Cubeben induces autophagy via PI3K-AKT-mTOR pathway to protect primary neurons against amyloid beta in Alzheimer's disease

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Abstract Autophagy is a lysosomal degradative process by which it recycles cytosolic components and degrades protein aggregates inside cells. Here, we identified cubeben as an inducer of autophagy in primary neuronal cells. Autophagy induction was accompanied the upregulation of autophagic proteins like Beclin, ATG5, ATG12 and lipidation of LC3-II in primary neuronal cells. Cubeben induces autophagy by the inhibition of PI3K-AKT pathway in a dose dependent manner. Constitutive active P110 α abrogates cubeben induced autophagic induction in primary neuronal cells. Furthermore, cubeben inhibits amyloid beta induced toxicity in primary neuronal cells. Thus our data suggests that cubeben as a potential anti-Alzheimer therapeutic lead.

Keywords Autophagy · Alzheimer's disease · Amyloid beta · Primary neuronal cells · Neuroprotection

Introduction

Autophagy is a lysosomal mediated degradation of protein aggregates and is involved in the recycling of

cytosolic components inside the cells (Eskelinen and Saftig 2009). The cellular function of cell especially neuronal survival and growth of dendrites is regulated by autophagy (Yue et al. 2009). Autophagy clears intracellular and extracellular amyloid beta from AD brain (Funderburk et al. 2010). Several reports suggest that defects in autophagy are linked with the pathogenesis of various neurodegenerative diseases like Alzheimer's disease (Nah et al. 2015). Slowdown of autophagy leads the accumulation of amyloid beta and tauopathy which leads to the destruction of neurons (Benito-Cuesta et al. 2017). It has been reported that genetic level of Beclin 1 is low in AD brain that tends to slow down of autophagy (Pickford et al. 2008). Moreover, slowdown of autophagy leads the accumulation of amyloid beta and neurodegeneration in transgenic mouse model of AD disease (Shimizu 2018). In transgenic mouse model it has been established that autophagy helps in the secretion of amyloid beta while as autophagic defects leads to the accumulation of extracellular amyloid beta (Barbero-Camps et al. 2018; Nilsson 2014). Several studies have shown that pharmacological intervention leads to reduction in accumulation of amyloid beta and maintains the neuronal health. It has been shown that rapamycin induces autophagy, clears amyloid beta and provides neuroprotection in mouse model of Alzheimer's disease (Cai and Yan 2013; Richardson et al. 2015). Keeping this in view, we have tested cubeben for autophagic induction in primary neuronal cells. Moreover, cubeben induces autophagy in a

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concentration dependent manner for 24 h. Induction of autophagy by cubeben protects the neurons against amyloid beta toxicity in primary neuronal cells. Interestingly, cubeben induced autophagy through inhibition of PI3K-AKT pathway. Upregulation of P110 α abrogates cubeben induced effect on PI3K-AKT pathway in primary neuronal cells. Surprisingly, cubeben protects neurons against amyloid beta toxicity in primary neuronal cells. Overall, in this study we demonstrated that cubeben induced autophagy through the inhibition of PI3K-AKT pathway and protects neurons against amyloid beta toxicity.

Material and methods

Chemicals and reagents

Dulbecco's Minimal Essential medium, Phosphate buffered saline, trans-retinoic acid, BSA, Penicillin G, Streptomycin sulphate, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DMSO, RIPA (Radioimmunoprecipitation assay buffer), Fluo-3AM, HEPES, Fetal bovine serum (FBS), L-15 media, neurobasal media, Glutamax, B27 were purchased from Invitrogen. Amyloid β was purchased from Sigma. Immobilon Western Chemiluminescent HRP substrate and PVDF membrane were obtained from Millipore. An antibody was purchased from cell signaling technology.

Isolation of primary neuronal cells

Primary neurons were isolated from the embryo of 18 days pregnant female mice in compliance with Institutional Animals Ethics Committee IAEC LSH/864795/9E ZiBo Central Hospital, Zibo, Shandong Province, China and grown in neurobasal media containing glutamax and B27 supplement. Briefly, the mice were anesthetized with chloroform and its lower abdomen exposed to isolate the embryos. The brain was dissected in hibernate media. The connective tissue and debris was digested in the neurobasal media without supplement but containing papain in humidified incubator at 37 °C for about half an hour. The debris was removed by centrifugation at 250 g for 5 min. The cells were suspended in fresh neurobasal media for the cell count and appropriate density were seeded and kept in incubator with 98% humidity and

5% CO₂ at 37 °C. Treatment to the cells was given in a concentration dependent manner.

Cell viability assay

Primary neuronal cells were seeded in 96 well plates for 24 h and kept in a humidified incubator at 37 °C and containing 5% CO₂. The cells were given the treatment with desired concentration of compound after 24 h. MTT dye (2.5 mg/ml) was added 4 h prior the termination of the experiment in each well. The media was discarded and the formazan crystals were dissolved in 150 μ l of DMSO. The plate was incubated for 15 min at 37 °C and the absorbance was measured at 570 nm using plate reader.

Neuroprotection assay

Primary neurons were seeded at appropriate density in 96 well plates and kept in incubator at 37 °C, 98% humidity and 5% CO₂ for 24 h. The cells were pretreated with desired concentration of compound for 12 h and A β (20 μ M) was added for next 12 h. MTT dye (2.5 mg/ml) was added for 4 h and then the experiment was terminated. The supernatant was discarded and 150 μ l of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using plate reader. The cell viability of the untreated control was taken as 100% and the effect of A β induced toxicity on cell viability was assessed.

Immunofluorescence of primary neuronal cells

Primary neurons were grown on coverslips in 6 well plates at required density for 24 h and kept in humidified incubator at 37 °C. The primary neuronal cells were fixed with 4% paraformaldehyde for 5 min after which 0.2% triton X was added for 10 min. The neurons were stained with primary antibody MAP2c overnight at 4 °C and Alexa 488 were added at room temperature for 2 h. Cells were washed three times with PBST (1 $\times$ PBS, 0.1 Triton X), followed by DAPI staining for 10 min. Slides were prepared using mounting media and images were taken under fluorescence microscope.

Transfection of pCAG-Myr-p110-IH in primary neuronal cells

pCAG-Myr-p110-IH was purchased from addgene (Catalog no. #15689). Primary neuronal cells were seeded into 60 mm dishes for 15 days and transient transfection was done by adding 10 µl of fugene and plasmid 1 µg into each well for 12 h. These cells were treated with cubeben at a concentration of 5 µM, 10 µM and 20 µM for 24 h and were analysed for immunoblotting.

Morphological changes in primary neuronal cells

Morphological changes in primary neuronal cells by different concentrations of cubeben were studied by phase contrast microscopy. Primary neuronal cells were incubated in six well plates and treated with amyloid beta 20 µM and different concentrations (5 µM, 10 µM, 20 µM) of cubeben for 48 h. Then cells were observed and photographed for any morphological changes under phase contrast microscope attached to the DP-12 camera (1X70, Olympus).

Western blot analysis

Primary neuronal cells were seeded in 60 mm dishes and kept in incubator with 37 °C temperature, 5% CO₂ and 95% humidity. After 24 h, the cells were treated with the cubeben at 5 µM, 10 µM and 20 µM for 24 h. The cells were collected and lysed with RIPA buffer which contains protease inhibitor cocktail (2%), 1 mM PMSF, 5 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, and 1 mM Na₃VO₄ for 1 h. The cells were kept on ice and vortexed after every 10 min. The cells were centrifuged at 400 g at 4 °C and supernatant were collected. The protein estimation was done using Bradford method. The proteins were then subjected to SDS electrophoresis for their separation. About 70 µg/µl of protein sample was loaded into each well of SDS PAGE and were run for 3 h at 100 V; the gels were transferred into PVDF membrane for 2 h at 100 V 4 °C. The membranes were blocked with 5% skimmed milk for 1 h at room temperature. The primary antibodies were added and incubated overnight at 4 °C followed by washing with TBST for 5 min thrice. The secondary antibodies were added for 1 h at room temperature and washed two times with TBST for 5 min each. The membrane bound antibodies were

detected using ECL chemiluminescence kit and the analysis of proteins was done using X-ray film. Quantification of western blot was done by ImageJ software (v.1.48, National Institutes of Health, USA).

Results

Cubeben induces autophagy in primary neuronal cells without toxicity

In order to evaluate the autophagy potential of cubeben in primary neuronal cells, the primary neuronal cells were checked for the expression of neuronal marker (Map2c) before each experiment (Fig. 1a) and were treated with cubeben in a concentration dependent manner (5 µM, 10 µM and 20 µM) for 24 h. Western blotting revealed that the expression of LC3-II was increased by cubeben treated primary neurons through concentration dependent manner as compared with control. The expression of adaptor protein P62 was also decreased in a concentration dependent manner (Fig. 1b). We further checked the toxicity of cubeben at 5 µM, 10 µM and 20 µM concentrations in primary neuronal cells. Interestingly cubeben was non-toxic up to 20 µM compared with control group for 24 h in primary neuronal cells (Fig. 1c).

Cubeben inhibits PI3K-AKT pathway to induce autophagy in primary neuronal cells

The various autophagic proteins like Beclin, ATG5 and ATG12 were up regulated by cubeben at a concentration dependent manner (5 µM, 10 µM and 20 µM) for 24 h (Fig. 2). The phosphorylation level of p-mTOR (S2448) was significantly downregulated by cubeben treatment (Fig. 2). In order to investigate the mechanism of autophagy induction through mTOR, we have analysed the expression level of AKT. Interestingly, the phosphorylation of p-AKT (Ser 473) and Thr 308 was downregulated by cubeben in a concentration dependent manner as compared to untreated cells. Moreover, the expression of p110α was also inhibited in a dose dependent manner, suggesting that cubeben inhibits PI3K/AKT/mTOR pathway by the down regulation of P110α expression (Fig. 2). Thus our results clearly indicated that

Fig. 1 Cubeбен induces autophagy without inducing toxicity. **a** Immunofluorescence of neuronal marker (Map2c) in primary neuronal culture. **b** Western blot analysis depicts that cubeбен induced autophagy in a concentration dependent manner for 24 h in primary neuronal cells. **c** Bar graphs represents the

viability of primary neuronal cells after treated with cubeбен for 24 h. Data represented here are the mean $\pm$ standard deviation of three independent experiments. Bonferroni's method was used for Statistical comparisons. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ versus 0 μ M groups

cubeбен induces autophagy through PI3K AKT pathway.

Upregulation of pCAG-Myr-p110-IH abolishes cubeбен effect on PI3K-AKT pathway in primary neuronal cells

In order to validate the mechanism of cubeбен, we have transfected the primary neuronal cells with pCAG-Myr-p110-IH overexpression plasmid. The p110 α overexpressed cells hampers the cubeбен induced inhibition of PI3K/AKT/mTOR in a concentration dependent manner. Interestingly, the expression of autophagy proteins like ATG5 and ATG12 and LC3-I in pCAG-Myr-p110-IH overexpressed cells treated with cubeбен remains unchanged compared with untreated cells (Fig. 3a). The overexpression of pCAG-Myr-p110-IH compared with control was checked by western blot (Fig. 3b).

Morphological observations in primary neuronal cells

The protective effect of cubeбен was confirmed by morphological observation using phase contrast microscope. Primary neuronal cells treated with 20 μ M of amyloid beta displayed altered neuronal out-growth and dendrite network which leads to floating of cells indicating cytotoxicity (Fig. 4). Furthermore, treatment with cubeбен at a concentration of 5, 10 and 20 μ M showed improved neuronal out-growth and intact neuritis formation. Moreover, cells treated with desired concentrations of cubeбен showed healthy morphology which was attenuated by amyloid beta (Fig. 4).

Protective effect of cubeбен against amyloid beta in primary neuronal cells

Amyloid beta disrupts the function of primary neurons that leads to the toxicity of cells. Amyloid beta at a concentration of 20 μ M were treated to primary

Fig. 2 Cubeбен inhibits of P110 α to induce autophagy in primary neuronal cells. Western blot analysis shows that cubeбен inhibits P110 α at a concentration dependent manner for 24 h to induce autophagy. Quantification of each western blot was done by using ImageJ software (v.1.48, National

Institutes of Health, USA). Data represented here are the mean $\pm$ standard deviation of three independent experiments. Bonferroni's method were used for statistical comparisons. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ versus 0 μM groups

neuronal cells for 24 h and were treated with cubeбен at a concentration 20 μM for 24 h. Amyloid beta provides significant toxicity which was attenuated by cubeбен in a dose dependent manner (Fig. 5).

Discussion

Alzheimer's disease is a neurodegenerative disease which happens in an old age (Johnson 2015). Accumulation of amyloid beta and hyperphosphorylation of tau is the main pathological feature of AD (Huang and Jiang 2009). Amyloid beta is cleared from the brain by various mechanism viz. blood brain clearance, interstitial fluid bulk-flow clearance, perivascular system, paravascular system, autophagy lysosomal

degradation, etc. (Marchi et al. 2016). Amyloid beta stay normal in healthy volunteers but its clearance is decreased that leads to the accumulation of amyloid beta which triggers cell death (Baranello et al. 2015). Autophagy plays a vital role in clearance of amyloid beta and provides the neuronal plasticity (Boland et al. 2008). Therefore, we have planned to explore the possibility of cubeбен as an inducer of autophagy. Cubeбен inhibits cell proliferation of vascular smooth muscles by inhibiting platelet-derived growth factor (PDGF) which abrogates cardiovascular symptoms (Park et al. 2017; Kingsley and Plopper 2005). However, we are exploring for the first time that cubeбен induces autophagy in primary neuronal cells and cubeбен was nontoxic up to 20 μM for 24 h. Autophagy is a cellular lysosomal degradation

Fig. 3 Effect of cubeben on P110 α overexpressed primary neuronal cells. **a** *pCAG-Myr-p110-IH* overexpressed primary neuronal cells treated with cubeben does not induced autophagy as depicted by western blot. **b** Western blot analysis shows overexpression of P110 α in primary neuronal cells.

Quantification of western blot was done by ImageJ software (v.1.48, National Institutes of Health, USA). Data represented here are the mean $\pm$ standard deviation of three independent experiments. Bonferroni's method were used for Statistical comparisons. *** $P < 0.001$ versus WT groups. WT wild type

Fig. 4 Cubeben reverts morphological change in primary neuronal cells. Amyloid beta at a concentration of 20 μM damages primary neuronal cells which were retained by cubeben in a concentration dependent manner

Fig. 5 Cubeбен protects neurons against amyloid beta toxicity. Amyloid beta induced toxicity in primary neuronal cells which were protected by cubeбен treated primary neuronal cells. Data represented here are the mean $\pm$ standard deviation of three independent experiments. Bonferroni's method were used for statistical comparisons. ** $P < 0.01$ versus control and *** $P < 0.001$ versus 0 Aβ group. Aβ amyloid beta

pathway which involves multistage process to recycle cytosolic components and amyloid beta degradation (Eskelinen 2005). With this view, we have established molecular mechanism of cubeбен in a concentration dependent manner. We further analysed the autophagic proteins, surprisingly Beclin, ATG5 and ATG12 were unregulated in a concentration dependent manner for 24 h in primary neuronal cells. We have further focused mTOR a major autophagy inducer (Jung et al. 2010). Interestingly, cubeбен inhibits mTOR in a concentration dependent manner. Further the upstream proteins like pAKT (ser473) and pAKT (T308) was also inhibited. This made us believe that cubeбен inhibits P110 α in a dose dependent manner for 24 h in primary neuronal cells. To further validate cubeбен inhibits P110 α , we have overexpressed primary neuronal cells by pCAG-Myr-p110-IH that remains constitutive active. Cubeбен was unable to inhibit P110 α which leads to the activation of pAKT (ser473), pAKT (T308) and mTOR. Activation of this PI3K-AKT pathway inhibits autophagy as the lipidation of LC3 does not take place moreover ATG5 and ATG12 expression remains same in cubeбен treated primary neuronal cells. Amyloid beta is known to induce cell death by loss of mitochondrial membrane potential loss and increase in reactive oxygen species (Abramov et al. 2004). Interestingly, autophagy induction by cubeбен protects primary neuronal cells

against amyloid beta toxicity in primary neuronal cells.

In conclusion, we have explored the potential of cubeбен on autophagy and established a molecular mechanism of cubeбен in primary neuronal cells. We have also demonstrated that cubeбен protects the neuronal health through autophagy. Cubeбен can be further investigated as a therapy for Alzheimer's disease.

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