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MODULATING AUTOPHAGY AFFECTS NEUROAMYLOIDOGENESIS IN AN *IN VITRO* ISCHEMIC STROKE MODEL

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Abstract—To explore the effects of modulating autophagy on neuroamyloidogenesis in an ischemic stroke model of cultured neuroblastoma 2a (N2a)/Amyloid precursor protein (APP)695 cells.

Methods: **The ischemic stroke model of N2a/APP695 cells was made by 6 h oxygen–glucose deprivation/12 h reperfusion (OGDR). Drug administration of 3-methyladenine (3-MA), rapamycin or DL-3-*n*-butylphthalide (NBP) was started at the beginning of the OGDR and lasted until the end of reperfusion, in order to explore their effects on N2a/APP695 cells under OGDR conditions. Then the cells were incubated in the drug-free and full culture medium under normoxic conditions for 12 h. Cell viability and injury were investigated. The key proteins of nuclear factor kappa B (NF-κB) pathway and a key component of autophagy Beclin 1 were detected by Western blotting; immunofluorescence double-staining of amyloid-β $(Aβ)_{1–42}$ with Beclin 1 was performed to investigate their cellular co-localization relationship; β-secretase and γ-secretase activity assay and $Aβ_{1–42}$ enzyme-linked immunosorbent assay were performed to investigate the amyloidogenesis.**

Results: **The results showed that, OGDR enhanced cell injury, autophagy activity, neuroinflammation and Aβ generation in N2a/APP695 cells; down-regulating autophagy by 3-MA and NBP increased cell viability, decreased lactate dehydrogenase (LDH) production, inhibited the activation of NF-κB pathway, suppressed β- and γ-secretase activities and Aβ generation; while up-regulating autophagy by rapamycin got the opposite results; immunofluorescence double-staining results showed elevated $Aβ_{1–42}^{+}$ signal was co-localized with Beclin 1^{+} signal.**

Conclusion: **Our data suggested that down-regulating autophagy may inhibit ischemia-induced neuroamyloidogenesis via suppressing the activation of NF-κB pathway. This study might help us to find a new therapeutic strategy to prevent brain ischemic damage and depress the risk of post-stroke dementia.** © **2014 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: amyloid-β, autophagy, DL-3-*n*-butylphthalide, oxygen and glucose deprivation/reperfusion, post-stroke dementia.

INTRODUCTION

Post-stroke dementia (PSD) is a dementia syndrome occurring after stroke; its leading cause is vascular, degenerative or mixed factors. In community-based studies, the prevalence of PSD in stroke survivors ranges from 6% to 32% (van Kooten and Koudstaal, 1998). Because of the declined mortality after stroke and ageing of populations, the incidence of PSD is likely to increase in future, and it will become one of the major causes of disability after stroke (Mackowiak-Cordoliani et al., 2005). About one third of PSD patients bear an Alzheimer's disease (AD) type of pathology (Ravona-Springer et al., 2003), Amyloid-β (Aβ) accumulation and plaque formation were also found in rat brains after stroke (van Groen et al., 2005). Amyloid precursor protein (APP) cleavages through β- and γ-secretases produces Aβ; ischemic conditions can modify amyloid protein metabolism through activating β- and γ-secretase cleavage of APP, increases the generation of Aβ (Pluta et al., 2013). Our previous study found that diabetes could exacerbate PSD through synergistic increases of BACE1 activation and Aβ generation in rat brains (Zhang et al., 2009).

Autophagy, which includes the formation of autophagosomes and autophagolysosomes, is a key and highly conserved self-degradation pathway for the turnover of dysfunctional organelles or aggregated proteins in cells (Uchiyama et al., 2008). It is essential for survival, differentiation, development and homeostasis. Autophagy also plays an important role in many acute and chronic neurological disorders including stroke, brain trauma, AD, vascular dementia and other neurodegenerative diseases (Chu et al., 2009). In pathological autophagy dysregulation process, abnormalities of autophagy always exhibit marked accumulations of autophagy-related vesicular compartments in affected neurons, and over-activated autophagy would lead to autophagic cell death

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Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; DMEM, Dulbecco's-Modified Essential Medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; LDH, lactate dehydrogenase; L-NBP, L-3-*n*-butylphthalide; 3-MA, 3-methyladenine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; N2a, neuroblastoma 2a; NBP, DL-3-*n*-butylphthalide; NF-κB, nuclear factor kappa B; OGD, oxygen and glucose deprivation; OGDR, oxygen and glucose deprivation/reperfusion; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PSD, post-stroke dementia.

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(Baehrecke, 2005). Beclin1, the homolog of the yeast autophagy gene 6, is a component of the phosphatidylinositol type III 3-kinase (PI3K) complex that is required for autophagy, and recruitment of PI3K-Beclin1 complexes together with Atg12-Atg5 is an initial step in autophagosome formation (Rami et al., 2008). As Beclin1 promotes autophagy, enhanced Beclin1 expression has been used as a marker of autophagy to estimate dynamic change on activation of autophagy. It has been reported that local cerebral ischemia induces the upregulation of Beclin 1 expression and autophagy-like cell death, and a subpopulation of Beclin 1-upregulating cells is also expressing the active form of caspase-3 (Rami et al., 2008). Large numbers of autophagic vacuoles have been found accumulating in the brains of AD patients (Boland et al., 2008); *in vitro* study reported that a defect in autophagic regulation may impair the clearance of Aβ and enhance neuronal death (Hung et al., 2009). Hence, whether autophagy is a friend or foe during PSD formation remains to be seen.

The L-3-*n*-butylphthalide (L-NBP) was extracted as a pure component from seeds of Apium graveolens Linn, Chinese celery. Synthesized DL-NBP received approval by the State Food and Drug Administration of China for clinical use in stroke patients in 2002. Previous *in vivo* and *in vitro* studies have reported that NBP shows beneficial effects in ameliorating cognitive dysfunction (Peng et al., 2010), reducing tau phosphorylation (Peng et al., 2012), and regulating APP processing (Peng et al., 2011), attenuating Aβ-induced cell death (Peng et al., 2008). In our previous study, we found that NBP could inhibit Caspase-3-mediated apoptosis in diabetic rat brain via enhancing vascular endothelial growth factor expression (Zhang et al., 2010b), and could attenuate neuronal autophagy and amyloid-beta expression in diabetic mice brain subjected to ischemia (Zhang et al., 2011). Although the positive effects of NBP on brain vascular and neurodegenerative diseases have been verified in many previous experiments, the effects of NBP in autophagy associated area are still unclear, we would investigate the effects of NBP on autophagy *in vitro*.

Inflammation plays an essential role in the pathophysiology of ischemic stroke, the nuclear factor kappa B (NF-κB) pathway can be activated by ischemia, and then leads to many inflammatory processes (Jin et al., 2010). However, the dynamics and function of NF-κB pathway are also under the control of the autophagy process. Autophagy can clear the cellular components, such as inflammasomes and cytokines, and modulate the production of the proinflammatory cytokines. Autophagy deficiency has been found associated with susceptibility to several autoimmune and inflammatory disorders by genetic association studies (Jones et al., 2013). Autophagic activities are significantly increased by cerebral ischemia mainly in the lesion area where inflammation occurs (Wen et al., 2008); however, excessive stimulation of autophagy is associated with cell death.

Using a combination of techniques and models, the purposes of this study were, therefore, to investigate the activation of autophagy in cultured mouse neuroblastoma 2a cells stably transfected with a human APP695 gene (N2a/APP695 cells) after oxygen and glucose deprivation/reperfusion (OGDR, an *in vitro* model of ischemia) combined with autophagy modulating drugs and/or NBP treatment; and its contribution to Aβ generation and the potential mechanisms related to the activation of NF-κB pathway. This study might help us to find a new therapeutic strategy to prevent brain ischemia damage; and depress the risk of PSD through treatment of NBP and modulating neuronal autophagy.

EXPERIMENTAL PROCEDURES

Materials

Mouse N2a/APP695 cells were generously provided by Prof. Chunjiu Zhong at the Shanghai Medical College, Fudan University. Dulbecco's-Modified Essential Medium (DMEM), glucose/glutamine-free DMEM and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). L-NBP was offered by the Shijiazhuang Pharma Group NBP Pharmaceutical Co.Ltd with a purity of more than 99%. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-methyladenine (3-MA) and rapamycin were purchased from Sigma–Aldrich Company Ltd (St. Louis, MO, USA). 3-MA was dissolved in distilled water. Rapamycin was dissolved in ethanol. All other reagents were from commercial suppliers and of highest purity available.

Cell cultures, OGDR and drug exposure

N2a/APP695 cells were maintained in DMEM/F12 supplemented with 10% FBS and 100 U/mL penicillin/streptomycin at 37 °C in 5% CO_2. Serial subcultivation was performed every other day. Rapamycin (5 nM), 3-MA (0.5 mM) or NBP (10 μM) were added to the medium to explore their effects on N2a/APP695 cells under OGDR conditions. The concentrations were chosen for the drug intervention based on the results of preliminary experiments (data not shown) and references (Zhang et al., 2010a; Peng et al., 2011; Lin et al., 2012). For OGDR, briefly, the cell cultures were washed three times with glucose-free DMEM media and incubated in the same media. The cell cultures were placed in an airtight chamber (≈12 L vol.) and equilibrated for 10 min with a continuous flux of gas (95% N_2/5% CO_2). The chamber was sealed and placed in an incubator at 37 °C for 3, 6 or 12 h of oxygen and glucose deprivation (OGD). Then the chamber was opened and the cells were returned to the normal culture media in a normal oxygen incubator for 6, 12 or 24 h of reperfusion. Control cell cultures that were not deprived of oxygen and glucose were placed in normal oxygenated DMEM containing glucose. Each experiment was repeated at least six times.

Assessment of cell viability by MTT

Neuronal cell viability was monitored using the colorimetric MTT assay, as previously described