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Research report

Inhibition of AMP-activated protein kinase alleviates focal cerebral ischemia injury in mice: Interference with mTOR and autophagy

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ABSTRACT

Ischemic stroke is one of the most frequent acute cerebrovascular events worldwide. This study evaluated the variability of AMPK and mTOR and their relevance on LC3 and Beclin-1 expression, and further expounded the possible protective mechanism of inhibiting AMPK activity in the cerebral cortex after permanent focal cerebral ischemia injury in mice. Western blot and immunohistochemistry showed that p-AMPK expression was low in the cerebral cortex of the sham group; whereas it was significantly increased at 3 h and 6 h and peaked at 3 h after pMCAO in the cerebral ischemic cortex, and was decreased at 12 h and 24 h. The expression patterns of LC3 and Beclin-1 were the same as that of p-AMPK after occlusion, and the variability pattern between p-AMPK and p-mTOR levels was completely inverted. After treatment with the AMPK inhibitor Compound C, p-AMPK/LC3/Beclin-1 expression was decreased significantly, whereas p-mTOR level was increased significantly. Deficiency of Nissl bodies was reduced compared with that in the vehicle group at all times points after occlusion. Neurological deficits, infarct areas, and brain water content were also significantly reduced 24 h after occlusion with compound C treatment. The results suggested that the AMPK-autophagy pathway was activated, concomitant with mTOR inhibition in cerebral cortex after ischemic injury in mice. Moreover, inhibition of AMPK activity by Compound C inhibited autophagy and conferred protection against brain damage by restoring mTOR activity.

1. Introduction

Ischemic stroke is an acute cerebrovascular event that usually results from a local brain blood circulation obstacle-induced acute nerve deficiency. The obstacle is mainly caused by a brain infarct, which is the result of brain disorder-induced hypoxia and ischemia, brain tissue softening, and necrosis. Ischemic stroke accounts for 80% of stroke cases with extremely complex pathological mechanisms, including excitatory toxic injury after blood flow interruption-induced energy attenuation, oxidative stress injury, inflammation response, depolarization around the infarct tissue, apoptosis, and programmed cell death of the neurons (Lo et al., 2003). Among these, neuronal apoptosis could be divided into apoptosis and autophagy, which are mutually independent, but closely related, processes (Li et al., 2015).

Recent studies have demonstrated that autophagy can be activated by ischemia, followed by the activation of autophagy-lysosomes and autophagy-related genes (Atg) under an electronic spectrum (Wen et al., 2008). Similar to cancer cells, autophagy occurs in focal cerebral ischemic neuronal injury due to energy stress, but the underlying mechanism remains to be determined. At the early stage, LC3 I/II, the ortholog of Atg8 in mammals, translocates from the cytoplasm to the autophagic bubble film and the autophagosome surface with the modification of Atg4 and a series of ubiquitination events by Atg7 (Lockshin and Zakeri, 2004; Huang et al., 2015). Meanwhile, Beclin-1 participates in autophagy activation through the Beclin-1/PI3K-III complex. Autophagy helps neurons to transiently overcome the energy crisis caused by an infarct (Petiot et al., 2000). Thus, autophagy may be a potential target for developing a novel therapy for ischemic stroke.

AMP-activated protein kinase (AMPK) is a trimeric enzyme consisting of three subunits (α, β, γ), and a well-accepted cellular energy sensor that is reported to regulate autophagy (Hardie, 2011). When energy is sufficient, the regulatory γ subunit of AMPK is bound to ATP, leading to inhibition of the trimeric holoenzyme.

Abbreviations: **AMPK**, AMP-activated protein kinase; **Atg**, autophagy related gene; **DAP1**, death associated protein 1; **4EBP1**, eukaryotic initiation factor 4E binding protein 1; **FGF**, fibroblast growth factor; **HIF-1**, hypoxia-inducible factor-1; **FIP**, FAK family kinase-interacting protein; **mTOR**, mammalian target of rapamycin; **pMCAO**, permanent middle cerebral artery occlusion; **p70S6K**, p70 ribosomal protein S6 kinases; **PI3K**, phosphatidyl inositol 3-kinase; **PIKK**, phosphatidyl inositol kinase-related kinase; **TTC**, triphenyltetrazolium chloride; **TSC2**, tuberous sclerosis complex2; **ULK**, unc-51 like autophagy activating kinase 1; **VEGF**, vascular endothelial cell growth factor

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Under energy crises such as ischemia, hypoxia, and oxidative stress, mitochondrial ATP synthesis decreases and the free AMP level or the AMP/ATP ratio increases. AMP then binds to the γ-subunit, causing allosteric activation of the catalytic α-subunit and subsequent phosphorylation at threonine 172 by the upstream kinases, which facilitates catabolism and inhibits anabolism (Novikova et al., 2015). Early in the 1990s, each subunit of the AMPK holoenzyme was reported to be expressed in the human brain (Verhoeven et al., 1995). AMPK commonly regulates cellular metabolism, proliferation, apoptosis, and autophagy through the regulation of multiple cellular factors such as mTOR, p53, and autophagic proteins (Ha et al., 2015; Kim et al., 2011).

Compound C, also named as dorsomorphin, is a small chemical compound that was originally isolated as an inhibitor of BMP signaling and is now found to be a selective and reversible inhibitor of AMPK. It is a widely used AMPK inhibitor in the scenarios where AMPK is activated and blocks the effects of AMPK signaling. In the central nervous system, Compound C is commonly used to inhibit AMPK activity at 20 mg/kg either in vivo or in vitro (Xu et al., 2014; An et al., 2015).

mTOR is an evolutionarily conserved serine/threonine protein kinase, classified as a member of the phosphatidylinositol kinase-related kinase (PIKK) family. mTOR exists in cells as two macromolecular units: mTORC1 and mTORC2 (Hoeffer and Klann, 2010). mTORC1 regulates cell growth and proliferation through the phosphorylation of p70S6K and 4EBP1, whereas mTORC2 participates in regulating the cell cycle, cytoskeletal dynamics, and survival by increasing HIF-1, VEGF, and cyclin D1 levels (Dazert and Hall, 2011; Cota, 2014). Moreover, studies have indicated three mechanisms of the AMPK-induced of mTOR pathway: 1) AMPK directly phosphorylates mTOR (Tao et al., 2010), 2) AMPK phosphorylates TSC2 to activate TSC1/2, which further inhibits Rheb, and negatively regulates mTOR (Corradetti et al., 2004), and 3) AMPK impedes the binding between raptor and mTOR by enhancing raptor binding to 14-3-3 protein (Gwinn et al., 2008).

Notably, many studies have demonstrated that mTOR activation plays a protective role in ischemic cerebral injury. Using permanent middle cerebral artery occlusion (pMCAO), which damages basal ganglia by blocking the flow proximal to the lenticulo-striate arteries, studies have found that melatonin prevents neuronal apoptosis and decreases the infarct area via mTOR/p70S6K; conversely, the mTOR inhibitor, rapamycin, increases the brain infarct area (Koh, 2008; Mehrjerdi et al., 2013; Xie et al., 2013). These data indicate that mTOR activation of ischemia reperfusion in rats can reduce the brain infarct area, thus ameliorating neurological deficiency. Thus, we hypothesize that inhibition of AMPK benefits mTOR expression, which regulates autophagy in permanent focal cerebral ischemia. To test this hypothesis, we have examined trend variability and causality of AMPK and mTOR activity in ischemic cerebral injury after pMCAO, further exploring the therapeutic effect of Compound C-mediated inhibition of AMPK.

2. Results

2.1. AMPK/mTOR pathway was activated after pMCAO in a time-dependent manner

To determine the activation state of AMPK/mTOR signaling in the ischemic cerebral cortex, we established a model of pMCAO in adult, male mice. We randomly assigned the mice to one of three groups: vehicle, Compound C (20 mg/mL), and sham. A time-course study after pMCAO showed that the expression of p-AMPK increases and peaks at 3 h (0.88 ± 0.03), and the gradient then decreases at 6 h (0.74 ± 0.02), 12 h (0.50 ± 0.03), and 24 h (0.44 ± 0.01) in the vehicle group (Fig. 1A, Table 1). A significant

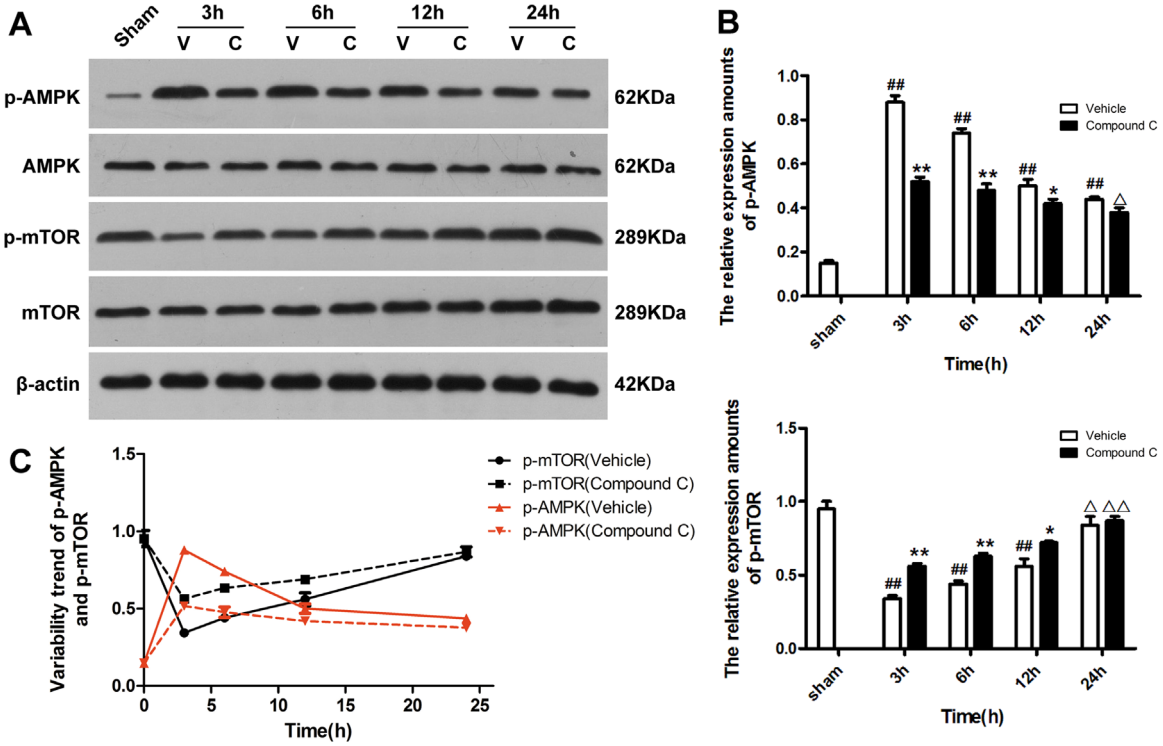

Fig. 1. The expression of p-AMPK and p-mTOR after pMCAO in mice. A, extracts from the entire ischemic and sham-operated cortex were determined with the expression of p-AMPK and p-mTOR by immunoblotting. V means Vehicle group, C means Compound C group. B, quantitative analysis of p-AMPK and p-mTOR were described above. Bar represents mean ± SE from 3 mice in each group. $^{\#\#}p < 0.01$ vehicle group compared with the sham operated group. $^{*}p < 0.05$ ($^{**}p < 0.01$) Compound C group compared with the vehicle group each time point after pMCAO. $^{\Delta\Delta}p > 0.1$ vehicle group compared with the sham operated group. $^{\Delta}p > 0.05$ Compound C group compared with the vehicle group each time point after pMCAO. C, the variability trend of p-AMPK and p-mTOR in vehicle group and Compound C group in 24 h.