# Rapamycin rescues lactacystin-induced dopaminergic neuron injury in vivo

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## ABSTRACT

**Objective:** To investigate if autophagy enhancement by rapamycin alleviates dopamine (DA) neuron injury induced by proteasome inhibitor lactacystin. **Background:** Rapamycin, which is a lipophilic, macrolide antibiotic, induces autophagy by inactivating the protein mammalian target of rapamycin (mTOR) to enhance autophagy activity. **Methods:** To investigate the role of autophagy in reversing DA neuron injury, we use stereotactic injection with lactacystin into the median forebrain bundle (MFB) of mice to produce an *in vivo* model of substantia nigra (SN) injury, which mimic the pathology of Parkinson's disease (PD). The mice started to receive rapamycin (4mg/kg body weight, twice a week) one week after lactacystin injection (1.25μg/1.25μl) for another three weeks. DA neurons in the SN of mice were evaluated by immunostaining with rabbit anti-TH antibody. The striatal DA levels were measured by HPLC. Glial activation and possible inflammation in the SN induced by lactacystin were demonstrated with anti-CD11b antibody and the astrocytes were detected with anti-GFAP antibody. **Results:** Our results demonstrate that rapamycin administration increases protein levels of LC3, a marker of autophagy, and inhibits p-mTOR and p70S6K phosphorylation, reduces microglia activation. Post-treatment of mice with rapamycin significantly attenuated the lactacystin-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 lactacystin-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.

## BACKGROUND

PD is one of the most neurodegenerative disorders, characterized with loss of dopaminergic neurons accompanied by aggregated/misfolded protein. There are two most important protein degradation systems: ubiquitin proteaome system (UPS), and autophagy-lysosome pathway (ALP). It has been reported that once UPS is inhibited, autophagy is up-regulated and the remaining aggregated proteins are degraded by autophagy, indicating that autophagy is a default pathway for UPS. But with the pathogenic condition deterioration, this compensatory auto-regulative mechanism finally is unable to clear the excessive misfolded proteins and then is unable to maintain the cellular balance and eventually results in neuronal death. Furthermore, it has been reported that autophagy is an important process in a variety of human diseases caused by toxic, aggregate-prone, intracytosolic proteins, which become inaccessible to the proteasome when they form oligomers. Thus, we hypothesized that induction of autophagy may be beneficial for the survival of cells under the condition of UPS dysfunction. In this study, we propose to investigate the neuroprotective effect of autophagy enhancement by rapamycin on lactacystin-induced neuronal injury and on the removal of misfolded protein aggregates caused by UPS dysfunction on C57BL/6 mice *in vivo*. We concluded that rapamycin rescues lactacystin-induced dopaminergic neuron injury in vivo. The possible mechanism involved in the neuroprotection can be either from enhanced autophagy or from its anti-inflammatory action. As a chemical compound, many other characteristics of rapamycin are needed to investigate. Considering the role of rapamycin in degradation of aggregated ubiquitinated proteins, we propose that compounds, which may enhance the autophagy, may be a promising strategy to prevent the neuronal injury related to PD.

## 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, age of 12 weeks) were randomly divided into four groups of 10 mice each and housed under conditions of constant temperature and controlled lighting (light period 12–12 h). Mice were microinjected with lactacystin (1.25 μg in 1.25 μl PBS) according to a previously described protocol. One week after lactacystin microinjection, the mice were injected i.p. with rapamycin at the dose of 4mg/kg body weight/day, twice a week for another 3 weeks. Rapamycin was dissolved in 0.1% DMSO immediately before i.p. injection. The mice were sacrificed by terminal anesthesia followed by transcardial perfusion with ice-cold 0.1 M PBS and decapitated. The brain tissues were either kept in -80ºC or fixed with 4% paraformaldehyde (PFA) for further use.

***Immunohistochemistry*** Serial frozen sections of the entire midbrain (30 μm) were systematically picked at 150 μm intervals and subjected to free-floating immunohistochemistry with following primary antibodies: rabbit anti-tyrosine hydroxylase (TH, 1:1500) 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, the secondary biotinylated anti-rabbit IgG antibodies (1:200) were added followed by ABC elite kit and 3,3′ -diaminobenzidine tetrachloride chromogen. For quantitative evaluation, the dissector technique was used to estimate the number of nigral 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 5 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 mammalian tissue lysis/extraction reagent according to the manufacture's protocol. Equal amounts of lysate protein were loaded and separated SDS−PAGE gel. The resulted pellets were resuspended in SDS sample buffer and boiled for 10 min till the samples becoming 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 0.3% Tween-20 for 45 min. Membranes were then incubated in the presence of respective primary antibodies, PARP and cleavage (1:500), p-mTOR (1: 1000), phospho-Thr389-specific 70-kDA ribosomal protein S6 kinase (p-p70S6K) (1: 1000), LC3B (1: 5000), a specific marker of autophagy, or a rabbit polyclonal to ubiquitin antibody (1: 1000). Chemilluminescence enhancement was performed using the corresponding secondary antibody (1:2000) and signals were detected using ECL. Immunoblot of β -actin (1:2000) was performed to demonstrate equal protein loading. The protein levels were quantified by densitometric analysis using a GS-700 Densitometer where it was necessary.

***Determination of striatal DA levels*** The concentration of DA was quantified in striatal tissues by HPLC according to the method previously described. Briefly, 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 and the supernatants were collected and filtered through acro-disc filters (mesh size, 0.25 μm Fisher, Scientific, Houston, TX) and subjected to HPLC (Eicom HTEC-50) with the column (EICOMPAK SC-3ODS). The mobile phase consisted of 0.1 mM citric acid, 0.1 M sodium acetate, 220mg/L Octane sulfate sodium, 5 mg/L EDTA, and 20% methanol (PH = 3.5).

## RESULTS

***Rapamycin enhanced the autophagy in mice***

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. 1A). The LC3 expression was increased by both rapamycin and lactacystin infusion (Fig. 1B). Rapamycin-induced increase of LC3 was accompanied by a decrease of p-p70S6K and p-mTOR, while lactacystin-induced increase was not related to the changes of p70S6K and p-mTOR (Fig. 1B). LC3 expression was highest in the midbrain of mice with both lactacystin and rapamycin treatment (Fig. 1B). To test the hypothesis that autophagy may be upregulated during the UPS dysfunction, the mice were injected with lactacystin once and stayed for another 1, 7, 14, and 28 days. Our results showed that 7 days after lactacystin micro-injection, the LC3 protein level was increased by 50% as compared with it vehicle control (Fig. 1C).

***Neuroprotective effect of rapamycin on lactacystin-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 the number of TH-positive neurons was decreased by 60% in SN of mice micro-injected with lactacystin as compared with controls micro-injected with PBS (Fig. 2B) ($P < 0.01$). Post-treatment of mice with rapamycin (one week after lactacystin) significantly reduced this loss to 24% ($P < 0.05$) (Fig. 2B). Furthermore, lactacystin caused a significantly reduction of striatal DA levels by 61% as compared with controls, whereas the reduction was decreased to 30% after rapamycin post-treatment (Fig. 2C), which was significantly restored by 51% with rapamycin post-treatment ($P < 0.05$) (Fig. 2C). Furthermore, our result showed that lactacystin caused an increase of high molecular weight band of ubiquitinated proteins in the insoluble fraction of midbrain tissue in vivo as determined by the immunoblotting assay (Fig. 2D). This increase was partially reduced in tissues from mice post-treated with rapamycin (Fig. 2D).

***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). Post-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 activation by 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). The number of GFAP-positive cells was reduced by 61% with post-treatment of mice with rapamycin (Fig. 3D).

## FIGURES

**Figure 1**

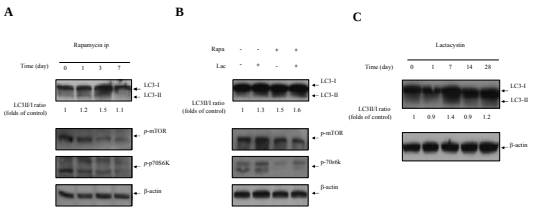



**Figure 2**

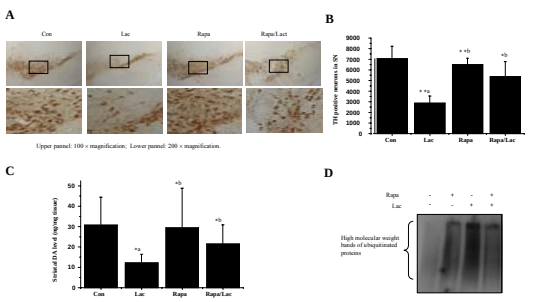
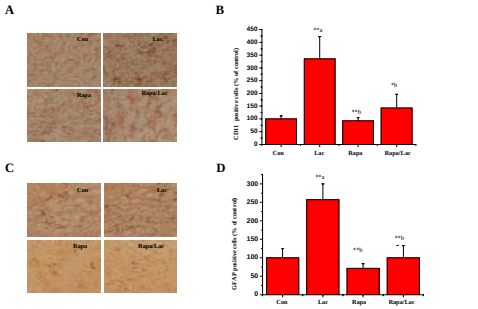



**Figure 3**




## LEGENDS

**Fig. 1**. Induction of autophagy by rapamycin in C57BL/6 mice. The protein levels of LC3 from midbrain tissues of mice injected with one dose of rapamycin and stayed for 1, 3, and 7 days (A), or microinjected with lactacystin with or without rapamycin treatment (B), or microinjected with lactacystin for different time durations (C) were evaluated by immunoblotting assay with anti-LC3 antibody. The density of the LC3 bands was evaluated by densitometric analysis. The ratio of LC3B-II/I was caculated and the data were expressed as folds of control. The protein levels of P70S6K, p-mTOR were also detected with anti-p-70S6K and anti-p-mTOR antibodies. β -actin was used as an equal loading of proteins.

**Fig. 2**. Rapamycin rescued the lactacystin-induced DA neuron injury in C57BL/6 mice. One week after lactacystin injection, the mice were administrated with rapamycin (ip) twice a week for another three weeks. Representative photomicrographs of SN with TH immunohistochemistry were shown in A. Quantitative analysis of TH immunopositive neurons in the SN was shown in B. Each value was presented by the mean ± SD based on the number of TH immuno-positive neurons in right hemispheric nigral slices. Striatal DA levels were determined by HPLC assay (n = 8) shown in C. Data were the means ± SD. *: $P < 0.05$; **: $P < 0.01$ as comopared with control (a) or lactacystin (b). The midbrain tissues were lysed in the lysis buffer and the resulting pellets were further dissolved in SDS sample buffer to get the insoluble fraction. The ubiquitinated proteins were detected from the insoluble fraction of midbrain by immunoblotting assay with anti-ubiquitin anti-body (D). Con:Control; Lac:Lactacystin; Rapa:Rapamycin;

**Fig. 3**. Rapamycin decreased activation of microglia and astrocytes caused by lactacystin in mice. Glial activation and possible inflammation in the SN induced by lactacystin were estimated by immunohistochemistry. The changes of microglial activation in SN were demonstrated with CD11b immunohistochemistry (A, 200 x). Quantification of CD11b-positive cells (indication of microglia) was shown in B. Astrocytes were detected by GFAP staining (C, 100 x). Quantification of GFAP-positive cells (indication of astrocyte) in SN was shown in D.

## CONCLUSIONS

- Rapamycin treatment partially rescued the loss of dopaminergic neurons in SN, reduction of DA levels in striatum, and the increase of protein aggregation caused by lactacystin.
- The neuroprotective effects of rapamycin shown in the present study may be contributed to the induction of autophagy and the anti-inflammatory effects of rapamycin.
- 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.

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