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Rapamycin induces of protective autophagy in vascular endothelial cells exposed to oxygen–glucose deprivation

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ABSTRACT

The protective potential of rapamycin has been reported in a few experimental models of brain ischemia, both *in vivo* and *in vitro*. Although the precise cellular processes underlying the neuroprotective effects of rapamycin in experimental models of stroke remain unknown, the current experimental data suggest that the mechanism of action of the drug may result from the mTOR-mediated autophagy induction. However, it is unclear whether the activation of autophagy acts as a pro-death or pro-survival factor in vascular endothelial cells in ischemic brain damage. It seems to be very important, since stroke affects not only neurons and astrocytes but also microvessels. In the present study, we used human umbilical vein endothelial cells (HUVEC) subjected to ischemia-simulating conditions (combined oxygen and glucose deprivation, OGD) for 6 h to determine potential effect of rapamycin-induced autophagy on HUVEC damage. The drug at concentrations of 100 and 1000 nM increased the expression of Beclin 1 and LC3-II together with a significant increase in the p62 degradation in ischemic HUVEC. Treatment with rapamycin in OGD significantly increased the cell viability, indicating that the drug exerts cytoprotective effect. The inhibition of Beclin 1 by siRNAs significantly attenuated the expression of autophagy-related proteins and reduced HUVEC viability following OGD and rapamycin treatment. Our findings demonstrated that toxicity of simulated ischemia conditions were enhanced in HUVEC when autophagy was blocked, and that rapamycin effectively prevented OGD-evoked damage by induction of protective autophagy via inhibition of the mTOR pathway.

Abbreviations: BafA1, bafilomycin A1; CNS, central nervous system; CQ, chloroquine; DMSO, dimethyl sulfoxide; 4EBP1, eukaryote initiation factor 4E binding protein 1; FBS, fetal bovine serum; FKBP12, FK506-binding protein 12; FOXO1, forkhead box protein O1; HUVEC, human umbilical vein endothelial cells; LC3, microtubule-associated protein light chain 3; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; OGD, oxygen and glucose deprivation; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol- 3,4,5-triphosphate; S6K, ribosomal S6 kinase; TSC, tuberous sclerosis complex; ULK1, unc-51-like kinase 1

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1. Introduction

Autophagy is a lysosome-mediated process that is responsible for the digestion of excess, old, and unneeded cytoplasmic macromolecules and organelles, such as mitochondria, the Golgi apparatus, endoplasmic reticulum, and peroxisomes (Uchiyama et al., 2008). During autophagy, bulk cytoplasm and organelles are sequestered into double-membrane vesicles called autophagosomes. Autophagosomes ultimately fuse with lysosomes to generate single-membrane autophagolysosomes, the luminal content of which is degraded (Rami et al., 2008). Autophagy occurs at a low, basal level in most cells and, from a physiological point of view, is important for the constitutive turnover of proteins or cytoplasmic components and selective removal of damaged organelles (e.g., mitochondria or peroxisomes) (Uchiyama et al., 2008). In the central nervous system (CNS), autophagy is further activated by various stressors, including cerebral ischemia, hypoxia, nutrient deprivation, neurotoxins or excitotoxic stimuli (Gabryel et al., 2012; Li et al., 2013a, 2013b). Such induced autophagy is considered a cellular defense process that provides cytosolic component turnover to promote cell survival (Carloni et al., 2010). Otherwise, excessive autophagy can also lead to cell death through self-digestion and degradation of essential cellular constituents (Rami et al., 2008). For this reason, autophagic death is described as programmed cell death type II (PCD II) (Uchiyama, 2001).

Beclin 1, microtubule-associated protein 1 light chain 3 (LC3) and p62 are the main autophagy markers (Yorimitsu and Klionsky, 2005; Wang et al., 2010). Beclin 1 (the mammalian homolog of yeast Atg6) is a part of a protein complex with the class III phosphoinositide 3-kinase (PI3K) that participates in the formation of autophagosomes, mediating the localization of the other autophagy proteins to the preautophagosomal membrane (Glick et al., 2010; Høyer-Hansen and Jäättelä, 2007). LC3 is the mammalian homolog of yeast Atg8 that exist in two molecular forms: LC3-I (18 kDa) and LC3-II (16 kDa). During autophagy, the cytoplasmic LC3-I form is converted into the lipidated LC3-II form, which is localized on autophagosomal membranes (Gabryel et al., 2012). The level of LC3-II is closely correlated with the number of autophagosomes and, for that reason, it is considered a good indicator of autophagosome formation (Yorimitsu and Klionsky, 2005). However, it has been shown that LC3-II level itself does not indicate the autophagy flux (Mizushima and Yoshimori, 2007). The p62 protein, an LC3 interaction partner, is incorporated into the completed autophagosome and degraded in autolysosomes (Wang et al., 2010). For this reason, a measure of p62 (SQSTM1/sequestosome 1) degradation is commonly used for characterizing an increase in autophagic flux.

Increasing evidence suggest that autophagy is also involved in ischemic brain damage. An accelerated number of autophagosomes and expression of autophagic proteins, mainly Beclin 1 and LC3-II, were observed in experimental models of focal and transient cerebral ischemia (Adhami et al., 2006; Liu et al., 2010; Wen et al., 2008). Similar results were obtained in *in vitro* experiments performed on primary cortical neuronal and astrocyte cultures challenged with ischemia (Meloni et al., 2011; Qin et al., 2010). However, the mechanisms that underlie autophagy activation in the ischemic brain are far from clear. Currently, due to the functional duality of autophagy it is unknown whether ischemia-induced autophagy is beneficial (Carloni et al., 2008; Liu et al., 2010; Zhou et al., 2011) or detrimental (Kubota et al., 2010; Wang et al., 2011; Wen et al. 2008). Furthermore, the findings are mainly restricted to neurons and astrocytes, and thus it is unclear whether the activation of autophagy acts as a pro-death or pro-survival factor in vascular endothelial cells in ischemic brain damage. It seems to be very important, since stroke affects not only neurons and astrocytes but also microvessels (Lee et al., 2004). Recently, the role of the neurovascular unit which is comprised of neurons, astrocytes, vascular endothelial cells, and a number of stromal elements (i.e. microglia, oligodendrocytes, pericytes, basal membranes) has received great attention in the field of stroke (Moroni and Chiarugi, 2009; Li et al., 2013a, 2013b). Within the neurovascular unit, endothelial cells are critical for the delivery of oxygen, the regulation of cerebral microcirculation, providing trophic support to neurons, as well as tissue repair after brain injury (Curin et al., 2006; Xie et al., 2012). Endothelial cells damage after ischemia often leads to the blood–brain barrier (BBB) disruption, dysregulation of vascular tonus and exacerbation of ischemic injury (Sandoval and Witt, 2008). Therefore, there is no doubt that determining the molecular mechanisms of autophagy in ischemic vascular endothelial cells may contribute to the development of new approaches to stroke therapy.

The data gathered so far unquestionably show that autophagy is negatively regulated by serine–threonine protein kinase mTOR (mammalian target of rapamycin) (Zeng et al., 2006; Gabryel et al., 2012). mTOR plays multiple role in CNS and is involved in regulation of cell viability, differentiation, transcription, translation, protein degradation, actin cytoskeletal organization and autophagy (Harris and Lawrence, 2003). In mammalian cells mTOR assembles into two functionally distinct protein complexes: mTORC1 and mTORC2. The first one acts as a regulator of autophagy and is highly sensitive to rapamycin. The latter one is insensitive to rapamycin and its role is to regulate actin cytoskeletal organization and activity of PKCα and Akt kinases. Both complexes consist of mTOR kinase and mLST8/GβL protein (G protein α-subunit like protein). Additionally, mTORC1 contains Raptor (regulatory associated protein of mTOR) and mTORC2 Rictor (rapamycin-insensitive companion of mTOR) and mSin1 (Wullschleger et al., 2006).

One well-characterized signaling pathway that contributes to mTOR activation involves the insulin-induced phosphatidylinositol 3-kinase (PI3K)/Akt pathway. PI3K stimulates production of phosphatidylinositol-3,4,5-triphosphate (PIP3) and PIP3 in turn recruits phosphoinositide-dependent kinase-1 (PDK1) and Akt to the membrane (Wullschleger et al., 2006). The Akt kinase is activated through phosphorylation at two sites: threonine (Thr308) by PDK1 and serine (S473) by mTORC2 complex (Sarbassov et al., 2005). Activated Akt phosphorylates and thus inhibits the activity of hamartin (TSC1) and tuberin (TSC2) protein complex (tuberous sclerosis complex, TSC1/TSC2), the main negative regulator of mTOR (Perycz et al., 2007). Besides insulin signaling pathway, mTOR