Original Article

3-methyladenine, an autophagic inhibitor, attenuates therapeutic effects of sirolimus on scopolamine-induced cognitive dysfunction in a rat model

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Abstract: Previous studies have demonstrated that sirolimus has therapeutic effects for Alzheimer’s disease which characterized by cognitive dysfunction. However, its underlying mechanisms have not been fully elucidated. In the present study, we aimed to investigate the mechanisms of therapeutic effects of sirolimus for cognitive dysfunction rat model which induced by chronic administration of scopolamine. Forty Wistar rats were randomly divided into 4 groups (n=10 each): saline group and scopolamine group, sirolimus plus scopolamine group and 3-methyladenine pretreatment group. Morris water maze test was applied to measure the cognitive function of rat. After behavioral test, rats were sacrificed and prefrontal cortex and hippocampus were harvested for measuring amyloid-β (Aβ), Beclin-1 and mammalian target of rapamycin (mTOR). Compared with saline group, scopolamine administered significantly decreased the cognitive performance of rats during the Morris water maze test and changed Aβ, Beclin-1 and mTOR levels in rat prefrontal cortex and hippocampus (P<0.05); In addition, rats in sirolimus plus scopolamine group significantly reversed scopolamine-induced effects (P<0.05). Most importantly, 3-methyladenine abrogated the effects of sirolimus on scopolamine-induced cognitive dysfunction (P<0.05). In conclusion, the mechanism of sirolimus exerting therapeutic effects for scopolamine-induced cognitive dysfunction is likely related to the activation of autophagy.

Keywords: 3-methyladenine, autophagy, sirolimus, scopolamine, cognitive dysfunction

Introduction

Cognition is the body awareness and an access to intelligent processing of knowledge, involving learning, memory, language, thinking, mental, emotional and a series of psychological and social behaviors [1-3]. Cognitive dysfunction refers to the learning and memory as well as critical thinking about the brain abnormalities in senior intelligence process, serious learning and memory impairment, which accompanied by pathological processes such as aphasia, apraxia, agnosia, etc. [4, 5]. Cognitive function depends on normal cortical function and structures, while any abnormal factors can lead to cognitive impairment [6]. Cognitive dysfunction and the pathogenesis of Alzheimer’s disease have similar characteristics, which are both related to the increased levels of amyloid-β (Aβ) in the central nervous system. Unfortunately, until now, the pathogenesis has not been fully elucidated [7].

Spilman and colleagues [8] have suggested that macrolide immunosuppressant sirolimus has a therapeutic effect for Alzheimer’s disease and other neurodegenerative diseases. In this study, they concluded its therapeutic mechanism is probably ascribed to the inhibition of mammalian target of rapamycin (mTOR), which is a major signaling pathway that integrates nutrient and growth factors in cell metabolism through two distinct subunits, mTORC1 and mTORC2. mTORC1 acts as a nutrient and energy sensor which controls protein synthesis inhibits autophagy. Besides, it has been reported that sirolimus pretreatment, but not post-treatment, could ameliorates cognitive deficits by inhibiting the formation of plaques and tangles [9]. However, several other studies have
considered that sirolimus exerting protective effects for cognitive dysfunction is probably, at least partially, related to its effect to enlarger lifespan, which results in reducing aging-related cognitive dysfunction [10, 11]. Taken together, these findings validated that sirolimus has exact therapeutic effects for cognitive dysfunction. However, there is little literature reporting the effects of sirolimus on scopolamine-induced cognitive dysfunction. In the present study, we aimed to investigate it and try to elucidate the mechanisms of therapeutic effects of sirolimus for cognitive dysfunction in a rat model which induced by chronic administration of scopolamine.

**Materials and methods**

**Animals and drugs**

The experimental procedures were approved by the Institutional Animal Ethics Committee of Soochow University. Forty male Wistar rats weighing 180-220 g were purchased from Shanghai Animal Center, Shanghai, China. Five rats were housed per cage with food and water available ad libitum and maintained on a 12-h light/dark cycle (lights on at 07:00 AM). Rats were randomly divided into 4 groups (n=10 each): saline group and scopolamine group, sirolimus plus scopolamine group and 3-methyladenine pretreatment group. Rats in scopolamine group and sirolimus plus scopolamine group were administrated either with 0.8 scopolamine (No. 120502, Shanghai Hefeng Pharmaceutical Company, China) or 3.5 mg/kg sirolimus (No. RO395, Sigma Company, US) plus 0.8 scopolamine for consecutive 14 days, separately. Rats in 3-methyladenine pretreatment group were pretreated with 3-methyladenine at a dose of 2 μl for 14 d consecutively, while 30 m later, rats were treated with 3.5 mg/kg scopolamine plus 0.8 scopolamine. Rats in saline group were intraperitoneally injected with the same volume of saline.

**Morris water maze**

According to our previous study [12], Morris maze test was applied to measure cognitive function of rats. The water maze model was used in a circular tank (diameter 1 m) filled with water. A platform was submerged below the water’s surface in the center of the target quadrant. The swimming path of the mice was recorded by a video camera and analyzed by Videomot software (Huabei Zhenghua Company, China). The rats were placed into the maze consecutively from distinct four random points of the tank and were allowed to search for the platform for 60 s. If rats did not find the platform within 60 s, it was gently placed on it and left there for 10 s. The latency to the platform and the proportion of time spent in the target quadrant were recorded.

**Test of Aβ and Beclin-1 levels**

Total RNA was isolated from frozen muscle biopsy tissues using TRIzol reagent (Tiangen, China) according to manufacturer’s instruction. The concentration of total RNA was measured...
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by a spectrophotometry and reverse-transcribed with a RT-PCR kit (Tiangen, China). The real-time PCR was performed using the SYBR Green I kit (Tiangen, China). Sequences of Aβ primers were as follows: forward, 5'-CCAGCC-AATACGAAAATGA-3' reverse, 5'-TGATGTTTGCAGCCAGAA-3'. Sequences of Beclin-1 primers were as follows: forward, 5'-TTGAGATGAGATGCTTTGT-3' reverse, 5'-TAGAGTGAGGACAGAGTG-3'. Sequences of β-actin primers were as follows: forward, 5'-CCTGCTCTCACCAGGAC-3' reverse, 5'-GACCCGTCCTCCTCAGGATCCATC-3'. The PCR reaction conditions were as follows: 50°C, 2 min, 95°C, 10 min, 40 cycles at 95°C for 15 s and 60°C for 60 s.

**mTOR measurement**

Animals were sacrificed immediately by decapitation. Protein concentrations were determined by using BCA method assay kit (Beyotime P0012S, Haimen, Jiangsu, China). After that, samples were centrifuged at 3000G at 4°C for 30 min to obtain the supernatants. Protein was separated by SDS-PAGE. The proteins were then transferred onto polyvinylidene difluoride membrane. After blocking with 5% non-fat milk, membranes were incubated with the primary antibodies: rabbit anti-mTOR (1:100, Sigma, USA). Subsequently, membranes were incubated for 1 hour at room temperature with second-
ary antibody of anti-rabbit HRP-conjugated IgG (1:20000, CWBIO, Beijing, China). Labeled protein was detected using chemiluminescence reagents (ECL; Amersham Bio-sciences, Little Chalfont, Buckinghamshire, UK) and the band intensity was analyzed (Image J software).

**Statistical analysis**

Data are expressed as mean ± S.D. Statistical analyses were made by one-way analysis of variance and post hoc analyses were performed by Least Significant Difference (LSD) tests. These statistical analyses were conducted by Statistical Product for Social Sciences (SPSS version 17.0). Differences were considered to be significant at P<0.05.

**Results**

**Behavioral performance of rats in Morris water maze**

*Figure 1* showed that chronic administration of scopolamine for consecutive 14 d significantly decreased the latency to the platform and increased the proportion of time spent in the target quadrant as compared with control group (P<0.01). Moreover, compared with scopolamine group, rats administrated with scopolamine plus sirolimus significantly increased the latency to the platform and decreased the proportion of time spent in the target quadrant (P<0.05). However, 3-methyladenine pretreatment significantly abrogated sirolimus-exerted protective effects (P<0.05) (*Figure 1A* and 1B).

**Expression of Aβ, Beclin-1 and mTOR in rat prefrontal cortex and hippocampus**

The expression of Aβ in rat prefrontal cortex and hippocampus both showed a significant increase in rats undergoing consecutive 14 d scopolamine administration as compared with the saline group (P<0.01). Compared with scopolamine group, rats administrated with scopolamine plus sirolimus significantly decreased the Aβ levels in rat prefrontal cortex and hippocampus (P<0.05). Compared with scopolamine plus sirolimus group, 3-methyladenine pretreatment significantly increased the Aβ levels in rat prefrontal cortex and hippocampus (P<0.05) (*Figure 2A* and 2B).

As to Beclin-1 expression, rats in scopolamine group significantly decreased the Beclin-1 levels in rat prefrontal cortex and hippocampus (P<0.01). Compared with scopolamine group, rats administrated with scopolamine plus sirolimus significantly increased the Beclin-1 levels.
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in rat prefrontal cortex and hippocampus (P<0.05). While compared with scopolamine plus sirolimus group, 3-methyladenine pretreatment significantly decreased the Beclin-1 levels in rat prefrontal cortex and hippocampus (P<0.05) (Figure 3A and 3B).

Our western blot result showed that the expression of mTOR in rat prefrontal cortex and hippocampus both showed a significant increase in rats undergoing consecutive 14 d scopolamine administration as compared with the saline group (P<0.01). Compared with scopolamine group, rats administrated with scopolamine plus sirolimus significantly decreased the mTOR levels in rat prefrontal cortex and hippocampus (P<0.05). Compared with scopolamine plus sirolimus group, 3-methyladenine pretreatment significantly increased the mTOR levels in rat prefrontal cortex and hippocampus (P<0.05) (Figure 4A and 4B).

Discussion

Morris water maze test is one of the commonly used methods to test learning and memory by observing animal behaviors [13]. The results of the present study showed that intraperitoneal injection of scopolamine for consecutive 14 d significantly decreased the latency to the platform and increased the proportion of time spent in the target quadrant as compared with control group, and sirolimus could abrogate scopolamine-induced behavioral changes. This result suggested that sirolimus has therapeutic effects for scopolamine-induced cognitive dysfunction in rat model. Most importantly, our results also showed that 3-methyladenine acts as an autophagic inhibitor which could attenuate therapeutic effects of sirolimus. These findings implied that autophagic pathway may mediate sirolimus exerting therapeutic effects for cognitive dysfunction. And most importantly, we found that 3-methyladenine acts as an autophagic inhibitor which could attenuate therapeutic effects of sirolimus. These findings implied that autophagic pathway may mediate sirolimus exerting therapeutic effects for cognitive dysfunction. Our study is consistent with results of previous study, but the only difference is that we use the scopolamine-induced cognitive dysfunction animal model.

Cognitive dysfunction and AD can be summarized as the same type of neurodegenerative diseases. The therapeutic mechanism of sirolimus is likely related to the regulation of neuron degeneration proteins [16]. Cai et al. [17] have concluded that sirolimus applied to treat cognitive dysfunction could be ascribed to the activation of mTOR-dependent autophagic pathway. As we know, sirolimus is a selective inhibitor for mTOR. In the present study, our results have demonstrated that mTOR signaling also involved in the therapeutic effects of sirolimus for scopolamine-induced cognitive dysfunction. Meanwhile, we also used autophagic inhibitor 3-methyladenine to observe sirolimus's therapeutic effects, and the results also demonstrated as we previously expected.

Beclin-1 is a protein that is encoded by the BECN1 gene [18]. Beclin-1 participates in the regulation of autophagy and has an important role in neuron development, tumorigenesis, and neurodegeneration [19]. In the present study, our results not only showed Beclin-1 involved in therapeutic effects of sirolimus, but also it is negatively related with the expression of mTOR. This result suggested that sirolimus enhancing autophagy has been associated with the clearance of Aβ and cognitive dysfunction improvement. In this regard, our results
confirmed and were consistent with previous studies.

In conclusion, the results of this study showed that the mechanism of therapeutic effects of sirolimus on scopolamine-induced cognitive dysfunction is probably associated with the activation of mTOR-dependent autophagy.

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Disclosure of conflict interest

None.

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