International Immunopharmacology 20 (2014) 221–228

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Short communication

# Madecassoside prevents Aβ25–35-induced inflammatory responses and autophagy in neuronal cells through the class III PI3K/Beclin-1/Bcl-2 pathway

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ARTICLE INFO

*Article history:*
Received 11 January 2014
Received in revised form 13 February 2014
Accepted 25 February 2014
Available online 12 March 2014

*Keywords:*
Madecassoside
Autophagy
Aβ deposition
Inflammatory responses
PI3K/Beclin-1/Bcl-2 pathway
Alzheimer's disease

ABSTRACT

Inflammatory responses and autophagy have been implicated in the amyloid-β (Aβ) aggregation in Alzheimer's disease (AD) due to recycling cellular waste and eliminating toxic protein aggregates. Madecassoside (Mad), a triterpenoid saponin compound, has been found to improve impaired cognitive function. However, little was known about the protection of Mad nerve cells against inflammation response and autophagy, as well as their underlying mechanism. In the present study, we investigated whether Mad could prevent $A\beta_{25-35}$-induced inflammatory responses and autophagy, as well as the possible mechanism. Transmission electron microscopy results showed that Mad could significantly reduce $A\beta_{25-35}$-induced autophagosomes in neural cells. Mad could also increase cell viability whereas decrease remarkably LDH leakage in $A\beta_{25-35}$-induced neural cells. Both ELISA assay and western blot showed that Mad attenuated inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-10, IL-6 and COX-2 production. Moreover, western blot results showed that Mad could block the conversion of light chain3-I (LC3-I) to light chain3-II (LC3-II), reduce Beclin-1, whereas increase anti-apoptotic protein Bcl-2 level. The levels of Beclin-1 and hVps34 in control vector-transfected NG108-15 neural cells but not in Bcl-2 transfected NG108-15 neural cells were reduced by Mad. The levels of inflammatory cytokines including TNF-α and IL-6 productions decreased significantly by the treatment with Mad. These results demonstrated that Mad protected neural cells against inflammation and autophagy induced by $A\beta_{25-35}$ through the class III PI3K/Beclin-1/Bcl-2 pathway. Our findings provide evidences for the beneficial effect of Mad on the treatment of AD.



## 1. Introduction

Alzheimer's disease (AD) has been regarded as one of the most common neurodegenerative disorders, which was characterized by neuronal inflammation, neuronal cell loss, decline of memory and learning [1]. The pathogenesis of AD has revealed that the accumulation of abundant senile plaques in the brains, mainly composed of amyloid-β (Aβ) peptide, contributes to inflammatory responses. $A\beta_{25-35}$ is one of the potential contributors to inflammatory responses of AD [2].

Autophagy, a ubiquitous lysosomal degradative pathway which is responsible for eliminating abnormal protein aggregates and damaging organelles prevalent in neuronal cells, has been implicated in the inflammatory responses of many neurodegenerative diseases including AD [3]. Inflammatory responses have been demonstrated to induce autophagy and increase autophagic vacuoles [4]. Accumulating evidence confirmed that excessive Aβ could trigger the secretion of inflammatory cytokines and then resulted in autophagy [5].

The class III PI3K (hVps34)/Beclin-1 signaling, known to be a positive regulator of autophagy, has been found to play an important role in inflammation-mediated autophagy [6]. Many studies showed that the induction of Aβ on autophagy is involved in the inflammatory responses of AD [7]. Furthermore, the stimulation of inflammatory cytokines can up-regulate Bcl-2 expression and inhibit the interaction of Beclin-1 and hVps34 [8].

*Centella asiatica* (CA) has been used as an alternative medicine for memory improvement in the Indian Ayurvedic system of medicine for a long time. Studies have revealed its beneficial effect on ameliorating the cognitive impairment in AD rat models. Prakash's research showed that CA could alleviate aluminum-induced cognitive dysfunction and oxidative damage [9]. Previous studies have indicated that Mad, one of triterpene compounds in CA herbs, possesses various biological activities, including preventing myocardial ischemia–reperfusion injury, neuroprotective and anti-inflammation [10]. Moreover, Mad could inhibit pro-inflammatory mediators including COX-2, PGE(2), TNF-α and IL-6 levels [11]. Although, pleiotropic mechanisms have been suggested

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http://dx.doi.org/10.1016/j.intimp.2014.02.036
1567-5769/

to contribute to Mad's effects, its activities on preventing Aβ-induced inflammatory and autophagy are still not fully understood.

The aim of this study was to investigate whether Mad could ameliorate $A\beta_{25-35}$-induced inflammatory responses and autophagy in neural cells, and also explore the possible mechanism on regulating the class III PI3K/Beclin-1/Bcl-2 pathway.

## 2. Materials and methods

### 2.1. Materials and reagents

Madecassoside (Fig. 1A, purity ≥ 98%), 3-methyladenine (3-MA), fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), and $A\beta_{25-35}$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco by Invitrogen (Carlsbad, CA, USA). In vitro toxicology assay kit (LDH) was purchased from Nanjing KeyGEN Biotech Co., Ltd. (Nanjing, China). LC3-I/II, Beclin-1, class III PI3K (hVps34), Bcl-2, TNF-α, IL-6, IL-10, COX-2 and GAPDH antibodies, rabbit polyclonal antibody against Beclin-1 (G-11), and monoclonal anti-IL-6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ELISA kits for TNF-α, IL-6, IL-10 and COX-2 were obtained from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China). The other chemicals and reagents were of analytical grade.

### 2.2. Cell culture and drug administration

Mouse NG108-15 neural cell line was purchased from ATCC and the cells were cultured in DMEM, supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U/l penicillin–streptomycin. The cells were maintained at 37 °C in an atmosphere of 5% $CO_2$. Stock solution of Mad was prepared in 0.1% DMSO at the concentration of 30 mM, and Mad (1, 5, 10 μM) was used in the whole experiment. 3-MA (5 mM), a specific inhibitor of autophagosome formation, was chosen as a positive control [12]. The cells were treated with $A\beta_{25-35}$ at a final concentration of 10 μM in the presence or absence of Mad and/or 3-MA [13]. For the inhibitory experiments, the cells were pre-incubated with a selective PI3K inhibitor (LY2940002, final concentration of 10 μM), and then treated with $A\beta_{25-35}$ at a final concentration of 10 μM and/or Mad and/or 3-MA.

Human neuroblastoma cell SH-SY5Y was ordered from Nanjing KeyGEN Biotech Co., Ltd. (Nanjing, China) and routinely cultured in 25 $cm^2$ tissue culture flasks (Costar, Corning, NY, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and then maintained at 37 °C in a 5% $CO_2$ humidified atmosphere. At 85% confluence, the cells were used for further experiments.

### 2.3. Transmission electron microscopy of autophagosomes

To study the effects of the Mad and 3-MA on autophagosomes and morphologic changes in the organelles by TEM, the cells were pretreated with Mad (10 μM) or 3-MA (5 mM) for 24 h followed by $A\beta_{25-35}$ (10 μM) for another 12 h. These cells were fixed in PBS with 2.5% cold glutaraldehyde. After being washed twice in PBS, the cells were post-fixed in 1% osmium tetroxide in PBS, dehydrated with a graded series of ethanol and propylene oxide. Then, the samples were infiltrated with EPON Resin/acetone (1:1) mixture overnight and embedded in EPON Resin. The blocks were cut into 0.5 μM sections and stained with uranyl acetate and Reynolds' lead citrate. Finally, they were observed with a transmission electron microscope (H-7650, Hitachi, Japan).

### 2.4. LDH assay

Lactate dehydrogenase (LDH) leakage occurs not only during necrosis but also during apoptosis. To examine the protective effect of Mad on Aβ-induced death of mouse NG108-15 neural cells, cells were exposed to Mad (1, 5, 10 μM) or 3-MA (5 mM) for 24 h followed by an additional 12 h treatment of $A\beta_{25-35}$. The supernatants and cell lysates were prepared using the toxicology assay kit in accordance with the manufacturer's instructions.

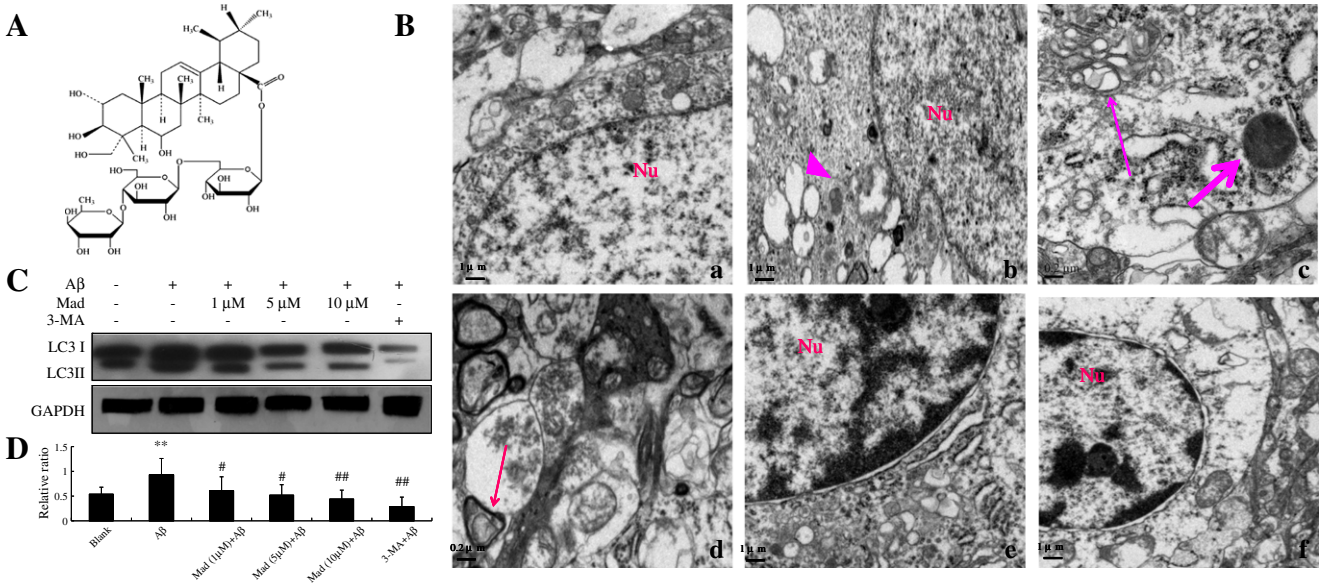


**Fig. 1.** Chemical structure of madecassoside (Mad) (A), and the effect of Mad on autophagy by transmission electron microscopy (B) and the conversion of LC3-I to LC3-II (C and D). Transmission electron microscopy showing normal morphology of cytoplasm, cell organelles, and nuclei of mouse NG108-15 neural cells in the absence (a) or presence of 10 μM $A\beta_{25-35}$ for 12 h (b, c, d): characteristic ultrastructural morphology of autophagy and a large number of autophagic vacuoles (b); the lysosomes were darkly stained, indicating the activation of lysosomes (c, as indicated by thick arrows), and double-membrane (d). Sharply decreased autophagic vacuoles of mouse NG108-15 neural cells pretreated with Mad of 10 μM (e) or 3-MA of 5 mM (f) for 24 h (a, b, f, ×8000; c, d, e ×40,000). Arrowheads represent autophagic vacuoles, thick arrows represent lysosomes, and thin arrows represent double-membrane. Nu: Nucleus. For the conversion of LC3-I to LC3-II, NG108-15 cells were pretreated with Mad or 3-MA (5 mM) for 24 h and then harvested for western blot analysis at various time points after $A\beta_{25-35}$ incubation. The data are taken from individual experiment and expressed as means ± SD (n = 3). $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. $A\beta_{25-35}$.