Global analysis of ginsenoside Rg1 protective effects in \(\beta\)-amyloid-treated neuronal cells

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Background: A number of reports have described the protective effects of ginsenoside Rg1 (Rg1) in Alzheimer’s disease (AD). However, the protective mechanisms of Rg1 in AD remain elusive.

Methods: To investigate the potential mechanisms of Rg1 in \(\beta\)-amyloid peptide-treated SH-SY5Y cells, a comparative proteomic analysis was performed using stable isotope labeling with amino acids in cell culture combined with nano-LC-MS/MS.

Results: We identified a total of 1,149 proteins in three independent experiments. Forty-nine proteins were significantly altered by Rg1 after exposure of the cells to \(\beta\)-amyloid peptides. The protein interaction network analysis showed that these altered proteins were clustered in ribosomal proteins, mitochondria, the actin cytoskeleton, and splicing proteins. Among these proteins, mitochondrial proteins containing HSD17B10, AARS2, TOMM40, VDAC1, COX5A, and NDUF4 were associated with mitochondrial dysfunction in the pathogenesis of AD.

Conclusion: Our results suggest that mitochondrial proteins may be related to the protective mechanisms of Rg1 in AD.

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1. Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disorder, usually occurring in people older than 65 years [1]. AD is pathologically characterized by brain shrinkage, amyloid-rich senile plaques, and neurofibrillary tangles [2]. The clinical symptoms of AD are loss of memory, poor recognition ability, changes in personal character, and loss of body control [3,4]. It has been reported that the main cause of AD is the accumulation of \(\beta\)-amyloid peptides (A\(_\beta\)) [5]. The A\(_\beta\) is produced from the amyloid precursor protein, which is cleaved by \(\beta\)-secretase and \(\gamma\)-secretase [6]. The cleaved A\(_\beta\) gives rise to oligomeric forms, which form plaques in the brain [7]. The plaque formations trigger the pathological cascade in AD [4]. A\(_\beta_{25-35}\), a very short fragment of A\(_\beta\), exhibits large \(\beta\)-sheet fibrils and retains the toxicity of the full-length peptides. Thus, A\(_\beta_{25-35}\) represents the biologically active region of A\(_\beta\) [8]. It has been reported that A\(_\beta_{25-35}\) leads to mitochondrial dysfunctions by blocking the entrance of nuclear-encoded proteins into mitochondria, which diminishes the mitochondrial membrane potential and alters mitochondrial morphology [9].

Ginseng has been a popular and widely used traditional herbal medicine in Asia for thousands of years. Previous studies have suggested that the components of ginseng exert beneficial effects on neurodegenerative diseases [10]. Ginseng suppresses oxidative stress, regulates apoptotic genes, and inhibits the toxicity of excitatory amino acids [11,12]. Ginseng is classified into two groups: American ginseng and Asian ginseng. More than 38 ginsenosides, which are known as the major biologically active components, have been identified in Asian ginseng, and one of the major bioactive components is the ginsenoside Rg1 (Rg1) [13]. A number of studies have demonstrated the neuroprotective effects of Rg1 in neurodegenerative disorders such as AD [14]. It has been reported that \(\beta\)-secretase activity and A\(_\beta\)-induced cytotoxicity is inhibited by Rg1 [15]. Although numerous studies have been conducted to assess the
neuroprotective effects of Rg1, the protective mechanisms of Rg1 in AD remain elusive.

In the present study, we investigated the protective effects of Rg1 on SH-SYSY cell damage induced by Aβ_{25–35}. We performed an MS-based proteomic experiment using stable isotope labeling with amino acids in cell culture (SILAC) to explore the differential proteome expression associated with the protective effects of Rg1 in Aβ-treated neuronal cells. Our data may elucidate the neuroprotective molecular mechanism of Rg1 in AD.

2. Materials and methods

2.1. Aβ_{25–35} aggregation

Aβ_{25–35} (AnaSpec, Fremont, CA, USA) was dissolved in distilled water at a concentration of 2.5 mM and the solutions was incubated at 37°C for one week before use [16,17].

2.2. SILAC

SILAC was performed as previously reported [18]. Briefly, SH-SYSY cells were cultured for at least five cell divisions in either light SILAC medium containing 12C_6-Arg and 12C_6, 14N_2-Lys or heavy SILAC medium containing 13C_6-Arg and 13C_6, 15N_2-Lys supplemented with 10% dialyzed fetal bovine serum, 50 IU/mL penicillin, 50 μg/mL streptomycin. The labeled cells were treated with 10% dialyzed fetal bovine serum, 50 IU/mL penicillin, 50 μg/mL streptomycin. The labeled cells were treated with (light medium) or without (heavy medium) 100μM Rg1 for 24 h in 25mM of Aβ_{25–35}-treated cells for 24 h. The cells were lysed in buffer containing 1% Triton X-100, 150mM NaCl, 1mM EDTA, 50mM Tris-HCl (pH 8.0), 1mM sodium orthovanadate, 5mM NaF, 5mM sodium pyrophosphate, 1mM phenylmethylsulfonyl fluoride, apro tinin (1.5 μg/mL), antipain (10 mg/mL), leupeptin (10 μg/mL), and benzamidine (100 μg/mL). The lysates were centrifuged at 160,000g and mixed at a 1:1 protein ratio. The mixed lysates were separated by 10% SDS-PAGE and visualized with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA, USA).

2.3. In-gel digestion

Each gel was cut into 10 bands of equal size, de-stained with 50% acetonitrile (ACN) in 25mM ammonium bicarbonate and dried in a speed vacuum concentrator. The dried gel pieces were re-swollen with 25mM ammonium bicarbonate and dried in a speed vacuum concentrator. The dried peptide samples were re-resolved in 2% ACN/0.1% FA and concentrated on a large-capacity HPLC Chip (Agilent Technologies). The HPLC chip incorporated an enrichment column (9 mm, 75 mm-inner diameter, 160 nL) and a reverse-phase column (15 cm, 75 mm-inner diameter, packed with Zorbax 300SB-C18 5 mm resins). The peptide separation was performed using a 70-min gradient of 3–45% buffer B (buffer A contained 0.1% FA/dry weight, and buffer B contained 90% ACN/0.1% FA/dry weight) at a flow rate of 300 nL/min. The MS and MS/MS data were acquired in positive ion mode and stored in centroid mode. The chip spray voltage was set at 1950 V and maintained under chip conditions.

The drying gas temperature was set at 325°C with a flow rate of 3.5 L/min. A medium isolation (4 m/z) window was used for precursor isolation. A collision energy with a slope of 3.7 V/100 Da and an offset of 2.5 V were used for fragmentation. Additionally, while the MS data were acquired over a mass range of 300–3000 m/z, the MS/MS data were acquired over a mass range of 50–2500 m/z. Reference mass correction was performed using a reference mass of 922.0098. Precursors were set in an exclusion list for 0.5 min after two MS/MS spectra. The MS and MS/MS spectra were extracted using the Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies) with default parameters. Database searches using the X! Tandem search engine were performed with a peptide mass tolerance of 20 ppm, an MS/MS tolerance of 0.5 Da, and a strict tryptic specificity (cleavage after lysine and arginine) allowing two missed cleavage sites; carbamidomethylation of Cys was set as a fixed modification, whereas oxidation (M) was considered a variable modification.

2.5. Protein validation and quantification

The MS database search results were analyzed using the Trans-Proteomic Pipeline (Systems Biology, Seattle, WA, USA), all assigned peptides were validated by PeptideProphet and the results filtered with an error rate of 0.05 (FDR of 5%) [19]. Peptide quantification was performed using the X! Tandem search engine and stored in centroid mode. The chip spray voltage was set at 1950 V and maintained under chip conditions.

2.6. Bioinformatics analysis

Functional protein–protein network analysis was performed using the STRING web-based database Cytoscape plug-in [20]. The subcellular locations of the identified proteins were analyzed using the Uniprot database.

3. Results and discussion

To investigate the mechanisms associated with the neuroprotective activity of Rg1 in AD, we performed a comparative proteomic analysis of SH-SYSY cells, which were treated with Rg1 after Aβ exposure using nano-LC-MS/MS combined with SILAC. We cultured SH-SYSY cells in either light SILAC medium containing 12C_6-Arg and 12C_6, 14N_2-Lys or heavy medium containing 13C_6-Arg and 13C_6, 15N_2-Lys to label the cells with the stable isotopes [18]. Previous studies have shown that fresh and aged Aβ_{25–35} decreases the cell survival rate based on the results of the MTT reduction assay [21]. Therefore, we used aged Aβ_{25–35} to induce neuronal toxicity in SH-SYSY cells. Cells cultured in light medium were exposed to aged Aβ_{25–35} and then treated with 100μM Rg1 [22], whereas cells cultured in heavy medium were treated with DMSO exposure to aged Aβ_{25–35} (Fig. 1A).

Three independent SILAC combined with LC-MS/MS experiments were performed. Fig. 1B shows a representative ion chromatogram of the AVIFCLSADKX peptide of destrin, and the monoisotopic peaks in the MS spectra of AVIFCLSADKX showed an expected 1:1 ratio (Fig. 1C). A total of 2,476 proteins were identified in two different physiological conditions. Of these, 1,149 proteins were common to the three data sets, and 592 proteins were
quantiﬁed in at least two of the three sets (Fig. 2A and Table S1). Among the quantiﬁed proteins, 49 were signiﬁcantly changed more than 1.5-fold; four proteins were downregulated and 45 proteins were upregulated in Aβ-treated SH-SY5Y cells exposed to Rg1 (Fig. 2B and Table 1).

Next, we analyzed the protein-protein interaction networks among the proteins that were changed 1.5-fold using the STRING web-based protein database [20]. Protein-protein interaction clustering was distributed into four cellular locations: mitochondrial proteins (AARS2, ETFB, HSD17B10, COX5A, BCKDK, and TOMM40), actin cytoskeleton proteins (CAP1, CAPZA1, ARPC2, CAP22B, and VCL), ribosomal proteins (RPL22, RPL29, PPP2R1A, RPSAP58, ENO3, and RPSA), and regulation of splicing proteins (SRSF1, SNRPD1, SRSF3, SNRNP70, and PRMT1; Fig. 3A). Interestingly, we have previously reported that Rb1 is linked to the actin cytoskeleton in AD [18].

Fig. 1. Stable isotope labeling with amino acids (SILAC) analysis of treatment with Rg1 in Aβ-treated cells. (A) Schematic diagram of the SILAC procedures used to quantify protein changes following treatment with Rg1 in β-amyloid peptide-treated SH-SY5Y cells. The lysate of cells cultured in SILAC medium was obtained, and the same amounts of proteins were combined and separated via SDS-PAGE followed by in-gel tryptic digestion. The tryptic peptides were analyzed by nano-LC MS/MS. (B) Paired extracted ion chromatograms and (C) monoisotopic mass peaks of destrin peptide (AVIFCLSADKK) from cells cultured in SILAC medium showed an approximate ratio of 1:1. The m/z difference between SILAC-labeled destrin peptides shows the triple charge of the destrin peptides.

Fig. 2. Analysis of differentially expressed proteins following exposure of Aβ-treated cells to Rg1. (A) The Venn diagram shows the overall distribution of the proteins identiﬁed in three independent biological experiments. A total of 2,476 proteins were identiﬁed from three replicates, with 1,149 overlapping proteins. (B) Quantitative analysis of the proteins that were differentially expressed following the treatment of Aβ-treated cells with Rg1. Forty-ﬁve proteins were upregulated, and four proteins were signiﬁcantly downregulated >1.5-fold.
Our previous study showed that mitochondrial proteins were dramatically downregulated in response to MPP\textsuperscript{+}-induced neuronal cells based on a quantitative proteomic analysis. Furthermore, the translocase of the outer membrane (TOM) complex has been reported to be the main entrance for most cytoplasmically synthesized mitochondrial proteins, and a decrease in TOMM40 levels causes mitochondrial dysfunction associated with neurodegenerative diseases such as AD [23]. Furthermore, the expression level of TOMM40 was found to be significantly downregulated in blood samples of patients with AD [24,25]. By contrast, our data showed the opposite results following the treatment of Aβ1-42-treated SH-SY5Y cells with Rg1. Fig. 3B shows the relative quantification of TOMM40 expression using an ion chromatogram from MS, which showed that TOMM40 was dramatically increased following treatment with Rg1.

Our previous study showed that mitochondrial proteins were dramatically altered in MPP\textsuperscript{+}-induced neuronal cells based on a quantitative proteomic analysis. Furthermore, the mitochondrial proteins HSD17B10 and AARS2 containing TOMM40 were dramatically downregulated in response to MPP\textsuperscript{+}-induced neuronal damage, which is known to be associated with neurodegeneration [19,26]. However, our results showed that HSD17B10 and AARS2 were noticeably increased by the treatment with Rg1 in Aβ1-42-treated SH-SY5Y cells (Table 1). These data indicate that Rg1 might have a protective role in the regulation of mitochondrial functions in AD.

To further investigate the functional role of Rg1 in relation to mitochondrial proteins in AD, all of the mitochondrial proteins identified in this study were further explored in the available literature. We found 14 mitochondrial proteins that have been reported to be associated with AD (Table S2), and we analyzed the protein interaction network among the mitochondrial proteins using the STRING database plug-in Cytoscape. Fig. 3C shows that 14 mitochondrial proteins exhibited a strong interaction network. The thickness of the black connection lines indicates the protein–protein interaction scores. The list of these proteins with a fold change > 1.5 is shown in Table 1.

| Accession No. | Gene name | Protein description | L/H log₂ ratio |
|---------------|-----------|---------------------|---------------|
| G3V28         | HS990A1   | Heat shock protein HSP 90-α (fragment) | -5.64         |
| O43432-3      | EIF4G3    | Isoform 3 of eukaryotic translation initiation factor 4 γ-3 | -1.12         |
| Q9VJ30        | RTC8      | tRNA-splicing ligase RtcB homolog | -1.12         |
| Q15369-2      | TCEB1     | Isoform 2 of Transcription elongation factor B polypeptide 1 | -0.92         |
| P18669        | PGAM1     | Phosphoglycerate mutase 1 | 0.58          |
| P52907        | CAPZA1    | F-actin-capping protein subunit α-1 | 0.58          |
| P37802-2      | TAGLN2    | Isoform 2 of transgelin-2 | 0.59          |
| P61970        | NUTF2     | Nuclear transport factor 2 | 0.59          |
| Q02616        | GCN1L1    | Translational activator GCN1 | 0.59          |
| Q15144        | ARPC2     | Actin-related protein 2/3 complex subunit 2 | 0.61          |
| P08865        | RPSA      | 40S ribosomal protein SA | 0.62          |
| O14880        | MGST3     | Microsomal glutathione S-transferase 3 | 0.63          |
| P04908        | HIST1H2B  | Histone H2A type 1B/E | 0.64          |
| P84103-2      | SRSF3     | Isoform 2 of serine/arginine-rich splicing factor 3 | 0.64          |
| Q5JT29        | AARS2     | Alanine-tRNA ligase, mitochondrial | 0.64          |
| P08621-2      | SNRP70    | Isoform 2 of U1 small nuclear ribonucleoprotein 70 kDa | 0.65          |
| P61086-1      | UBE2K     | Isoform 2 of ubiquitin-conjugating enzyme E2 K | 0.65          |
| P62314        | SNRP1D     | Small nuclear ribonucleoprotein Sm D1 | 0.61          |
| Q07955-2      | SRSF1     | Isoform ASF-2 of serine/arginine-rich splicing factor 1 | 0.67          |
| Q09598        | TSNAX     | Translin-associated protein X | 0.67          |
| Q15056-2      | EIF4H     | Isoform short of eukaryotic translation initiation factor 4H | 0.69          |
| Q0UNF1        | MAGED2    | Melanoma-associated antigen D2 | 0.69          |
| Q7Z6G7-2      | HUWE1     | Isoform 2 of 3 ubiquitin-protein ligase HUWE1 | 0.72          |
| P18041        | HIST1H1E  | Histone H1.4 | 0.73          |
| P18206-2      | VCL       | Isoform 1 of vinculin | 0.75          |
| Q96C7N        | ISOC1     | Isocitrate/isocitrate domain-containing protein 1 | 0.75          |
| P51665        | PSMD7     | 26S proteasome non-ATPase regulatory subunit 7 | 0.76          |
| P62095        | CDC42     | Cell division control protein 42 homolog | 0.77          |
| P68036-2      | UBE2L3    | Isoform 2 of ubiquitin-conjugating enzyme E2 L3 | 0.77          |
| P38117-2      | ETER      | Isoform 2 of electron transfer flavoprotein subunit β | 0.79          |
| Q97974        | HSD17B10  | 3-hydroxyacyl-CoA dehydrogenase type-2 | 0.80          |
| P30153        | PPP2R1A    | Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A α isoform | 0.82          |
| P20674        | COX5A     | Cytochrome c oxidase subunit 5A, mitochondrial | 0.84          |
| P28072        | PSMB6     | Proteasome subunit β type-6 | 0.85          |
| P47756-2      | CAP2B     | Isoform 2 of F-actin-capping protein subunit β | 0.85          |
| O60888-2      | CUTA      | Isoform A of protein CutA | 0.86          |
| Q19479        | KPNB1     | Importin subunit β-1 | 0.86          |
| P47914        | RPL29     | 60S ribosomal protein L29 | 0.86          |
| Q01518        | CAP1      | Adenyl cyclase-associated protein 1 | 0.93          |
| Q99873-2      | PRMT1     | Isoform 2 of protein arginine N-methyltransferase 1 | 0.94          |
| Q0PF3        | ABRACL    | Costars family protein ABRACL | 0.98          |
| O14874-2      | BCKDK     | Isoform 2 of [3-methyl-2-oxobutanate dehydrogenase (lipoamide)] kinase, mitochondrial | 1.22          |
| P16948-2      | STMN1     | Isoform 2 of stathmin | 1.48          |
| P26583        | HMGB2     | High mobility group protein B2 | 1.66          |
| P19399-2      | ATIC      | Isoform 2 of bifunctional purine biosynthesis protein PURH | 1.67          |
| P13526        | RPL22     | 60S ribosomal protein L22 | 1.98          |
| P13929-2      | EN03      | Isoform 2 of β-enolase | 2.58          |
| A6NE09        | RPSAP58   | 40S ribosomal protein SA | 4.39          |
| Q09008        | TOMM40    | Mitochondrial import receptor subunit TOMM40 homolog | 4.75          |

1) Accession Nos. are from the Uniprot database. The fold change was calculated using Rg1 treated/control (unlabeled/labeled) ratios quantitated from integrated proteomics software. Ratios were obtained from n = 3.

We noted the identification of mitochondrial proteins because a number of studies have shown that mitochondrial dysfunction is associated with the pathogenesis of AD [3]. Interestingly, the translocase of the outer membrane (TOM) complex has been reported to be the main entrance for most cytoplasmically synthesized mitochondrial proteins, and a decrease in TOMM40 levels causes mitochondrial dysfunction associated with neurodegenerative diseases such as AD [23]. Furthermore, the expression level of TOMM40 was found to be significantly downregulated in blood samples of patients with AD [24,25]. By contrast, our data showed the opposite results following the treatment of Aβ1-42-treated SH-SY5Y cells with Rg1. Fig. 3B shows the relative quantification of TOMM40 expression using an ion chromatogram from MS, which showed that TOMM40 was dramatically increased following treatment with Rg1.
protein interaction score. Interestingly, VDAC1 has been shown to be significantly increased in AD and transgenic mice [27,28], and abnormally high levels of Aβ interact with VDAC and interrupt metabolite transport via pore blockade [29]. VDAC1 protein expression was not altered by Rg1 treatment after Aβ exposure, in contrast to previous results showing increased levels of VDAC1 in AD. COX5A is the terminal oxidase of the mitochondrial electron transport chain and binds to Aβ, resulting in a malfunction of COX5A in cellular energy metabolism in AD and amyloid precursor protein transgenic mice [30]. Interestingly, the interaction between COX5A and TOMM40 is mediated by VDAC1, and the expression of these proteins exhibits a pattern opposite to that observed in Rg1-treated AD (Fig. 3C). A previous study has shown that NDUFA4 is highly expressed in AD [31]. However, Rg1 treatment decreased NDUFA4. Taken together, our data suggest that Rg1 might play a critical role in regulating the functions of mitochondrial proteins in AD.

In conclusion, numerous studies have been performed to investigate the potential mechanisms of ginsenosides in neuronal diseases such as AD. To explore the neuronal protective mechanism of Rg1 in AD, we performed a global proteomic analysis using SILAC together with LC-MS/MS. In total, 1,149 proteins were identified in the three replicated experiments. A total of 45 proteins showed significant alterations in response to Rg1 treatment in AD. Our data showed that these proteins were associated with mitochondrial proteins, actin cytoskeleton proteins, ribosomal proteins, and proteins that regulate splicing. In particular, the results showed that mitochondrial proteins containing HSD17B10, AARS2, TOMM40, VDAC1, COX5A, and NDUFA4 might be related to the protective mechanisms of Rg1 in AD. Additional studies are needed to investigate the detailed molecular mechanisms that link Rg1 to these proteins in AD.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2016.12.003.

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