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NEURONAL HYPOXIA DISRUPTS MITOCHONDRIAL FUSION

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Abstract—Brain ischemia/reperfusion injury results in death of vulnerable neurons and extensive brain damage. It is well known that mitochondrial release of cytochrome *c* (cyto *c*) is a hallmark of neuronal death, however the molecular events underlying this release are largely unknown. We tested the hypothesis that cyto *c* release is regulated by breakdown of the cristae architecture maintenance protein, optic atrophy 1 (OPA1), located in the inner mitochondrial membrane. We simulated ischemia/reperfusion in isolated primary rat neurons and interrogated OPA1 release from the mitochondria, OPA1 oligomeric breakdown, and concomitant dysfunction of mitochondrial dynamic state. We found that ischemia/reperfusion induces cyto *c* release and cell death that corresponds to multiple changes in OPA1, including: (i) translocation of the mitochondrial fusion protein OPA1 from the mitochondria to the cytosol, (ii) increase in the short isoform of OPA1, suggestive of proteolytic processing, (iii) breakdown of OPA1 oligomers in the mitochondria, and (iv) increased mitochondrial fission. Thus, we present novel evidence of a connection between release of cyto *c* from mitochondria and disruption of the mitochondrial fusion. Published by Elsevier Ltd. on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Key words: cytochrome *c*, ischemia, reperfusion, mitochondrial dynamics, oxidative stress, brain.

INTRODUCTION

Brain ischemia and reperfusion injury continue to be a leading cause of loss of cerebral function following cardiac arrest or stroke, often resulting in mortality. In neurons, post-ischemic cell signaling often culminates on the mitochondria resulting in the detrimental release of cytochrome *c* (cyto *c*) from mitochondria (Perez-Pinzon et al., 1999; Sugawara et al., 1999; Prakasa Babu et al., 2000; Borutaite et al., 2003). Many investigators have proposed and tested a number of hypotheses centered on mitochondrial rupture, pro-apoptotic pore formation, and/or mitochondrial permeability transition as the basis of cyto *c* release into the cytosol following brain ischemia (Borutaite et al., 2003; Hirakawa et al., 2003; Zhao et al., 2005; Endo et al., 2006). Although there has been a great deal of research on this topic, there is little consensus in the field regarding the specific mechanism of cyto *c* release from mitochondria following cerebral ischemia.

The emergent field of mitochondrial dynamics has revealed that mitochondria are dynamic and continuously exist in a balance of fission and fusion phenotypes in response to stimuli in the cellular environment (Chen and Chan, 2004; Chan et al., 2006). While mitochondria typically exist as both thread-like and granular structures, genetic manipulation of eukaryotic cells that push mitochondrial phenotypes into either extreme allowed the identification of key proteins that regulate mitochondrial dynamics. Mitochondrial fission and fusion are highly regulated processes that are controlled by a wide array of signaling mechanism including post-translational modifications, cellular localization changes, and proteolytic cleavage of the key regulatory proteins. Interestingly, recent studies have implicated severe alterations in mitochondrial dynamic phenotype as a key regulator of the apoptotic program (cyto *c* release) (Nguyen et al., 2011). Considering alterations in mitochondrial dynamics occur in response to physiological changes in cells and severe cellular stress can unbalance mitochondrial dynamics to favor cell death, we investigated the concept that mitochondrial dynamics could play a key role in neuronal apoptosis following ischemia/reperfusion.

We, like others, have observed cyto *c* release following global brain ischemia (Perez-Pinzon et al., 1999; Namura et al., 2001; Sanderson et al., 2008, 2013). To clearly observe if alterations in mitochondrial morphology and dynamics play a role in cyto *c* release, we chose to interrogate a key regulator of mitochondrial fusion, optic atrophy 1 (OPA1), and mitochondrial dynamics in primary hippocampal and cortical rat neurons subjected to oxygen–glucose deprivation. This model allows

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Abbreviations: BN-PAGE, blue native-polyacrylamide gel electrophoresis; cyto *c*, cytochrome *c*; Drp1, dynamin related protein 1; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ETC, electron transport chain; GADPH, glyceraldehyde 3-phosphate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IMM, inner mitochondrial membrane; LDH, lactate dehydrogenase; Mfn1, mitofusin 1; MIB, mitochondria isolation buffer; mitoGFP, mito green fluorescent protein; OGD, oxygen and glucose deprivation; OPA1, optic atrophy 1; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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for clear visualization in alterations of mitochondria of neurons in a relatively homogenous environment. Here we report that oxygen–glucose deprivation in primary neuronal culture results in release of cyto *c* from mitochondria, with concomitant release of OPA1 into the cytosol, breakdown of OPA1 complexes, excessive mitochondrial fragmentation, and neuronal death.

EXPERIMENTAL PROCEDURES

Preparation of cortical neurons

All experiments conformed to Wayne State University's animal care program as well as International Guidelines on the ethical use of animals and that all efforts were made to minimize the number of animals used and their suffering. Primary cultures of cortical and hippocampal neurons were isolated from embryonic day 18 Sprague–Dawley rats using a modification of Hilgenberg and Smith (2007). In brief, cerebral cortices and hippocampi were isolated in ice-cold dissection buffer, and incubated in papain. The tissue was then gently triturated in ice-cold Hibernate E medium (Invitrogen). After the tissue settled, the supernatant was aspirated, and the cells were resuspended in Neurobasal Media with B27 supplement (Invitrogen). Cells were plated on poly-D-lysine-coated plates and kept at 37 °C in a 5% CO_2 incubator. After 4–6 days *in vitro*, half the media was replaced and cultures were fed every 3–4 days. All experiments were performed with a mixture of cortical and hippocampal neurons (subsequently referred to as primary neurons) and were used at 10–15 days *in vitro*. These cultures were 91–95% neuronal, as estimated by immunocytochemical staining according to the manufacturer's protocols with anti-MAP2 antibody (1:10,000; #ab5392; Abcam Cambridge, MA, USA).

Oxygen–glucose deprivation model

To model ischemia-like conditions *in vitro*, cells were exposed to transient oxygen and glucose deprivation (OGD) (modification of (Scorziello et al., 2001)). In brief, the culture medium was replaced two times with serum- and glucose-free medium bubbled with 95% nitrogen and 5% CO_2, resulting in a final glucose concentration of <1 mM. The glucose-deprived cultures were then placed in a Billups-Rothenberg (Del Mar, CA, USA) modular incubator chamber, which was flushed for 10 min with 95% nitrogen and 5% CO_2 and then sealed. The chamber was placed in a water-jacketed incubator at 37 °C for 60 min and then returned to 95% air, 5% CO_2 and glucose-containing medium for the period of time indicated in each experiment. Control glucose-containing cultures were incubated for the same periods of time at 37 °C in humidified 95% air and 5% CO_2.

Cellular fractionation

Mitochondria were isolated from cells according to previously described methods with modifications (Almeida and Medina, 1997, 1998; Kristian et al., 2006). In brief, primary neurons were rinsed with phosphate-buffered saline (PBS) and collected in mitochondria isolation buffer (MIB) containing 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.5, 1 mM EGTA, 1 mM EDTA, 100 mM KCl, protease and phosphatase inhibitors. Neurons were homogenized using a Teflon-homogenizer to break open the cells, and centrifuged at 1000*g* for 10 min. The supernatant was transferred to be centrifuged at 10,000*g* for 15 min to collect the mitochondria. The remaining supernatant was collected as the cytosolic fraction and stored at −80 °C. The mitochondrial pellet was resuspended in MIB containing 1% triton X-100 and stored at −80 °C.

Western blots

Protein concentration was determined using the Coomassie protein assay (#1856209; Thermo scientific, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of protein were denatured in sodium dodecyl sulfate (SDS) sample buffer (Boston BioProducts, Ashland, MA, USA; #BP-111R) and resolved by SDS–polyacrylamide gel electrophoresis (8–12% polyacrylamide), transferred to nitrocellulose membranes and analyzed for OPA1 (1:1,000; #612607; BD Biosciences, San Jose, CA, USA) and cyto *c* (1:1000; 556433; BD Biosciences, San Jose, CA, USA), glyceraldehyde 3-phosphate dehydrogenase (GADPH) (1:2000; #G8795; Sigma, St. Louis, MO, USA), and adenosine triphosphate (ATP) synthase (1:1000; #ab14730; Abcam, Cambridge, MA, USA) by Western blotting using the enhanced chemiluminescence technique (#32132; Pierce, Rockford, IL, USA). In the figures, each lane represents one experiment. The data were represented as mean ± SD from three experiments. Relative band densities were determined by densitometry and groups were compared using a one-way ANOVA followed by Tukey's HSD test for post hoc analysis to statistically evaluate differences between groups.

Blue Native-Polyacrylamide Gel Electrophoresis (BN-PAGE)

Blue native electrophoresis was utilized to isolate membrane protein complexes and analysis of molecular masses and oligomeric states of protein complexes, as described (Hornig-Do et al., 2009). Linear 5–13% (w/v) polyacrylamide-gradient gels were formed with a 4% overlay. Proteins were extracted from mitochondrial samples using Lauryl maltoside (2:1 wt:wt sample protein). Samples were supplemented with a fivefold concentrated loading dye (5% Serva Blue G, 750 mM 6-amino-n-caproic acid and 100 mM bis-Tris, pH 7.0). The electrophoresis was started at 100 V for and increased to 240 V once samples entered into the overlay gel at room temperature. Native gels were transferred to nitrocellulose membranes and de-stained of Coomassie brilliant blue with methanol prior to immunoblotting. Immunoblotting was performed as described in Western blot section above using anti-OPA1 antibodies.

Immunofluorescence

Primary neurons were transfected with a mitochondrial marker, the pAcGFP1-Mito Vector (#632432, Clonetech Laboratories, Inc., Mountain View, CA, USA) referred to