Rapamycin rescues lactacytin-induced dopaminergic neuron injury in vivo

Tianhong Pan; Wenjie Xie; Joseph Jankovic; Weidong Le

Parkinson Disease Research Laboratory, Parkinson’s Disease Center and Movement Disorder Clinic, Department of Neurology, Baylor College of Medicine, Houston, 77030, Texas, USA

Serial frozen sections of the entire midbrain (30 µm) were systematically picked at 150

Anti-CD11b antibody (Fig. 3A). Compared with vehicle control, there was a 3.4 folds increase in CD11b-positive cells in SN of mice microinjected with lactacystin (P < 0.01, Fig. 3B). Post-immunohistochemistry with following primary antibodies: rabbit anti-tyrosine hydroxylase (TH, 1:1500) at 4

Objective:

Treatment of mice with rapamycin reduced microglial activation by 57% as determined by counting CD11b-positive cells (P < 0.01; Fig. 3B). We next examined whether rapamycin treatment affected astrocytes as a marker for activated astrocytes (Fig. 3C). Compared with vehicle control, there was a 2.6 folds increase in GFAP-positive cells in SN of mice microinjected with lactacystin (P < 0.01; Fig. 3D).

Background:

Rapamycin, which is a lipophilic, macrolide antibiotic, induces autophagy by activating mammalian target of rapamycin (mTOR) to enhance autophagy activity. Methods: To investigate the role of autophagy in PD, we used lactacystin, a lysosomotropic agent, and the non-selective mTOR inhibitor rapamycin.

Immunohistochemistry Serial frozen sections of the entire midbrain (30 µm) were systematically picked at 150 µm intervals and subjected to free-floating immunohistochemistry with primary antibodies anti-tyrosine hydroxylase (TH, 1:1000) at 4°C overnight. The sections were then incubated in the secondary antibody for 2 h at room temperature. For avidin-biotin-peroxidase method of immunostaining, avidin biotinylated anti-rabbit (IgG) antibodies (1:200) were added followed by ABC elite kit and 3,3'-diaminobenzidine tetrahydrochloride. For quantitative evaluation, the dissected tissue was used to estimate the number of signal neurons immunoreacted with TH antibody in animal midbrain. The number of TH-positive neurons in a specific region was estimated by multiplying the total number of TH-positive neurons counted in all section pairs by 3 to reflect the total number of sections from which the section pairs were chosen.

Immunoblot Analysis Total proteins were isolated from midbrain of mice with rapamycin significantly increased the lactacytin-induced loss of DA neurons in SN and the reduction of DA levels in the striatum. Conclusions: Our data suggest that rapamycin is neuroprotective against lactacytin-induced DA neuron injury. As a drug used in the clinic for other indications, we proposed that further studies on rapamycin should be conducted in order to consider it as a novel therapy for PD.

RESULTS

The protein levels of LC3 in midbrain of mice were increased after a single dose injection with rapamycin, accompanied by the decrease of p-p70S6K and p-mTOR (Fig. 1B). Rapamycin induced increase of LC3-II expression related to the changes of p70S6K and p-mTOR (Fig. 1B). LC3 expression was highest in the midbrain of mice with both lactacytin and rapamycin treatment (Fig. 1B). To test the hypothesis that autophagy may be upregulated during the UPS dysfunction, the mice were injected with lactacytin once and stayed for another 1, 7, 14, and 28 days. Our results showed that 7 days after lactacytin micro-injection, the LC3 protein level was increased by 50% as compared with vehicle control.

Neuroprotective effect of rapamycin on lactacytin-induced injury in mice

The TH-positive dopaminergic neurons in the SN of mice were determined by immunostaining assay with rabbit anti-TH antibody (Fig. 2A). Our results showed that TH-positive dopaminergic neurons were significantly decreased in 1, 7, 14, and 28 days after lactacytin micro-injected with lactacytin as compared with controls micro-injected with PBS (Fig. 2B) (P < 0.01). Post-treatment of mice with rapamycin (one week after lactacytin) significantly reduced this loss to 24% (P < 0.05) (Fig. 2B). Furthermore, the number of TH-positive neurons in a specific region was estimated by multiplying the total number of TH-positive neurons counted in all section pairs by 3 to reflect the total number of sections from which the section pairs were chosen.

Rapamycin inhibits the activation of microglia and astrocytes caused by lactacystin

The changes of microglial activation in SN were demonstrated by immunohistochemistry with anti-CD11b antibody (Fig. 3A). Compared with vehicle control, there was a 3.4 folds increase in CD11b-positive cells in SN of mice microinjected with lactacystin (P < 0.01, Fig. 3B). Treatment of mice with rapamycin reduced microglial activation by 57% as determined by counting CD11b-positive cells (P < 0.01; Fig. 3B). We next examined whether rapamycin treatment affected astrocytes using anti-GFAP antibody as a marker for activated astrocytes (Fig. 3C). Compared with vehicle control, there was a 2.6 folds increase in GFAP-positive cells in SN of mice microinjected with lactacystin (P < 0.01; Fig. 3D).

METHODS

Animals and treatment All animal procedures were approved by the Laboratory Animal Care and Use Committee of Baylor College of Medicine. C57BL/6 mice (Male, 12 weeks old) were randomly divided into four groups of 10 mice each and housed under conditions of constant temperature and controlled lighting (light period, 12 h). Mice were microinjected into the nigrostriatal system of unilateral intracerebral injection of saline or lactacytin in C57BL/6 mice in vivo as determined by the immunohistochemistry assay (Fig. 2D). This increase was partially reduced in tissues from mice post-treated with rapamycin (Fig. 2D).

Figure 1

Induction of autophagy by rapamycin in C57BL/6 mice. The protein levels of LC3-II in midbrain of mice were increased after a single dose injection with rapamycin (Rapa), and microinjected with lactacytin (Lac). The mice started to receive rapamycin (4mg/kg body weight, twice a week) one week after lactacytin injection (1.25µg/1.25µl) for another 3 weeks. DA neurons in the SN of mice were determined by immunostaining with rabbit anti-TH antibody. The striatal DA levels were determined by HPLC assay. Data were expressed as means ± SD. *: P < 0.05; **: P < 0.01 as compared with control (C) or lactacystin (Lac). B: microinjection pellets were resuspended in SDS sample buffer and boiled for 10 min till the samples became clear for detecting ubiquitinated aggregated proteins immunoblot assay. Proteins were electrophoretically transferred to a nitrocellulose membrane and the nonspecific sites were blocked in 6% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 45 min. Membranes were then incubated in the presence of respective primary antibodies, PARP and cleavage (1:500), p-p70S6K (1:1000), phospho-Thr63-specific 70kDa ribosomal S6 kinase (p70S6K) (1:1000), LC3B (1:3000), a specific marker of autophagy, or a rabbit polyclonal to ubiquitin antibody (1:1000). Chloroquine enhancement was performed using the corresponding secondary antibody (1:2000) and signals were detected using ECL. Immunoblot of 70 kDa (1-2000) was performed to demonstrate equal protein loading. The protein levels were quantified using NIH Image software.

Figure 2

Determination of striatal DA levels Concentration of DA was quantified in striatal tissues from mice were homogenized in ice-cold 0.1 M perchloric acid (1:30 weight (mg)/volume (µl) by sonication. Homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and filtered through a disc-fiber (memb, 0.22 µm, Fastgram, Schleicher & Schuell, USA) and subjected to HPLC (Exxon H-TCE-50) with the column (ECPOMPK SC-30DS). The mobile phase consisted of 0.1 mM citric acid, 0.1 M sodium acetate, 220mM LG M sodium sulfate, 3 mg/l L-ascorbic acid and 0.05% methanol (PH 3.5).

Figure 3

Neuroprotective effect of rapamycin on lactacytin-induced injury in mice The thioflavine-S positive neurons in midbrain of mice were injected with saline or lactacytin, accompanied by the decrease of P70S6K and p-mTOR (Fig. 3B). LC3 expression was highest in the midbrain of mice with both lactacytin and rapamycin treatment (Fig. 3B). To test the hypothesis that autophagy may be upregulated during the UPS dysfunction, the mice were injected with lactacytin once and stayed for another 1, 7, 14, and 28 days. Our results showed that 7 days after lactacytin micro-injection, the LC3 protein level was increased by 50% as compared with vehicle control.

REFERENCES


Study supported by Diana Heli Harvey Medical Research Foundation (2007)