RESEARCH ARTICLE

Cytoprotective effects of hesperetin and hesperidin against amyloid β-induced impairment of glucose transport through downregulation of neuronal autophagy

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Scope: This study investigated whether flavonoids, such as hesperetin and hesperidin, inhibited amyloid β (Aβ)-impaired glucose utilization through regulating cellular autophagy in insulin-stimulated neuronal cells.

Methods and results: In this study, we used a toxic Aβ1-42 peptide to impair insulin-stimulated glucose utilization in Neuro-2A cells, and this study also hypothesized that Aβ-induced autophagy might be emerging as a key process regulating neuronal glucose uptake. Additionally, hesperetin and hesperidin were used to test the neuroprotective effect against Aβ-induced impairment of glucose utilization. Our data found that Aβ-stimulated autophagy activation promoted the phenomenon of impairment of neuronal energy metabolism, including glucose uptake, glucose transporters (GLUTs), and insulin signaling cascades. In this study, confocal images of autophagy punctate further confirmed that downregulation of Aβ-stimulated autophagy could increase insulin-stimulated neuronal glucose uptake. Moreover, treatment with hesperetin and hesperidin improved Aβ-impaired glucose utilization by inhibiting Aβ-induced autophagy in neuronal cells.

Conclusion: These findings suggest that downregulation of autophagy may be one of the approaches to control the impairment of energy metabolism leading to neuronal injury in the early development of Alzheimer's disease, and hesperetin or hesperidin may be a potential agent in the preventing of Alzheimer's disease progression.

Keywords:
Amyloid β / Autophagy / Glucose uptake / Hesperetin / Hesperidin

1 Introduction

Alzheimer's disease (AD) is pathologically characterized by abnormal deposition of senile plaques, which are primarily composed of amyloid β (Aβ) protein. Aβ is a physical peptide derived from sequential proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretase [1]. In addition, increasing studies show that not only insoluble amyloid fibrils but soluble oligomers of Aβ may be directly neurotoxic or indirectly toxic, which increase neuronal degeneration, death, and synaptic dysfunction [2].

In recent years, accumulating evidence from multiple clinics has suggested that metabolic diseases such as diabetes and obesity increase a risk for development of AD [3]. At least as early as 1980, researchers have speculated that metabolic abnormalities including impaired glucose consumption and energy metabolism commonly exist in Alzheimer's patients, which there may be a possible relationship between metabolic diseases and AD [4]. In addition, much evidence indicates that age-related impairment of brain energy utilization occurs prior to cognitive impairment, and impaired energy metabolism in brain is one of the earliest important hallmarks of AD [5].

The significant relationship between insulin, glucose transporters (GLUTs), and glucose utilization is well

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Abbreviations: AD, Alzheimer's disease; **APP**, amyloid precursor protein; **BBB**, blood-brain barrier; **GLUTs**, glucose transporters; **HIF-1α**, hypoxia-inducible factor 1α; **IRS**, insulin receptor substrate; **MAPKs**, mitogen-activated protein kinases; **PI3K**, phosphatidylinositol 3-kinase

established in many clinical and pathological studies [6]. In human brain, insulin–insulin receptor complex is crucial for neural synaptic activities which are essential for learning and memory. However, the levels of insulin and GLUTs are decreased in the brains of AD patients compared with healthy people. Recent evidence indicates that Aβ could inhibit insulin signaling in neurons by binding to synapses and decreasing activity of the membrane insulin receptor (IR) and/or by interfering Akt activation [7, 8]. Thus, Aβ accumulation and Aβ-impaired glucose transport may be the initiation factor in the development of AD.

In various cells, particularly under extra- or intracellular stress or injury condition, autophagy is a catabolic process for organelle turnover, which is involving the self-degradation of normal and aggregated protein through the lysosomal machinery. A large number of studies have indicated that autophagy may play an important role in human pathologies [9, 10]. Recent evidence has shown that Aβ accumulation induces cellular autophagy, supporting a role of autophagy on the neuropathologic hallmark of AD [11]. Modulation of autophagy might be a targeting strategy for AD therapy.

As mentioned above, Aβ deposition might be a major contributor to the process of impaired energy metabolism in human brain, which is one of the earliest important hallmarks of AD. Moreover, recent studies indicated that regulation of Aβ-induced autophagy should be a targeting strategy for neuropathologic therapy. However, the relationship between Aβ-induced autophagy and impaired energy metabolism in neuronal cells remains unclear.

Flavonoids are widely distributed in various vegetables and fruits, and possess many physiological and pharmacological functions. Many studies have suggested that dietary flavonoid supplements could reduce risk of neurodegenerative disorders in human being and neurotoxic effects of Aβ in animal models [12–14]. There is also evidence showing that flavonoids could reduce Aβ deposition in AD, which provides a therapeutic approach for Aβ pathology. Plant flavonoids are widely implicated to treat human pathology. Some citrus flavonoids such as hesperetin and hesperidin have been shown to possess cytoprotective effects by regulating cellular signaling pathways, including phosphatidylinositol 3-kinase (PI3K), tyrosine kinases, and mitogen-activated protein kinases (MAPKs) [15]. In addition, studies suggest that hesperetin and hesperidin can cross the blood-brain barrier (BBB) and possess good bioavailability [16, 17]. Although it is now known that flavonoids could provide a benefit to prevent the development of AD, the underlying neuroprotective effect of hesperetin and hesperidin in Aβ-induced neuron cells has not yet been reported.

In this study, we tested the hypothesis that the abnormalities of basal glucose utilization were caused by Aβ through the impairment of protein expression of GLUTs and insulin signaling pathway in insulin-stimulated neuronal cells, which resulted in more rapid development of AD. Moreover, the neuroprotective effects of hesperetin and hesperidin on Aβ-impaired glucose uptake as well as the insulin signaling cascades in insulin-stimulated neuronal cells were also investigated. Our results are the first evidence that natural flavonoids, such as hesperetin and hesperidin, might be a potential agent to improve Aβ-impaired glucose metabolism in insulin-stimulated neuronal cells.

2 Materials and methods

2.1 Chemicals

Sodium bicarbonate, trypsin, protease inhibitor, polyethylene glycol sorbitan monolaurate (Tween-20), dimethyl sulfoxide (DMSO), insulin, glycine, sodium chloride (NaCl), and Trizma® base were purchased from Sigma-Aldrich (St. Louis, MO). Tri-Isolation Reagent was purchased from MoBioPlus (Taipei, Taiwan). Sodium hydroxide (NaOH) was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Penicillin-streptomycin (PS) solution, fetal bovine serum (FBS), alpha-minimum essential medium (α-MEM), SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR kit (where PCR is polymerase chain reaction), SYBR® GreenER™ qPCR SuperMix for ABI PRISM® were purchased from Invitrogen (Carlsbad, CA). Isopropyl alcohol was purchased from Union Chemical Works Ltd. (Hsin Chu, Taiwan). 2-[^{14}C(U)] deoxy-D-glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). Radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Upstate (Billerica, MA). Disodium dihydrogen ethylenediaminetetraacetate dehydrate (EDTA-2Na) was purchased from Show chemical (Tokyo, Japan). Anti-phospho-Akt (anti-pAkt), anti-Akt antibodies, and anti-GLUT4 antibody were purchased from Cell signaling Technology (Beverly, MA). Anti-GLUT3 antibody was purchased from Santa Cruz (Santa Cruz, CA). Anti-LC3 antibody was purchased from Abgent (San Diego, CA).

2.2 Preparation of Aβ1-42

Amyloid β-peptide (Aβ1-42) was purchased from AnaSpec (Fremont, MO). Aβ1-42 was dissolved in calcium-free phosphate-buffered saline (PBS) to 1 mg/mL (a final concentration of 200 μM) and incubated at 37°C for 4 days to form Aβ aggregates. This solution was stored at −20°C.

2.3 Cell cultures

The Neuro-2A neuroblastoma cell line was obtained from the Bioresource Collection and Research Center (BCRC 60026, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in α-MEM medium, supplemented with 10% FBS and 1% streptomycin/penicillin (100 units/mL and 100 g/mL) at 37°C in a humidified atmosphere at 5% CO_2.

2.4 Glucose uptake

The Neuro-2A cells were seeded into six-well plates and cultured in DMEM plus 10% FBS. After 24 h, cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) for 48 h, followed by incubation with 100 nM insulin for 30 min. To clarify the relationship between Aβ1-42-induced glucose-uptake impairment and autophagy and assess the protective effects of hesperetin and hesperidin, cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) for 24 h, or treated with rapamycin (2 μM, an autophagic inducer) only and co-treated with 3-methyladenine (5 mM, an autophagic inhibitor) and Aβ1-42 (500 nM) for 24 h, respectively. Thereafter, cells were washed with PBS and kept in pre-warmed Hank's buffered salt solution (HBSS) solution (HBSS solution containing 5.4 mM KCl, 0.3 mM $Na_2HPO_4{\cdot}7H_2O$, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.6 mM $MgSO_4{\cdot}7H_2O$, 137 mM NaCl, 5.6 mM D-glucose, and 0.02% phenol red, and adjust to pH 7.4 with 1 M HCl or 1 M NaOH). 2-[^{14}C(U)]-deoxy-D-glucose was dispensed into each well for a final concentration of 0.1 Ci/mL HBSS and incubated for 1 h, and the reaction was terminated by three washes with ice-cold PBS. Cell-associated radioactivity was determined by lysing the cells with 1N NaOH, followed by liquid scintillation counting (Tri-carb 2800TR Liquid Scintillation Analyzer PerkinElmer Life and Analytical Sciences, Waltham, MA). Data are expressed as counts per minute 2-[^{14}C(U)]-deoxy-D-glucose per milligram protein.

2.5 RNA extraction and real-time PCR

The Neuro-2A cells were seeded into six-well plates and cultured in DMEM plus 10% FBS. After 24 h, cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) for 24 and 48 h, followed by incubation with 100 nM insulin for 30 min. After treatment, cellular RNA was extracted with a Tri-Isolation Reagent kit (MoBioPlus) as described in the manufacturer's manual. The forward and reverse primers for IR were 5′-acagccaccacactcacacttc-3′ and 5′-gccacactgcacctctcatctg-3′, respectively. The 5′ and 3′ primers for GLUT3 were 5′-gggcatcgttgttgggattctg-3′ and 5′-gcaggatctctgtagcttggtc-3′, respectively. The 5′ and 3′ primers for Hypoxia-inducible factor 1α (HIF-1α) were 5′-agctgccggcgacaccatcatc-3′ and 5′-gtgcaggatcagcactacttcg-3′, respectively. The 5′ and 3′ primers for housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GADPH) were 5′-tcaacggcacagtcaagg-3′ and 5′-actccacgacatactcagc-3′, respectively. Briefly, from each sample, cDNA corresponding to 1 μg of RNA was reverse-transcribed, using RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada) as described in the manufacturer's manual. PCR analyses were performed on the aliquots of the cDNA preparation to detect IR, GLUT3, HIF-1α, and GADPH (as an internal standard) gene expression in Neuro-2A cells using Smart Quant Green Master Mix with dUTP and ROX kit (Protech Technology, Taipei, Taiwan). All PCRs were performed using the 7300 Real-Time PCR System (Applied Biosystems, Foster city, CA). Quantification of a given gene, expressed as a relative mRNA level compared to the control, was calculated after normalization to GAPDH.

2.6 Western blot analysis

Neuro-2A cells were pretreated with hesperetin or hesperidin for 6 h then exposed to Aβ1-42 (500 nM) for 24 h, followed by incubation with 100 nM insulin for 30 min. To clarify the relationship between Aβ1-42-induced glucose-uptake impairment and autophagy and assess the protective effects of hesperetin and hesperidin, cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) for 24 h, or treated with rapamycin (2 μM, an autophagic inducer) only and co-treated with 3-methyladenine (5 mM, an autophagic inhibitor) and Aβ1-42 (500 nM) for 24 h, respectively. After treatment, the total proteins were extracted by adding RIPA lysis buffer (Upstate) and protease inhibitor to the cell pellets on ice for 10 min, followed by centrifugation at 12 000 rotations per minute for 15 min at 4°C. The cytosolic fraction (supernatant) proteins were boiled at 100°C for 5 min with 5× protein loading dye (2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 2.5% glycerol, 60 mM Tris-HCl [pH 6.8], and 14 mM 2-mercaptoethanol). Total cytosolic extracts were separated on 10% SDS-polyacrylamide minigels for pAkt, Akt, GLUT3, GLUT4, LC3, and β-actin protein detection, and then transferred to nitrate cellulose membrane (NC; Sartorius Stedim Biotech, Goettingen, Germany). The membrane was blocked in StartingBlock™ (PBS) blocking buffer (Thermo Scientific, Rickford, IL) for 30 min at room temperature and then incubated overnight at 4°C with indicated primary antibodies (1:1000 dilutions). After hybridization with primary antibodies, the membrane was washed with Tris buffered saline Tween-20 (TBST) three times, incubated with horseradish peroxide-linked secondary antibody (1:8000 dilutions) for 1 h at room temperature, and washed with TBST four times. Final detection was performed with enhance chemiluminescence (ELC) Western blotting reagents (Millipore, Billerica, MA) and detected by Biospectrum AC Imaging System (UVP, Upland, CA). The protein expression was normalized against β-actin. The intensity of the chemiluminescence signal was quantified using UVP VisionWorksLS Image Acquisition and Analysis Software (UVP).

2.7 Immunofluoresence staining

Cells were transfected with pSELECt-green fluorescent protein (GFP)-LC3 plasmid, and then treated with hesperetin or hesperidin (1 and 20 μM) for 6 h following Aβ1-42 (500 nM)

treatment for 24 h, or treated with rapamycin (2 μM, an autophagic inducer) only and co-treated with 3-methyladenine (5 mM, an autophagic inhibitor) and Aβ1-42 (500 nM) for 24 h, respectively. After treatment, the cells were seeded onto coverslips, treated with or without 1 μM pemetrexed for 48 h and harvested. The cells were washed twice with PBS, incubated with 0.2 μM MitoTracker probe at 37°C for 1 h, and then fixed with 2% paraformaldehyde at room temperature for 20 min. The cells were permeabilized with 0.1% Triton X-100/PBS solution at room temperature for 30 min. After air-drying, the cells were incubated with a monoclonal antibody against LC3 at 37°C for 1 h. The coverslips were then washed three times with PBS and detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. In addition, an aliquot of the suspension (200 μL) for each treatment was loaded into a 96-well plate for densitometry analyses of LC3 immunofluoresence staining. The LC3 fluorescence intensity was detected using a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies, Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. After detection of fluorescence intensity, the total protein of cells was measured by Bradford assay for internal control in each well.

2.8 Statistical analysis

Each experiment was performed in triplicate and repeated three times. The results were expressed as means ± standard deviation (SD). Statistical comparisons were made by one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered significant when the p values were <0.05.

3 Results

3.1 Effects of hesperetin and hesperidin against Aβ1-42-induced glucose uptake defection in insulin-stimulated Neuro-2A cells

Growing evidence has demonstrated that an increased production of Aβ could lead to impaired energy metabolism, which might be an early hallmark in the progression of AD [1]. In this study, results showed that Aβ1-42 attenuated glucose uptake of insulin-stimulated Neuro-2A cells in a concentration-dependent manner (100–500 nM) (Fig. 1A). However, a significant increase in glucose uptake was observed with the treatment of hesperetin or hesperidin (1 and 20 μM) in Aβ-induced Neuro-2A cells (Fig. 1B), suggesting that hesperetin and hesperidin might be able to improve impaired energy metabolism to decrease Aβ-stimulated neuronal injury. In our preliminary test, the cell viability of Neuro-2A cells was tested using the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay and was not significantly affected when incubated with Aβ1-42 (100–500 nM) and hesperetin or hesperidin (1 and 20 μM) alone.

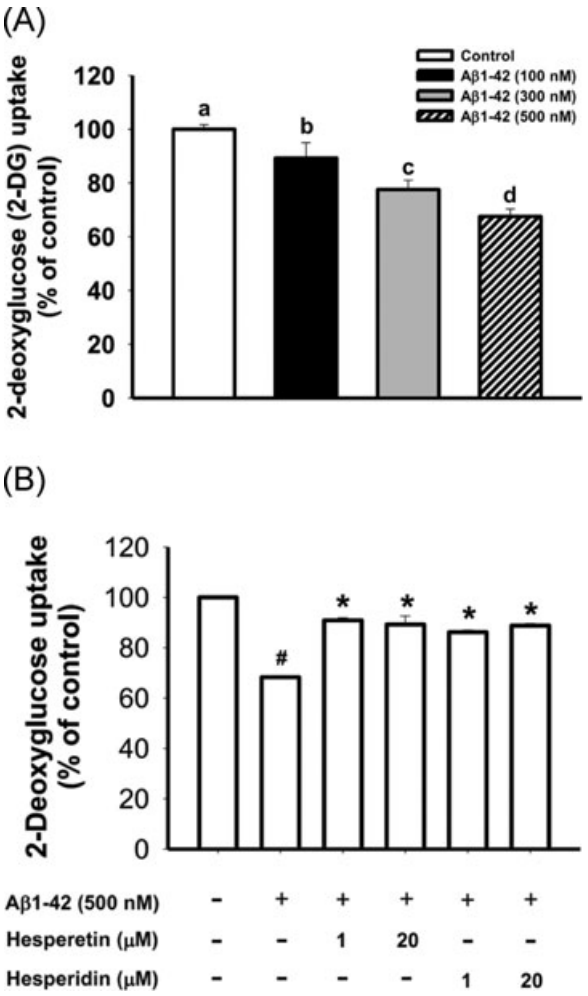

Figure 1. Effects of hesperetin and hesperidin against Aβ1-42-induced glucose uptake defection in insulin-stimulated Neuro-2A cells. (A) Cells were treated with or without Aβ1-42 (100–500 nM) for 48 h, followed by insulin (100 nM) treatment for 30 min. (B) Cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) for 48 h, followed by insulin (100 nM) treatment for 30 min. Results are means ± SD for $n = 3$. Data with different letters (a, b, c, d) indicate significant difference ($p < 0.05$) by Duncan's test. #, significantly different from control group ($p < 0.05$). *, significantly different from Aβ treatment only ($p < 0.05$).

3.2 Effects of hesperetin and hesperidin on the mRNA expression of IR in Aβ1-42-treated Neuro-2A cells

Clinical research has suggested that people with AD have a lower content of GLUT3 and HIF-1α protein as compared to normal people [18]. As shown in Fig. 2A, treatment with Aβ for 24 and 48 h significantly inhibited IR gene expression in insulin-stimulated Neuro-2A cells. However, the gene expression of GLUT3 and HIF-1α were not significantly affected by Aβ treatment in insulin-stimulated Neuro-2A cells (Fig. 2B

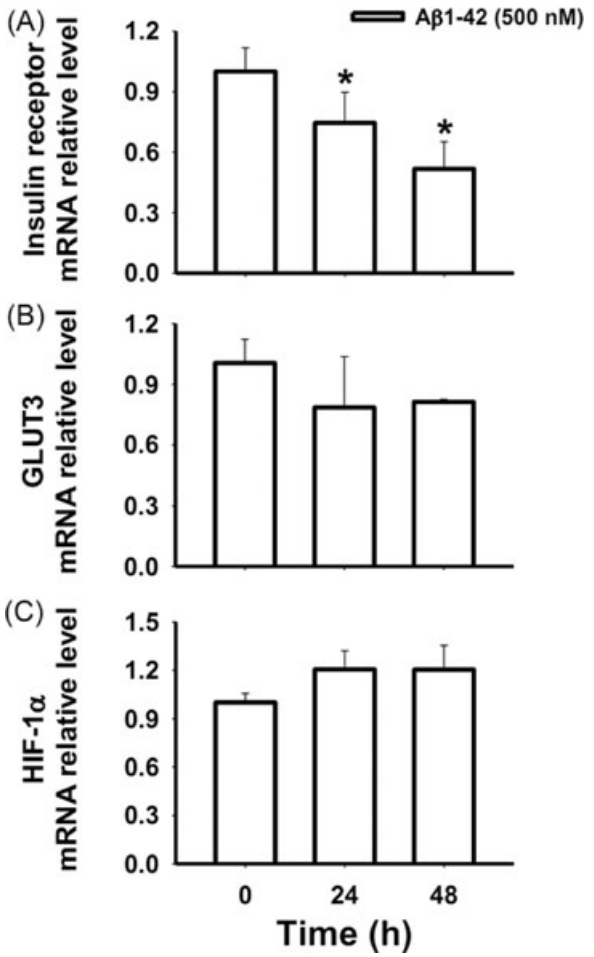

Figure 2. Effect of Aβ1-42 on the mRNA expressions of IRs (A), GLUT3 (B), and HIF-1α (C) in insulin-treated Neuro-2A cells. Cells were treated with or without Aβ1-42 (500 nM) for 24 and 48 h, respectively, followed by insulin (100 nM) treatment for 30 min. Results are means ± SD for $n = 3$. *, significantly different from control group ($p < 0.05$).

and C). Additionally, the data indicated that hesperetin or hesperidin treatment of Neuro-2A cells for 24 h could significantly stimulate the gene expression of IR in Aβ-stimulated Neuro-2A cells, suggesting that hesperetin and hesperidin might regulate insulin action to increase glucose utilization (Fig. 3).

3.3 Effects of hesperetin and hesperidin on regulators (Akt, GLUT3, and GLUT4) of insulin signaling pathway in Aβ1-42-induced Neuro-2A cells

Akt is a key signaling molecule of the IR pathway downstream of insulin receptor substrate (IRS) and PI3 kinase, which participates in insulin-stimulated GLUT4 translocation [8]. As shown in Fig. 4, treatment with Aβ1-42 of Neuro-2A cells for 24 h attenuated the protein expression of GLUT4 as well as the protein phosphorylation of Akt in insulin-stimulated Neuro-2A cells. Aβ1-42 also decreased the protein expression of GLUT3 that was the main facilitative GLUT in neurons. However, a significant increase in the protein expression of GLUT3 and GLUT4 as well as the protein phosphorylation of Akt was observed after treatment with hesperetin or hesperidin of Neuro-2A cells for 24 h, indicating that hesperetin and hesperidin might be able to improve Aβ1-42-stimulated impairment of insulin signaling cascade through regulating IRS-PI3K-Akt signal transduction.

3.4 Effects of hesperetin and hesperidin on Aβ1-42-induced autophagy associated with impairment of glucose uptake in insulin-stimulated Neuro-2A cells

Aβ has been implicated in many studies to have an effect on neurodegenerative diseases; however, Aβ is also a substrate of cellular autophagy. In this study, we found that Aβ1-42 caused the impairment of glucose uptake in insulin-stimulated Neuro-2A cells, and this phenomenon might relate to cellular autophagy. As shown in Fig. 5A, treatment with rapamycin (2 μM, an autophagic inducer) of Aβ-induced Neuro-2A cells for 24 h, significantly induced LC3 protein conversion (LC3-I to LC3-II; a process of autophagy [9]), respectively, treatment with 3-methyladenine (5 mM, an autophagic inhibitor) significantly eliminated LC3 protein conversion. The data indicated that regulating autophagy function might be involved in the progression and/or

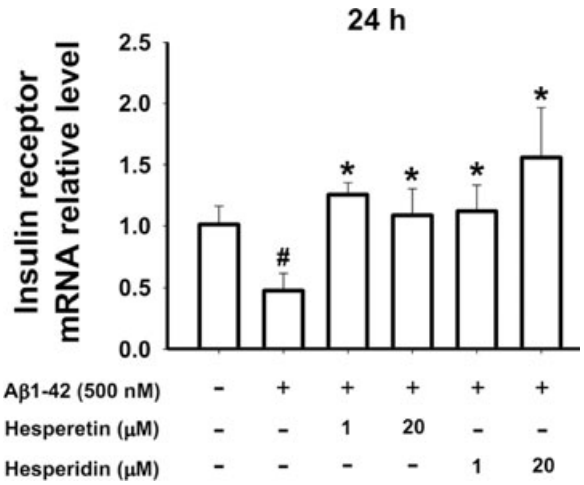

Figure 3. Effects of hesperetin and hesperidin on the mRNA expression of IRs in Aβ1-42 treated Neuro-2A cells. Cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) for 24 h, followed by insulin (100 nM) treatment for 30 min. Results are means ± SD for $n = 3$. #, significantly different from control group ($p < 0.05$). *, significantly different from Aβ treatment only ($p < 0.05$).

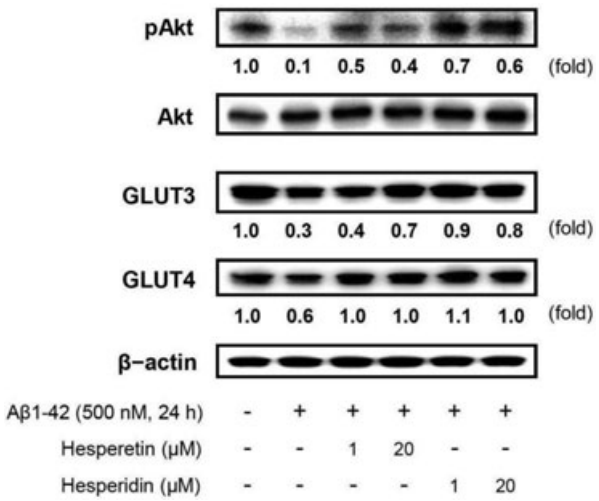

Figure 4. Effects of hesperetin and hesperidin on insulin signaling (Akt, GLUT3, and GLUT4) in Aβ1-42-induced Neuro-2A cells. Cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) for 24 h, followed by insulin (100 nM) treatment for 30 min. Total cell lysates were prepared after treatment. Proteins separated by SDS-PAGE (polyacrylamide gel electrophoresis) were immunoblotted and probed with antibodies of phospho-Akt, Akt, GLUT3, GLUT4, and β-actin. The relative density was compared with untreated control. Reported values are the means ± SD ($n = 3$).

development of neurodegenerative diseases. The intracellular images of GFP-LC3 punctate formation were also confirmed by confocal microscopy shown in Fig. 5B and 5C. Moreover, inhibition of autophagy could increase insulin-stimulated glucose uptake in Aβ-induced Neuro-2A cells, respectively, insulin-stimulated glucose uptake inhibited by activation of autophagy (Fig. 5D). However, treatment with hesperetin or hesperidin could stimulate glucose uptake by inhibiting Aβ-induced autophagy in insulin-stimulated Neuro-2A cells (Fig. 5D).

4 Discussion

Alzheimer's disease (AD) is a common neurodegenerative disorder, which is pathologically characterized by Aβ deposition in human brain. The amyloidogenesis is an alternative process for APP which leads to Aβ peptides production. Aβ is produced through the endoproteolytic cleavage of the parental APP by a group of sheddases named α-, β-, and γ-secretase [18]. Most Aβ peptide generated by secretases is predominantly the 40-residue peptide (Aβ1-40) and smaller amounts of a 42-residue peptide (Aβ1-42). The Aβ1-42 peptide with additional hydrophobic residues is more hydrophobic and more prone to formation of toxic amyloid fibril than Aβ1-40 peptide. Moreover, Aβ1-42 aggregates more readily than Aβ1-40, suggesting that the high level of Aβ1-42 in brain is a major risk of AD development [19].

Insulin is a well-known biological agent to promote glucose uptake in muscle, endothelial and neuron cells [20], etc. In vitro work has shown that Aβ is an inhibitor of insulin binding to its receptor, and an increased production of Aβ could lead to impaired energy metabolism, which might be an early hallmark in the progression of AD. Moreover, recent studies have reported that metabolic diseases, such as diabetes and obesity associated with impaired energy metabolism, would increase a risk for the development of AD [21]. In agreement with these findings, there might be a possible relationship between impaired glucose utilization and pathology of AD. In this study, we used a toxic Aβ1-42 peptide to test the hypothesis that Aβ might lead to an impairment of insulin-stimulated 2-deoxyglucose (2DG) uptake in Neuro-2A cells. As shown in Fig. 1A, Neuro-2A cells were treated with Aβ1-42 (100–500 nM) for 48 h, followed by 100 nM of insulin treatment for 30 min. Compared with control condition, Aβ treatment of Neuro-2A led to a significant decrease in insulin-stimulated glucose uptake. Our results indicated that toxic Aβ could interfere with glucose metabolism or insulin action in normal neuronal cells.

The physiological consequences of Aβ accumulation have been widely implicated in the accelerated neuronal injury, which eventually results in the pathogenesis of AD [22, 23]. There is a growing evidence of Aβ-induced neuronal injury, which may be due to Aβ-impaired energy metabolism in neuronal cells [24]. In this study, a significant increase in insulin-stimulated glucose uptake was observed with the treatment of hesperetin and hesperidin in Aβ1-42-treated Neuro-2A cells (Fig. 1B), suggesting that hesperetin and hesperidin might be able to improve impaired energy metabolism to decrease Aβ1-42-stimulated neuronal injury.

Glucose is an essential metabolic substrate of all mammalian cells for energy utilization. Glucose enters the cells by facilitated diffusion, which is mediated by membrane transporter system named GLUTs. GLUT family can be divided into two types, the facilitative and the sodium-dependent GLUTs. Among the facilitative GLUT family, six functional isoforms, including GLUT1–4, GLUT5, and GLUT7, have been identified and characterized [25]. Glucose binds to the membrane facilitative GLUTs that is an essential process for glucose uptake [26]. Evidence has indicated that glucose uptake in the brain is partly mediated by GLUT3 which is the main facilitative GLUT in neurons [27]. Moreover, previous studies have reported that levels of such facilitative GLUT are decreased in AD brain, hypothesizing that impaired GLUTs may cause energy dysfunction leading to neuronal injury [28].

HIF-1α is one of the transcription factors of GLUT3, it can regulate the expression of GLUT3 in glucose sensing in many cells. Following the study [29], the authors indicated that people with AD had a lower content of GLUT3 and HIF-1α protein as compared to normal people. In this study, we found that Aβ1-42 significantly inhibited IR gene expression in insulin-stimulated Neuro-2A cells (Fig. 2A). However, the gene expression of GLUT3 and HIF-1α were not significantly

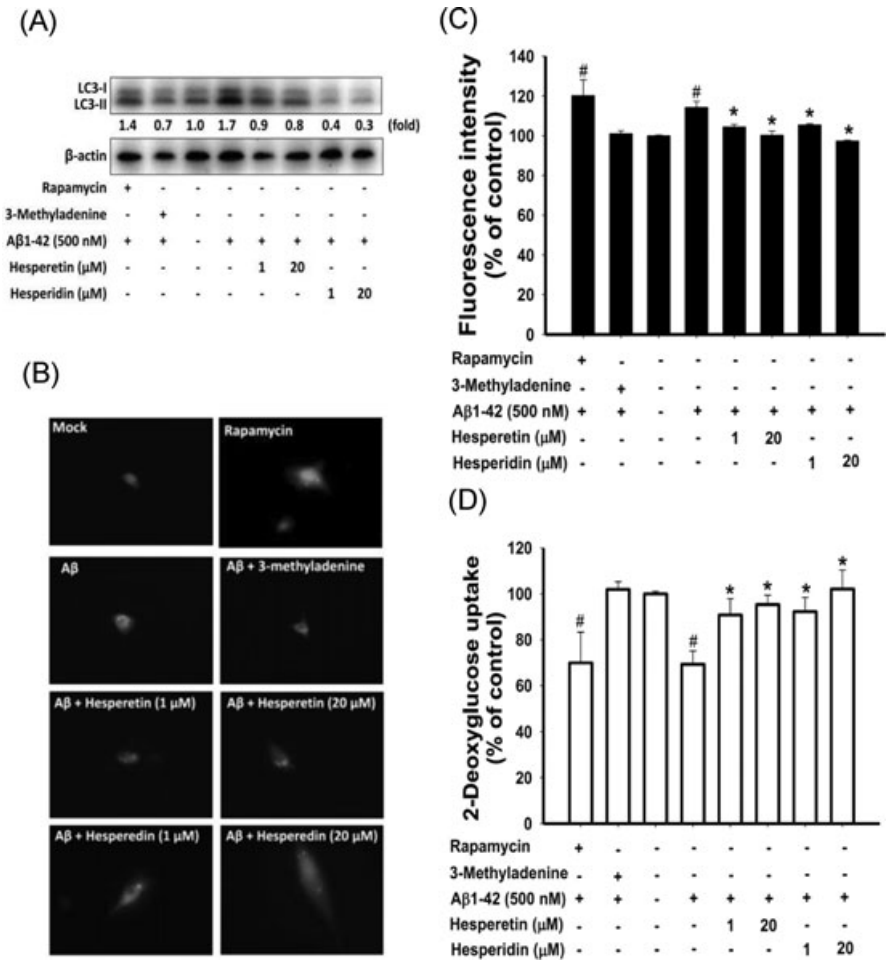

Figure 5. Effects of hesperetin and hesperidin on Aβ1-42-induced autophagy associated with impairment of glucose uptake in insulin-stimulated Neuro-2A cells. Cells were pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) only, or co-treated with Aβ1-42 and rapamycin (2 μM, an autophagic inducer) for 24 h; co-treated with 3-methyladenine (5 mM, an autophagic inhibitor) and Aβ1-42 (500 nM) for 24 h, respectively. After treatment, the cells were subjected to (A) probed with antibodies of LC3 complex and β-actin by Western blot. (B) Cells were transfected with pSELECt-GFP-LC3 plasmid, and then pretreated with hesperetin or hesperidin (1 and 20 μM) for 6 h then exposed to Aβ1-42 (500 nM) only, or co-treated with Aβ1-42 and rapamycin (2 μM, an autophagic inducer) for 24 h; co-treated with 3-methyladenine (5 mM, an autophagic inhibitor) and Aβ1-42 (500 nM) for 24 h, respectively. Intarcellular images of GFP-LC3 punctate were analyzed using confocal microscopy. Magnification, ×40. (C) The results of densitometry analyses from panel (B). Results are means ± SD for $n = 3$. # and * mean significantly different from control group and Aβ-treated alone group at $p < 0.05$, respectively. (D) Measurement of glucose utilization followed by ^{14}C-labeled 2DG uptake.

affected by treating Aβ1-42 in insulin-stimulated Neuro-2A cells. Our data suggest that Aβ1-42 might directly compete for insulin binding to IRs and contribute to the impaired glucose utilization in neurons. In this study, hesperetin and hesperidin significantly increased glucose uptake in Aβ1-42-stimulated Neuro-2A cells (Fig. 1B). Moreover, we also found that hesperetin and hesperidin could significantly stimulate the gene expression of IR in Aβ1-42-stimulated Neuro-2A cells, suggesting that hesperetin and hesperidin might regulate insulin action to increase glucose utilization.

Besides the GLUT3 isoform, GLUT4 is of major importance for the glucose transport under the influence of insulin. In response to insulin action, GLUT4 enhanced glucose uptake by translocation from the intracellular storage sites to membrane plasma [30]. Evidence indicates that GLUT4 can be found and mediate insulin-stimulated glucose uptake in specific neuronal tissue within brain, which appears to correspond to the possibility of GLUT4 regulation by insulin action [31]. Recently, a defect in GLUT4 activity has been implicated in insulin resistance or pre-type 2 diabetes that may be linked to progression of AD. Supporting evidence demonstrated that Aβ induces impairment of neuronal PI3K-Akt insulin signaling cascades [32]. Akt is a key signaling molecule of the IR pathway downstream of IRS and PI3 kinase, which participates in insulin-stimulated GLUT4 translocation. In this study, we found that Aβ1-42 decreased the protein expression of GLUT3, GLUT4, and the protein phosphorylation of Akt in insulin-stimulated Neuro-2A cells (Fig. 4), suggesting that impaired insulin signaling pathway was response to Aβ1-42. However, a significant increase in protein expression of GLUT3, GLUT4, and the protein phosphorylation of Akt was observed after treatment with hesperetin and hesperidin, indicating that hesperetin and hesperidin might be able to improve Aβ1-42-stimulated impairment of insulin signaling cascade through regulating IRS-PI3K-Akt signal transduction.

Autophagy is a catabolic process for organelle turnover through the lysosomal machinery, which is involving the self-degradation of normal and aggregated protein in response to cellular stress and starvation. Recent studies have indicated that Aβ is a substrate of cellular autophagy, suggesting that regulating autophagy function might play a critical role in the progression and/or development of neurodegenerative diseases. In this study, we found that inhibition of autophagy could increase insulin-stimulated glucose uptake in Aβ-induced Neuro-2A cells, whereas insulin-stimulated

glucose uptake inhibited by promoting autophagy (Fig. 5A-C). Besides, treatment with hesperetin and hesperidin also stimulated glucose uptake by inhibiting Aβ-induced autophagy in Neuro-2A cells (Fig. 5D). However, for instance [33, 34], autophagy plays a role of scavenger in response to Aβ generation, and that presenilin-1, a part of γ-secretase complex related to Aβ formation, promotes autophagic dysfunction. In fact, it is still unclear why Aβ generation might both stimulate and impair cellular autophagy at the same time. According to clinical treatment, a growing body of research has suggested that autophagy should be carefully regulated in specific steps of targeting AD therapy [35, 36]. In this study, our evidence confirmed that deregulation of Aβ-induced autophagy could promote insulin-stimulated glucose uptake in neuronal cells, suggesting that inhibition of autophagy might be one approach to improve the impairment of glucose metabolism in the early stage of metabolic diseases, such as prediabetes and obesity, leading to AD progression. Additionally, in our ongoing study (data not shown), we have found that Aβ-induced impairment of glucose uptake might affect neuronal growth. Interestingly, our ongoing study further hypothesizes that neuronal death or growth might participate in the regulating process between Aβ-induced autophagy and Aβ-impaired energy metabolism, and it might also be an early hallmark in the progression of AD.

In conclusion, the present study shows that Aβ-induced autophagy leads to the impairment of glucose metabolism in insulin-stimulated Neuro-2A cells. Natural flavonoids, especially hesperetin and hesperidin, are able to improve Aβ-impaired glucose uptake partly by inhibiting autophagy. Our evidence provides two useful implications. First, downregulation of autophagy may be one of the ways to control the impairment of energy metabolism leading to neuronal injury in the early stage of AD development. Second, selected hesperetin or hesperidin may be a potential agent in the prevention of AD progression.

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5 References

[1] Querfurth, H. W., LaFerla, F. M., Alzheimer's disease. *N. Engl. J. Med.* 2010, *362*, 329–344.

[2] Hardy, J., Selkoe, D. J., The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 2002, *297*, 353–356.

[3] Ott, A., Stolk, R. P., Hofman, A., van Harskamp, F. et al., Association of diabetes mellitus and dementia: the Rotterdam Study. *Diabetologia* 1996, *39*, 1392–1397.

[4] Hoyer, S., Brain glucose and energy metabolism abnormalities in sporadic Alzheimer disease causes and consequences: an update. *Exp. Gerontol.* 2000, *35*, 1363–1372.

[5] de la Monte, S. M., Wands, J. R., Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. *J. Alzheimers Dis.* 2005, *7*, 45–61.

[6] Park, C. R., Cognitive effects of insulin in the central nervous system. *Neurosci. Biobehav. Rev.* 2001, *25*, 311–323.

[7] Ma, Q. L., Yang, F., Rosario, E. R., Ubeda, O. J. et al., Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin. *J. Neurosci.* 2009, *29*, 9078–9089.

[8] Lee, H. K., Kumar, P., Fu, Q., Rosen, K. M. et al., The insulin/Akt signaling pathway is targeted by intracellular beta-amyloid. *Mol. Biol. Cell.* 2009, *20*, 1533–1544.

[9] Eskelinen, E. L., Saftig, P., Autophagy: a lysosomal degradation pathway with a central role in health and disease. *Biochimica. Biophysica. Acta* 2009, *1793*, 664–673.

[10] He, C., Klionsky, D. J., Regulation mechanisms and signaling pathways of autophagy. *Annu. Rev. Genet.* 2009, *43*, 67–93.

[11] Wong, E., Cuervo, A. M., Autophagy gone awry in neurodegenerative diseases. *Nat. Neurosci.* 2010, *13*, 805–811.

[12] Bu, L., Lephart, E. D., Soy isoflavones modulate the expression of BAD and neuron-specific beta III tubulin in male rat brain. *Neurosci. Lett.* 2005, *385*, 153–157.

[13] Yoon, I., Lee, K. H., Cho, J., Gossypin protects primary cultured rat cortical cells from oxidative stress- and beta-amyloid-induced toxicity. *Arch. Pharm. Res.* 2004, *27*, 454–459.

[14] Weinreb, O., Mandel, S., Amit, T., Youdim, M. B., Neurological mechanisms of green tea polyphenols in Alzheimer's and Parkinson's diseases. *J. Nutr. Biochem.* 2004, *15*, 506–516.

[15] Rainey-Smith, S., Schroetke, L. W., Bahia, P., Fahmi, A. et al., Neuroprotective effects of hesperetin in mouse primary neurones are independent of CREB activation. *Neurosci. Lett.* 2008, *438*, 29–33.

[16] Manach, C., Morand, C., Gil-Izquierdo, A., Bouteloup-Demange, C. et al., Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *Eur. J. Clin. Nutr.* 2003, *57*, 235–242.

[17] Youdim, K. A., Dobbie, M. S., Kuhnle, G., Proteggente, A. R. et al., Interaction between flavonoids and the blood-brain barrier: in vitro studies. *J. Neurochem.* 2003, *85*, 180–192.

[18] De Strooper, B., Vassar, R., Golde, T., The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat. Rev. Neurol.* 2010, *6*, 99–107.

[19] Li, Q. X., Fuller, S. J., Beyreuther, K., Masters, C. L., The amyloid precursor protein of Alzheimer disease in human brain and blood. *J. Leukoc. Biol.* 1999, *66*, 567–574.

[20] Mielke, J. G., Wang, Y. T., Insulin, synaptic function, and opportunities for neuroprotection. *Prog. Mol. Biol. Transl. Sci.* 2011, *98*, 133–186.

[21] Schubert, D., Glucose metabolism and Alzheimer's disease. *Ageing Res. Rev.* 2005, *4*, 240–257.

[22] Dumurgier, J., Paquet, C., Peoc'h, K., Lapalus, P. et al., CSF Aβ1–42 levels and glucose metabolism in Alzheimer's disease. *J. Alzheimers Dis.* 2011, *26*, 1–7.

[23] LaFerla, F. M., Green, K. N., Oddo, S., Intracellular amyloid-beta in Alzheimer's disease. *Nat. Rev. Neurosci.* 2007, *8*, 499–509.

[24] Dumurgier, J., Paquet, C., Benisty, S., Kiffel, C. et al., Inverse association between CSF Aβ 42 levels and years of education in mild form of Alzheimer's disease: the cognitive reserve theory. *Neurobiol. Dis.* 2010, *40*, 456–459.

[25] McEwen, B. S., Reagan, L. P., Glucose transporter expression in the central nervous system: relationship to synaptic function. *Eur. J. Pharmacol.* 2004, *490*, 13–24.

[26] Duelli, R., Kuschinsky, W., Brain glucose transporters: relationship to local energy demand. *News Physiol. Sci.* 2001, *16*, 71–76.

[27] Haber, R. S., Weinstein, S. P., O'Boyle, E., Morgello, S., Tissue distribution of the human GLUT3 glucose transporter. *Endocrinology* 1993, *132*, 2538–2543.

[28] Mooradian, A. D., Chung, H. C., Shah, G. N., GLUT-1 expression in the cerebra of patients with Alzheimer's disease. *Neurobiol. Aging* 1997, *18*, 469–474.

[29] Mobasheri, A., Richardson, S., Mobasheri, R., Shakibaei, M. et al., Hypoxia inducible factor-1 and facilitative glucose transporters GLUT1 and GLUT3: putative molecular components of the oxygen and glucose sensing apparatus in particular chondrocytes. *Histol. Histopathol.* 2005, *20*, 1327–1338.

[30] Liu, Y., Liu, F., Iqbal, K., Grundke-Iqbal, I. et al., Decreased glucose transporters correlate to abnormal hyperphosphorylation of tau in Alzheimer disease. *FEBS Lett.* 2008, *582*, 359–364.

[31] Foley, K., Boguslavsky, S., Klip, A., Endocytosis, recycling, and regulated exocytosis of glucose transporter 4. *Biochemistry* 2011, *50*, 3048–3061.

[32] Reagan, L. P., Glucose, stress, and hippocampal neuronal vulnerability. *Int. Rev. Neurobiol.* 2002, *51*, 289–324.

[33] Yu, W. H., Cuervo, A. M., Kumar, A., Peterhoff, C. M. et al., Macroautophagy-a novel beta-amyloid peptide-generating pathway activated in Alzheimer's disease. *J. Cell. Biol.* 2005, *171*, 87–98.

[34] Wilson, C. A., Murphy, D. D., Giasson, B. I., Zhang, B. et al., Degradative organelles containing mislocalized alpha and beta-synuclein proliferate in presenilin-1 null neurons. *J. Cell. Biol.* 2004, *165*, 335–346.

[35] Martinez-Vicente, M., Cuervo, A. M., Autophagy and neurodegeneration: when the cleaning crew goes on strike. *Lancet Neurol.* 2007, *6*, 352–361.

[36] Jaeger, P. A., Wyss-Coray, T., All-you-can-eat: autophagy in neurodegeneration and neuroprotection. *Mol. Neurodegener.* 2009, *4*, 16–37.

