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Original Article

Polydatin prevents Aβ-induced neuron cytotoxicity via enhancing autophagy and decreasing oxidative stress

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Abstract: Amyloid beta (Aβ) accumulation is a key pathological feature of Alzheimer's disease (AD). Aβ leads to increased oxidative stress and mitochondrial damage of neuron cells. The ATP production is insufficient in dysfunctional mitochondria, and then causes the release of cytochrome C, ultimately leading to the activation of caspase enzyme and neuron apoptosis. Nevertheless, Aβ-induced neurotoxicity can be weakened by the increase in autophagy. Polydatin, a derivative of resveratrol, was reported to alleviate myocardial ischemia-reperfusion injury by upregulating autophagy and removing the dysfunctional mitochondria. Polydatin was also shown to be a mitochondria protector in acute ischemic neuronal injury. However, whether polydatin could prevent Aβ-induced neuron cell apoptosis through regulating autophagy and dysfunctional mitochondria clearance is still unclear. This study is to explore the effect and mechanism of polydatin in protecting neuron cells. Neuron cells were treated with polydatin and Aβ. Flow cytometry analysis of Annexin V-PI cells were conducted to measure cell apoptosis. The proteins related to mitochondria apoptosis were measured by western blot. The activation of autophagy pathway in neuron cells was assessed by evaluating the expression levels of autophagy marker proteins. The role of autophagy in the anti-apoptotic function of polydatin was evaluated through using autophagy inhibitor. The determinations of mitochondrial membrane potential, ATP concentration, and reactive oxygen species (ROS) were used to evaluate the change of integrity and function of mitochondria. A preliminary mitochondrial autophagy was assessed by western blot analysis of the loss of mitochondria related proteins. Polydatin strongly inhibited Aβ-induced neuron cell apoptosis, which was related with the repression of mitochondrial apoptosis. Polydatin induced cell autophagy through the activation of AMPK/mTOR pathway. Autophagy inhibition partially abolished the anti-apoptotic function of polydatin. Treatment with polydatin effectively prevented the Aβ-induced reduction of mitochondrial membrane potential and ATP, and the production of ROS, H_2O_2, and superoxide anion, suggesting that polydatin could prevent the structure and function of mitochondria from being damaged. In addition, polydatin treatment led to the loss of Aβ-induced mitochondrial related proteins, which means polydatin might promote mitochondria autophagy (mitophagy) and facilitate the clearance of damaged mitochondria, further prevented dysfunctional mitochondria-induced neuron cell apoptosis. In conclusion, polydatin prevented Aβ-induced neuron cell apoptosis by promoting autophagy, mitochondria clearance, and oxidative stress reduction, serving as a potential natural product for AD prevention.

Keywords: Polydatin, amyloid beta, mitochondria autophagy, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease and the most common cause of dementia. The typical clinical feature of AD is progressive cognitive changes including memory loss, personality changes, impaired executive function, and a progressive inability to perform the activities of daily living [1-3]. AD has been hypothesized to begin decades before the first symptoms manifest [4]. Therefore, studies on well-validated biomarkers of AD and the development of effective therapeutics are urgently required. The neuropathological hallmarks of AD are extracellular amyloid-β (Aβ) proteins accumulation in the form of senile plaques and intraneuronal hyperphosphorylated tau (microtubule-associated protein tau, MAPT) aggregates (neurofibrillary tangles) followed by neuronal cell death [1-3]. Aβ plays a central role in the pathogenesis and progression of AD [5]. Accumulation of Aβ is an important issue in AD progression, because intracellular Aβ has toxic effects in neuron cells. Since accumulation of amyloid in the brain takes place over many years and typically precedes tau tangles by a decade or more, controlling Aβ-induced neuronal toxicity opens the gate for AD therapy [6-9].

Ab is a 4 kDa peptide, generated by abnormal cleavage of amyloid-precursor protein (APP) in AD neurons [10]. Accumulation of Aβ aggregates eventually triggers a cascade of cellular changes, including mitochondrial oxidative damage, the hyperphosphorylation of tau, synaptic failure and inflammation. Recent studies proved that Aβ is associated with mitochondria dysfunction in AD [11-15]. Moreover, Aβ is shown to accumulate in mitochondria of both human and model mice AD brains [16-24]. Mitochondrial failure and dysfunction are an early sign of AD [25]. Aβ increases cellular reactive oxygen species (ROS) level, which particularly leads to mitochondria damage [26]. Aβ accumulates in synapse and synaptic mitochondria, leading to mitochondrial dysfunction and synaptic degeneration in AD neurons. Mitochondria dysfunction leads to increased ROS production, abnormal intracellular calcium levels and reduced mitochondrial ATP [15]. Mitochondria are both generators of and targets for reactive species [27]. AD cell lines with increased Ab production and mitochondrial dysfunction exhibited lower cytochrome oxidase activity, elevated free radical production and oxidative stress markers, altered calcium homeostasis, reduced mitochondrial membrane potential, and changed apoptosis pathways [28]. In healthy cells, mitochondrial dynamics is well maintained and essential for cell survival. However, in AD cells with Aβ-induced oxidative stress and mitochondrial dysfunction, the dynamics of mitochondria is imbalanced, resulting in structural and functional abnormalities leading to neuron cell apoptosis [15]. Removal of dysfunctional mitochondria or reduced oxidative stress is essential for mitochondrial dynamics maintenance and neuron cell protection.

Mitochondria turnover is dependent on autophagy, which is frequently dysfunctional in neurodegenerative disease [29]. Autophagy is a dynamic and protective cellular process for the lysosomal degradation and continuous removal of protein aggregates and damaged cell organelles to maintain cellular homeostasis [30]. It has been proven that autophagy protects the degenerating neurons via the removal of toxic proteins and defects in autophagy contribute to neurodegeneration [31, 32]. Autophagy was demonstrated to play critical role in AD-pathogenesis by affecting neuronal death [33, 34]. Finding a proper way to activate autophagy leading to effective dysfunctional mitochondria clearance holds great promise for AD therapy.

Polydatin, a derivative of resveratrol, was reported to alleviate myocardial ischemia-reperfusion injury by upregulating autophagy and removing the dysfunctional mitochondria [35]. Polydatin was also shown to be a mitochondria protector in acute ischemic neuronal injury [36]. However, whether polydatin could prevent Aβ-induced neuron cell apoptosis through regulating autophagy and dysfunctional mitochondria clearance is still unclear. In this study, we investigated how polydatin protected neuron cell from Aβ-induced oxidative stress and mitochondria dysfunction.

Methods and materials

Cell culture

Human neuroblastoma cell lines SH-5Y5Y and SK-N-SH were obtained from the American Type Culture Collection (ATCC). SH-5Y5Y was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen). SK-N-SH was cultured in MEM (Gibco, Invitrogen). Both medium were supplemented with 10% (v/v) FBS (Gibco, Invitrogen), 2 mM GlutaMAX (Gibco, Invitrogen), and 1% penicillin-streptomycin (Gibco, Invitrogen). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

Apoptosis analysis

Apoptotic cells were collected and detected by Annexin V Apoptosis Detection Kit APC (eBioscience) and analyzed by FACS Calibur. Briefly, 10^6 cells were washed and resuspended in 1× Binding Buffer. Fluorochrome-conjugated Annexin V was added to the cell suspension and incubated at room temperature for 10 minutes. Cells were washed with 1× Binding Buffer. Propidium Iodide was added before flow cytometry analysis.

Cellular fractionation

Cells were collected are mitochondria were isolated using a Mitochondria Isolation Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, cells were resuspended with Mitochondria Isolation Reagent A, vortexed and incubated on ice for 2 minutes. Then cells were homogenized, and Mitochondria Isolation Reagent C added to the homogenates, followed by centrifuged at 700 g for 10 min. The supernatant fractions were centrifuged at 3000 g for 15 min and the resultant pellets stored as mitochondrial fractions.

Western blot

The cytosol and mitochondrial protein extractions were separated by SDS-PAGE and transfected to a PVDF membrane (Millipore). The membrane was blocked with 5% (w/v) reagent-grade nonfat milk (Cell Signaling Technology) and incubated with primary antibodies at 4°C overnight followed by secondary antibody incubation. The protein bands were visualized using Clarity™ Western ECL substrate (Bio-Rad). The protein level was quantified using Image J software. Cytosol and mitochondrial fractions were normalized with β-actin (cytosol) and Hsp60 (mitochondria), respectively.

Mitochondrial membrane potential detection

The change in mitochondrial membrane potential (MMP) was measured by JC-1 Mitochondrial Membrane Potential Detection Kit (Molecular Probes Eugene, USA). The JC-1 accumulates in intact mitochondria to form J-aggregates (red fluorescence) indicating high or normal MMP. Low MMP was indicated when JC-1 remains in the cytoplasm in monomeric form in the cytoplasm to show green fluorescence. Cells were incubated in culture medium containing 10 μM JC-1 at 37°C for 15 min, washed with PBS, and then transfected to a 96-well plate. JC-1 aggregate fluorescent emission was measured at 583/26 nm with an excitation at 488 nm. JC-1 monomer fluorescence intensity was measured with excitation and emission at 488 nm and 525/30 nm respectively.

Cellular ATP determination

The cellular ATP levels were measured by CellTiter-Glo kit (Promega, USA) according to the manufacturer's instructions. CellTiter-Glo reagent was added to cell suspensions and incubated for 10 min to stabilize the luminescent signal. The luminescence was measured by an automatic microplate-reader (SpectraMax, CA).

Measurement of intracellular ROS content

Intracellular ROS was determined with 2,7-dichlorofluoresein diacetate (DCFH-DA) (Sima-Aldrich, USA). ROS mediates the conversion of non-fluorescent DCFH-DA into fluorescent DCFH. Cells were incubated with culture medium containing 20 μM DCFH-DA for 30 min at 37°C and washed with PBS three times. The cells were collected and analyzed by FACS Calibur (Becton Dickinson).

Determination of hydrogen peroxide

H_2O_2 was measured by an Amplex red hydrogen peroxide assay kit (Molecular Probes). In brief, the collected cells were lysed by repeat freeze-thawzing. The supernatant was collected after centrifugation, and reacted with Amplex red (100 μM) and horseradish peroxidase (0.2 unit/ml) for 30 min at room temperature. The absorbance was measured at 560 nm.

Superoxide anion release analysis

Superoxide anion production was measured using the superoxide dismutase-inhabitable (SOD-inhabitable) cytochrome reduction. 500 μl culture medium was mixed with 50 μl 40 μM cytochrome c (Sigma-Aldrich, USA), and then 250 μl Hank's balanced salt solution was added. The mixture was incubated with or without 50 μl SOD (100 μg/ml) at room temperature for 10 min. The absorbance was detected spectrophotometrically at 550 nm.

Statistical analysis

All data are presented as the mean ± SD and derived from at least three independent experiments. Statistical analysis was performed by SPSS 18.0 software (SPSS, Chicago, IL) and GraphPad Prism Software (GraphPad Software, InC., San Diego, CA). For all comparisons, differences were considered significant when $P<0.05$.

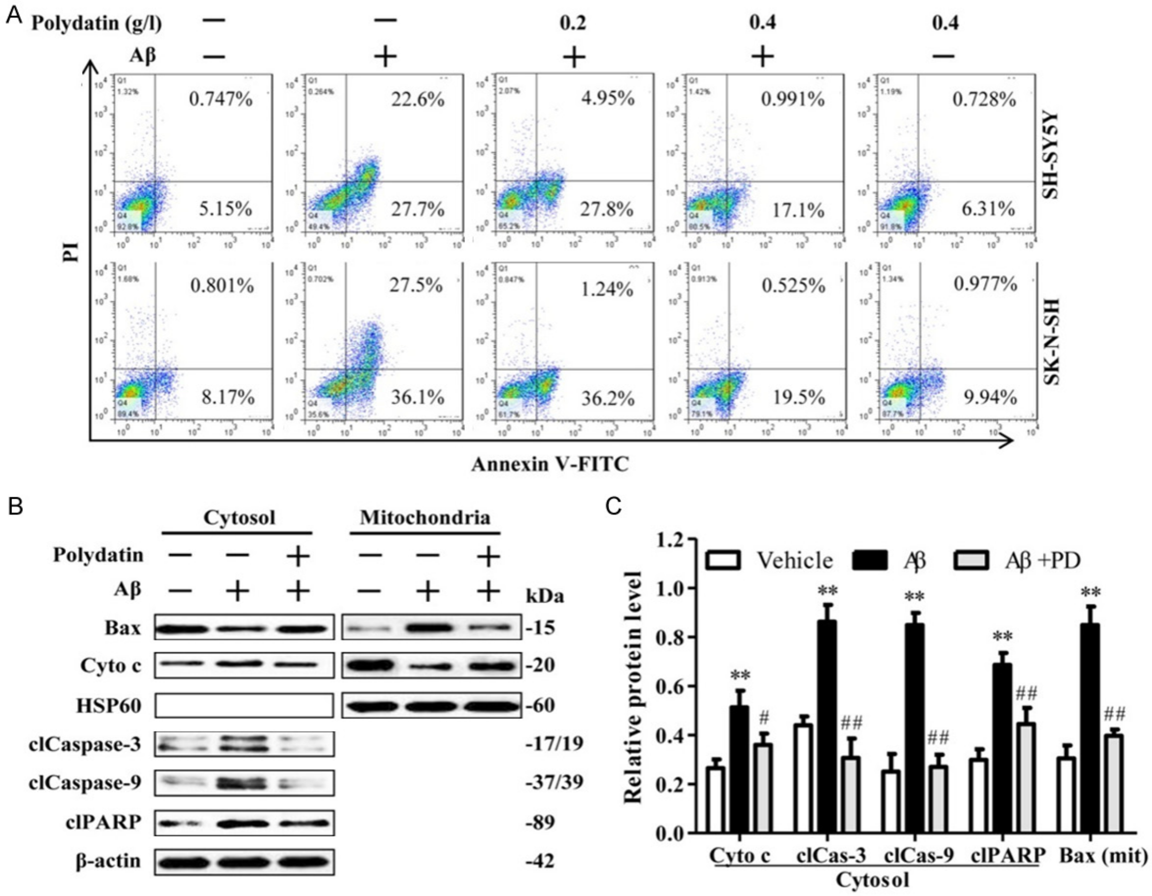

Figure 1. Polydatin treatment inhibits Aβ-induced apoptotic signaling by regulating mitochondrial apoptotic pathways. A: Neuronal cell lines SH-SY5Y and SK-N-SH were pre-treated with polydatin for 12 h in a dose-dependent manner (0.2 and 0.4 g/l), then exposed to 10 μm Aβ (25-35) for 6 h. Cell death was measured by FACS analysis of Annexin V-PI staining. B: The subcellular fractions were separated from cytosol and mitochondria. The apoptotic proteins were analyzed by Western blot assay. The cytosol proteins were normalized with β-actin, while the mitochondria proteins were normalized with HSP60. C: The protein quantification results were shown. Error bars indicate s.d.. **: $P<0.01$, *: $P<0.05$, Student's *t*-test.

Results

Polydatin treatment inhibits Aβ-induced apoptotic signaling by regulating mitochondrial apoptotic pathways

To uncover the function of polydatin (PD) in Ab-induced neuron cell degeneration, we pretreated SH-SY5Y and SK-N-SH neuronal cells with polydatin (12 h) in a dose-dependent manner followed by exposure to 10 μM Aβ (25-35) for another 6 hours. The apoptotic cells were measured by Annexin V-PI assay. As the results shown, Aβ-treated cells underwent prominent cell apoptosis (**Figure 1A**). Meanwhile, pretreatment with polydatin largely reduced Aβ-induced apoptosis, which showed a dose-dependent manner (**Figure 1A**). To further elucidate the mechanism by which polydatin reduced Aβ-induced apoptosis, we isolated proteins from cytosol and mitochondria respectively and analyzed apoptotic-related protein levels after polydatin treatment. As shown by western blot, cleaved-capase3/9, Cyto c, cleaved-PARP in cytosol were up-regulated by Aβ and then reduced upon polydatin treatment, suggested polydatin indeed inhibited Aβ-induced apoptotic signaling pathway (**Figure 1B** and **1C**). Intriguingly, polydatin treatment significantly alleviated mitochondrial Bax level rather than which in cytosol (**Figure 1B** and **1C**). Taken together, polydatin inhibits Aβ-induced apoptotic signaling possibly by regulating mitochondrial apoptotic pathways.

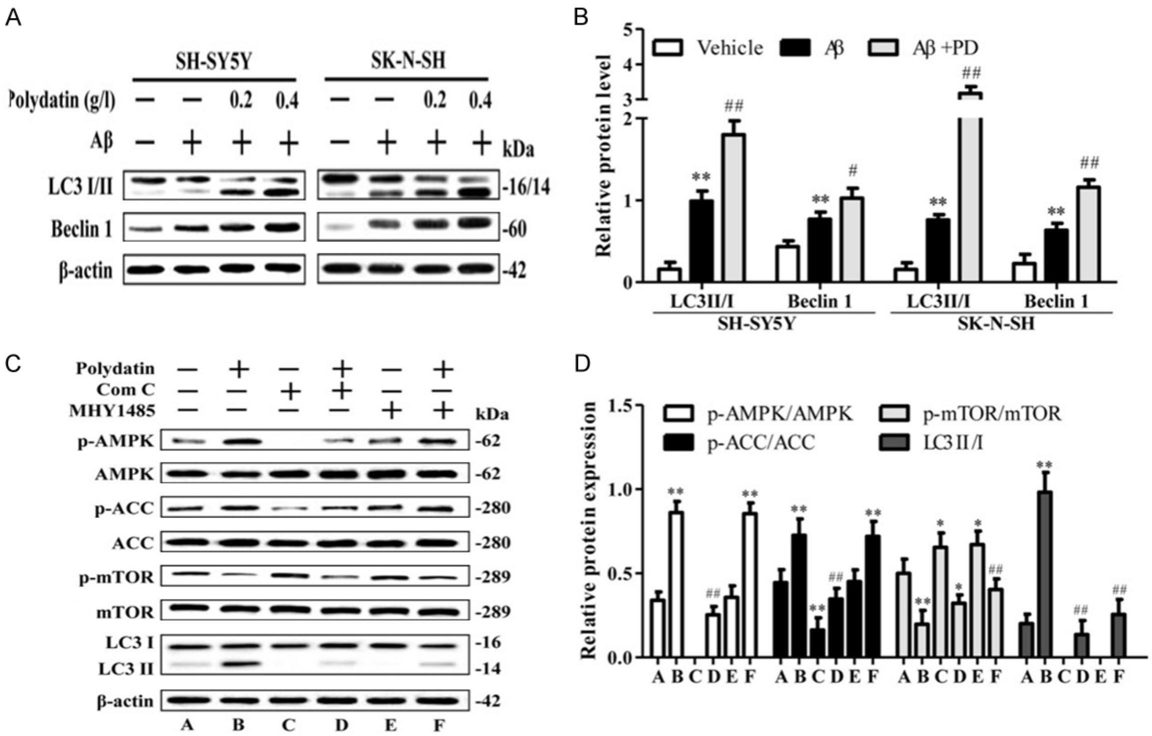

Figure 2. Polydatin induces autophagy via AMPK/mTOR pathway. A: The autophagy-related proteins were measured by western blot assay. Cells were pretreated with polydatin and then with Aβ. B: The protein quantification results were shown. Error bars indicate s.d.. **: *P*<0.01, *: *P*<0.05, Student's *t*-test. C: The phosphorylation of AMPK and mTOR was determined by western blot to study the activation of AMPK/mTOR pathway with or without AMPK inhibitor Com C and mTOR activator MHY1485 upon polydatin treatment. D: The protein quantification results were shown. Error bars indicate s.d.. **: *P*<0.01, *: *P*<0.05, Student's *t*-test.

Polydatin induces autophagy via AMPK/mTOR pathway

Polydatin was previously found to inhibit mitochondrial apoptotic pathway by enhancing autophagy in multiple myeloma [37], we next asked whether polydatin activated autophagy in Aβ-treated neuronal cells. To study the effect of polydatin on autophagy, the autophagy-related proteins LC3I/II and Beclin 1 were detected by Western blot. We found that polydatin treatment elevated LC3II and Beclin 1 (**Figure 2A** and **2B**), indicating that polydatin could induce autophagy. Since AMPK/mTOR signaling pathway played a crucial role in regulating autophagy, we further investigated the effect of polydatin on AMPK/mTOR pathway. As determined by Western blot, after polydatin treatment, p-AMPK was up-regulated but p-mTOR was do wnregulated (**Figure 2C** and **2D**). Together treatment with AMPK inhibitor compound C (Com C) reduced AMPK activation and abolishes polydatin induced autophagy indicated by LC3II downregulation (**Figure 2C** and **2D**). Moreover, when the cells were treated with MHY1485, an mTOR activator, polydatin induced autophagy was again reduced (**Figure 2C** and **2D**). These results indicated that polydatin induced autophagy by activating AMPK and inactivating mTOR signaling pathways.

Autophagy-mediated neuroprotection by polydatin

We next identified whether polydatin protect neuronal cell apoptosis via inducing autophagy. The autophagy inhibitors 3MA (10 mM) and bafilomycin A1 (Baf, 200 nM) were used to further verify the effect of PD on Aβ-treated neuronal cells. Both 3MA and Baf blocked PD-induced autophagy as shown by Annexin V-PI (**Figure 3A** and **3B**). Western blot indicated Baf reversed PD-induced mitochondria Bax upregulation, alleviated cytosol Bax levels (**Figure 3C** and **3D**), while the effect on Cyto C expression was the opposite (**Figure 3C** and **3D**). Baf also abolished PD-induced apoptotic proteins alleviation (**Figure 3C** and **3D**). These data con-

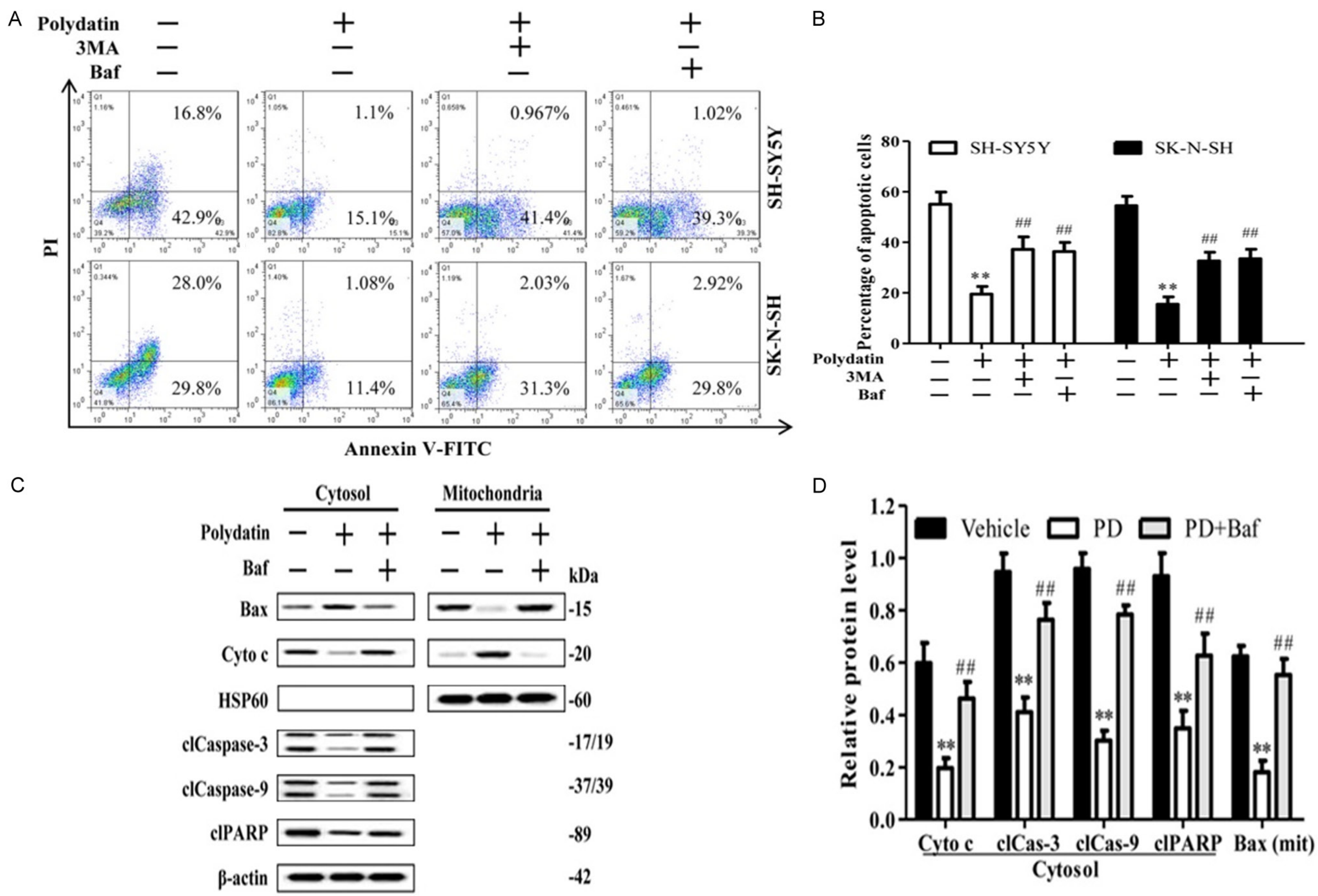

Figure 3. Autophagy-mediated neuroprotection by polydatin. A: Neuronal cell lines SH-SY5Y and SK-N-SH were treated with polydatin, Aβ together with autophagy inhibitor 3MA or bafilomycin (Baf). Cell death was measured by FACS analysis of Annexin V-PI staining. B: Cell death quantification was shown. C: The apopotic proteins were measured by western blot assay. Cells were treated with polydatin, Aβ, together with 3MA or Baf. D: The protein quantification results were shown. Error bars indicate s.d.. **: *P*<0.01, Student's *t*-test.

firmed that polydatin protected neuronal cells from apoptosis by inducing autophagy.

Polydatin reduces mitochondria dysfunction and oxidative stress through promoting mitophagy

We sought to investigate the effect of polydatin on dysfunctional mitochondria and oxidative stress. Aβ-treated neuron cells exhibited impaired mitochondrial membrane potential indicated by increased JC-1 monomer level (**Figure 4A**). Polydatin treatment protected damaged mitochondria shown by prominently JC-1 monomer reduction (**Figure 4A**), which was reversed when autophagy was inhibited (**Figure 4A**). Polydatin treatment also increased ATP which was down-regulated by Aβ through regulating autophagy (**Figure 4B**). Aβ treatment significantly up-regulated the level of oxidative products, including ROS, H_2O_2, and superoxide (**Figure 4C-E**). Polydatin alleviated Aβ-induced oxidative production by activating autophagy (**Figure 4C-E**). Furthermore, polydatin resulted in the loss of mitochondrial proteins in Aβ-treated cells, which was reversed by Baf (**Figure 4F** and **4G**), suggested that polydatin induced mitophagy. Taken together, we found polydatin reduces mitochondria dysfunction and oxidative stress through promoting mitophagy.

Discussion

AD is a progressive, neurodegenerative disorder. Oxidative stress and synaptic damage are known to have an essential role in AD pathogenesis. Aβ accumulation at synapses and mitochondrial dysfunction lead to synaptic damage, impair neurotransmission and cause cognitive decline. It has been shown that Aβ accumulation promoted mitochondria dysfunction, gradually leading to neuronal cell apoptosis and degeneration. It is necessary to find a solution to alleviate Aβ-induced neuron toxicity. In this study, we found polydatin prevented Aβ-induced neuron cell apoptosis by promoting autophagy, dysfunctional mitochondria clearance, and oxidative stress reduction.

Neurons contain the highly-specialized structures for intercellular communication with limited proliferation ability. Intracellular Aβ accumulates in cell organelles, including mitochondria, and leads to neuronal cell death by increasing oxidative stress, causing mitochondria damage and cellular toxicity. Demonstrating the interface between stress adaptation and cell death is important for neurons maintenance [26]. Autophagy has been proved to be a major sensor of redox signaling and key regulator for protecting neurons from death. Autophagy is essential for preserving the balance between organelle biogenesis, protein synthesis and their clearance. The cellular mitochondria are regulated in a highly dynamic way. There is a balance between functional and dysfunctional mitochondria [14]. In AD patients, mitochondrial dynamics are impaired [38]. The quick clearance of dysfunctional mitochondria is important for keeping the normal mitochondrial dynamics. Autophagy is induced by various stimuli and is considered as a survival mechanism activated in adverse conditions to maintain cell integrity. It has been demonstrated that autophagy has neuroprotective effect in neurodegenerative diseases. In our study, Aβ induced neuron cell death, which was abolished upon polydatin treatment. The results showed that polydatin reduced the expression of apoptotic proteins including Bax, clCaspase3/9. By further analysis, we found polydatin treatment activates autophagy through regulating AMPK and mTOR pathway. The AMPK pathway was shown to be activated during resveratrol-induced autophagy process in cellular models of PD [39]. Polydatin was reported to regulate autophagy in multiple myeloma cells through suppressing mTOR pathway [37]. These data collectively proved that polyatin could induce autophagy via regulating AMPK and mTOR pathway in several disease systems. Autophagy inhibitors 3MA and bafilomycin reversed polydatin-induced autophagy indicated by upregulation of apoptotic related proteins. These results together showed polydatin protect neuron cells from Ab-induced apoptosis via inducing autophagy.

Mitochondrial dysfunction has been implicated in AD pathogenesis. Recent research revealed that Aβ accumulates in synaptic mitochondria, leading to abnormal mitochondrial dynamics and synaptic degeneration in AD neurons. Drugs regulating mitochondrial dynamics will help to improve AD. Our study showed that polydatin enhanced the clearance of damaged mitochondria by inducing mitophagy. We found that treatment with polydatin effectively pre-

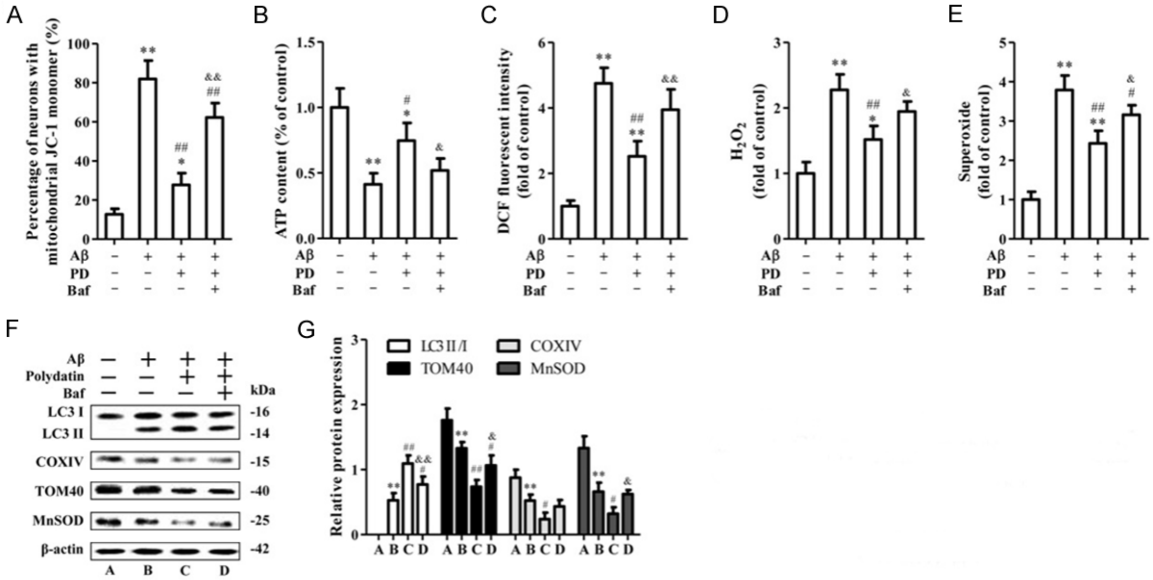

Figure 4. Polydatin reduces mitochondria dysfunction and oxidative stress through promoting mitophagy. A: Mitochondrial membrane potential was measured by the quantification of JC-1 monomer. B: Cellular ATP content was quantified by CellTiter Glo. C: ROS level was determined using FACS analysis of DCFDA staining positive cells. D: Intracellular H_2O_2 production was measured and quantified. E: Superoxide anion release was quantified. F: Mitochondrial proteins were isolated and autophagy-related proteins were detected by western blot. G: The protein quantification results were shown. Error bars indicate Error bars indicate s.d.. **: $P<0.01$, *: $P<0.05$, Student's *t*-test.

vented the Aβ-induced reduction of mitochondrial membrane potential and ATP, and the production of ROS, H_2O_2, and superoxide anion, suggesting that polydatin could protect mitochondria dynamics by inducing mitophagy.

Conclusion

In summary, our study demonstrated that polydatin prevented Aβ-induced neuron cell apoptosis by promoting autophagy, mitochondria clearance, and oxidative stress reduction, serving as a potential natural product for AD prevention. Polydatin was shown here to be a natural antioxidant and effective mitochondrial therapeutic to protect neuron cells from Aβ accumulation induced cell death and neurodegeneration. We also elucidated the mechanism how polydatin works, by which polydatin induces autophagy via regulating AMPK/mTOR pathways. Taken together, our study provided a new approach for AD therapy.

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Disclosure of conflict of interest

None.

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