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Mitochondrial dynamics following global cerebral ischemia

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ABSTRACT

Global brain ischemia/reperfusion induces neuronal damage in vulnerable brain regions, leading to mitochondrial dysfunction and subsequent neuronal death. Induction of neuronal death is mediated by release of cytochrome *c* (cyt *c*) from the mitochondria though a well-characterized increase in outer mitochondrial membrane permeability. However, for cyt *c* to be released it is first necessary for cyt *c* to be liberated from the cristae junctions which are gated by Opa1 oligomers. Opa1 has two known functions: maintenance of the cristae junction and mitochondrial fusion. These roles suggest that Opa1 could play a central role in both controlling cyt *c* release and mitochondrial fusion/fission processes during ischemia/reperfusion. To investigate this concept, we first utilized *in vitro* real-time imaging to visualize dynamic changes in mitochondria. Oxygen-glucose deprivation (OGD) of neurons grown in culture induced a dual-phase mitochondrial fragmentation profile: (i) fragmentation during OGD with no apoptosis activation, followed by fusion of mitochondrial networks after reoxygenation and a (ii) subsequent extensive fragmentation and apoptosis activation that preceded cell death. We next evaluated changes in mitochondrial dynamic state during reperfusion in a rat model of global brain ischemia. Evaluation of mitochondrial morphology with confocal and electron microscopy revealed a similar induction of fragmentation following global brain ischemia. Mitochondrial fragmentation aligned temporally with specific apoptotic events, including cyt *c* release, caspase 3/7 activation, and interestingly, release of the fusion protein Opa1. Moreover, we uncovered evidence of loss of Opa1 complexes during the progression of reperfusion, and electron microscopy micrographs revealed a loss of cristae architecture following global brain ischemia. These data provide novel evidence implicating a temporal connection between Opa1 alterations and dysfunctional mitochondrial dynamics following global brain ischemia.

1. Introduction

Patients who are successfully resuscitated following cardiac arrest often suffer serious neurologic complications that limit survival and/or functional recovery. The cerebral consequences of transient global ischemia are often quite severe and selectively damage vulnerable brain regions, including the pyramidal neurons of the CA1 hippocampus. The specific molecular mechanisms by which CA1 neurons die are not completely understood, however it is well-established that mitochondria play a central role in cell demise (Sims & Pulsinelli, 1987; Fiskum et al., 2004; Niizuma et al., 2010).

Release of cyt *c* from the intermembranous space of the mitochondria is a critical step in apoptosis induction and is generally thought to be controlled by increased permeability of the outer mitochondrial membrane to allow cyt *c* to cross into the cytosol. Release of cyt *c*, however, is a highly regulated process that requires more than outer membrane permeabilization (Yamaguchi et al., 2008). Under normal conditions, over 85% of cyt *c* is confined within the cristae folds of the inner mitochondrial membrane by protein complexes that maintain cristae junctions.

The cristae junctions are composed of complex oligomers of short and long isoforms of Opa1 and the balance of these isoforms dictates the structure or "tightness" of the cristae junction (Arnoult et al., 2005a; Frezza et al., 2006). Disruption of cristae junctions (*i.e.* Opa1 complexes) has been shown to be triggered by intrinsic apoptosis activation and is central for cyt *c* release independent of outer membrane permeabilization (Arnoult et al., 2005a; Frezza et al., 2006; Olichon et al., 2003). We have previously shown that in neurons Opa1 is released into the cytosol following both oxidative injury and OGD/reoxygenation *in vitro* (Sanderson et al., 2015a; Sanderson et al., 2015b). In these studies, proteolytic processing of Opa1, Opa1 oligomeric breakdown, and

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Opa1 release was observed with cyt *c* release. Evidence of a central role for Opa1 regulation in cerebral injury has also been shown in models of focal ischemic insults to the neonate (Baburamani et al., 2015) and adult brain (Varanita et al., 2015).

In addition to the role of Opa1 in maintenance of the cristae junction, it is also well-established that Opa1 is an indispensable component of the mitochondrial fusion machinery. Proteolytic processing and altered expression of Opa1 isoforms have been shown to play key roles in mitochondrial fusion, with disruptions in Opa1 isoforms promoting a mitochondrial fission phenotype (Yamaguchi et al., 2008; Ehses et al., 2009). Evidence supporting a post-ischemic increase in mitochondrial fragmentation was provided by mitochondrial-YFP reporter mice exposed to brain ischemia (Owens et al., 2015). Indeed, mitochondrial fragmentation was observed following ischemia in the regions surrounding the CA1 pyramidal neurons in the striatum radiatum and striatum oriens of the CA1. However, due to imaging limitations, mitochondrial dynamics in the pyramidal neuron cell bodies of the CA1 were not reported. These data from our group and others lead us to hypothesize that Opa1 could be playing a central role in regulating cell death after global brain ischemia, and could be involved in post-ischemic mitochondrial fragmentation. Here, we proposed that the release of cyt *c* following global brain ischemia would be associated with disruption of Opa1 oligomers, thereby disrupting the balance of fusion and fission to promote mitochondrial fission and cell death.

2. Methods

2.1. Cell culture

HT22 murine hippocampal neurons were used for all *in vitro* experiments and were kindly provided by Dr. David Schubert (The Salk Institute, La Jolla, *CA*). Cells were transfected with mito-GFP plasmids (mitochondria-GFP: AcGFP1-Mito 632432, ClonTech) for 24 h utilizing opti-MEM media and 239fectin transfection reagent (Invitrogen). Cells were plated on glass-bottom culture dishes and experiments were conducted in a microscope chamber (Zeiss/PeCon). Baseline measurements were taken under normoxic conditions and cells were subjected to 45 min of oxygen-glucose deprivation (OGD) by bubbling glucose-free media in a sealed flask with 95% N_2/5% CO_2, then perfusing the media through a sealed system into the culture dish, exchanging the normal media with ischemic media at a rate of 0.5 mL/min. Pericellular O_2 was further reduced by active gas exchange using gas-permeable tubing (Teflon AF2400) connected to a gas mixer. Importantly, control experiments confirmed that the gas exchange protocol did not cause bubbling of the media or mechanical disruption of the cells. After the 45 min OGD insult, a second controlled exchange was performed, restoring normoxic and high-glucose media to the cultures. To monitor cell death caused by apoptosis, the substrate-based caspase-3/7 assay Magic Red® (MR-$(DEVD)_2$) was added to media. MR-$(DEVD)_2$ fluoresces red (emission at 610 nm) when it is cleaved by active caspase-

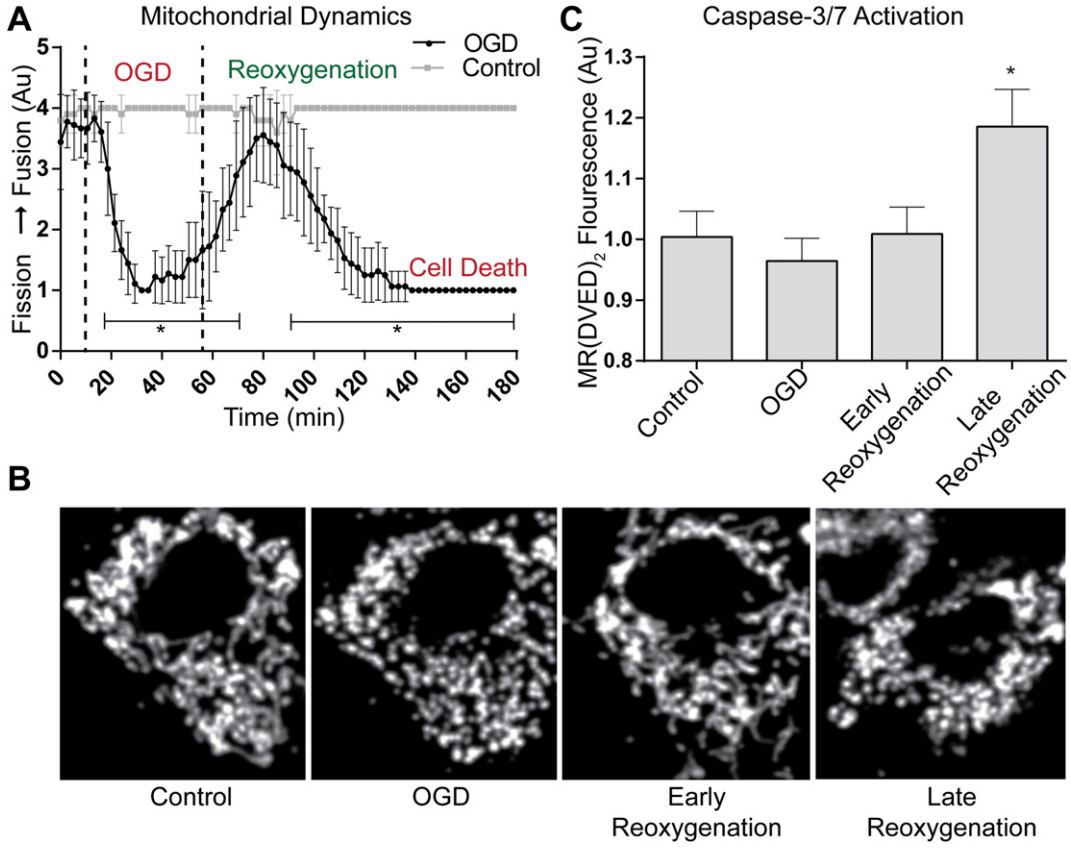

Fig. 1. Mitochondrial dynamics and caspase-3/7 activation during OGD and reoxygenation. (A) Graphical representation of fission/fusion index (Supplementary Fig. 1) during the progression of OGD/Reoxygenation (Mean ± SD). (A & B) Baseline measurements of mitochondrial morphology include both small, fragmented and long, tubular mitochondrial. During OGD induction, rapid fragmentation occurs. Upon reoxygenation mitochondrial morphology returns to a control state (Early Reoxygenation). As reoxygenation is prolonged, mitochondria undergo rapid and irreversible fragmentation, coinciding with cell death (Late Reoxygenation) [n = 18 significant differences in control *vs.* OGD shown in brackets ($p < 0.05$)]. (C) Caspase activation was measured at designated stages of the experiment. During late reoxygenation (60 min after reoxygenation), MR-$(DVED)_2$ fluorescence was significantly increased *versus* control (*$p < 0.05$).