Neuroprotective effects of sarsasapogenin-AA13 via autophagy and MAPK pathway

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Abstract: Alzheimer’s disease is a growing global health concern with huge burdens to individuals and society. Due to the complex etiology of Alzheimer’s disease, there is currently no effective treatment in clinical practice. Rhizoma Anemarrhena, a traditional Chinese medicine, has been reported with multiple potential neuroprotective effects in neurodegenerative diseases including Alzheimer’s disease. Sarsasapogenin-AA13 (AA13) is a novel synthetic derivative of Sarsasapogenin extracted from Rhizoma Anemarrhena. Our previous studies revealed that AA13 possesses potential anti-inflammatory and neuroprotective activities. Targeting autophagy is recently emerging as a promising strategy in many diseases including neurodegenerative diseases. This study aims to investigate whether AA13 protects SH-SY5Y cells when exposed to H$_2$O$_2$, which has been used as an AD model, via regulating autophagy, MAPKs pathway and NF-κB. Cell viability correlated with oxidative damage was determined by MTT assay. Expression levels of LC3, Akt, p-Akt, Erk, p-Erk, p38, p-p38, JNK, and p-JNK were evaluated by Western blot. Our results showed that the cell viability decreased in SH-SY5Y cells exposed to H$_2$O$_2$, which has been used as an AD model, via regulating autophagy, MAPKs pathway and NF-κB. Cell viability correlated with oxidative damage was determined by MTT assay. Expression levels of LC3, Akt, p-Akt, Erk, p-Erk, p38, p-p38, JNK, and p-JNK were evaluated by Western blot. Our results showed that the cell viability decreased in SH-SY5Y cells exposed to H$_2$O$_2$. Both phosphorylation of mitogen activated protein kinase (MAPK) and Akt in H$_2$O$_2$-stimulated SHSY5Y cells pretreated with AA13 were decreased compared to cells exposed to H$_2$O$_2$ alone. Furthermore, AA13 also inhibited the H$_2$O$_2$-induced activation of nuclear factor NF-κB. These findings suggest that AA13 can protect the neuronal cells from oxidative injury which could be related to autophagy, MAPKs pathway and NF-κB.

Keywords: Neuroprotection, sarsasapogenin-AA13, autophagy, MAPKs, NF-κB

Introduction

Neurodegenerative diseases severely affect the central nervous system, leading to progressive nervous system dysfunction. Alzheimer’s disease is considered to be the most representative neurodegenerative disease [1]. In Western and eastern countries, the incidence and mortality of Alzheimer’s disease account for a large proportion, with an increasing trend. However, there is currently no effective treatment. Rhizoma Anemarrhena, a traditional Chinese herb, has been often used as an anti-diabetic and antidepressant agent in traditional medicine [2-4]. Rhizoma Anemarrhena has been reported to have neuroprotective effect [5, 6], and the extracts of Rhizoma Anemarrhena improved the memory deficit induced by amyloid β-peptide [7]. Sarsasapogenin-AA13 (AA13) is a novel synthetic derivative of sarsasapogenin, which was screened for neuroprotective effect (Figure 1). Our previous study has shown that AA13 has significant anti-inflammatory effects in RAW264.7 cells and peritoneal macrophages [8]. In that study, the potential neurotrophic activity of AA13 was demonstrated in vitro. Researches implied that there was a close relationship between inflammatory pathways and neurodegenerative diseases [9-11].

The neuronal cell death induced by oxidative stress is involved in apoptosis and necrosis. Autophagy is an important adaptive and regulatory mechanism during the growth, proliferation, differentiation and senescence. Studies have shown that the autophagy pathway is important for the maintenance of cell survival, differentiation and the development of tissue [12]. According to previous reports, the pathogenesis of AD is often accompanied by the occurrence of autophagy.
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LC3 protein is associated to autophagosomal membranes upon autophagy induction [13]. The mitogen-activated protein kinase (MAPK) family, such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (Erk1/2), have been shown to be related to neuronal apoptosis, as well as PI3K/Akt pathway [14-17]. NF-κB p65 is a critical signaling molecule in H\textsubscript{2}O\textsubscript{2}-induced damage, which was an important redox-sensitive transcriptional factor that regulates the transcription of genes encoding inflammatory cytokines and chemokines [18]. NF-κB is activated by a wide variety of agents, including interleukin-1, interleukin-17, interleukin-18, H\textsubscript{2}O\textsubscript{2}, ceramide and lipopolysaccharide [15, 19]. H\textsubscript{2}O\textsubscript{2} induced the tyrosine phosphorylation of I-κBα which is needed in NF-κB activation [19].

Here we investigated whether AA13 protects H\textsubscript{2}O\textsubscript{2}-stimulated SHSY5Y cells via autophagy, MAPKs pathway and NF-κB in vitro and also investigated potential changes in the level of related proteins under the condition.

Materials and methods

Drugs and reagents

Ham’s/F12 (F12) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS), protease inhibitor cocktail and Phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich (St Louis, MO, USA). Electrochemiluminescence (ECL) reagent kit, Phosphatase inhibitor complex III, and 3-(4,5-Dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sangon Biotech (Shanghai, China). Hoechst 33342 was bought from Beyotime Company (Nantong, China). Isoflurane was obtained from RWD Life Science Company (Shenzhen, China).

2-(4-Amidinophenyl)-6-indolecarbamidine (DAPI) dihydrochlorid was bought from Beyotime Company (Nantong, China). Rabbit anti-phospho-Akt (Ser473) antibody, Rabbit anti-LC3-I/II antibody, MAPK family antibody sampler kit and Rabbit anti-NF-κB p65 antibody were provided by Cell signaling Technology (Boston, MA, USA). Secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Sarsasapogenin-AA13 (AA13) (Figure 1) was provided by Lei Ma’s lab (School of Pharmacy, East China University of Science and Technology) as a gift.

Cell culture

SHSY5Y cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured with Dulbecco’s modified Eagle media: Ham’s/F12 (D/F12) supplemented with 10% fetal bovine serum at 37°C atmosphere of 5% CO\textsubscript{2}. Cells were seeded in cell culture plates at a density of 1x10\textsuperscript{5}/mL before experiments. Experiments were carried out 24-48 h after cells were seeded.

Cell viability assays

The effects of H\textsubscript{2}O\textsubscript{2} and AA13 on cell viability were determined by MTT assay, which depends on the activity of mitochondrial enzymes. The cells were cultured in 96-well plates at a density of 1x10\textsuperscript{5}/mL. After adhered, cells were pre-treated with AA13 (0.1, 1, 5 and 10 μM) for 2 h, followed by H\textsubscript{2}O\textsubscript{2} (300 μM) stimulation for 24 h. MTT solution was added to the medium at 0.5 mg/mL and then cells were incubated for another 4 h at 37°C. The formazan crystals formed in cells were dissolved with 150 μL DMSO, and the absorbance at 490 nm was measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT).

Western blot analysis

SHSY5Y cells were cultured in 6-well plates at a density of 1x10\textsuperscript{5}/mL. After adhered, the cells were treated with H\textsubscript{2}O\textsubscript{2} (300 μM) in the presence or absence AA13 (0.1, 1, 5 and 10 μM) for 30 min, and then cells were washed three times with pre-cooling D-Hanks (137.93 mM NaCl, 5.33 mM KCl, 0.441 mM KH\textsubscript{2}PO\textsubscript{4}, 4.17 mM NaHCO\textsubscript{3}, 0.338 mM Na\textsubscript{2}HPO\textsubscript{4}, 100 μL lysing buffer (1.0 mmol/L EDTA, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS and 1% sodi-

Figure 1. Structure of AA13.
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**Immunofluorescence staining**

SHSY5Y cells were plated at a density of 1×10⁵/mL in 12-well plates. After 24 h, cells were pretreated with AA13 (10 μM) for 2 h, followed by H₂O₂ (300 μM) stimulation for 1 h. After washing with cold PBS, cells were fixed with 4% paraformaldehyde (PFA) solution for 15 min at room temperature. After fixation, PFA were removed by washing with PBS and cells were permeated with 0.1% Triton-X100 for 30 min at room temperature. After 1 h blocking with 5% BSA at room temperature, the cells were incubated with rabbit anti-NF-κB p65 antibody (1:300) at 4°C for at least 12 h. After washing by PBS, fluorescent secondary antibody (Anti-rabbit 1:5000) was added to the cells and incubated for 2 h at room temperature. DAPI (0.1 μg/ml) was used for nuclear staining. The images were captured by fluorescence microscope.

**Hoechst staining assay**

SHSY5Y cells were plated at a density of 1×10⁵/mL in 12-well plates. After 24 h, cells were pretreated with AA13 (10 μM) for 2 h, followed by H₂O₂ (300 μM) stimulation for 6 h. After washing with cold PBS, cells were incubated with Hoechst 33342 solution for 15 min. And then cells were washed with cold PBS for three times. The images were captured by fluorescence microscope.

**Statistical analysis**

Data were expressed as mean ± SEM and evaluated by One-way analysis of variance (ANOVA) test, followed by a post hoc (Student-Newman-Keuls) test for multiple comparisons. P value of <0.05 was considered significant.

**Results**

**Sarsasapogenin-AA13 protects against H₂O₂-induced cytotoxicity in SHSY5Y cells**

To detect the effects of AA13 on H₂O₂-induced cell death, SHSY5Y cells were treated with AA13 (10 μM) for 2 h, followed by H₂O₂ (300 μM) stimulation for 24 h. Cell viability was detected by MTT assay. The results were expressed as percentage of the control group. Data are presented as mean ± SEM. All experiments were repeated 3 times with triplicated in each assay. Differences with p value less than 0.05 were considered statistically significant. ***p<0.001 compared with control; #p<0.05, ##p<0.01, ###p<0.001 compared with H₂O₂ treatment cells.

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**Sarsasapogenin-AA13 Inhibits H₂O₂-induced apoptosis of SHSY5Y cells**

To confirm the neuroprotective effect of AA13 on H₂O₂-induced cell injury, further detection was implemented. Morphological data showed (Figure 3A) that AA13 pretreatment ameliorated the cellular damage induced by H₂O₂. Hoechst 33342 staining assay was used to evaluate the morphological characteristics of apop-
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Figure 3. AA-13 improved H₂O₂ induced cytotoxicity. SH-SY5Y cells were pretreated with AA13 for 2 h before exposure to 300 μM H₂O₂ for 24 h. Cell morphology was captured under phase contrast microscope (A). The morphological characteristics of apoptosis were evaluated by Hoechst 33342 staining assay captured by fluorescence microscope (B).

Figure 4. Effect of Sarsasapogenin-AA13 on H₂O₂-induced autophagy. The ratio of LC3-II/LC3-I was used to estimate autophagy. SHSY5Y cells were cultured in 6-well plates at a density of 1×10⁵/mL. Cells were pretreated with AA13 for 2 h prior to the exposure of 300 μM H₂O₂ for 30 min. After preparation of the protein samples, the LC3-II and LC3-I were determined by Western blot analysis. The results were expressed as the percentage of the control group. Data are presented as mean ± SEM (n=3). Differences with p value less than 0.05 were considered statistically significant. *p<0.05, **p<0.01 compared with control; #p<0.05, ##p<0.01, ###p<0.001 compared with H₂O₂ treatment cells.

tosis in SHSY5Y cells. As shown in Figure 3B, the cells, which were treated with 300 μM H₂O₂ for 6 h, showed shrinkage of nuclear. However, pretreatment with AA13 (1, 5, 10 μM) obviously decreased the number of cells with nuclear shrinkage. This result revealed that AA13 could reduce the apoptosis of SHSY5Y cells induced by H₂O₂.

Effect of sarsasapogenin-AA13 on H₂O₂-induced increase of LC3-II/LC3-I ratio values

LC3 is a well-recognized autophagy marker. When autophagy is formed, cytosolic LC3 (LC3-I) will enzymatically cleave a small segment of the polypeptide into an autophagosome type LC3-II. The ratio of LC3-II/I can be used to estimate the level of autophagy [20]. Intracellular ROS induced the autophagic response amplification. The cells will increase their autophagy due to self-regulation mechanism, resulting in an increase in the ratio of LC3-II/LC3-I [21]. In our study, H₂O₂ exposure increase the LC3-II/LC3-I ratio compared to untreated control. Pretreatment with low concentrations (0.1 and 1 µM) AA13 did not reverse the LC3-II/LC3-I ratio, but 0.1 µM of AA13 enhanced the ratio. In contrast to lower concentrations, higher concentrations of AA13 (5, 10 µM) could down-regulate LC3-II/LC3-I ratio to the normal level. The results indicated that AA13 did affect the H₂O₂ induced autophagy. Our results also suggested that the concentration might be one of factors for the efficacy (Figure 4).

Effect of sarsasapogenin-AA13 on H₂O₂-induced phosphorylation of Akt in SHSY5Y cells

To further investigate the possible mechanisms of the protective effects of AA13 against H₂O₂-
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induced injury, the phosphorylation of Akt was measured by Western blot analysis. As the result shown, H$_2$O$_2$-induced the phosphorylation of Akt. After AA13 pretreatment, the H$_2$O$_2$-induced phosphorylation of Akt was significantly decreased (Figure 5A).

Sarsasapogenin-AA13 reverses H$_2$O$_2$-induced activation of MAPK pathways

The effect of AA13 on the phosphorylation of Erk, p38 and JNK in SHSY5Y cells treated with H$_2$O$_2$ was examined. After H$_2$O$_2$ exposure, the
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phosphorylation of Erk, p38 and JNK were significantly increased. As shown in Figure 5B, H$_2$O$_2$-induced phosphorylation of Erk was inhibited by AA13 at all concentrations. However, AA13 inhibited the phosphorylation of p38 (Figure 5C) at concentrations of 1, 5 and 10 μM, and inhibited the phosphorylation of JNK (Figure 5D) at concentrations of 5 and 10 μM.

Sarsasapogenin-AA13 inhibits H$_2$O$_2$-induced activation of NF-κB p65

NF-κB p65 is a critical transcription factor that responses to H$_2$O$_2$-induced damage. In order to investigate if the antioxidant effect of AA13 is related to the activation of NF-κB p65, the translocation of NF-κB p65 was detected using immunofluorescent staining. The result indicated that NF-κB p65 translocated from the cytoplasm to nucleus after stimulated by H$_2$O$_2$. AA13 pretreatment inhibited the H$_2$O$_2$-induced NF-κB p65 translocation at the concentration of 10 μM (Figure 6).

Discussion

In our previous study, from dozens of synthetic sarsasapogenin derivatives, we found that AA13 had a significant protective effect in SHSY-5Y cells undergoing many different damages [22]. However, the mechanisms of neuroprotective effects still maintain unclear. In this study, we first showed that AA13 dose-dependently inhibited the H$_2$O$_2$-induced injury of SHSY-5Y cells at concentrations of 0.1, 1, 5 and 10 μM. And, the autophagy disorder contributed to H$_2$O$_2$-induced cell injury. AA13 (5, 10 μM) pretreatment reversed the disordered autophagy of H$_2$O$_2$ treated cell to the normal level. Meanwhile, both phosphorylation of Akt and activation of MAPK pathway were inhibited. Nuclear factor κB (NF-κB) is a nuclear transcription factor that regulates expression of a large number of genes critical for the regulation of apoptosis and inflammation. The activation of NF-κB is thought to be part of a stress response as it is activated by a variety of stimuli including oxidative stress. When activated, NF-κB p65 is dissociated from the binding of IκB protein family and translocated to the nuclei. Using immunofluorescent staining, we found that when oxidative stress occured the NF-κB p65 was translocated to nucleus, and translocation of NF-κB p65 was inhibited by...
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AA13 pretreatment. Since NF-κB p65 is one of the downstream transcription factors of MAPK pathway, we speculated that the protective effects of AA-13 might be related to regulation of MAPK pathway. Several studies have shown that autophagy represents a potential target for neurodegenerative diseases. Our research indicates that the protection of AA13 for neuronal injury induced by H$_2$O$_2$ could be related to autophagy. The activation of the MAPKs induced by H$_2$O$_2$ contributes to the neuronal cell death, thus it could be partially attenuated by inhibiting the activation of MAPKs [16]. The study further demonstrated that treatment with AA13 induced the cytoprotective autophagy and inhibited the activation of MAPKs which may contribute to the protective effect of AA13 on H$_2$O$_2$-induced injury. Activation of Akt could promote cell survival and prevent apoptosis to some extent, and as the figure shows, H$_2$O$_2$ increased phosphorylation of Akt [23, 24]. However, the phosphorylation of Akt was inhibited by AA13. We speculate that the phosphorylation of Akt may be a protective mechanism when cells were injured. Under the circumstances, other signal pathways could be involved in this process. Therefore, the phosphorylation of Akt was suppressed. Activation of NF-κB was involved in the signaling pathways regulated by H$_2$O$_2$ [25], which plays a key role in the cell survival. As the study shown, AA13 attenuating the neuronal injury was also correlative with the inhibition of NF-κB.

In conclusion, this study suggests that AA13 has a potential neuroprotective activity. Meanwhile, we demonstrate that inhibition of MAPKs pathway and NF-κB p65 activation might contribute to the neuroprotective effect of AA13. Likewise, autophagy might be involved in the protective effects of AA13. Our findings not only provide a new insight into the underlying mechanism of the neuroprotection of AA13, but offer a novel potential therapy for prevention and treatment of AD.

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Disclosure of conflict of interest

None.

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