Comparative Proteomics Reveals Silver Nanoparticles Alter Fatty Acid Metabolism and Amyloid Beta Clearance for Neuronal Apoptosis in a Triple Cell Coculture Model of the Blood–Brain Barrier

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ABSTRACT

Silver nanoparticles (AgNPs) enter the central nervous system through the blood–brain barrier (BBB). AgNP exposure can increase amyloid beta (Aβ) deposition in neuronal cells to potentially induce Alzheimer’s disease (AD) progression. However, the mechanism through which AgNPs alter BBB permeability in endothelial cells and subsequently lead to AD progression remains unclear. This study investigated whether AgNPs disrupt the tight junction proteins of brain endothelial cells, and alter the proteomic metabolism of neuronal cells underlying AD progression in a triple cell coculture model constructed using mouse brain endothelial (bEnd.3) cells, mouse brain astrocytes (ALT), and mouse neuroblastoma neuro-2a (N2a) cells. The results showed that AgNPs accumulated in ALT and N2a cells because of the disruption of tight junction proteins, claudin-5 and ZO-1, in bEnd.3 cells. The proteomic profiling of N2a cells after AgNP exposure identified 298 differentially expressed proteins related to fatty acid metabolism. Particularly, AgNP-induced palmitic acid production was observed in N2a cells, which might promote Aβ generation. Moreover, AgNP exposure increased the protein expression of amyloid precursor protein (APP) and Aβ generation-related secretases, PSEN1, PSEN2, and β-site APP cleaving enzyme for APP cleavage in ALT and N2a cells, stimulated Aβ40 and Aβ42 secretion in the culture medium, and attenuated the gene expression of Aβ clearance-related receptors, P-gp and LRP-1, in bEnd.3 cells. Increased Aβ might further aggregate on the neuronal cell surface to enhance the secretion of inflammatory cytokines, MCP-1 and IL-6, thus inducing apoptosis in N2a cells. This study suggested that AgNP exposure might cause Aβ deposition and inflammation for subsequent neuronal cell apoptosis to potentially induce AD progression.

Key words: silver nanoparticles; proteomic profiling; blood–brain barrier; tight junction protein; amyloid beta clearance; Alzheimer’s disease.

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Silver nanoparticles (AgNPs) have antibacterial properties and are widely used in consumer products, including wound dressings, textiles, toothpastes, food containers, and cosmetics. AgNPs can dissociate into Ag particles and Ag ions in suspension solutions (Speranza et al., 2013), and both of them exert cytotoxic effects on cells (Chen et al., 2016; Foldbjerg et al., 2009).

Oral administration of AgNPs (60 nm) in rats resulted in the dose-dependent accumulation of Ag in many tissues, including the blood, liver, lungs, kidneys, stomach, testes, and brain (Li et al., 2007). Obedorster et al. (2004) indicated that AgNPs can translocate from the olfactory mucosa of the respiratory tract to the central nervous system (CNS) through the olfactory nerve. In a mouse model, AgNP exposure (50 nm) induces blood–brain barrier (BBB) destruction and astrocyte swelling, and causes neuronal degeneration (Tang et al., 2009). In an in vitro BBB model constructed using primary rat brain microvessel endothelial cells, AgNPs (25 nm) induce the secretion of proinflammatory cytokines, interleukin (IL)-1β, IL-2, and tumor necrosis factor alpha, and increase BBB permeability (Trömpler et al., 2010). In neuronal cells, AgNPs (20 nm) induce the degradation of cytoskeleton components, perturbations of postsynaptic proteins, and mitochondrial dysfunction to stimulate cell apoptosis (Xu et al., 2013).

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder. Amyloid beta (Aβ) 40/42-induced extracellular amyloid plaques and tau protein-induced intraneuronal neurofibrillary tangles are the two major pathological mechanisms of AD (Bloom, 2014). Aβ40/42 is generated from Aβ precursor protein (APP) through β- and γ-secretase-mediated enzymatic digestion (Sinha and Lieberburg, 1999). β-Secretase is also referred to as β-site APP cleaving enzyme (BACE), which is responsible for initiating Aβ generation. Enhanced β-secretase activity increases Aβ deposition in the brain of patients with AD (Hampel and Shen, 2009). Presenilin (PSEN) is a multiple-pass transmembrane protein that forms the catalytic core of γ-secretase (Smolarkiewicz et al., 2013). Citron et al. (1997) demonstrated increased Aβ42 levels in the brain of PSEN1/PSEN2 transgenic mice. Aβ can be eliminated from the brain by p-glycoprotein (P-gp), an adenosine triphosphate (ATP)-binding cassette transporter, and low-density lipoprotein (LDL) receptor-related protein-1 (LRP-1), a member of the LDL receptor family (Basak et al., 2012; Deane et al., 2009; Yamada et al., 2008). Both P-gp and LRP-1 can transport Aβ from the brain to the bloodstream (Lam et al., 2001; Shibata et al., 2000). Moreover, decreased Aβ clearance activity increases Aβ accumulation in the brain of patients with AD (van Assema et al., 2012).

The BBB allows the passage of nutrients and prevents the toxic substances through the CNS (Liu et al., 2012). Zonula occludens-1 (ZO-1), occludin, and claudin-5 are the main components of the tight junctions that maintain BBB integrity in brain endothelial cells (Yang and Rosenberg, 2011). ZO-1 is an important link between tight junction proteins (claudin and occludin) and perijunctional actin (Musch et al., 2006). Aberrant tight junctions are associated with neurological disorders, including AD, Parkinson disease, and multiple sclerosis (Bednarczyk and Lukasiuk, 2011).

Our previous study revealed that AgNP-exposed mouse neuroblastoma neuro-2a (N2a) cells have increased Aβ40/42 deposition, which potentially leads to the development of neurodegenerative disorders (Huang et al., 2015). However, the mechanism through which AgNPs modulate proteomic metabolism to facilitate passage through the BBB and consequently cause neurodegenerative disorders remains unclear. Therefore, this study constructed a triple cell coculture model using mouse brain endothelial (bEnd.3) cells, mouse brain astrocytes (ALT), and mouse neuroblastoma N2a cells to simulate AgNP exposure in the brain. After AgNP exposure, in vitro BBB integrity was evaluated by examining transmembrane permeability and tight junction proteins in bEnd.3 cells and tracing the localization of AgNPs in ALT and N2a cells. To determine the potency of AgNP exposure in AD development, the mechanisms of aberrant Aβi generation, Aβi clearance, and apoptosis in N2a cells underlying AgNP-induced inflammation were investigated through proteomic profiling and gene expression studies.

**MATERIALS AND METHODS**

**AgNP exposure in triple cell coculture model.** The 3–5 nm uncoated AgNPs (Gold Nanotech Inc., Taipei, Taiwan) were used in this study were maintained in deionized (DI) water. The AgNPs remained in a uniform spherical shape in DI water. Based on the measurements recorded by a JEM2100 transmission electron microscope (JEOL, Tokyo, Japan), the average size of the AgNPs was 4.5 nm. The size of the AgNPs as measured according to dynamic laser scattering and their zeta potential in Dulbecco’s modified Eagle medium (DMEM; Corning Inc., Corning, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, Canada) and DI water were 105.6 ± 20.26 and 114.5 ± 2.75 nm and −5.5 ± 1.67 and 6.28 ± 0.338 mV, respectively. The Ag ion concentration in the AgNP stock solution was measured as 0.17 μg/ml using a perfecION comb Ag/S2 combination electrode (Mettler-Toledo Inc., Columbus, OH).

ALT and bEnd.3 cells were cultured in DMEM (Corning Inc.) supplemented with 10% FBS (Invitrogen), 1% penicillin and streptomycin (Biowest, Loire Valley, France), and 1% L-glutamine (Invitrogen) in a cell incubator at 5% CO2 and 37°C. N2a cells were cultured in a medium identical to that for ALT cells, and 1% sodium pyruvate (Invitrogen) was added. A modified version of the construction procedure for the triple cell coculture model by Li et al. (2010) was used. The bottom side of transwell filters (pore size, 0.4 μm; Corning Inc.) was coated with collagen type I (8 μg/cm²) in 0.02 N acetic acid; Corning Inc.) for 1 h. The filters were washed with phosphate-buffered saline (PBS; Geneset Biotech, Shanghai, China). ALT cells (2.44 × 10⁴ cells/cm²) were seeded on the bottom side of the transwell filters for 2 h. After incubation, bEnd.3 cells (10⁵ cells/cm²) were seeded on the top side of the transwell filters and incubated for 3 days. In addition, N2a cells (2.1 × 10⁶ cells/cm²) were seeded on a new six-well plate for 16 h. After incubation, the medium was replaced with a differentiation medium (DMEM/10% FBS containing 200 μM isobutylmethylxanthine and 30 μM forskolin) for 48 h. Finally, the transwell filters were combined with the six-well plate containing differentiated N2a cells to construct a triple cell coculture model (Figure 1). After model assembly, the bottom medium was replaced with 2 ml of DMEM/1% FBS. Subsequently, 2 ml of AgNPs at final concentrations of 1, 2, 3, and 4 μg/ml in the DMEM/1% FBS medium was added to the upper chamber of the transwell filters for 24 h incubation.

Detection of transendothelial electrical resistance and tight junction proteins and Evans blue determination. Transendothelial electrical resistance (TEER) values were measured using Millicell ERS-2 (Millipore, Bedford, MA) according to the manufacturer’s instructions. In brief, after 24 h AgNP exposure, the medium in the upper and bottom chambers were replaced with fresh DMEM/10% FBS. Subsequently, the electrode was placed in the...
upper and bottom chambers to measure TEER (Ω·cm²) values. Results were corrected with blanks (DMEM/10% FBS).

\[
\text{TEER} (\Omega \cdot \text{cm}^2) = \frac{|\text{TEER}_{\text{sample}}(\Omega) - \text{TEER}_{\text{blank}}(\Omega)|}{\text{area of membrane (cm}^2\rangle}
\]

After exposing the triple cell coculture model to AgNPs, ALT cells on the bottom side of the transwell filter were removed using cotton swabs. The filter membrane with bEnd.3 cells was removed from the chamber, fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100/PBS solution, blocked in 2% horse serum, incubated with the rabbit anti-mouse ZO-1 (cat. 617300; Invitrogen) or claudin-5 primary antibody (cat. 341600; Invitrogen) for 1 h and the fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (IgG) secondary antibody for 1 h (cat. 12-507; Merck Millipore), and finally transferred to a coverslip and mounted in the mounting medium (Invitrogen). Immunofluorescence images were captured using an inverted microscope with fluorescence filters (Axio Observer A1/D1, Zeiss, Oberkochen, Germany).

In Evans blue determination, the upper and bottom chambers were washed with Hank’s balanced salt solution (HBSS) after exposing the triple cell coculture model to AgNPs for 24 h. HBSS (1.5 ml) was added to the bottom chamber, and 0.5 ml of Evans blue dye was added to the upper chamber and incubated at 37°C for 5 min. The solution in the bottom chamber was collected, and absorbance at 610 nm was measured using a UV/vis spectrometer (Shimadzu UV-2550, Japan).

Reverse transcription and quantitative real-time polymerase chain reaction. Total RNA was extracted from bEnd.3, ALT, and N2a cells in the triple cell coculture model after 24 h AgNP exposure by using RNA Trizol (Invitrogen). The quantity and purity of RNA samples were assessed using NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE). The absorbance ratios of acceptable RNA purity were established as A260/A280 > 1.8 and A260/A230 > 1.5.

Complementary DNA (cDNA) was synthesized from 3 μg of total RNA by using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). A real-time polymerase chain reaction (PCR) was performed to amplify 100 ng of cDNA in 40 cycles using the 2× power SYBR green PCR master mix (Applied Biosystems). The primer sequences used in this study were as follows: β-actin sense 5'-ATG CTC CCC GGG CTG TAT-3' and antisense 5'-GCT GTG GGC CTC GTG ACC-3'; BACE sense 5'-CAA AAC TGG GAA AGG CAG AAC TC-3' and antisense 5'-CGACTACA ACC ACA GCC ACA TT-3'; claudin-5 sense 5'-TGG ATG TGC GTG GTG-3' and antisense 5'-GCC TGG GTC ATC TTG GGT G-3'; P-gp sense 5'-TTT CAT TTC CTG TCT TCT GTG TGA CCA GAA C-3' and antisense 5'-TTT CAT TTC CTG TCT TCT GTG TGA CCA GAA C-3'; and ZO-1 sense 5'-TGTTTGGTATGCACCCTCCGTCG-3' and antisense 5'-GGT TAA GGT AAA AAA GAG TGC C-3'. Quantitative analysis of PCR products was conducted using a sequence detector (Model 7300, Applied Biosystems), according to the manufacturer’s instructions. The signal of SYBR green was measured at 530 nm during the extension phase and detected and analyzed using SDS 1.0 software (Applied Biosystems). The relative level of mRNA expression was defined as the optical density of the experimental groups to that of an endogenous house-keeping gene β-actin.

Determination of total Ag concentration and AgNP localization in neuronal cells. One milliliter of the medium or harvested ALT and N2a cells was pretreated with 0.5 ml of pure nitrate (Sigma-Aldrich, St. Louis, MO) and digested at 75°C for 16 h. DI water was added to the treated samples to attain a volume of 5 ml. The total Ag concentration in these samples was measured using an inductively coupled plasma mass spectrometer (ELAN...
DRC II, PerkinElmer, Norwalk, CT). AgNO₃ was used as a standard with concentrations ranging from 0.5 to 1000 µg/l to plot a standard curve.

In the AgNP localization experiment, N2a cells were cultured and differentiated on glass coverslips before constructing the triple cell coculture model. After the triple cell coculture model was exposed to AgNPs (4 µg/ml) for 24 h, bEnd.3 cells in the upper chamber were removed using cotton swabs, and the membrane was cut off from the chamber. The membrane with ALT cells and glass coverslips with N2a cells were rinsed with a mixture of tripotassium hexacyanoferrate (III) (10 mM; Sigma-Aldrich) and sodium thiosulfate pentahydrate (10 mM; Sigma-Aldrich) to remove AgNPs from the cell surface. The cells were fixed in 4% PFA and covered with ProLong antifade mountant and 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies Inc., Carlsbad, CA) for nuclear staining. Images were captured using a confocal laser scanning microscope (LSM 780; Carl Zeiss, Göttingen, Germany) with a ×63 oil-immersion objective; a 561-nm laser was used for imaging AgNPs in the reflectance mode, and a 405-nm laser was used for DAPI.

Protein extraction for quantitative proteomic analysis and fatty acid determination. N2a cells (25 µl cell pellet from the six-well plate) were lysed by mixing them with 150 µl of lysis buffer containing 5% sodium deoxycholate, 10 mM dithiothreitol, and 0.1 M triethylammonium bicarbonate with 1% protease inhibitor cocktail (Sigma-Aldrich) and sodium thiosulfate pentahydrate (10 mM; Sigma-Aldrich) to remove AgNPs from the cell surface. The cells were fixed in 4% PFA and covered with ProLong antifade mountant and 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies Inc., Carlsbad, CA) for nuclear staining. Images were captured using a confocal laser scanning microscope (LSM 780; Carl Zeiss, Göttingen, Germany) with a ×63 oil-immersion objective; a 561-nm laser was used for imaging AgNPs in the reflectance mode, and a 405-nm laser was used for DAPI.

Protein quantification by Western blotting. MD, N2a and ALT cells were lysed with the radiolow immune precipitation assay buffer (Cell Signaling, Danvers, MA) containing a protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 14 000 × g for 10 min at 4 °C. The total protein concentration in samples was determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich). The protein samples were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride membrane (Millipore). The membrane-bound proteins were immunostained with 1:1000 rabbit anti-mouse APP (cat. EPR 5118-34; Abcam, Cambridge, UK), BACE1 (cat. EPR5118-34; Abcam, Cambridge, UK), and β-actin primary antibody (cat. sc-4778; Santa Cruz Biotechnology, Santa Cruz, CA), followed by treatment with the anti-rabbit IgG horseradish peroxidase secondary antibody (cat. sc-2301; Santa Cruz Biotechnology). The tagged proteins were detected using a chemiluminescence reagent (Thermo Fisher Scientific) and photographed in a G:Box ChemiXT16 system (Syngene, Frederick, MD). The densitometric analysis of Western blot results was conducted using Image J software.

Quantification of Aβ40, Aβ42, and inflammatory cytokines. The Aβ40 concentration of the medium in the upper and bottom chambers was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (AnaSpec, Fremont, CA). First, 50 µl of a dilute detection antibody and 100 µl of the medium were added to a 96-well plate, and the plate was incubated at 4 °C for 16 h. After incubation, the wells were washed seven times with 1× wash buffer, and 100 µl of tetra methyl benzidine (TMB) color substrate solution was added to each well and incubated at room temperature for 20 min. Finally, 50 µl of stop solution was added to each well. The absorbance values were measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The Aβ42 concentration was determined using an Aβ42 ELISA kit (cat. EM0864; FineTest, Wuhan, China). Two hundred microliters of the medium was added to an Aβ42 antibody-coated 96-well plate and incubated at 37 °C for 90 min. The plate content was discarded, 100 µl of a biotin detection antibody was added, and the plate was incubated at 37 °C for 60 min. The plate was washed three times with wash buffer, 100 µl of SABC solution was added, and the plate was incubated at 37 °C for 30 min. The plate was washed five times with wash buffer, 90 µl of TMB substrate was added, and the plate was incubated at 37 °C in the dark for 15 min. Finally, 50 µl of stop solution was added to the plate, and the absorbance values were measured at 450 nm using the microplate reader (Molecular Devices). The concentration of inflammatory...
cytokines in the medium in the upper and bottom chambers were measured using a Bio-PlexMouse Cytokine 23-Plex panel (Bio-Rad Laboratories Inc., Hercules, CA).

Cell apoptosis assay. Cell apoptosis was observed using an Annexin-V detection kit (Invitrogen) in N2a cells after the triple cell coculture model was exposed to AgNPs. One million cells were washed with PBS and resuspended in binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂). Annexin V-FITC (100 ng/ml) was added to the cell solution and incubated in the dark for 10 min, washed with PBS, and resuspended in 500 µl of binding buffer. Prior to flow cytometric analysis, 10 µl of propidium iodide (1 mg/ml; Invitrogen) was added to each sample. The cells were analyzed using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA). In each sample, 10,000 cells were counted. Data analysis was performed using WinMDI software (version 2.9; Scripps Research Institute, San Diego, CA).

Statistical analysis. Statistically significant differences in the gene expression, protein expression, Evans blue concentration, cytokine concentration, and total Ag concentration of the AgNP exposure and control groups were analyzed through one-way analysis of variance (GraphPad 6.0). A two-tailed P value < .05 was considered to indicate statistical significance.

RESULTS

Tight Junctions in bEnd.3 Cells After AgNP Exposure

After the triple cell coculture model was exposed to AgNPs (2, 3, and 4 µg/ml) for 24 h, the TEER values of bEnd.3 cells decreased...
in a dose-dependent manner (Figure 2A). Evans blue determination revealed that AgNPs at 3 and 4 μg/ml increased the permeability of the triple cell coculture model (Figure 2B). The integrity of tight junction proteins, ZO-1 and claudin-5, was disrupted after AgNP exposure at 4 μg/ml (Figure 2C). Furthermore, AgNP (3 and 4 μg/ml) exposure reduced the gene expression of ZO-1 and claudin-5 in bEnd.3 cells (Figure 2D).

**AgNP Distribution in Astrocytes and Neuronal Cells**

After exposure of the triple cell coculture model to AgNPs (1, 2, and 4 μg/ml), the total Ag was detected to move from the medium to ALT and N2a cells (Table 1). After exposure to AgNPs at 4 μg/ml, the percentage of AgNPs concentrations in the bottom medium, ALT cells, and N2a cells were 4.82 ± 0.26%, 0.51 ± 0.07%, and 1.31 ± 0.31%, respectively. Confocal microscopy imaging revealed that AgNPs were located in the cytoplasm of ALT and N2a cells after AgNP exposure at 4 μg/ml (Figure 3).

**Proteomic Metabolism in N2a Cells After AgNP Exposure**

Total protein in N2a cells was labeled with TMTs after the triple cell coculture model exposure to AgNPs. In proteomic analysis, 298 DEPs (upregulated, n = 38; downregulated, n = 260) were identified from 5953 proteins (Figure 4A; Supplementary Table 1). Molecular function analysis (Supplementary Figure 1A) revealed that these proteins were involved in catalytic activity, binding, structural molecular activity, and receptor activity. In addition, biological function analysis (Supplementary Figure 1B) indicated that these DEPs were associated with metabolic process, cellular process, localization cellular component organization of biogenesis and biological regulation. Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Table 2) demonstrated that DEPs were involved in ribosome biogenesis, oxidative phosphorylation, insulin signaling pathways, fatty acid elongation, and neuronal diseases, such as Parkinson disease, Huntington disease, and AD. Cytoscape gene-network analysis revealed that DEPs were associated with ribosome biogenesis, protein localization to the endoplasmic reticulum, posttranslational protein modification, endoplasmic reticulum organization, mitochondrion organization, ribonucleoside metabolism, Parkinson’s disease, and Huntington’s disease (Figure 4B). Subnetwork analysis showed that AgNPs modulated fatty acid metabolism-related proteins, including insulin, peroxisome proliferator-activated receptor alpha (PPARα), protein kinase adenosine monophosphate (AMP)-activated catalytic subunit γ2, malonyl-CoA decarboxylase, protein kinase AMP-activated noncatalytic subunit gamma (PRKAG) 1, PRKAG2, and peroxisome proliferator-activated receptor gamma coactivator 1α (PPARGC1α), to activate cytochrome c, somatic (CYCS), and caspase 3 (CASP3) for cell apoptosis (Figure 4C).

**Fatty Acid Composition in Neuronal Cells After AgNP Exposure**

After exposure of the triple cell coculture model to AgNPs, the total fatty acid composition in ALT and N2a cells was determined through gas chromatography and mass selective detection. After AgNP exposure at 4 μg/ml, the palmitic acid concentration significantly increased (fold change, 1.35 ± 0.02) in N2a cells, and no apparent changes were observed in ALT cells (Table 3).

**Aβ Determination After AgNP Exposure**

After the triple cell coculture model was exposed to AgNPs, gene and protein expression results revealed that APP was induced in N2a cells in a dose-dependent manner (Figs. 5A and B). AgNPs at 2 μg/ml significantly increased the secretion of Aβ40 (113 ± 4%) and Aβ42 (107 ± 3%) into the bottom medium compared with the control group, and no apparent changes were observed in the upper medium (Figure 5C). Moreover, the gene expression of Aβ generation-related secretases, namely PSEN1, PSEN2, and BACE, in ALT and N2a cells (Figure 5D) was significantly increased after AgNP exposure. The protein expression of BACE in ALT cells and that of PSEN1 and PSEN2 in N2a cells was also increased after AgNP exposure (Figure 5E). The gene expression of Aβ clearance-related receptors, namely LRP-1 and P-gp, in bEnd.3 cells was significantly decreased after AgNP exposure at 2 and 4 μg/ml (Figure 5F).

**Inflammatory Cytokine Secretion and Cell Apoptosis in Neuronal Cells After AgNP Exposure**

In the triple cell coculture model, AgNP exposure at 4 μg/ml induced IL-6 and MCP-1 secretion and decreased IL-1β secretion in the upper medium containing bEnd.3 cells and MCP-1 secretion in the bottom medium containing ALT and N2a cells (Figs. 6A and B). AgNPs (4 μg/ml) induced early and late apoptosis in 10.8% and 26.2% of N2a cells, respectively (Figure 6C).

**DISCUSSION**

AgNPs have been used in consumer products for many years. However, their effects on neurodegenerative disorders remain unclear. Our previous studies have revealed that AgNPs can induce inflammation and enhance Aβ accumulation in monocultured N2a cells (Huang et al., 2015; Lin et al., 2016). The present study used molecular and proteomic approaches to investigate the adverse effects of AgNPs on neuronal cells. This study demonstrated that AgNPs can traverse through the
in vitro triple cell coculture BBB to reach neuronal cells and induce palmitic acid generation for Aβ accumulation in neuronal cells, leading to inflammatory cytokine secretion and apoptosis and potentially inducing AD progression.

**AgNPs Disrupt Tight Junctions in bEnd.3 Cells**

Tight junction proteins play a major role in protecting and maintaining brain homeostasis. This study observed that AgNPs disrupted tight junction proteins, ZO-1 and claudin-5, in bEnd.3 cells to enhance BBB permeability in the triple cell coculture model (Figure 2), and AgNPs were absorbed by ALT and N2a cells (Figure 3). Similar results were observed in in vitro double- and triple cell coculture models. Tang et al. (2010) revealed that AgNPs can cross the rat BBB to accumulate inside brain microvascular endothelial cells (BMVECs) and astrocytes in a double cell coculture model. Xu et al. (2015) showed that AgNPs reduce the gene expression of ZO-1, disrupt the tight junctions of brain endothelial cells, and induce nuclear atypia and mitochondrial shrinkage in astrocytes of a triple cell (BMVECs, astrocytes, and pericytes) coculture model. Accordingly, AgNP exposure might enhance BBB permeability in inner neuronal cells by altering the protein expression of ZO-1 and claudin-5 to disrupt the tight junctions in brain endothelial cells.

**AgNPs Modulated Fatty Acid Metabolism in Neuronal Cells**

In this study, the concentration of AgNPs (1–4 μg/ml) converted to human exposure levels was 0.05–0.2 mg/kg/d. In a human study, the blood Ag concentration was 1.6 ± 0.4 mg/l after exposure to AgNPs (5–10 nm) at 10 μg/ml for 14 days (Munger et al., 2014). Armitage et al. (1996) revealed that the Ag concentration in blood samples of workers with occupational Ag exposure (n = 19) and a control group (n = 15) was 1.3–20 and <0.1 μg/l, respectively. AgNPs influence the proteomic metabolism of LOVO human colon cancer cells (Verano-Braga et al., 2014). In this study, Cytoscape gene-network analysis of DEPs indicated that AgNPs upregulate PPARα and PPARγ1α to activate CASP3 for apoptosis in N2a cells (Figure 4C). Furthermore, flow cytometric analysis revealed early and late apoptosis in N2a cells after AgNP exposure (Figure 6C). PPARα and PPARγ1α have been reported to regulate fatty acid metabolism through β-oxidation in C57BL/6J mouse liver mitochondria (Aharoni-Simon et al., 2011; Poulsen et al., 2012). Furthermore, PPARα activation has been reported to increase CASP3 activation and induce

![Figure 3. AgNP uptake in ALT and N2a cells of the triple cell coculture model.](image-url)
hepatocyte apoptosis in mice (Richardson-Burns et al., 2002; Xiao et al., 2006). In addition, fatty acid determination in this study revealed that AgNP exposure increased the palmitic acid concentration in N2a cells (Table 3). Kwon et al. (2014) indicated that palmitic acid increases the phosphorylation of extracellular signal-regulated kinase 1/2, c-Jun aminoterminal kinase, and nuclear factor kappa B to induce inflammatory signaling in N2a cells. Palmitic acid can activate CASP3 and increase apoptosis in
neuron-like cells differentiated from PC12 rat adrenal medulla cells (Ulloth et al., 2003). Hsiao et al. (2014) demonstrated that palmitic acid induces endoplasmic reticular stress and cell cycle arrest in the G2/M phase and accelerates Aβ accumulation and cell apoptosis through protein palmitoylation in SH-SY5Y human neuroblastoma cells. In C57BL/6 mice, palmitic acid increases reactive oxygen species levels, reduces neural progenitor cell concentration, disrupts hippocampal neurogenesis (Park et al., 2011), increases membrane localization of protein kinase C-theta, and induces hypothalamic insulin resistance (Benoit et al., 2009). These findings suggested that AgNP exposure might modulate fatty acid metabolism to increase the palmitic acid concentration and induce neuronal cell apoptosis.

**AgNPs Induced Aβ Deposition and Inflammation for Neuronal Cell Apoptosis**

Aβ accumulation in the brain is a pathological hallmark of AD progression. This study observed that AgNPs induced Aβ40 and Aβ42 secretion into the bottom medium with ALT and N2a cells (Figure 5C); however, Aβ40 and Aβ42 were not detected inside ALT and N2a cells (data not shown). Aβ40 and Aβ42 are common isoforms of Aβ, which is generated by β- and γ-secretase-mediated APP cleavage. Aβ40 and Aβ42 are typically produced in the trans-Golgi network and endoplasmic reticulum, respectively (Hartmann et al., 1997), and can be secreted into the cerebrospinal fluid (CSF) of the brain (Maia et al., 2015). In addition, the gene expression of Aβ clearance-related receptors, P-gp and LRP-1, was attenuated after AgNP exposure (Figure 5F), suggesting that AgNPs might impair Aβ clearance activity, resulting in Aβ accumulation in neuronal cells and subsequent cell apoptosis. P-gp is an ATP-binding cassette transporter that transports Aβ40/42 out of the brain endothelium (Lam et al., 2001). In the brain of P-gp null mice, the Aβ clearance rate is reduced after Aβ40/42 injection into the CNS (Cirrito et al., 2005). P-gp function is decreased significantly in patients with AD compared with non-AD patients (van Assema et al., 2012). LRP-1 acts as an important Aβ transporter from the brain to the blood through the blood-brain barrier, increased brain Aβ42 levels, and impair learning ability and recognition.

### Table 3. Fatty Acid Profile of Neuronal Cells After 4 μg/ml AgNPs Exposure

<table>
<thead>
<tr>
<th>Number</th>
<th>RT (min)</th>
<th>Ratio of AgNPs/Control</th>
<th>Common Name</th>
<th>Lipid Name</th>
<th>Molecular Formula</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT Cells</td>
<td>N2a Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30.04</td>
<td>1.02±0.03</td>
<td>Palmitic acid</td>
<td>C16:0</td>
<td>C16H32O2</td>
<td>370.330</td>
</tr>
<tr>
<td>2</td>
<td>34.11</td>
<td>1.01±0.02</td>
<td>Octadecanoic acid</td>
<td>C18:0</td>
<td>C18H36O2</td>
<td>398.358</td>
</tr>
</tbody>
</table>

Fatty acid profile of ALT cells and N2a cells were determined by GC-MSD.

*P < .05 compared with control group.

Figure 5. Detection of Aβ generation-related proteins and genes. After the triple cell coculture model was treated with AgNPs (1, 2, 3, and 4 μg/ml), the protein and gene expression of APP in ALT and N2a cells was determined through (A) real-time PCR (n = 6) and (B) Western blotting (n = 4), respectively. (C) The protein levels of Aβ40 and Aβ42 in the upper and bottom medium were evaluated using an ELISA kit. Results were represented in percentages compared with control group (n = 4). D, Gene expression and E, protein expression of Aβ generation-related secretases, namely PSEN1, PSEN2, and BACE, in ALT and N2a cells were examined through real-time PCR (n = 6) and Western blotting (n = 4), respectively, and F, gene expression of Aβ clearance-related receptors, LRP-1 and P-gp, in bEnd.3 cells was evaluated using real-time PCR (n = 6). Data were presented as the mean ± SD. These reactions were performed in duplicate in three independent experiments. *P < .05 and **P < .01, significantly different from the control group. #P < .05 and ##P < .01, significantly different between the two exposure groups.
Figure 6. Inflammatory cytokine secretion and cell apoptosis in the triple cell coculture model after AgNP exposure. After the triple cell coculture model was exposed to AgNPs at 4 μg/ml for 24 h, cytokine levels in the (A) upper medium and (B) bottom medium were determined using a cytokine assay kit (n = 2). Data were presented as the mean ± SD. C, Early and late apoptosis in N2a cells was detected using flow cytometry in the triple cell coculture model after AgNP exposure (1, 2, 3, and 4 μg/ml) for 24 h (n = 3). Results were presented as percentages of early apoptosis cells, late apoptosis cells, and normal cells. These assays were performed in duplicate in two independent experiments. *P < .05, significantly different from the control group.

Figure 7. Schematic diagram of AgNP exposure in cerebral vascular endothelial cells, astrocytes, and neuronal cells inducing AD progression. AgNPs disrupt the tight junction proteins ZO-1 and claudin-5 in cerebral vascular endothelial cells to enhance BBB permeability. AgNPs can enter the brain and can be internalized by astrocytes and neuronal cells. AgNPs increase the palmitic acid concentration and enhance APP accumulation to promote Aβ generation in neuronal cells. Moreover, AgNPs induce the protein expression of Aβ generation-related secretases (PSEN1, PSEN2, and BACE) for digesting APP in astrocytes and neuronal cells into Aβ peptide fragments, which were subsequently released into the CSF. AgNPs also induce IL-6 secretion to attenuate the gene expression of Aβ clearance-related receptors (LRP-1 and P-gp) in brain endothelial cells, which impair Aβ clearance ability, resulting in Aβ accumulation in the CSF. These AgNP-induced Aβ proteins can further aggregate on the neuronal cell surface to stimulate an MCP-1-mediated inflammatory response, potentially leading to neuronal cell apoptosis and thus AD progression.
memory (Jaeger et al., 2009). LRP-1 inhibitor-induced attenuation of LRP-1 protein activity increases Aβ40 accumulation in the brain of C57BL/6 mice (Shibata et al., 2000). Furthermore, Aβ accumulation increases inflammatory responses in the brain of APP transgenic mice (Patel et al., 2005), and Aβ-induced inflammation increases neuronal cell apoptosis (Palop and Mucke, 2010). In addition, this study revealed that AgNP exposure induces early and late apoptosis in N2a cells (Figure 6C).

This study also demonstrated that AgNPs enhanced MCP-1 and IL-6 secretion and attenuated IL-1β secretion into the upper medium with bEnd.3 cells. In addition, AgNPs induced MCP-1 secretion into the bottom medium with ALT and N2a cells in the triple cell coculture model (Figs. 6A and B). Inflammation is a key factor in AD pathogenesis (Shadfar et al., 2015). Miklossy and McGeer (2016) indicated that chronic bacterial infection induces inflammation and plays a crucial role in AD progression. IL-1β expression in human brain endothelial cells can prevent toxic mycobacteria from entering the brain (Corsini et al., 1999). The present study revealed that AgNP exposure reduced IL-1β secretion in brain endothelial cells, which may reduce the activities for defense against bacterial infections in the brain and increase the risk of AD progression. MCP-1 has been demonstrated to recruit monocytes into the foci of active inflammation (Ajuebor et al., 1998). MCP-1 secretion can be induced by Aβ40/42 in primary rat astrocytes (Johnstone et al., 1999). Furthermore, increased MCP-1 levels might induce neuronal cell death (Katayama et al., 2002). Sawyer et al. (2014) indicated that MCP-1 influences neuronal loss, which is integral to the progression of neurological disorders, such as AD and Parkinson disease, through BBB leakage and macrophage polarization. In addition, MCP-1 has been reported to induce the redistribution and disruption of ZO-1 and claudin-5 to increase BBB permeability in mouse monocultured brain endothelial cells (Stamatovic et al., 2003) and a coculture model of mouse brain endothelial cells and astrocytes (Stamatovic et al., 2005). IL-6 attenuates the expression of claudin-5 and occludin to enhance BBB permeability in the brain of adult sheep (Cohen et al., 2013). Furthermore, IL-6 reduces LRP-1 expression in human microvascular endothelial cells (Kitazawa et al., 2016) and attenuates P-gp activity in the cultured brain endothelial cells of fetal guinea pigs (Iqbal et al., 2016). Moreover, increased MCP-1 and IL-6 levels have been observed in patients with mild AD (Cojocaru et al., 2011; Galimberti et al., 2006) and a rat AD model (El Dayem et al., 2013; Patel et al., 2005).

These findings indicate that AgNPs can disrupt tight junctions to reach neuronal cells, and AgNP-induced palmitic acid generation might increase Aβ secretion to induce an inflammatory response and attenuate Aβ clearance to stimulate neuronal cell apoptosis.

AgNP Exposure Potentially Induced AD Progression

Figure 7 depicted the potential pathway through which AgNPs induced AD progression according to the findings of this study. After entering the blood circulation system, AgNPs might disrupt the tight junction proteins of brain endothelial cells, ZO-1 and claudin-5, and enter through astrocytes to reach neuronal cells. These AgNPs may modulate fatty acid metabolism to increase palmitic acid generation, which could enhance Aβ accumulation, inflammation, and apoptosis in neuronal cells. In addition, Aβ is generated through β- and γ-secretase-mediated APP cleavage in neuronal cells, and is secreted into the CSF. AgNP exposure increased APP accumulation and induced the protein expression of Aβ generation-related secretases, PSEN1, PSEN2 (encoded for γ-secretases), and BACE (encoded for β-secretases), for APP cleavage in astrocytes and neuronal cells. Furthermore, AgNPs induced IL-6 secretion to attenuate the gene expression of Aβ clearance-related receptors, P-gp and LRP-1. Aβ can translocate from the CSF to the blood circulation system through the LRP-1 and P-gp of brain endothelial cells. Decreased LRP-1 and P-gp activity might promote Aβ accumulation in the CSF, and increased Aβ would aggregate on the neuronal cell surface to stimulate an MCP-1-mediated inflammatory response, leading to neuronal cell apoptosis and potentially inducing AD progression.

CONCLUSION

This study is the first to demonstrate that AgNP exposure disrupts tight junctions in a triple cell coculture model to promote palmitic acid generation, Aβ40 and Aβ42 deposition, and inflammatory cytokine secretion and to attenuate gene expression of Aβ clearance-related receptors, leading to neuronal cell apoptosis. AgNPs have been widely used in medical applications and consumer products; therefore, these findings highlighted the potential adverse effects of AgNP exposure, which may cause neurodegenerative disorders such as AD.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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