[a] F. YUAN,[c] R. LIU[a] AND S.-H. YANG[a,b,c*]

[a] *Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, TX, USA*

[b] *Cardiovascular Research Institute, University of North Texas Health Science Center, Fort Worth, TX, USA*

[c] *Department of Neurosurgery, Beijing Tiantan Hospital, Beijing Neurosurgical Institute, Capital Medical University, Beijing 100050, China*

Abstract—Brain ischemia and reperfusion (I/R) injury occurs in various pathological conditions, but there is no effective treatment currently available in clinical practice. Methylene blue (MB) is a century-old drug with a newly discovered protective function in the ischemic stroke model. In the current investigation we studied the MB-induced neuroprotective mechanism focusing on stabilization and activation of hypoxia-inducible factor-1α (HIF-1α) in an *in vitro* oxygen and glucose deprivation (OGD)-reoxygenation model. *Methods:* HT22 cells were exposed to OGD (0.1% O_2, 6 h) and reoxygenation (21% O_2, 24 h). Cell viability was determined with the calcein AM assay. The dynamic change of intracellular O_2 concentration was monitored by fluorescence lifetime imaging microscopy (FLTIM). Glucose uptake was quantified using the 2-[N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino]-2-Deoxy-D-Glucose (2-NBDG) assay. ATP concentration and glycolytic enzyme activity were examined by spectrophotometry. Protein content changes were measured by immunoblot: HIF-1α, prolyl hydroxylase 2 (PHD2), erythropoietin (EPO), Akt, mTOR, and PIP5K. The contribution of HIF-1α activation in the MB-induced neuroprotective mechanism was confirmed by blocking HIF-1α activation with 2-methoxyestradiol-2 (2-$MeOE_2$) and by transiently transfecting constitutively active HIF-1α. *Results:* MB increases cell viability by about 50% vs. OGD control. Compared to the corresponding control, MB increases intracellular O_2 concentration and glucose uptake as well as the activities of hexokinase and G-6-PDH, and ATP concentration. MB activates the EPO signaling pathway with a corresponding increase in HIF-1α. Phosphorylation of Akt was significantly increased with MB treatment followed by activation of the mTOR pathway. Importantly, we observed, MB increased nuclear translocation of HIF-1α vs. control (about three folds), which was shown by a ratio of nuclear:cytoplasmic HIF-1α protein content. *Conclusion:* We conclude that MB protects the hippocampus- derived neuronal cells against OGD-reoxygenation injury by enhancing energy metabolism and increasing HIF-1α protein content accompanied by an activation of the EPO signaling pathway. © 2015 Published by Elsevier Ltd. on behalf of IBRO.

Key words: methylene blue, hypoxia-inducible factor, oxygen and glucose deprivation, neuroprotection, ischemia and reperfusion injury.

INTRODUCTION

Ischemic stroke is the leading cause of severe adult disability and the 4th leading cause of death in the U.S. While the incidence of stroke in the Medicare population ⩾65 years of age has dropped about 40% in the last two decades, possibly due to the preventive interventions such as anti-hypertension and diet control (Fang et al., 2014), there is no currently available treatment for ischemic stroke. Indeed, recombinant tissue plasminogen activator (rtPA) remains the only FDA approved treatment for ischemic stroke (O'Collins et al., 2006). Unfortunately, the therapeutic window of rtPA is less than 4.5 h, thus, less than 4 % of stroke patients have received rtPA thrombolytic therapy (Green, 2008; Hacke et al., 2008). Development of an alternative or combined therapy for ischemic stroke is urgently needed.

Hypoxia-inducible factors (HIF) are the most pertinent transcription factors in the maintenance of cellular homeostasis under ischemic/hypoxic conditions. Three isoforms of HIF have been identified: HIF-1, HIF-2, and HIF-3 (Majmundar et al., 2010). Although physiological functions and regulatory mechanisms of HIF-1 and HIF-2 are relatively well documented, the role of HIF-3 is less known, except as a negative regulator of HIF-1 and HIF-2 (Zhang et al., 2014). HIF-1 plays critical roles as a transcriptional activator regulating various proteins in energy metabolism and in maintenance of cellular homeostasis in low O_2 conditions (Lu et al., 2005; Majmundar et al., 2010). The importance of HIF can be

*Corresponding authors: Address: Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107-2699 USA. Tel: +1-817-735-256, fax: +1-817-735-2091 (M.-G. Ryou). Tel: +1-817-735-2250, fax: +1-817-735-2091 (S.-H. Yang).
E-mail addresses: myoung-gwi.ryou@unthsc.edu (M.-G. Ryou), shaohua.yang@unthsc.edu (S.-H. Yang).

Abbreviations: 2-NBDG, 2-[N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino]-2-Deoxy-d-Glucose; ARNT, aryl hydrocarbon nuclear translocator; EDTA, ethylenediaminetetraacetic acid; EPO, erythropoietin; FBS, fetal bovine serum; G6PDH, glucose-6-phosphodehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIF-1α, hypoxia- inducible factor-1α; HK, hexokinase; MB, methylene blue; OGD, oxygen and glucose deprivation; PBS, phosphate- buffered saline; PHD2, prolyl hydroxylase 2; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; ROS, reactive oxygen species; rtPA, recombinant tissue plasminogen activator.

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underscored with its primary functions in angiogenesis, hematopoiesis, energy metabolism, and anti-/pro-apoptosis (Forsythe et al., 1996; Marti, 2004; Semenza, 2007; Majmundar et al., 2010). All of the primary functions of HIF-1α directly or indirectly contribute to the increase the O_2 content in ischemic organs. HIF has two subunits: α and β (also known as aryl hydrocarbon nuclear translocator (ARNT)). Those proteins belong to the basic helix-loop-helix-per-ARNT (bHLH-PAS) protein family (Wang et al., 1995). Although both subunits are continuously synthetized, the O_2-regulated HIF-α subunit is recognized as a critical regulatory subunit because of short half-life (< 3 min) under normoxia. In the presence of O_2, iron, and ascorbate, the HIF-α subunit is rapidly degraded via proline hydroxylation in the O_2-dependent degradation domain, ubiquitination by von Hippel–Lindau protein (VHL), and the proteasomal degradation pathway. Interaction between HIF-1α and PHD2, which is a key enzyme catalyzing proline hydroxylation, occurs in both nucleus and cytoplasm, but recent reports state that a significant portion of HIF-1α proline hydroxylation occurs in the nucleus (Pientka et al., 2012). Many studies focusing on pharmacological inhibition of PHD2 to study the role of HIF-1α have been conducted (Demidenko and Blagosklonny, 2011; Chen et al., 2014; Ong et al., 2014).

A series of metabolic and pharmacological HIF-1α stabilizers under normoxia are proposed, such as pyruvate (Lu et al., 2002; Ryou et al., 2012), moderate level of reactive oxygen species (ROS) Guzy et al., 2005; Mansfield et al., 2005, and PHD inhibitor (Chen et al., 2014). Stabilization of HIF protects O_2-sensitive organs, such as the brain and heart, by mitigating inflammatory (Curtis et al., 2014) and apoptotic (Trollmann et al., 2014) responses, but a HIF-1-induced neuronal protective mechanism is not clear. Empirically, outcomes of HIF-1α stabilization and HIF-1 activation seem varied depending on the way HIF-1α is stabilized or activated.

Methylene blue, the first synthetic drug, has been used in clinics for various diseases for more than a century (Boylston and Beer, 2002; Schirmer et al., 2003; Meissner et al., 2006). Recently MB has been proposed as a potential treatment for cancer (Wondrak, 2007), hepatopulmonary syndrome (Schenk et al., 2000), and septic shock (Preiser et al., 1995; Kwok and Howes, 2006). Furthermore, the neuroprotective function of MB, which is able to traverse the BBB, has been reported (O'Leary et al., 1968). Our previous studies have demonstrated that MB increases ATP synthesis and minimizes ROS generation (Wen et al., 2011; Poteet et al., 2012). The MB-induced antioxidant function is unique compared to the traditional ROS scavengers. MB is not capable of detoxifying the glucose oxidase-generated H_2O_2 (Poteet et al., 2012), rather it reduces newly formed ROS by minimizing electron leakage from the mitochondrial electron transport chain (ETC) ,shuttling electrons from complex I to cytochrome c bypassing complex II and complex III, a primary source for superoxide generation (Poteet et al., 2012). Since MB increases byproducts of glucose metabolism and reduces ROS generation, it could be reasonably speculated that MB stabilizes HIF-1α under a normoxic environment.

Therefore, we hypothesized, based on previous reports including ours, that MB-induced neuroprotection is mediated by stabilizing HIF-1α and MB activates HIF-1 by increasing glucose metabolism along with activating the EPO-mTOR pathway and enhancing nuclear translocation.

EXPERIMENTAL PROCEDURES

OGD and reoxygenation stress model

Murine hippocampal cell line, HT22 (< 20 passages), was maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin (10,000 units/ml)–streptomycin (10,000 μg/ml). At 18 h prior to OGD and reoxygenation, HT22 cells (5000/well, 96-well plate) were seeded in high glucose DMEM supplemented with 10% FBS and 1% penicillin–streptomycin cocktail. OGD stress was introduced by replacing media with DMEM without FBS, glucose or pyruvate (Gibco, NY, USA) in which O_2 concentration is maintained at 0.1% with auto-controlled N_2 gas injection. After a 6-h OGD, reoxygenation was initiated by transferring the cells to normoxic 5% CO_2 cell culture incubator. At the beginning of reoxygenation, Dextrose (11 mM) and pyruvate (1 mM) were restored to simulate *in vivo* reperfusion.

Primary neuron preparation

Primary neurons were generated as described previously with modifications. Briefly, cortex tissue from postnatal day 0 C57BL/6 pups was dissociated by incubating in TrypLE Express (Invitrogen) at 37 °C for 20 min. Single- cell suspension was made by passing tissue through fire-polished glass pipettes and a cell strainer. Cells were re-suspended in neuron culture medium (neurobasal medium with 2% B27 and 1% Glutamax) and seeded on poly-L-lysine- coated coverslips. After incubation in a cell culture incubator at 37 °C for 3–6 h, old medium was removed and fresh warm neuron culture medium was added. Two days after plating, cytosine arabinoside (araC; 1-b-D-arabinofuranosylcytosine) was added to the medium to a final concentration of 5 μM to inhibit the proliferation of glia cells. After 24 h, old medium containing araC was replaced with fresh warm neuron culture medium. Cells were cultured for ~10 days before experiment.

Cell viability

After a 24-h reoxygenation, cell viability was tested with the calcein AM assay. Cells were washed with phosphate-buffered saline (PBS) (pH 7.0) and incubated with calcein AM (1 μM; Anaspec, Fremont, CA, USA) in PBS for 15 min at 37 °C. Fluorescence was measured using a Tecan Infinite F200 plate reader (Maennedorf, Switzerland) with 485/530-nm excitation/emission. Percent viability was calculated by comparing to the corresponding control. Treatment groups consisted of four independent trials, each in sextuplicate. After spectrophotometric analysis, green fluorescent images

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