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Curcumin mediates presenilin-1 activity to reduce β-amyloid production in a model of Alzheimer's disease

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Abstract:
Curcumin has been reported to inhibit the generation of Aβ, but the underlying mechanisms by which this occurs remain unknown. Aβ is thought to play an important role in the pathogenesis of Alzheimer's disease (AD). The amyloid hypothesis argues that aggregates of Aβ trigger a complex pathological cascade that leads to neurodegeneration. Aβ is generated by the processing of APP (amyloid precursor protein) by β- and γ-secretases. Presenilin 1 (PS1) is central to γ-secretase activity and is a substrate for GSK-3β, both of which are implicated in the pathogenesis of AD. The present study aimed to investigate the effects of curcumin on the generation of Aβ in cultured neuroblastoma cells and on the *in vitro* expression of PS1 and GSK-3β. To stimulate Aβ production, a plasmid expressing APP was transfected into human SH-SY5Y neuroblastoma cells. The transfected cells were then treated with curcumin at 0–20 μM for 24 h or with 5 μM curcumin for 0–48 h, and the extracellular levels of $A\beta_{40/42}$ were determined by ELISA. The levels of PS1 and GSK-3β mRNA were measured by RT-PCR, and the expression of the PS1 and GSK-3β proteins (including the phosphorylated form of GSK-3β, p-GSK-3β-Ser9) were evaluated by western blotting. Curcumin treatment was found to markedly reduce the production of $A\beta_{40/42}$. Treatment with curcumin also decreased both PS1 and GSK-3β mRNA and protein levels in a dose- and time-dependent manner. Furthermore, curcumin increased the inhibitory phosphorylation of GSK-3β protein at Ser9. Therefore, we propose that curcumin decreases Aβ production by inhibiting GSK-3β-mediated PS1 activation.

Key words:
Alzheimer's disease, β-amyloid, curcumin, GSK-3β, PS1

Abbreviations: Aβ – β-amyloid, AD – Alzheimer's disease, APP – amyloid precursor protein, BACE1 – β-site APP-cleaving enzyme, GSK-3β – glycogen synthase kinase-3β, PS1 – presenilin 1

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects nearly 5% of people over 65 years of age and approximately 30% of individuals aged 85 or over. Neuritic plaques, neurofibrillary tangles, and neuronal loss are the main histological hallmarks observed in the brain of a patient with AD. Amyloid β-protein (Aβ) is the central component of senile plaques. Aβ is derived by sequential endoproteolytic cleavage of the type 1 transmembrane glycoprotein amyloid precursor protein (APP) by β- and γ-secretases [5]. β-Secretase cleavage *in vivo* is thought to be mediated by the β-site APP-cleaving enzyme BACE1 [13]; however, the key cleavage step leading to Aβ formation is mediated by γ-secretase [18]. γ-Secretase is an atypical multimeric membrane-bound aspartyl protease composed of presenilin (PS1 or PS2), nicastrin (Nct), presenilin enhancer 2 (PEN-2), and anterior pharynx defective 1 homologs (APH-1aL, APH-1aS, or APH-1b). Although the expression

and activity of each of the components of the γ-secretase complex are tightly coordinated, PS1 is generally thought to harbor the catalytic core of the enzyme [10, 19].

PS1 has been reported to be an unprimed substrate for glycogen synthase kinase-3β (GSK-3β), leading to the phosphorylation of serine residues in the PS1 loop domain and modulation of γ-secretase activity [15]. GSK-3β, a serine/threonine kinase, was first identified as an enzyme that phosphorylates and inactivates glycogen synthase [3, 20]. The GSK-3β enzyme is involved in a wide range of cellular processes ranging from glycogen metabolism to cell cycle regulation and cell proliferation. Mutations affecting PS1 are a major cause of early-onset familial AD [9, 12], and elevated GSK-3β expression has been associated with AD development [21]. Although the precise mechanisms of neurodegeneration in AD remain controversial, the accumulation of Aβ is thought to play a pivotal role in the pathogenesis of AD.

Curcumin (diferuloylmethane) is a phenolic compound extracted from the plant *Curcuma longa*, and it has been used to treat various ailments, including inflammatory disease [8], cancer [7], AIDS [2], and other diseases [4]. Epidemiological studies in India, a country where curcumin consumption is widespread, have shown that it has one of the lowest rates of AD worldwide [1]. There is increasing evidence that curcumin inhibits the production of Aβ both *in vitro* and *in vivo* [17, 22], but the underlying mechanisms by which this occurs are not known.

In the present study, we examined the effects of curcumin on the generation of Aβ and investigated the underlying mechanism by which curcumin inhibits the production of Aβ. We report that curcumin inhibits GSK-3β-stimulated PS1 activity *in vitro* and suppresses the generation of Aβ. These data suggest that curcumin may be an effective anti-amyloid therapy for the prevention and treatment of AD.

Materials and Methods

Cell culture and transfection

The human neuroblastoma cell line SH-SY5Y (Department of Pathophysiology, Chongqing Medical University, China) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml of penicillin. The cells were maintained at 37°C in an incubator containing 5% CO_2. For transfection, the cells were grown in 75 cm^2 flasks to approximately 70% confluence, and each flask was transfected with 8 μg pAPPswe (from Prof. Weihong Song, University of British Columbia, Vancouver, BC, Canada) using 30 μl of Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Curcumin treatment

Curcumin (Sigma) was dissolved in 75% (v/v) ethanol. The transfected SH-SY5Y cells were treated with curcumin at 0, 1.25, 5.0, or 20.0 μM for 24 h, or at 5.0 μM for 0, 12, 24, or 48 h. The controls were treated with a vehicle solution. The cells and cell culture were collected separately and stored frozen before subsequent analysis.

Aβ determination by ELISA

To measure the levels of extracellular Aβ, conditioned media from transfected cells were treated with a cocktail of protease inhibitors including 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and the $Aβ_{40/42}$ levels were measured by a colorimetric ELISA assay using a commercial detection kit (Biosource International Inc., CA, USA) according to the manufacturer's instructions.

Measurement of mRNA levels by RT-PCR

RNA was isolated from cells using Biozol (BioFlux, Tokyo, Japan). PowerScript Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA according to the manufacturer's instructions, and the cDNA was further amplified by Platinum *Taq* NA polymerase (Invitrogen) in a 25 μl reaction using the following primers: PS1, forward 5'-GAC ATA CTT GTA CGC TCA CTT GC-3', reverse 5'-CCT AGA ATA ATG GGA CCA TCT GC-3' and GSK-3β, forward 5'-TCC CTC AAA TTA AGG CAC ATC-3', reverse 5'- CAC GGT CTC CAG TAT TAG CAT CT-3'. The internal control, β-actin, was amplified using 5'-CTC GTC ATA CTC CTG CTT GCT G-3' and 5'-CGG GAC CTG ACT GAC TAC C TC-3'. The RT-PCR products were analyzed on a 1.2% agarose gel.

Western blot analysis

The cells were lysed in PRO-PREP™ liquid (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2; Intron Biotechnology, Kyungi-Do, Korea) supplemented with a protease inhibitor cocktail (Roche, Welwyn Garden City, UK). Cell lysates were collected by centrifugation 13,000 × g × 10 min, 4°C), and the protein concentrations were determined by the Bradford method employing a Universal Microplate Reader (Gene Company, Hong Kong) at 595 nm. After gel electrophoresis (15% polyacrylamide in a Tris-glycine buffer system) in the presence of SDS, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MS, USA). After blocking, the membranes were incubated with primary antibodies directed against GSK-3β (46 kD, Cell Signaling, #9315, Beverly, MA, USA), p-GSK-3β (Ser9) (46 kD, Cell Signaling, #9336), PS1 (18 kD, Chemicon International Inc, MAB5232), or β-actin (43 kD, Beijing Biosynthesis Biotechnology Co., Ltd., bs-0061R) diluted in 0.1% (w/v) non-fat dry milk powder and incubated overnight at 4°C. After washing, the bound antibodies were detected by incubation for 1–2 h at room temperature with secondary peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Zhong Shan Golden Bridge Biotech Company, China). The membranes were then developed using a commercial enhanced chemiluminescence system (ECL; Bio-Rad, Hercules, CA, USA) and quantitated using Quantity One image analysis (Bio-Rad, USA).

Statistical analysis

All data were normally distributed and are presented as the means ± SD. The homogeneity test for variance was performed using Prism 5 software (GraphPad, San Diego, CA, USA). The significance of the differences between the groups was determined using the two-tailed Student's *t*-test.

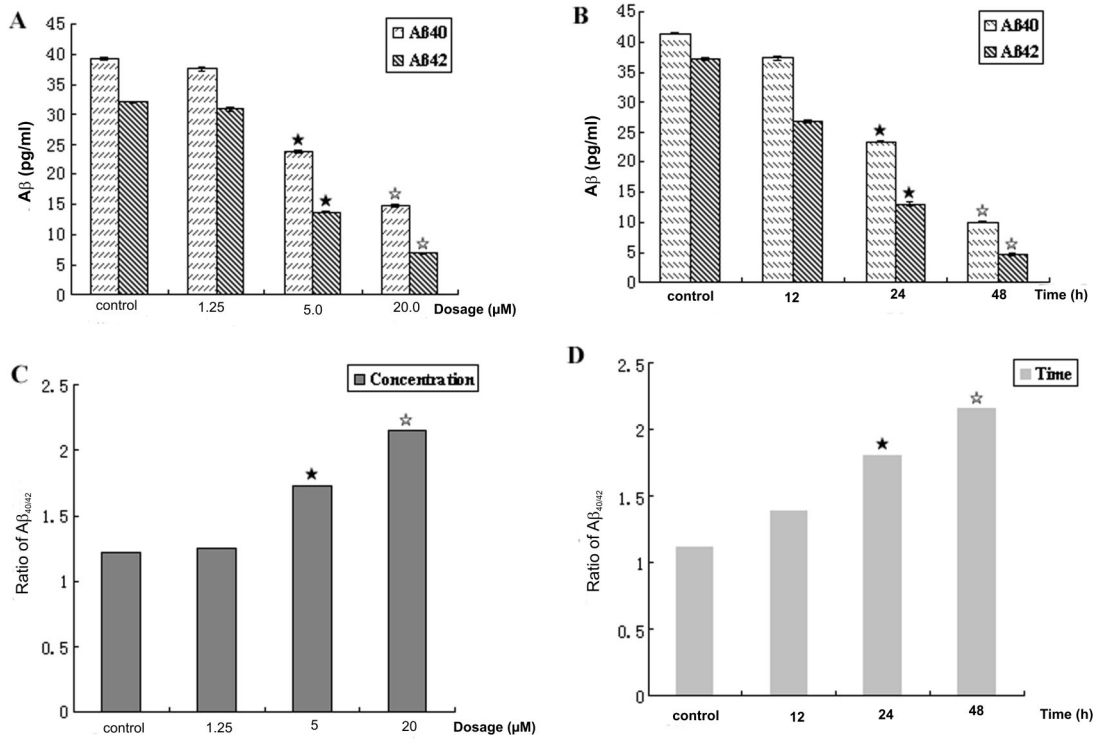

Fig. 1. Curcumin treatment significantly decreased the generation of $A\beta_{40/42}$. The transfected cells were treated with curcumin at 0, 1.25, 5.0, or 20.0 μM for 24 h for the dosage-dependent assay (**A**), or with curcumin at 5.0 μM for 0, 12, 24, and 48 h for the time course assay (**B**). The conditioned media from the cells were collected. The concentration of $A\beta_{40}/A\beta_{42}$ was measured by the ELISA kit. The values represent the mean ± SD (n = 3). The results show that curcumin inhibited the generation of $A\beta_{40}/A\beta_{42}$ in a dose- and time-dependent manner, and $A\beta_{42}$ was decreased more significantly than $A\beta_{40}$. The reduction in the $A\beta_{40}$ and $A\beta_{42}$ levels was both dose- and time-dependent, and the ratio of $A\beta_{40}/A\beta_{42}$ was significantly increased (**C**, **D**). (★ $p < 0.05$, compared with control; ☆ $p < 0.01$, compared with control)

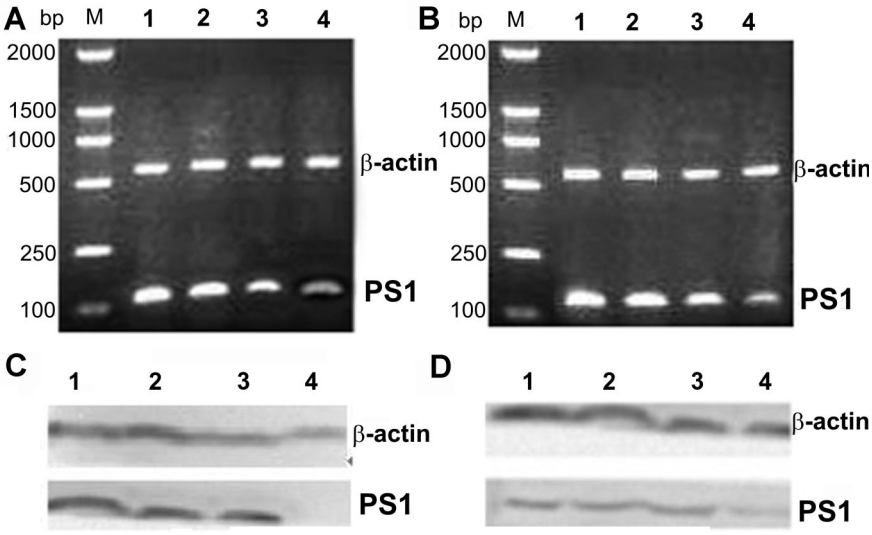

Fig. 2. Curcumin inhibited the expression of PS1 in the transfected cells. The transfected cells were treated with curcumin for 24 h at 1.25, 5.0, or 20.0 μM (**A**, **C**) or with curcumin at 5.0 μM for 12, 24, or 48 h (**B**, **D**). (**A**, **B**) RT-PCR analysis shows that curcumin significantly reduced the expression of PS1 mRNA in a concentration- and time-dependent manner. (**C**, **D**) Western blot analysis shows that curcumin also reduced PS1 protein in a concentration- and time-dependent manners

Tab. 1. Statistical analysis of the OD values of PS1/β-actin mRNA in the transfected cells after curcumin treatment (OD value = comparative density value of PS1/β-actin mRNA)

Concentration	OD values	*t*	Time	OD values	*t*
Control	0.93 ± 0.26		control	0.97 ± 0.32	
1.25 μM	0.81 ± 0.18	1.33	12 h	0.88 ± 0.24	0.75
5.0 μM	0.54 ± 0.17	3.90☆	24 h	0.71 ± 0.3	2.06☆
20.0 μM	0.32 ± 0.19	N6.45★	48 h	0.38 ± 0.29	4.72★

(Note: ☆ $p < 0.05$, compared with control; ★ $p < 0.01$, compared with control)

Tab. 2. Statistical analysis of the OD values of PS1/β-actin in the transfected cells after curcumin treatment (OD value = comparative density value of PS1/β-actin)

Concentration	OD values	*t*	Time	OD values	*t*
Control	0.53 ± 0.14		control	0.45 ± 0.11	
1.25 μM	0.47 ± 0.15	1.02	12 h	0.39 ± 0.12	1.17
5.0 μM	0.38 ± 0.17	2.34☆	24 h	0.31 ± 0.10	3.25☆
20.0 μM	0.10 ± 0.11	8.43★	48 h	0.24 ± 0.08	5.38★

(Note: ☆ $p < 0.05$, compared with control; ★ $p < 0.01$, compared with control)

Results

Curcumin inhibits the generation of $A\beta_{40/42}$ in transfected cells

The effects of curcumin on the generation of Aβ were examined in SH-SY5Y neuroblastoma cells. First, the SH-SY5Y cells were transfected with pAPPswe to promote the formation of Aβ. The cells were then treated with curcumin at different concentrations and for different time points, and the Aβ levels were determined. As shown (Fig 1, A, B), the $A\beta_{40}$ and $A\beta_{42}$ levels were strongly decreased by treatment with curcumin. When the cells were treated with 5–20 μM curcumin for 24 h, the production of $A\beta_{40}$ and $A\beta_{42}$ was decreased by 39–51% ($p < 0.01$). A comparable reduction in $A\beta_{40}$ and $A\beta_{42}$ was observed following incubation with 5 μM curcumin for 24–48 h (Fig. 1, A, B). The reduction in the $A\beta_{40}$ and $A\beta_{42}$ levels was both dose- and time-dependent, and the ratio of $A\beta_{40}/A\beta_{42}$ was significantly increased ($p < 0.05$) (Fig. 1, C, D).

Curcumin reduces the expression level of PS1

Aβ is produced by β- and γ-secretase-mediated cleavage of APP. Because $A\beta_{40/42}$ production was inhibited by curcumin, we investigated whether curcumin reduces γ-secretase activity. PS1 is the central catalytic component of γ-secretase; therefore, we studied whether curcumin treatment affects the expression levels of PS1. RT-PCR and western blotting were used to measure PS1 mRNA and protein levels following curcumin treatment in APP-overexpressing neuroblastoma cells. As shown in Figure 2 and Tables 1, 2, curcumin treatment significantly ($p < 0.05$) reduced both the PS1 mRNA and protein levels. The inhibitory effect occurred in a dose- and time-dependent manner, and the expression levels were most strongly reduced at 48 h of treatment with 20 μM curcumin ($p < 0.01$).

Curcumin downregulates GSK-3β expression

The activity of PS1/γ-secretase is modulated by GSK-3β; therefore, we studied whether the observed changes in the Aβ and PS1 levels are associated with changes in the expression and/or phosphorylation of GSK-3β. Curcumin-treated cells were analyzed by RT-PCR for GSK-3β mRNA levels and by western blotting for protein levels of GSK-3β and its phosphorylated form, p-GSK-3β-Ser9. As shown in Figure 3 and Tables 3–5, curcumin treatment had no significant effect on the mRNA levels of the internal control (β-actin) but significantly reduced the levels of both GSK-3β mRNA and protein ($p < 0.05$). Moreover, curcumin increased GSK-3β phosphorylation at Ser9 ($p < 0.05$; Fig. 3). All of the observed changes were in a time- and concentration-dependent manner.

Discussion

Accumulating evidence suggests that Aβ is a central element in the pathogenesis of AD [16], and Aβ production is a characteristic feature of both the sporadic and familial forms of AD. Aβ has two forms, $A\beta_{40}$ and $A\beta_{42}$ that are normally present in a ratio of 10:1. The minor form, $A\beta_{42}$ has been implicated in the pathogenesis of AD because of its propensity to form fibrous neurotoxic aggregates. In addition, excessive production of $A\beta_{42}$ leads to the formation of $A\beta_{42}$ aggregates and senile plaques. In this study, we have in-

Tab. 3. Statistical analysis of the OD values of GSK-3β/β-actin mRNA in the transfected cells after curcumin treatment (OD value = comparative density value of GSK-3β/β-actin mRNA)

Concentration	OD values	*t*	Time	OD values	*t*
Control	0.98 ± 0.21		control	0.76 ± 0.33	
1.25 μM	0.89 ± 0.37	0.73	12 h	0.66 ± 0.41	0.68
5.0 μM	0.65 ± 0.43	2.36☆	24 h	0.45 ± 0.31	2.38☆
20.0 μM	0.55 ± 0.27	4.34★	48 h	0.24 ± 0.27	4.24★

(Note: ☆ $p < 0.05$, compared with control; ★ $p < 0.01$, compared with control)

Tab. 4. Statistical analysis of the OD values of GSK-3β/β-actin in the transfected cells after curcumin treatment (OD value = comparative density value of GSK-3β/β-actin)

Concentration	OD values	*t*	Time	OD values	*t*
Control	0.99 ± 0.12		control	0.98 ± 0.27	
1.25 μM	0.85 ± 0.23	1.27	12 h	0.88 ± 0.17	1.13
5.0 μM	0.36 ± 0.43	9.11☆	24 h	0.61 ± 0.21	3.01☆
20.0 μM	0.23 ± 0.27	9.87★	48 h	0.37 ± 0.11	4.66★

(Note: ☆ $p < 0.05$, compared with control; ★ $p < 0.01$, compared with control)

Tab. 5. Statistical analysis of the OD values of GSK-3β-Ser9/β-actin in the transfected cells after curcumin treatment (OD value = comparative density value of GSK-3β-Ser9/β-actin)

Concentration	OD values	*t*	Time	OD values	*t*
Control	0.25 ± 0.19		control	0.31 ± 0.2	
1.25 μM	0.70 ± 0.27	4.69	12 h	0.61 ± 0.19	3.75☆
5.0 μM	0.89 ± 0.33	5.82☆	24 h	0.84 ± 0.23	6.02☆
20.0 μM	0.95 ± 0.32	6.37★	48 h	0.97 ± 0.31	6.47★

(Note: ☆ $p < 0.05$, compared with control; ★ $p < 0.01$, compared with control)

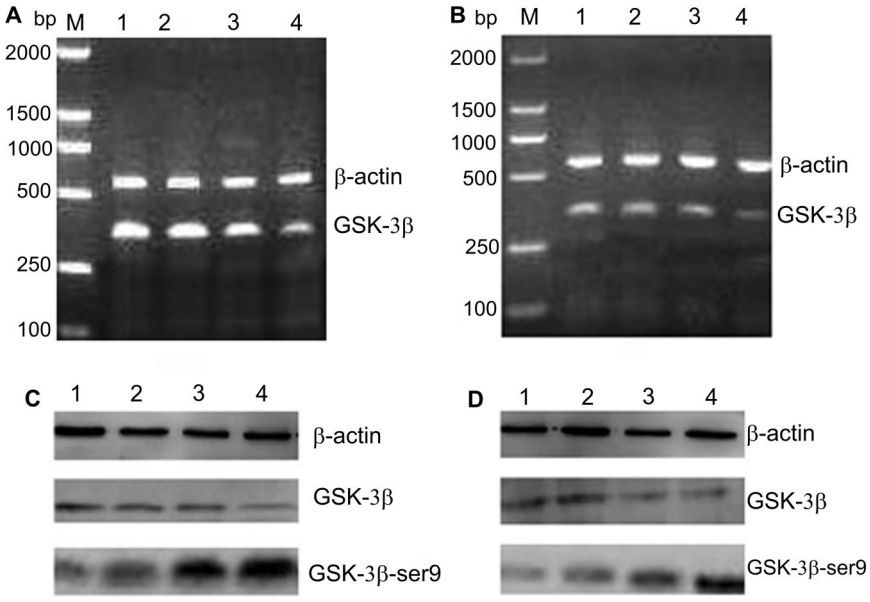

Fig. 3. Treatment with curcumin suppressed the activity of GSK-3β in cultured cells. SHSY5Y cells were transfected with expression plasmids and were treated with curcumin at the various concentrations indicated for 24 h or at 5.0 μM for different time points. In **A** and **B**, RT-PCR analysis showed that treatment with curcumin had no effect on the expression of β-actin. Treatment with a concentration of 5.0 μM curcumin for 24 h led to decreased expression of GSK-3β mRNA ($p < 0.05$). In **C** and **D**, the cell lysates were prepared and subjected to Western blot analysis for GSK-3β and its phosphorylated form GSK-3β-Ser9. The expression of β-actin was unchanged with curcumin treatment, while the expression of GSK-3β significantly decreased with the increasing concentration of curcumin and the extension of the time course ($p < 0.05$). GSK-3β-Ser9 levels were notably elevated with increasing concentrations and time points ($p < 0.05$). The changes all occurred in a concentration- and time-dependent manner

vestigated the potential effects of curcumin on the generation of Aβ and on the underlying mechanisms of Aβ reduction. SH-SY5Y neuroblastoma cells were transfected with pAPPswe to increase the Aβ content; the transfected cells were then treated with curcumin and the levels of Aβ were analyzed. This analysis revealed that curcumin treatment produced a concentration- and time-dependent reduction in the levels of both $A\beta_{40}$ and $A\beta_{42}$. The decrease in $A\beta_{42}$ was more significant than that of $A\beta_{40}$, as reported previously [17, 22].

Aβ is generated from APP cleavage by β- and γ-secretase, the latter being pivotal for the production of Aβ in AD [11]. The catalytic site of γ-secretase is within PS1and increased PS1 activity has been associated with AD [6]. Our results confirmed that curcumin inhibits the production of Aβ, but it was unclear which element of the amyloidogenic pathway was directly targeted. We theorized that curcumin inhibits Aβ formation through its effects on γ-secretase. We report that curcumin inhibits the expression of PS1 at both the mRNA and protein levels in a concentration- and time-dependent manner.

PS1 has been reported to be a target for GSK-3β-mediated phosphorylation [15]. GSK-3β is found in a tetrameric complex with β-catenin and with the C- and N-terminal fragments (CTF, NTF) of PS1 [14], and this complex is inferred to regulate the phosphorylation of PS1. We hypothesized that curcumin might inhibit the activity of GSK-3β by reducing the activity of PS1 and in turn, inhibit the formation of Aβ. Curcumin was found to reduce the expression of GSK-3β at both the mRNA and protein levels. We also report that the phosphorylated form of GSK-3β, p-GSK-3β (Ser9) was increased after curcumin treatment in a time- and concentration-dependent manner. GSK-3β is activated by phosphorylation at Tyr216 but is inactivated by phosphorylation at Ser9 [7], and the latter is thought to play a pivotal role in gating GSK-3β activity. Together, these data reveal that curcumin downregulates GSK-3β activity by regulating the gene expression and phosphorylation of the enzyme. Because GSK-3β-mediated phosphorylation of PS1 is thought to modulate its activity, it is plausible that curcumin-mediated inhibition of GSK-3β reduces γ-secretase activity.

In conclusion, we suggest that curcumin inhibits Aβ formation by inhibiting GSK-3β-mediated phosphorylation of PS1 and γ-secretase activity. Further research is needed to elucidate the molecular mechanisms of the curcumin-mediated inhibition of GSK-3β activity, and *in vivo* studies in animal models will be required to explore the potential of curcumin as a preventative or therapeutic agent in AD.

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