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ORIGINAL ARTICLE

Aβ-Induced Insulin Resistance and the Effects of Insulin on the Cholesterol Synthesis Pathway and Aβ Secretion in Neural Cells

Dema Najem[1,2] · Michelle Bamji-Mirza[1,2] · Ze Yang[3] · Wandong Zhang[1,2]

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Abstract Alzheimer's disease (AD) is characterized by amyloid-β (Aβ) toxicity, tau pathology, insulin resistance, neuroinflammation, and dysregulation of cholesterol homeostasis, all of which play roles in neurodegeneration. Insulin has polytrophic effects on neurons and may be at the center of these pathophysiological changes. In this study, we investigated possible relationships among insulin signaling and cholesterol biosynthesis, along with the effects of $A\beta_{42}$ on these pathways *in vitro*. We found that neuroblastoma 2a (N2a) cells transfected with the human gene encoding amyloid-β protein precursor (AβPP) (N2a-AβPP) produced Aβ and exhibited insulin resistance by reduced p-Akt and a suppressed cholesterol-synthesis pathway following insulin treatment, and by increased phosphorylation of insulin receptor subunit-1 at serine 612 (p-IRS-S612) as compared to parental N2a cells. Treatment of human neuroblastoma SH-SY5Y cells with $A\beta_{42}$ also increased p-IRS-S612, suggesting that $A\beta_{42}$ is responsible for insulin resistance. The insulin resistance was alleviated when N2a-AβPP cells were treated with higher insulin concentrations. Insulin increased Aβ release from N2a-AβPP cells, by which it may promote Aβ clearance. Insulin increased cholesterol-synthesis gene expression in SH-SY5Y and N2a cells, including 24-dehydrocholesterol reductase (DHCR24) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) through sterol-regulatory element-binding protein-2 (SREBP2). While $A\beta_{42}$-treated SH-SY5Y cells exhibited increased HMGCR expression and c-Jun phosphorylation as pro-inflammatory responses, they also showed down-regulation of neuro-protective/anti-inflammatory DHCR24. These results suggest that $A\beta_{42}$ may cause insulin resistance, activate JNK for c-Jun phosphorylation, and lead to dysregulation of cholesterol homeostasis, and that enhancing insulin signaling may relieve the insulin-resistant phenotype and the dysregulated cholesterol-synthesis pathway to promote Aβ release for clearance from neural cells.

Keywords Alzheimer's disease · Insulin resistance · Aβ peptides · Insulin signaling · Cholesterol synthesis pathway · Pro-inflammatory response

Introduction

Alzheimer's disease (AD) is a progressive, age-related neurodegenerative disorder of the brain. It is the most common form of dementia that is incurable and terminal [1]. The prevalence of the disorder is increasing at an alarming rate, afflicting ~30 million people worldwide and is expected to quadruple by 2050 [2]. The neuropathological characteristics of AD include the presence of senile plaques, defective brain insulin signaling [3], dysregulated cholesterol homeostasis [4], decreased glucose utilization, increased oxidative stress, and inflammation leading to neurodegeneration [5]. The plaques are composed mostly of aggregated amyloid-β 40 ($A\beta_{40}$) and $A\beta_{42}$ peptides, which are derived by proteolysis of the amyloid-β precursor protein (AβPP) [6, 7]. In the amyloidogenic pathway, AβPP is cleaved by β-secretase (also

✉ Wandong Zhang
Wandong.Zhang@nrc.ca; wzhan2@uottawa.ca

[1] Faculty of Medicine, University of Ottawa, Ottawa, ON K1H8M5, Canada

[2] Human Health Therapeutics, National Research Council of Canada, Ottawa, ON K1A0R6, Canada

[3] Beijing Hospital, Institute of Geriatrics-Chinese Health Ministry, Beijing 100730, China

known as β-site AβPP-cleaving enzyme 1), releasing $sA\beta PP_{\beta}$ while retaining C99 within the membrane. The γ-secretase complex then cleaves C99 to produce $A\beta_{40}$ and $A\beta_{42}$ [8]. In AD, the production, accumulation, and aggregation of Aβ are considered to be the initial steps to neurodegeneration and a critical part of the progression of the disease [9]. $A\beta_{42}$ plays a more pivotal role in the pathogenesis of AD than $A\beta_{40}$ due to its higher aggregative ability [10, 11].

Recently, insulin resistance and insulin signaling deficiency have been considered important features in the progression of AD. Insulin is an important regulator of brain cell function and metabolism. It affects neuronal synaptic function and plasticity, and glucose/lipid/cholesterol metabolism in the brain [12]. Several studies have shown clinical correlations between the failure of signals in the insulin pathways and brain disorders including stroke, AD, and Parkinson's disease [13]. Impairments of insulin signaling have not only been described in human AD, but also in animal models of AD [14, 15] and in rodents receiving intracerebral streptozotocin injections [16]. Lower levels of insulin and insulin receptors (IRs) in AD brains further implicate insulin resistance in the neuropathology [17, 18]. One way to visualize insulin signaling in cells is to examine the phosphorylation of downstream signaling proteins such as pAkt-S473 and IR substrate 1 (IRS-1) at Y895 [19, 20]. IRS-1 contains multiple tyrosine-phosphorylation motifs and they mediate the metabolic and growth-promoting function of insulin [21–23]. IRS-1 also contains serine/threonine phosphorylation sites, such as IRS-1-S612, that when phosphorylated results in an inhibition of insulin signaling in the cell [24, 25] and may indicate insulin resistance [26].

Another recognized feature of AD is the dysregulation of cholesterol and lipid homeostasis in the brain [4]. Such dysregulation may increase Aβ production [4], and several studies have linked cholesterol, lipid, and lipoprotein dysregulation to the insulin-resistant state [27]. One study attributed the link between cognitive deficits in diabetic rodents and the impaired regulation of cholesterol synthesis in the brain to insulin resistance [28]. Insulin activates the transcription factors sterol-regulatory element-binding proteins (SREBPs), which are involved in cholesterol and fatty-acid synthesis [28]; SREBP1a and SREBP1c regulate fatty-acid synthesis while SREBP2 is specific for cholesterol biosynthesis [29–32]. There are two key enzymes along the cholesterol biosynthesis pathway, HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase), the rate-limiting enzyme of cholesterol biosynthesis, and DHCR24 (24-dehydrocholesterol reductase), catalyze the last step of cholesterol synthesis. The mechanisms of insulin resistance in the context of Aβ production and cholesterol metabolism in AD progression are not completely understood. In this study, we investigated the interplay between insulin resistance, cholesterol metabolism, and Aβ production *in vitro* in N2a, N2a-AβPP, and SH-SY5Y cells. We showed that $A\beta_{42}$ peptides can cause insulin-resistance phenotypes in N2a-AβPP and SH-SY5Y cells, and that treatment of the cells with insulin increases the expression of cholesterol synthesis genes and Aβ secretion.

Materials and Methods

Chemical Reagents

Dulbecco's modified Eagle's medium (DMEM), TRIzol, geneticin, 0.25% trypsin/EDTA, and gentamycin were from Life Technologies Inc. (Burlington, ON, Canada). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Hank's balanced salt solution (HBSS) was from Wisent Multicell (Montreal, QC, Canada). Dimethyl sulfoxide (DMSO), recombinant human insulin, protease inhibitor cocktail, bovine serum albumin (BSA), and penicillin-streptomycin were from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada). Eagle's minimum essential medium (EMEM) was from ATCC (Manassas, VA, USA). Ham's F12 medium was from Cellgro (Manassas, VA, USA). The BSA standard, Experion RNA StdSens analysis kit, SsoFast EvaGreen Supermix, iScript cDNA synthesis kit, and PVDF membranes were from Bio-Rad (Hercules, CA, USA). Recombinant $A\beta_{42}$ and scrambled $A\beta_{42}$ peptide ($A\beta_{42}$sc), with the same amino-acids as $A\beta_{42}$ but in a randomized order, were from r-Peptide Inc. (Bogart, GA, USA). The sequences of $A\beta_{42}$ and $A\beta_{42}$sc were DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM VGGVVIA and KVKGLIDGAHIGDLVYEFMDSNSAI-FREGVGAGHVHVAQVEF, respectively. Antibodies for β-actin, total c-Jun, phospho c-Jun S63, phospho IRS-1 (S612), phospho IRS-1 (Y895), total IRS, total AKT, phospho AKT (S473), and DHCR34/seladin-1 were from New England Biolabs (Pickering, ON, Canada). SREBP2 and HMGCR antibodies were from Abcam (Cambridge, MA, USA). Western-Lighting Plus-ECL was from Perkin Elmer Inc. (Waltham, Massachusetts, USA). Autoradiography film was from Mandel Scientific (Guelph, ON, Canada).

Cell Cultures

Immortalized, undifferentiated human SH-SY5Y cells were maintained in EMEM: Ham's F12 (1:1) supplemented with 10% FBS and 1% gentamycin at 37°C and 5% CO_2. Immortalized mouse neuroblastoma 2a cells (N2a) and these cells stably transfected with human AβPP695 gene (N2a-AβPP) were grown in EMEM:DMEM (1:1) supplemented with 5% FBS and 1% penicillin-streptomycin [4].

Geneticin was added to the N2a-AβPP medium to select for cells carrying the AβPP gene.

Insulin and $A\beta_{42}$ Treatment

Cells were plated in 60-mm dishes at 70% density in serum-deficient medium (to exclude the effects of serum insulin on the cells). Human recombinant insulin was solubilized in sterile water at pH 2, following the manufacturer's instructions. Cells were treated, 24 h after plating, with insulin at varying concentrations and for different treatment times. Negative control cells were treated with the vehicle solution (sterile water at pH 2). $A\beta_{42}$ and $A\beta_{42}$sc were reconstituted to 400 mmol/L in 0.25% sterile acetic acid, following the manufacturer's instructions. SH-SY5Y cells were treated, 24 h after plating, with 5 μmol/L $A\beta_{42}$ or $A\beta_{42}$sc for different times. Negative control cells were treated with $A\beta_{42}$ vehicle (0.25% sterile acetic acid).

Protein Isolation and Western Blotting

After treatment, the cells were washed twice with HBSS and lysed with western loading buffer (5% glycerol, 5% β-mercaptoethanol, 3% SDS, 0.03% bromophenol blue, 10 nmol/L Tris-HCl). The lysates were boiled at 100°C for 10 min, cooled on ice for 5 min, and spun at 14000 rpm for 15 min. The supernatant containing the solubilized proteins was transferred to new tubes and total protein levels were determined by the trichloroacetic acid assay. Proteins (15 μg) were resolved on 10% SDS-PAGE gels and transferred onto PVDF membrane. The blots were incubated in blocking buffer [5% skim milk powder in 1× Tris-buffered saline Tween 20 (TBST) or filtered 3% BSA powder in 1× TBST] at room temperature for 1–2 h. The blots were incubated with a primary antibody at 1:1000 dilution overnight at 4°C, washed with 1× TBST, incubated with an appropriate secondary antibody at 1:5000 dilution, and the protein bands were visualized with ECL Plus solution on X-ray film. The bands were analyzed using UN-SCAN-IT gel 6.1 software (Silk Scientific Inc., Orem, Utah) for densitometry and the treated samples were compared to controls by calculating the fold-change of treated sample to vehicle.

Enzyme-Linked Immunosorbent Assay (ELISA)

The $A\beta_{40}$ and $A\beta_{42}$ ELISA kits were from Life Technologies Inc. (Burlington, ON, Canada). Media from N2a-AβPP cells were harvested and stored at −80°C. Samples were centrifuged at 200 rpm to remove cell debris, and diluted 1:2 using the provided buffer supplemented with protease inhibitor cocktail to prevent protein degradation. Standards and samples were prepared as per the instructions with the kit. Results were normalized to the protein content of cells and are presented relative to vehicle.

RNA Isolation and Quantitative Polymerase Chain Reaction (QPCR)

Total RNA was isolated from treated cells using TRIzol reagent, following the manufacturer's instructions. Genomic DNA was removed from the samples using Ambion DNA-free kits, following the manufacturer's instructions (Life Technologies, Burlington, ON, Canada). The concentration of RNA in each sample was determined using a NanoDrop 1000 UV–Vis Spectrophotometer (Thermo Scientific Inc., Nepean, ON, Canada). The RNA integrity was checked using an Experion Automated Electrophoresis System from Bio-Rad (Berkeley, CA, USA). The mRNA was transcribed into cDNA using iScript kits, following the manufacturer's instructions. The QPCR primers were from IDT (Coralville, IA, USA; Table 1). Mixtures consisting of 10 μL Ssofast Eva Green Supermix (Bio-Rad), 2 μL of cDNA sample at 1/10 dilution (determined

Table 1 Primer sequences of genes involved in cholesterol synthesis.

Gene			Sequences
Hs. DHCR24	Human	Forward	5′-CTTGCTACCCTGCTCCTTC-3′
		Reverse	5′-CGCTCTCGCTTATCTTCGAT-3′
Hs. HMGCR		Forward	5′-CTGACATGCAGCCAAAGC-3′
		Reverse	5′-GTTTACCCTCGATGCTCTTGT-3′
Hs. SREBF2		Forward	5′-GGACACACAGAAGAATCCGT-3′
		Reverse	5′-TCCCTACTCCATTGACTCTGAG-3′
Mm. DHCR24	Mouse	Forward	5′-CGAAGAGGTAGCGGAAGATG-3′
		Reverse	5′-AGAACTACCTGAAGACAAACCG-3′
Mm. HMGCR		Forward	5′-ACTGACATGCAGCCGAAG-3′
		Reverse	5′-CACATTCACTCTTGACGCTCT-3′
Mm. SREBF2		Forward	5′-GACACATAAGAGGATTCGAGAGC-3′
		Reverse	5′-CCCTATTCCATTGACTCTGAGC-3′

Hs. human, *Mm*. mouse.

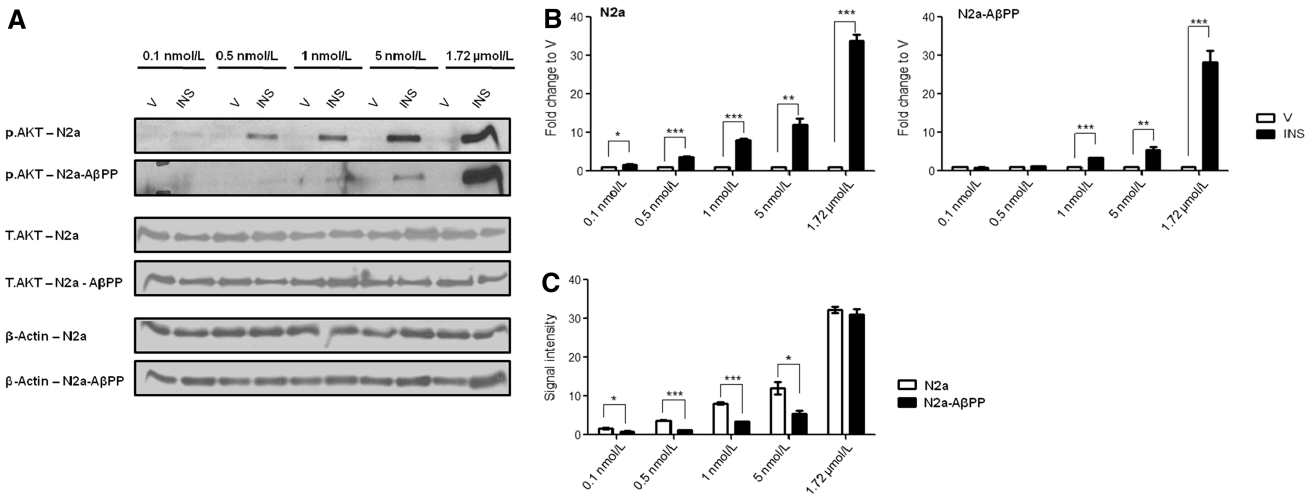

Fig. 1 Effects of insulin on Akt phosphorylation in N2a and N2a-AβPP cells. A, N2a and N2a-AβPP cells were treated with vehicle (V) or insulin (INS) for 1 h at 0.1, 0.5, 1, and 5 nmol/L, and 1.72 μmol/L. Cellular proteins were resolved by SDS-PAGE, and pAkt-S473 (p.AKT), total Akt (T.AKT), and β-actin protein levels were visualized by western blotting. B, Protein levels were quantified by densitometry, normalized to β-actin, and are presented relative to vehicle, which was set at 1 ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed *t*-test). C, Signal intensities of p-Akt in N2a and N2a-AβPP cells.

using standard curves for each primer), 2 μL of primers at a final concentration of 500 nmol/L, and 6 μL DNase/RNase-free water at a final volume of 20 μL were prepared for QPCR and run using a CFX96 Real-time PCR detection system (Bio-Rad). The reactions were carried out using the 2stepAmp+melt program with the conditions 95°C/10 min, 55°C/30 s, go to step 2 39×, 95°C/10 min. The PCR efficiency was assessed by performing standard curves using pooled cDNA material and plotting the log of the starting quantity of the template against the Cq values to determine the equation of the linear regression line. Quantification of each target gene was normalized against the β-actin gene and the fold-change for each gene was calculated using vehicle as control.

Statistical Analysis

All results are reported as mean ± SEM. Statistical significance was analyzed using the unpaired two-tailed *t*-test for two-group comparisons or by one-way ANOVA with Bonferroni post-hoc tests for multiple-group comparisons. Statistical analysis for all experiments was performed using GraphPad Prism version 5 (La Jolla, CA, USA).

Results

Compromised Insulin Signaling and Inhibition of Insulin-Dependent Up-Regulation of Cholesterol-Synthesis Enzymes in N2a-AβPP Versus N2a Cells

The effects of Aβ on insulin signaling in the N2a-AβPP cell line expressing AβPP and producing Aβ peptides were investigated by treating these cells and the parental N2a cells with insulin. N2a and N2a-AβPP cells were treated with insulin for 1 h at physiological concentrations 0.1, 0.5, and 1 nmol/L [33], higher concentration 5 nmol/L, and supra-physiological level 1.72 μmol/L. A comparison of the levels of insulin signaling in N2a and N2a-AβPP cells was carried out by visualizing the phosphorylation of Akt at S473 (pAkt) by western blot analysis (Fig. 1A). N2a cells displayed significant increases in pAkt at all insulin concentrations (Fig. 1B, left panel), while N2a-AβPP cells showed significant increases in the pAkt signal only at the higher concentrations of insulin (Fig. 1B, right panel). Accordingly, N2a-AβPP cells showed a significantly lower pAkt signal at all concentrations (except for 1.72 μmol/L) than the parental N2a cells (Fig. 1C). This indicates that insulin signaling is compromised in the N2a-AβPP cells, and suggests that insulin signaling is inhibited or interfered with in cells over-expressing AβPP and producing Aβ. Total Akt was also measured (Fig. 1A), and the levels were similar in both cell lines (data not shown).

To further support the finding that N2a-AβPP cells did not respond to physiological levels of insulin, N2a and N2a-AβPP cells were treated for up to 10 h with 0.1, 0.5, or 5 nmol/L insulin, and the expression levels of various cholesterol-synthesis genes were analyzed by QPCR. The SREBP2 gene expression level in N2a cells was significantly up-regulated from 1 h to 6 h, and was significantly down-regulated after 10 h of treatment at 0.1 and 0.5 nmol/L insulin (Fig. 2A, B, left panels). There was no significant change in SREBP2 expression in N2a-AβPP cells at these low concentrations (Fig. 2A, B, right panels), reflecting the poor response in insulin signaling as shown in Fig. 1. In

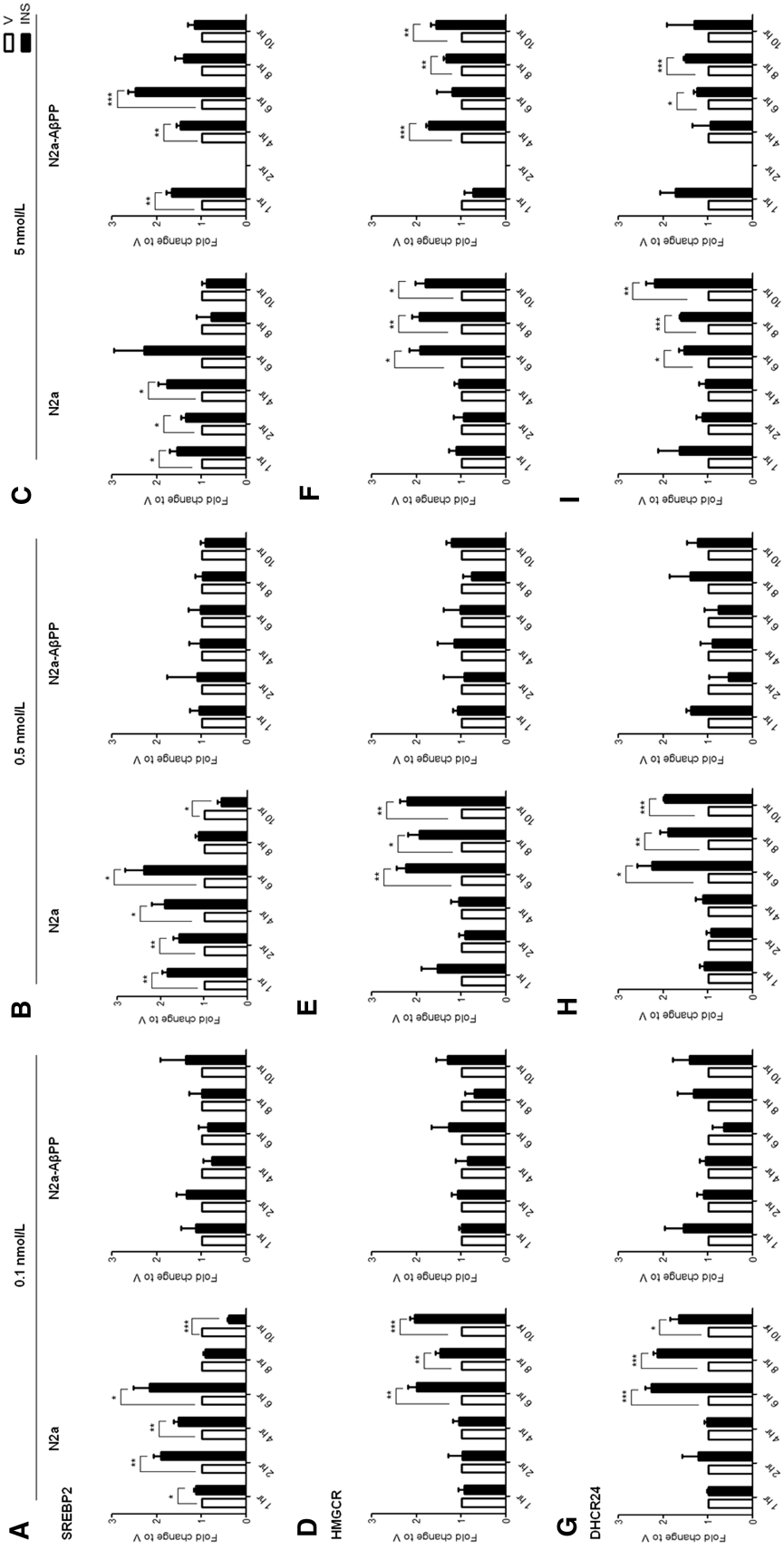

Fig. 2 Effects of insulin on the expression of cholesterol-synthesis genes in N2a and N2a-AβPP cells. N2a and N2a-AβPP cells were treated with 0.1, 0.5, and 5 nmol/L insulin (INS) or vehicle (V) for 1, 2, 4, 6, 8, and 10 h. Gene expression levels of SREBP2 (A–C), HMGCR (D–F), and DHCR24 (G–I) were determined by QPCR, normalized to β-actin, and are presented relative to vehicle, which was set to 1 ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed *t*-test).

cells treated with the higher concentration of 5 nmol/L insulin, the SREBP2 gene expression level was significantly up-regulated after 1, 2, and 4 h of treatment in N2a cells and after 1, 4, and 6 h in N2a-AβPP cells (Fig. 2C). Similarly, the HMGCR gene expression level was significantly up-regulated in N2a cells at all insulin concentrations after 6 and up to 10 h (Fig. 2D–F, left panels), but it was only up-regulated with 5 nmol/L insulin in N2a-AβPP cells after 4, 8 and 10 h of treatment (Fig. 2F, right panel). The expression level of HMGCR was not significantly increased at 0.1 or 0.5 nmol/L insulin after any treatment time in N2a-AβPP cells (Fig. 2 D, E, right panels). The DHCR24 gene expression level followed a similar trend, where the expression was significantly up-regulated in N2a cells at all insulin concentrations from 6 h up to 10 h (Fig. 2G–I, left panels), while it was only significantly up-regulated at 5 nmol/L in N2a-AβPP cells after 6 and 8 h of treatment (Fig. 2I, right panel). The expression level of DHCR24 was not significantly increased in N2a-AβPP cells with 0.1 or 0.5 nmol/L insulin at any treatment time (Fig. 2G, H, right panels). These results suggest that insulin resistance and the dysregulation of insulin-regulated cholesterol-metabolism genes occur in N2a-AβPP cells at physiological levels of insulin as compared to parental N2a cells.

Phosphorylation of IRS-1 at S612 Occurs in N2a-AβPP Cells at Basal Levels

Phosphorylation of IRS-1 at S612 represents the dysregulation of IRS activity [24, 25]. To determine how insulin signaling was dysregulated in N2a-AβPP cells at physiological levels of insulin, the phosphorylation state of IRS subunits was analyzed. N2a and N2a-AβPP cells were plated in serum-deprived medium for 24 h and cellular proteins were harvested for western blots (Fig. 3A). There was no significant difference in the levels of pAkt or pIRS-Y895 between N2a-AβPP and N2a cells (Fig. 3B), which indicated that there were no differences in insulin signaling between them. However, there was a significant increase in phosphorylation of IRS-1 at S612 in N2a-AβPP as compared to N2a cells. This suggests that IRS and the insulin signaling pathway are inhibited in N2a-AβPP cells.

Effects of Insulin on $A\beta_{40}$ and $A\beta_{42}$ Levels in N2a-AβPP Cells

The effects of insulin on Aβ metabolism were investigated in N2a and N2a-AβPP cells. The cells were treated with 5 nmol/L insulin for up to 8 h and the levels of $A\beta_{40}$ and $A\beta_{42}$ in the conditioned medium were determined by ELISA. A significant increase in $A\beta_{40}$ and $A\beta_{42}$ levels was found in the medium of N2a-AβPP cells at all treatment times (Fig. 4A, B). As expected, Aβ was not detected in the parental N2a cell line since it does not express human AβPP (data not shown). The levels of Aβ in the medium increased in a time-dependent manner after treatment with insulin, showing that insulin promotes the secretion of Aβ. The ELISA kits were not sensitive enough to detect Aβ levels within the cells to note any differences and to rule out increased production versus secretion of Aβ.

$A\beta_{42}$ Treatment of SH-SY5Y Cells Results in Phosphorylation of IRS-1 at S612 and c-Jun at S63 and Affects the Levels of Cholesterol-Synthesis Enzymes

In order to determine whether Aβ is a key factor causing insulin resistance in neural cells, SH-SY5Y cells were treated with 5 μmol/L $A\beta_{42}$ or scrambled $A\beta_{42}$ ($A\beta_{42}$sc) for 4, 6, and 10 h. Then the phosphorylation states of Akt, pIRS-S612, and c-Jun Ser63, as well as the levels of HMGCR and DHCR24 were determined by western blot analysis (Fig. 5A). The levels of pAkt did not change in treated cells versus controls (data not shown). The levels of pIRS-S612 were significantly higher in cells treated with $A\beta_{42}$ for 6 and 10 h than in vehicle- or $A\beta_{42}$sc-treated controls (Fig. 5B), suggesting a potential insulin-resistance effect in SH-SY5Y cells due to the presence of $A\beta_{42}$. The pIRS-S612 levels were also significantly increased relative to vehicle control after 4 h of treatment in $A\beta_{42}$- or $A\beta_{42}$sc-treated SH-SY5Y cells (Fig. 5B), suggesting a peptide effect at 4 h. Furthermore, the levels of phosphorylated c-Jun Ser63 were significantly higher after 6 and 10 h of treatment than in both controls (Fig. 5C). As we reported previously, increased c-Jun phosphorylation indicates the activation of JNK and AP-1 [34]. The level of DHCR24 protein was significantly decreased in SH-SY5Y cells after treatment with $A\beta_{42}$ for 4 and 6 h in comparison to both controls, and after 10 h in comparison to vehicle control (Fig. 5D). On the other hand, the level of HMGCR protein was significantly higher in response to 6 h of $A\beta_{42}$ treatment than both controls (Fig. 5E). These results indicate that $A\beta_{42}$ interferes with the levels of cholesterol-synthesis enzymes and cholesterol homeostasis in SH-SY5Y cells.

Insulin Signaling and Insulin-Dependent Up-Regulation of Cholesterol-Synthesis Enzymes in SH-SY5Y Cells

SH-SY5Y cells were treated for 1 h with insulin at 0.1, 0.5, 1, 5 nmol/L and 1.72 μmol/L, and insulin signaling was verified by analyzing the phosphorylation of Akt at serine 473 by western blot analysis (Fig. 6A). The results showed

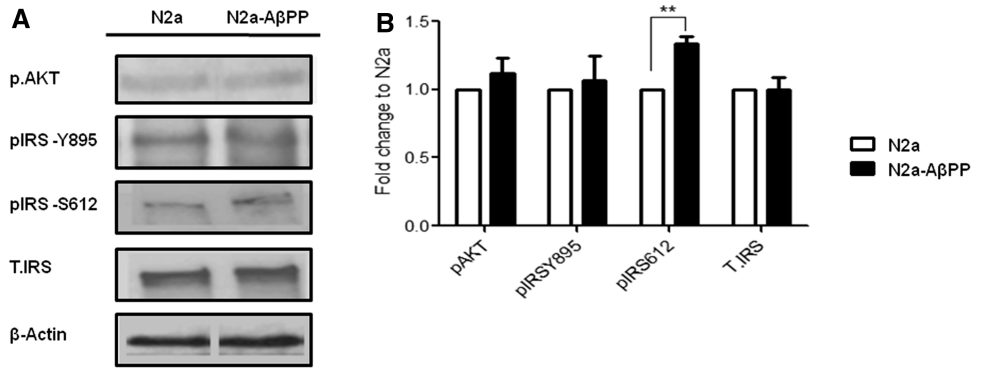

Fig. 3 N2a-AβPP cells exhibit higher IRS phosphorylation at S612 under basal conditions than N2a cells. (A) N2a and N2a-AβPP cells were plated in serum-free medium for 24 h and western blot was carried out for pAkt-S473 (p.AKT), pIRS-Y895, pIRS-S612, total IRS (T.IRS), and β-actin. (B) The levels were quantified by densitometry, normalized to β-actin and are presented relative to N2a cells, which were set to 1 ($n = 3$; $^{**}P < 0.01$, two-tailed *t*-test).

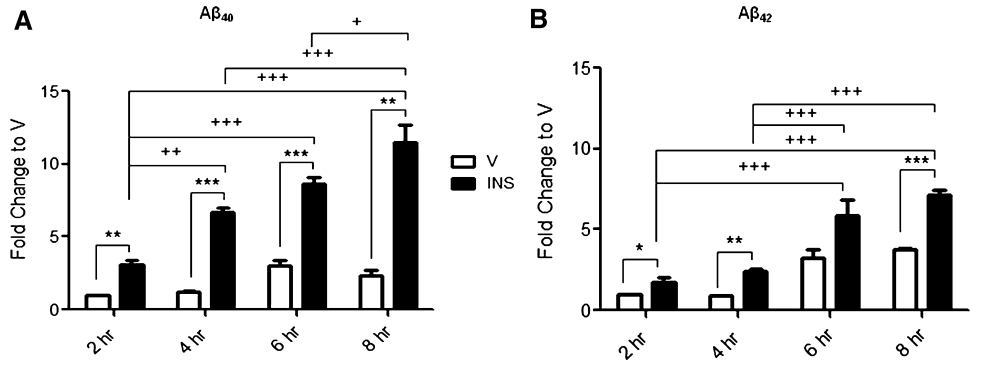

Fig. 4 Effect of insulin treatment on the levels of $A\beta_{40}$ and $A\beta_{42}$ secreted by N2a-AβPP cells. N2a-AβPP cells were treated with 5 nmol/L insulin for up to 8 h. The levels of $A\beta_{40}$ (A) and $A\beta_{42}$ (B) in the conditioned medium were normalized to cellular protein content and are presented relative to the average of vehicle at 2 h, which was set to 1 ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed *t*-test; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, one-way ANOVA).

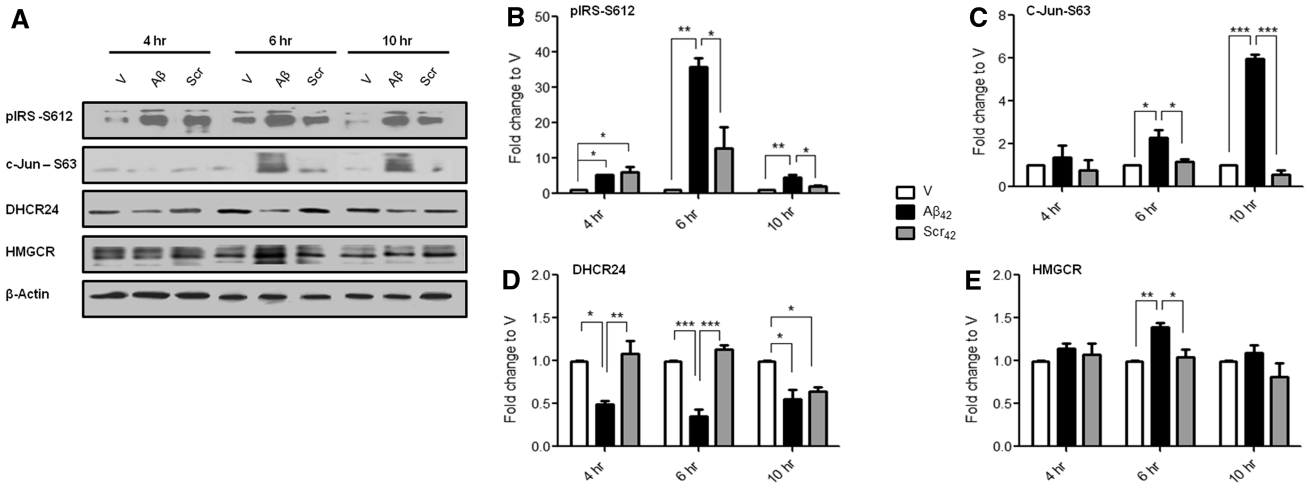

Fig. 5 Effects of $A\beta_{42}$ treatment on SH-SY5Y cells. SH-SY5Y cells were treated with vehicle (V), 5 μmol/L $A\beta_{42}$ (Aβ) or $A\beta_{42}$sc (Scr) for 4, 6, or 10 h. A, Cell proteins were harvested, resolved on SDS-PAGE, and the phosphorylation of IRS at S612 and c-Jun at S63, and the protein levels of DHCR24 and HMGCR were determined by western blotting. B–E, The levels of these proteins were quantified by densitometry, normalized to β-actin, and are presented relative to vehicle, set to 1 ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA).

a significant increase in Akt phosphorylation in cells treated with all doses of insulin as compared to cells treated with vehicle (Fig. 6B). There was no significant difference in total Akt levels between the cells treated with insulin or with vehicle (Fig. 6A). These data show that insulin signaling occurs in SH-SY5Y cells treated with insulin. The protein levels of HMGCR and DHCR24 were determined by western blotting after 8-h insulin treatment (Figure 6C). The levels of HMGCR protein were significantly up-regulated at all insulin concentrations, including physiological levels (0.1–1 nmol/L; Fig. 6D, left panel). Similarly, the levels of DHCR24 protein were significantly up-regulated at all insulin concentrations (Fig. 6D, right panel). The expression levels of the cholesterol-synthesis genes were also analyzed by QPCR at the same insulin concentrations after treatment from 1 to 10 h. After 1 h, there was a significant increase in SREBP2 gene expression level in cells treated with 0.1 to 5 nmol/L insulin and it continued to be upregulated for up to 2 h (Fig. 7A). Starting at 4 h, a significant decrease in SREBP2 gene expression occurred in cells treated with 5 nmol/L insulin, and at 6 h in cells treated with 0.1–1 nmol/L insulin (Fig. 7A). HMGCR gene expression was significantly increased at all insulin concentrations starting at 4 h and up to 10 h (Fig. 7B). Similarly, DHCR24 gene expression was significantly increased at 0.1 nmol/L from 6 h to 10 h and at 0.5, 1, and 5 nmol/L from 4 h to 10 h (Fig. 7C). The up-regulation of cholesterol-synthesis enzymes mirrors the increased gene expression in response to insulin treatment at physiological levels.

Discussion

A number of studies have shown that AD brain is insulin-resistant and/or deficient in insulin signaling; this has been coined "Type III diabetes" [17, 35]. Insulin resistance or deficiency in insulin signaling may represent important mechanisms underlying the neuronal dysfunction and neurodegeneration leading to dementia [35]; however, the molecular mechanisms are still not completely understood. We used the *in vitro* models of N2a, N2a-AβPP, and SH-SY5Y cells to investigate whether AβPP and Aβ peptides are involved in the development of this phenotype. Treatment of SH-SY5Y and N2a cells with insulin showed that insulin activated insulin signaling in these cells (as shown by Akt phosphorylation) and stimulated the expression of cholesterol synthesis enzymes. However, N2a-AβPP cells, which overexpressed AβPP and produced $A\beta_{40}$ and $A\beta_{42}$ peptides, displayed an insulin-resistant phenotype relative

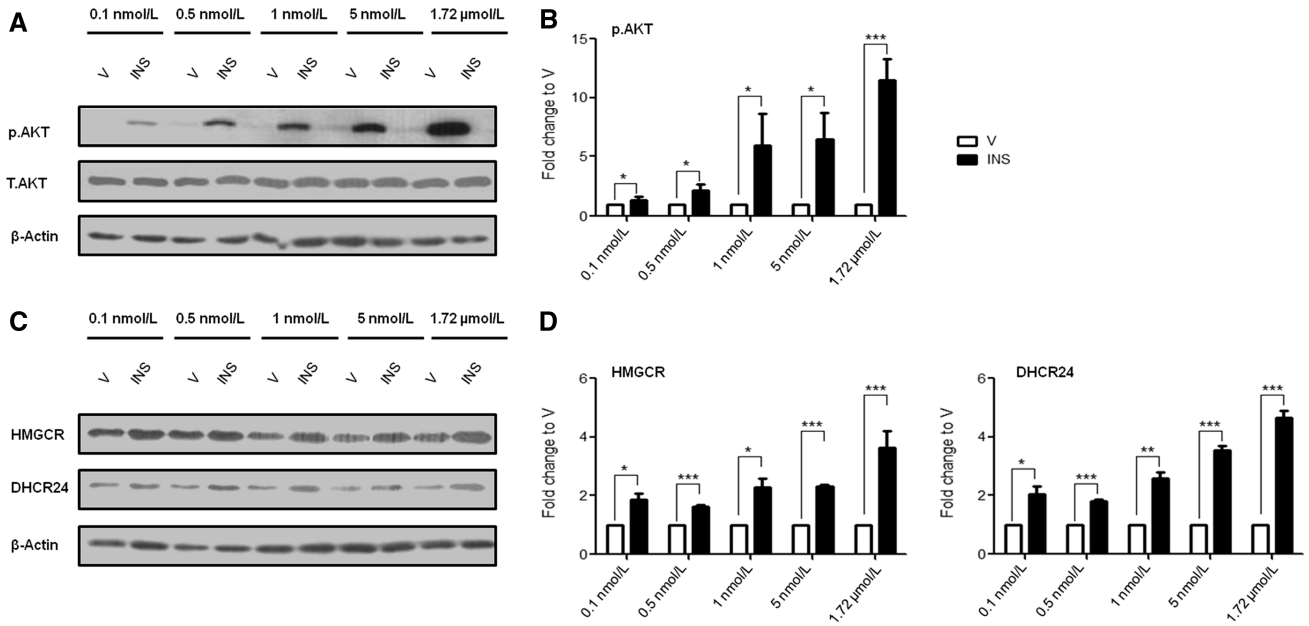

Fig. 6 Effects of insulin on Akt phosphorylation and cholesterol-synthesis enzymes in SH-SY5Y cells. A, B, SH-SY5Y cells were treated for 1 h with insulin at 0.1, 0.5, 1, and 5 nmol/L, and 1.72 μmol/L. Cell proteins were harvested and resolved by SDS-PAGE, and p-Akt at S473, total Akt, and β-actin protein levels were detected by western blotting (A). The levels of p-Akt were quantified by densitometry, normalized to β-actin, and are presented relative to vehicle, which was set to 1 ($n = 3$; $^*P < 0.05$, $^{***}P < 0.001$, two-tailed *t*-test) (B). C, D, SH-SY5Y cells were treated with insulin at the same concentrations as in (A) but for 8 h. The cell proteins were harvested and resolved by SDS-PAGE, and the protein levels of HMGCR and DHCR24 were analyzed by western blotting (C). The band intensities were quantified by densitometry, normalized to β-actin, and are presented relative to vehicle, which was set to 1 ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed *t*-test) (D).

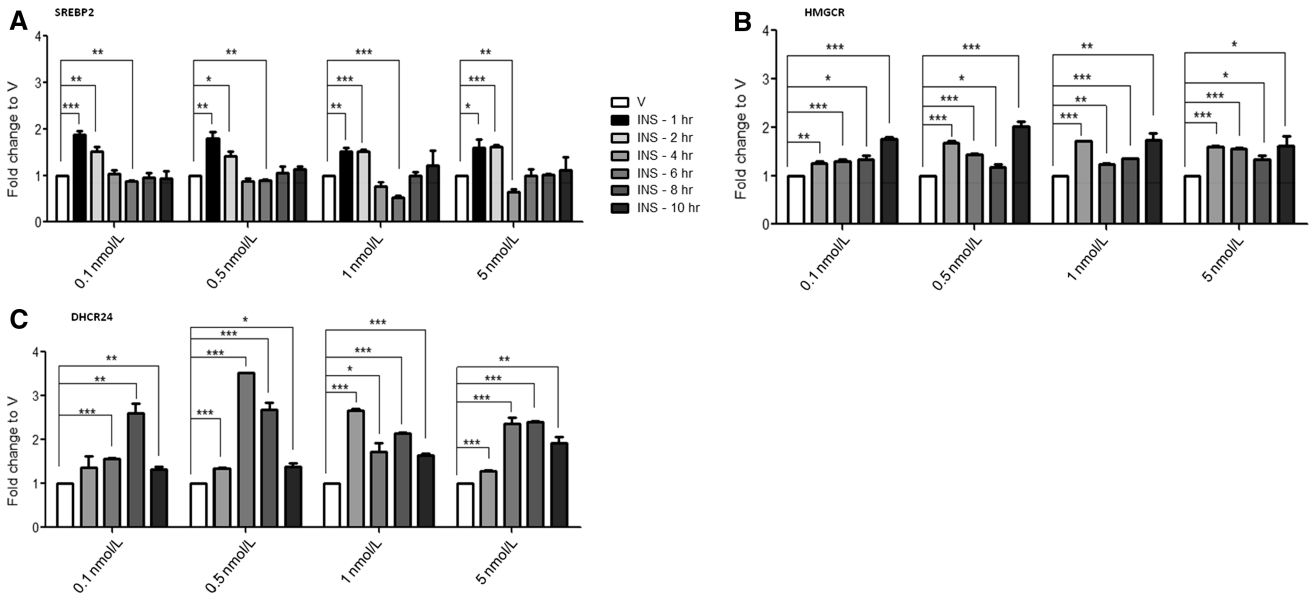

Fig. 7 Cholesterol genes are upregulated by insulin treatment. SH-SY5Y cells were treated with 0.1, 0.5, 1, and 5 nmol/L insulin for 1, 2, 4, 6, 8, or 10 h. The mRNA was extracted, reverse-transcribed to cDNA, and QPCR analysis was performed for SREBP2 (A), HMGCR (B), and DHCR24 (C). The levels were normalized to β-actin, and are presented relative to vehicle, which was set to 1 ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed *t*-test).

to parental N2a cells, as evidenced by reduced phosphorylation of Akt at S473 and disrupted insulin-dependent cholesterol-synthesis gene expression. It is attractive to speculate that the compromised insulin signaling in N2a-AβPP cells is due to the presence of Aβ peptides and/or AβPP. Increased phosphorylation of IRS-1 S612 in human neuroblastoma SH-SY5Y cells further substantiated that Aβ may cause insulin resistance. These results suggested that pIRS-1 S612 could be one of the sites of Aβ-induced insulin resistance in neural cells. Several mechanisms may be involved in the development of the insulin-resistant phenotype in these cells. Aβ peptides can cause c-Jun phosphorylation in SH-SY5Y cells as well as in human brain endothelial cells as we described previously [34, 36], indicating the activation of c-Jun N-terminal kinase (JNK). Activated JNK may associate with IRS-1 and inhibit the insulin-stimulated tyrosine phosphorylation of IRS-1 and thus block the downstream transmission of insulin signaling [37, 38]. SH-SY5Y cells treated with $A\beta_{42}$ showed an increase in phosphorylation of c-Jun at serine 63, indicating activation of JNK and AP-1 for the pro-inflammatory response. Our previous study found that JNK-AP1 signaling is activated in the AD brain and in Aβ-treated brain endothelial cells, and is responsible for increased expression of inflammatory cytokines and chemokines [39]. Interestingly, activation of the pro-inflammatory response in SH-SY5Y cells post-Aβ treatment was in the same timeframe as pIRS-1 phosphorylation at S612, suggesting that Aβ-activated JNK and other events are involved in the development of the insulin-resistant phenotype in these cells. Aβ peptides may also competitively bind to or interact with IRs [40], which desensitizes their response to insulin. All of these possibilities support the notion that overproduction or accumulation of Aβ peptides may be a primary cause of insulin resistance in neural cells.

Aβ dysregulated the expression of cholesterol-synthesis genes, not only in N2a-AβPP cells, but in SH-SY5Y cells as well. Treatment of SH-SY5Y cells with $A\beta_{42}$ up-regulated the expression of HMGCR, which coincided with c-Jun phosphorylation in the cells. There is no report on the HMGCR levels in AD brain. HMGCR is a rate-limiting enzyme upstream in the mevalonate pathway that produces cholesterol and other isoprenoids [41]. The expression of genes for the proteins that are involved in isoprenoid synthesis, along with isoprenoids themselves, have been reported to be elevated in the frontal cortex of AD patients [42, 43] and have been implicated in inflammation and oxidative stress [44–46]. HMGCR inhibitors (such as statins) have been shown to reduce Aβ generation by depleting isoprenoids independent of cholesterol levels [47–49]. This suggests that up-regulation of HMGCR in SH-SY5Y cells by $A\beta_{42}$ may increase the isoprenoid levels for an inflammatory response. DHCR24, also known as selective AD indicator 1 (seladin 1), is a multifunctional protein due to its enzymatic, antioxidant, anti-apoptotic, and neuroprotective activities [50, 51], and is ubiquitously expressed in endocrine glands and in the brain [52]. DHCR24 is down-regulated in AD-vulnerable brain

regions such as the inferior temporal cortex [52, 53], and the enzyme has been shown to protect neurons from Aβ-mediated toxicity and to decrease $A\beta_{42}$ production by counteracting the β-secretase cleavage of AβPP [54, 55]. Moreover, DHCR24-dependent cholesterol synthesis reduces the membrane aggregate interaction and cell damage associated with amyloid treatment [56]. Thus, our results of decreased DHCR24 protein levels in SH-SY5Y cells in response to $A\beta_{42}$ treatment are consistent with the literature. $A\beta_{42}$ increased HMGCR expression and decreased DHCR24 expression in SH-SY5Y cells, which may lead to dysregulation of the cholesterol-synthesis pathway or cholesterol homeostasis, an increased inflammatory response, and decreased neuroprotection. Overall, $A\beta_{42}$ induces pro-inflammatory c-Jun phosphorylation and HMGCR expression and down-regulates the expression of neuroprotective/anti-inflammatory DHCR24 in SH-SY5Y cells.

Interestingly, treatment of N2a-AβPP cells with insulin (5 nmol/L) increased the levels of $A\beta_{40}$ and $A\beta_{42}$ in the medium, in line with other studies [57, 58]. It has been reported that insulin-induced release of Aβ into the culture medium can reduce the intracellular accumulation of the peptide [58]. The release of Aβ into the extracellular compartment can lead to its degradation by membrane-associated and secreted insulin-degrading enzymes [59] or Aβ clearance. Along with increasing Aβ secretion into the medium, insulin favors α-secretase activity with increased neurotrophic action, inhibits the translocation of the AβPP intracellular domain into the nucleus, and phosphorylates GSK3β, inhibiting its enzymatic activity for reduced tau pathology [60]. Studies have also shown that insulin inhibits the nuclear activity of the AβPP intracellular domain, reduces the transcription of genes that encode pro-amyloidogenic enzymes such as β-secretase and GSK3β, and increases the transcription of anti-amyloidogenic enzymes such as α-secretase and insulin-degrading enzyme [60, 61]. Our results together with those of others suggest that enhancing insulin signaling can relieve insulin resistance, have positive effects on the suppressed cholesterol metabolism of neural cells, and promote Aβ release from the cells.

In summary, the results of this study demonstrated that Aβ may be a primary cause of developing an insulin-resistant phenotype in N2a-AβPP and SH-SY5Y cells as manifested by decreased response to insulin treatment, increased IRS-1 inhibition, and dysregulation of cholesterol-synthesis gene expression. Another study has provided direct evidence that intraneuronal expression of $A\beta_{42}$ inhibits both insulin-induced Akt phosphorylation and activity [62]. In addition, Aβ induced c-Jun phosphorylation and the expression of pro-inflammatory HMGCR, as well as inhibiting the expression of anti-inflammatory/neuroprotective DHCR24. All of these conditions dysregulate the function and metabolism of neural cells. Enhancement of insulin signaling by higher concentrations of insulin relieved the insulin-resistant phenotype and the dysregulated cholesterol synthesis pathway, increased the release of Aβ peptides from cells into the extracellular compartment, and inhibited the pro-inflammatory response. Alleviation of insulin resistance in AD brain may be a future direction of therapeutic development to relieve or delay the progression of the disease and neurodegeneration.

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