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Research report

Autophagy-regulated AMPAR subunit upregulation in *in vitro* oxygen glucose deprivation/reoxygenation-induced hippocampal injury

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ABSTRACT

Autophagy has been implicated to mediate experimental cerebral ischemia/reperfusion-induced neuronal death; the underlying molecular mechanisms, though, are poorly understood. In this study, we investigated the role of autophagy in regulating the expression of AMPAR subunits (GluR1, GluR2, and GluR3) in oxygen glucose deprivation/reperfusion (OGD/R)-mediated injury of hippocampal neurons. Our results showed that, OGD/R-induced hippocampal neuron injury was accompanied by accumulation of autophagosomes and autolysosomes in cytoplasm alongside a dramatic increase in expression of autophagy-related genes, LC3 and Beclin 1 and increased intracellular Ca^{2+} levels. Pre-treatment with autophagy inhibitor 3-methyladenine (3-MA) significantly reduced this effect. Moreover, the OGD/R-induced upregulation of mRNA and protein expressions of GluR1, GluR2, and GluR3 were also effectively reversed in cells pretreated with 3-MA. Our findings indicate that OGD/R induced the expression of GluRs by activating autophagy in *in vitro* cultured hippocampal neurons, which could be effectively reversed by the administration of 3-MA.

1. Introduction

Cerebral ischemia-reperfusion (I/R) injury is a pathological phenomenon that occurs after restoration of blood supply to brain tissues subsequent to ischemia or hypoxia (Carden and Granger, 2000). It typically occurs after therapeutic thrombolysis in patients with acute ischemic stroke. I/R injury aggravates neuronal insult and may lead to disability and death (Bai and Lyden, 2015; Pan et al., 2007). Treatment outcomes of I/R are typically poor; innovative approaches are required to prevent brain damage induced by I/R (Eltzschig and Eckle, 2011).

Recent studies have demonstrated a pivotal role of autophagy in the regulation of I/R-induced neuronal cell injury (Descloux et al., 2015; Gabryel et al., 2012; Xu et al., 2012, 2013; Zhang et al., 2013). Autophagy is an essential intracellular catabolic pathway responsible for the turnover of long-lived proteins and cellular components (Feng et al., 2014). Based on the mechanism of substrate-delivery to lysosome, three different forms of autophagic pathways are recognized: macroautophagy, microautophagy, and chaperone-mediated autophagy (Feng et al., 2014). Macroautophagy, hereinafter referred to as autophagy, initiates with the formation of double-membraned phagophore (also known as isolation membrane), which encapsulates intracellular materials and matures into an autophagosome (He and Klionsky, 2009). Autophagosome fuses with lysosome and degrades engulfed cargo for recycling.

Activation of autophagy in the reperfusion phase of I/R has been demonstrated in rodent brain as well as in cultured neurons following oxygen-glucose deprivation (OGD)/reperfusion (OGD/R) (Zhang et al., 2013). The autophagy inhibitor, 3-methyladenine (3-MA), has been shown to prevent death of hippocampal CA1 neurons in a rat model of I/R injury (Wang et al., 2011), which suggests that autophagic cell death may contribute to hippocampal damage induced by I/R. However, the regulatory role of autophagy in neuronal damage is yet to be fully elucidated.

Calcium overload, caused by increased Ca^{2+} influx from extracellular environment or release from intracellular stores, is known

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to be an initial event in ischemic neuronal damage in brain (D'Orsi et al., 2015; Pringle, 2004). Ca^{2+} influx through ion channels formed by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type (AMPA-type) glutamate receptor (AMPAR) are shown to be involved in hypoxic-ischemic brain injury (Deng et al., 2003; Gerace et al., 2015; Tang and Xing, 2013). Four subunits, namely GluR1-4, have been identified as the main components of Ca^{2+}-permeable AMPAR channels (Liu and Savtchouk, 2012). Degradation of GluR1 in hippocampal neurons has been shown to be regulated by autophagy (Shehata et al., 2012), which implies an association between autophagic pathway and GluR-modulated Ca^{2+} influx.

Hippocampus is particularly vulnerable to I/R injury (Kirino and Sano, 1984). Abundant expression of GluR1, GluR2, and GluR3 are found in hippocampal neurons (Shi et al., 2001). Based on this body of evidence, we investigated the role of autophagy in mediating OGD/R injury in cultured hippocampal neurons. The association between autophagy and AMPARs was also explored.

2. Results

2.1. OGD/R induced hippocampal neuron injury

Hippocampal neurons were subjected to OGD for 0.5, 1 or 2 h, followed by 24 h of reoxygenation. Increased neuronal damage was observed after OGD/R (Fig. 1A) CCK-8 assay revealed significantly reduced viability of cells exposed to OGD/R ($P < 0.05$) (Fig. 1B). These findings suggest that OGD/R significantly induced hippocampal neuron injury. In the following experiments, 0.5 h or 1 h OGD, followed by reoxygenation for indicated time duration (0–96 h) was used for establishment of OGD/R-like neuronal damage.

2.2. OGD-induced autophagic activity

In order to understand the mechanism of OGD/R-induced hippocampal neuron injury, TEM analysis was carried out. Compared with healthy control cells, OGD/R induced obvious ultrastructual change in neurons, with autophagosomes and autolysosomes being detected in the cytoplasm of damaged neurons (Fig. 2). Some of the autophagosomes and autolysosomes engulfed with damaged intracellular organelles. mRNA and protein levels of autophagy-related genes (Atgs), including microtubule-associated protein 1 light chain 3 (MAP1 LC3, or briefly LC3) and Beclin 1 (a mammalian ortholog of the yeast Atg6), were measured. As shown in Fig. 3A and B, the mRNA levels of both LC3 and Beclin 1 were significantly elevated following 0.5 h or 1 h of OGD ($P < 0.05$ compared with control). Persistent upregulation of mRNA expression of genes coding for LC3 and Beclin 1 was detected immediately after OGD and until 96 h after reoxygenation. When compared to 0.5 h of OGD, prolonged OGD treatment (1 h) upregulated the mRNA expression of genes coding for LC3 and Beclin 1.

Increased protein expressions of LC3-II and Beclin 1 were also detected in cells exposed to 0.5 h or 1 h of OGD following reoxygenation (Fig. 3C-E). The baseline levels of LC3-II and Beclin 1 were relatively low in the control hippocampal neurons. However, OGD treatment rapidly elevated LC3-II and Beclin 1 expression, both of which were continually upregulated until 96 h after reoxygenation. These findings suggest activation of autophagy in hippocampal neurons after OGD/R injury. Autophagy activation was more

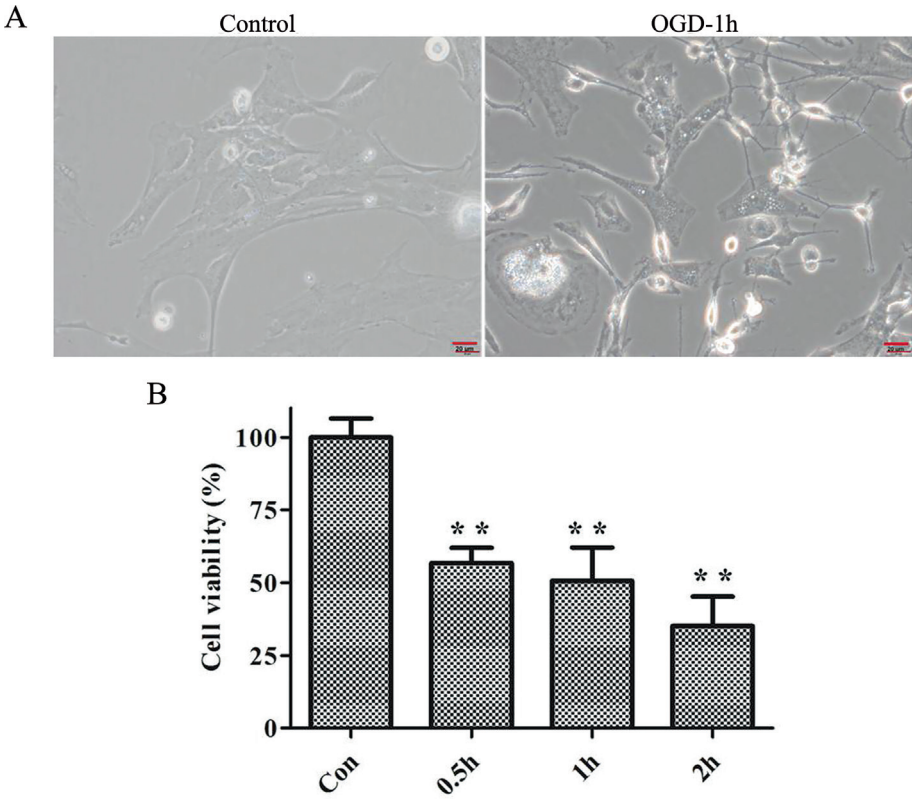

Fig. 1. OGD/R induced hippocampal neuron injury. Cells were subjected to 0 (Control, Con), 0.5, 1 or 2 h OGD, followed by 24 h of reoxygenation. (A) Representative micrographs of cell morphology. (B) Cell viability was measured by CCK-8 assay. OGD/R induced loss of cell viability and resulted in cell injury. $^{**}P < 0.01$ compared with Control. (n = 3) Comparisons between groups were evaluated using two-tailed Student's t-tests. *OGD/R, oxygen glucose deprivation/reperfusion; CCK-8, cell counting kit-8.*