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Research Paper

Lysophosphatidic acid induces neuronal cell death *via* activation of asparagine endopeptidase in cerebral ischemia-reperfusion injury

Chao Wang[a], Jie Zhang[a], Junchun Tang[b], Yi-yi Li[a], YanXia Gu[a], Ying Yu[a], Jing Xiong[a], Xueqing Zhao[a], Zheng Zhang[a], Ting-ting Li[a], Jutao Chen[a], Qi Wan[c], Zhaohui Zhang[a,*]

[a] Department of Neurology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, China
[b] Department of Physiology, Collaborative Innovation Center for Brain Science, School of Basic Medical Sciences, Wuhan University School of Medicine, Wuhan, China
[c] Institute of Neuroregeneration & Neurorehabilitation, Qingdao University, 308 Ningxia Street, Qingdao 266071, China

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ABSTRACT

Lysophosphatidic acid (LPA), an extracellular signaling molecule, influences diverse biological events, including the pathophysiological process induced after ischemic brain injury. However, the molecular mechanisms mediating the pathological change after ischemic stroke remain elusive. Here we report that asparagine endopeptidase (AEP), a lysosomal cysteine proteinase, is regulated by LPA during stroke. AEP proteolytically cleaves tau and generates tauN368 fragments, triggering neuronal death. Inhibiting the generation of LPA reduces the expression of AEP and tauN368, and alleviates neuronal cell death. Together, this evidence indicates that the LPA-AEP pathway plays a key role in the pathophysiological process induced after ischemic stroke. Inhibition of LPA could be a useful therapeutic for treating neuronal injury after stroke.

1. Introduction

Lysophosphatidic acid (LPA) has a simple structure, a ubiquitous phospholipid, and acts as an extracellular signaling molecule *via* binding to six receptors (LPA_1-LPA_6) (Goldshmit et al., 2010; Takara et al., 2017). These receptors mediate the function of LPA, which contributes to disease pathogenesis. LPA and its receptors have been suggested as therapeutic targets in multiple sclerosis (MS), ischemic stroke, and neurotrauma (Choi and Chun, 2013). Phospholipase A2 (PLA2) and autotaxin (ATX) are two major enzymes responsible for the generation of LPA (Sinderewicz et al., 2017; Fayyaz et al., 2017; Erstad et al., 2017), and autotaxin was widely used in the studies of inhibition of LPA generation (Fayyaz et al., 2017; Baader et al., 2017), what's more, some researchers reported that ATX-mediated LPA generation is involved in nerve injury-caused neuropathic pain (Ma et al., 2010; Inoue et al., 2008) and dorsal root demyelination (Nagai et al., 2010), and all these points imply that ATX inhibitor may become a drug for the treatment of nerve injury, which is similar to our study. In particular, Li ZG reported that plasma LPA levels were significantly increased in nonvalvular atrial fibrillation patients with silent brain infarctions (Li et al., 2010). Another study demonstrated that concentrations of LPA in the plasma of patients with ischemic stroke were higher than levels in normal subjects (Zhou et al., 2014). Moreover, our group found that the level of serum LPA increased in patients suffering from ischemic stroke, as well as in rat models of middle cerebral artery occlusion (MCAO). These studies demonstrate that LPA plays an important role in neuronal injury during ischemic stroke. However, the molecular mechanisms of LPA involved in ischemic stroke remain obscure.

Asparagine endopeptidase (AEP), also called legumain, is an endolysosomal cysteine protease that cleaves its substrate at the C-terminal side of asparagine residues (Zhang et al., 2017a). It has been reported that the expression of AEP is increased in rat models of MCAO (Ishizaki et al., 2010). Furthermore, AEP cleaves tau in a mouse model of Alzheimer's disease and in the human brain in an age-dependent manner. AEP-derived tau fragment (tau N368) induces the apoptosis of cortical neurons and blocking the cleavage of tau by AEP rescues neuronal loss (Zhang et al., 2014). Therefore, we hypothesized that hyperactivation of AEP after stroke cleaves tau and induces neuronal cell death.

Since both AEP and LPA increase after ischemic injury, and they likely play similar roles in the development and progression of tumors (Ha et al., 2018; Valdes-Rives and Gonzalez-Arenas, 2017; Qi et al., 2017; Zhu et al., 2017), meanwhile, some researchers have shown that tau deposition also appears after cerebral infarction, and it has been reported that AEP promotes the deposition of tau. We therefore hypothesize that LPA may be connected to AEP, and that connection contributes to neuronal injury during ischemic stroke.

* Corresponding author.
E-mail address: zhzhqing1990@163.com (Z. Zhang).

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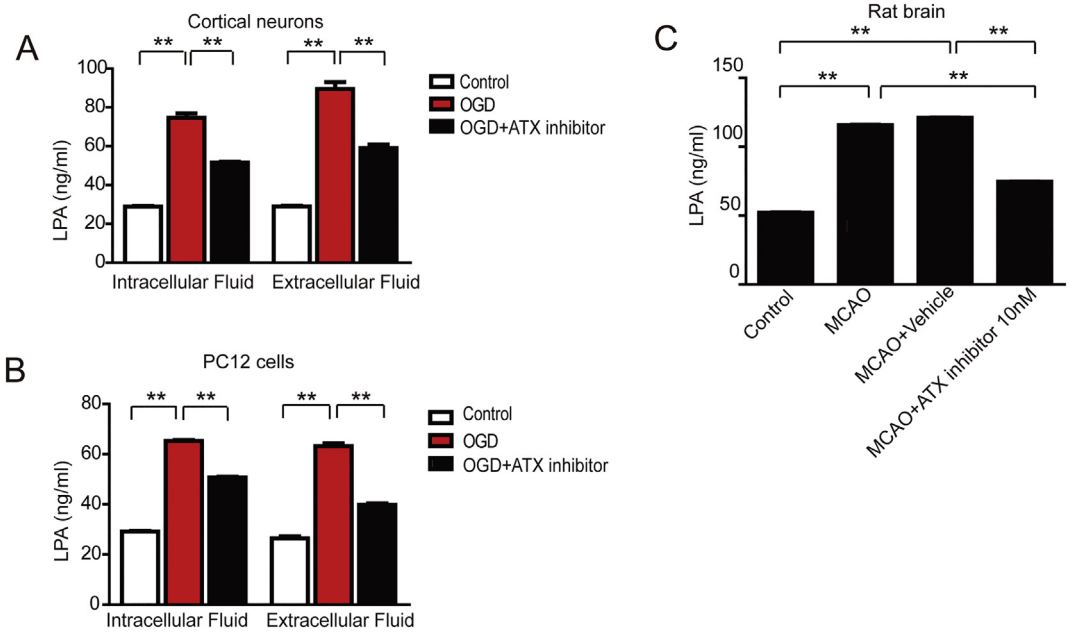

Fig. 1. LPA levels during ischemia *in vitro* and *in vivo*. ELISA detects LPA levels in the extracellular fluid and intracellular fluid of cortical neurons (A). ELISA measures the LPA levels in the extracellular fluid and intracellular fluid of PC12 cells (B). LPA of rat brain lysate was detected by ELISA in four groups (C). Data are mean ± s.e.m. of three independent experiments in cell experiments, and animal number is 6 per group in animal experiments, *P < 0.05, * *P < 0.01.

2. Methods

2.1. Cell culture

PC12 cells were obtained from the China Center For Type Culture Collection (CCFTCC). Primary rat cortical neurons were prepared from E16 embryos, and cultured as previously described (Liu et al., 2008) at 37 °C in 5% CO_2/95% air. Cortical neurons were cultured for 8 days *in vitro*. PC12 and cortical neurons were treated with appropriate concentrations of LPA (sigma L7260). For OGD (Oxygen-glucose deprivation) experiments, the medium was exchanged for glucose-free DMEM with or without autotaxin inhibitor (Apexbio PF8380) and incubated at 37 °C, 95% N_2/5% CO_2 for 4 h (Cortical neurons) or 12 h (PC12 cells), and then the medium was exchanged for DMEM with or without autotaxin inhibitor (100 nM), and the PC12 cells and cortical neurons were then reperfused for 24 h.

2.2. Western blot

The cell pellet or rat brain was lysed in lysis buffer (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na3VO4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, supplemented with protease inhibitors cocktail), and centrifuged for 15 min at 12,000 rpm. The supernatant was boiled in SDS loading buffer. Before the Western-blot, we used the BCA method to determine the protein concentrations of the samples. Equal amount of protein was loaded among samples. The western blot were implemented as described previously (Ning et al., 2004). Briefly, the proteins were then transferred to a PVDF membrane (Millipore). Western blot analysis was performed with the appropriate antibodies: anti-legumain (Abcam ab183028, 1:500), anti-N368 (Millipore, 1:5000), anti-Tubulin (Proteintech Group 10068-1-AP, 1:20000), anti-GAPDH (CST #2118, 1:20000).

2.3. Cell counting kit (CCK-8)

The CCK-8 assay was used to measure cell viability following treatment with LPA or autotaxin inhibitor. PC12 cells and cortical neurons were seeded at a density of suitable density cells/well in 96-well plates. Cell viability was assessed using Cell Counting Kit-8 (CCK-8) (Dojindo) according to the instructions, and the survival ratio = [(As − Ac)/(Ac − Ab)] * 100%. As represents experimental group, Ac represents control group, Ab represents blank group.

2.4. TUNEL staining

The staining procedures were the same as the fluorescence TUNEL method. TUNEL conversion reagent was added according to the manufacturer's instructions. Hydrated and permeabilized cross cryosections were treated for 1 h at 37 °C in TdT incubation buffer (ddH_2O 34 μL, 5× Equilibration Buffer 10 μL, Alexa Fluor 647-12-dUTP Labeling Mix 5 μL, Recombinant TdT Enzyme 1 μL). After incubation, sections were washed with PBS 3 times, and incubated with DAPI for 5 min. Four sections in each rat were collected from the ischemic zone and were examined in each group. In each section, four independent fields (400×) were selected for examination by observers. The percentage of TUNEL-positive nuclei in the region was calculated to evaluate apoptosis.

PC12 cells and cortical neurons were fixed with 4% paraformaldehyde for 25 mins and permeabilized in PBS 3 times and treated with 100 μL 20 μg/mL Proteinase K for 20 mins, and then washed with PBS. 100 μL 1× Equilibration Buffer was added to each sample for 30 mins. TdT incubation buffer (ddH_2O 34 μL, 5× Equilibration Buffer 10 μL, Alexa Fluor 647-12-dUTP Labeling Mix 5 μL, Recombinant TdT Enzyme 1 μL)was added to the sample slide for 60 mins. Then the stained cells were washed three times with PBS, stained with DAPI for 5 mins, and four independent fields (400×) were selected for examination by observers. The percentage of TUNEL-positive nuclei in the region was calculated to evaluate apoptosis. The marker index is measured by the number of dead cells per visual field/all the cells in the visual field, and the apoptotic index (AI) of each sample was equal to the mean value of each visual field marker index.

2.5. Intracerebroventricular injection (i.c.v.)

First, the rats were anaesthetized with a mixture of 4% isoflurane. We used ear bars and upper incisor bars to fix the head in a stereotaxic