



Curcumin inhibits BACE1 expression through the interaction between ER β and NF κ B signaling pathway in SH-SY5Y cells

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Received: 3 March 2019 / Accepted: 25 September 2019 / Published online: 8 October 2019
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Abstract

Alzheimer's disease (AD) is the leading cause of dementia, which characterized by toxic senile plaques is composed of amyloid- β (A β). β -site amyloid precursor protein-cleaving enzyme 1 (BACE1) is the rate-limiting protease in A β generation. Therefore, pharmacology BACE1 inhibition is one of the prime targets for potential treatment of AD. Curcumin, a yellow polyphenol derived from the rhizomes of the plant *Curcuma longa* Linn, has been reported to cross the blood–brain barrier and prevent A β aggregation in AD models. However, its neuroprotective mechanism is still unclear. In the present study, we find that curcumin markedly reduces A β levels in HEK293-APP_{sw} cells. Our results show that curcumin inhibits *BACE1* gene expression in SH-SY5Y cells at transcriptional and translational levels. Furthermore, we reveal that nuclear factor kappa B (NF κ B) signaling is involved in the regulation of curcumin on BACE1. Interestingly, the estrogenicity of curcumin is found to partially contribute to its protective action. Our data show that curcumin activates estrogen receptor β (ER β) selectively and the activation of ER β directly effects on the upstream factors of the NF κ B signaling pathway. The above results indicate that curcumin reduces BACE1 expression through ER β and NF κ B pathway, providing a novel mechanism for curcumin as a candidate for AD therapy.

Keywords Curcumin · AD · BACE1 · NF κ B · ER β

Abbreviations

AD	Alzheimer's disease
A β	Amyloid- β
BACE1	B-site amyloid precursor protein-cleaving enzyme1
NF κ B	Nuclear factor kappa B
ER	Estrogen receptor

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11010-019-03638-0>) contains supplementary material, which is available to authorized users.

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Introduction

Alzheimer's disease (AD) is characterized by two major histopathological hallmarks: the cerebral accumulation of senile plaques and intracellular neurofibrillary tangles [1]. A fertile area of research supports the amyloid cascade hypothesis that neuronal dysfunction, synaptic loss, neurofibrillary neurodegeneration, and the full manifestation of Alzheimer pathology are initiated by amyloid β (A β) [2]. A β results from sequential proteolysis of the amyloid- β precursor protein (APP) by β -secretase and γ -secretase, with β -cleavage being the rate-limiting step in this pathway [3]. β -site amyloid precursor protein-cleaving enzyme 1 (BACE1) is the major β -secretase and expresses highly in neurons [4]. It has been reported that BACE1 $^{-/-}$ mice dramatically abolishes A β generation [5]. In addition, BACE1 deficiency mice crossed with APP transgenic mice do not show A β -dependent memory loss [6]. Most importantly, the side effects of BACE1 inhibition are relatively moderate to γ -secretase inhibitors [7]. Herein, inhibition of BACE1 is currently one of the viable treatments for AD.

Mounting evidence suggests an early and substantial involvement of inflammation in AD pathogenesis. For

instance, elevated concentrations of proinflammatory cytokines can be found in AD brains [8]. Nuclear factor kappa B (NF κ B) signaling is the center of immune responses and inflammation [9]. NF κ B is a complex including five members: p50, p52, p65 (Rel A), c-Rel, and Rel B. In basic state, inhibitory protein I κ B α associates with NF κ B in the cytoplasm. Upon activated by stimuli, I κ B α undergoes phosphorylation, ubiquitination, and consequently degradation. This enables NF κ B dimers (the mainly abundant, p65:p50) transferring to the nucleus to induce target genes. The recent study revealed aberrant activation of NF κ B in AD patients [10]. Since the putative binding sites for NF κ B in BACE1 promoter have been identified, several groups reported that NF κ B signaling regulates BACE1 gene expression at multiple levels [11]. These observations suggest that the regulation of NF κ B might be a good target for investigation.

Curcumin (Fig. 1a) is a polyphenolic compound isolated from turmeric which possesses anti-carcinogenic, anti-inflammatory, and anti-oxidant properties [12]. The new study reported that curcumin has received much attention as a fluorochrome in retinal imaging for AD diagnostics [13]. Curcumin was shown to prevent A β aggregation from the formation of non-toxic aggregates by Thapa group [14]. Previous research showed that the protective effect of curcumin is associated with its property of CpG demethylation [15]. However, the research about the mechanism of curcumin in AD treatment is not well established, particularly from its anti-inflammatory aspect. In the present study, we aimed to explore the mechanism underlying curcumin's anti-amyloidogenesis action in AD cell models. Based on its potent modulatory role in the immune response, we reasoned that this process is mediated by NF κ B pathway. We further found that, selectively, activation of estrogen receptor β (ER β) is involved in curcumin's effects on NF κ B. The interaction between ER and NF κ B pathway is investigated in our neuroprotective study.

Materials and methods

Cell culture and drug treatment

SH-SY5Y (human neuroblastoma cells) and HEK293 (human kidney cells) were purchased from American Type Culture Collection (USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, BI) and 1% penicillin/streptomycin (Gibco). HEK293 cells stably transfected with a Swedish mutant APP695 (H293-APPswe) were cultured in the medium mentioned above with an additional 200 ng/ml G418 (Sigma, USA). All cells were cultured at 37 °C in a humidified incubator containing 5% CO₂. Curcumin (#C1386), DAPT (#D5942), ICI 182,780

(#V900926), DPN (#H5915), PPT (#H6036), and MTT (#M2128) were purchased from sigma. TNF α was from CST. For each experiment, drugs were diluted from its stock solution and added to the growth medium at the indicated concentrations.

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H293-APPswe cells were incubated with indicated doses of curcumin for 24 h. Or H293-APPswe cells were transfected with pcDNA5-FLAG-P65, and then treated with 10 μ M curcumin. Medium was removed and cells were treated with opti-MEM (Gibco, USA) for another 4 h. Medium samples were collected and A β levels were determined by ELISA kit (CUSABIO, China) according to the manufacturer's indication.

Real-time PCR

SH-SY5Y cells were seeded in 6-well culture plates per 10⁶ cells/well, then treated with various concentration of curcumin for 12 h and collected for RNA analysis. Total RNA was isolated with Trizol extraction kits (Cwbio, China). Single-strand cDNA was synthesized from 1 μ g of total RNA using reverse transcription system (Promega, USA). The number of transcripts in cDNA samples was measured with SYBR Green I master (TianGen) by quantitative real-time PCR in Bio-rad CFX instrument (USA). The primers used are provided in Supplementary Table 1.

Luciferase report assay and transfection

HEK293 cells were seeded at approximately 10⁵ cells/well in a 24-well plate. Each well was transfected with 400 ng target plasmids and 2 ng pCMV. pCMV was used as an empty vector. After transfection, drugs were added into the medium. Luciferase assays were conducted with the Dual-Luciferase reporter assay system (Promega, USA). Plasmid pBACE1, pBACE1-NF κ B, phage-ER α , pEGFP-ER β , and pGL3-ERE were used in the study. The siRNAs against p65 were synthesized by Genepharma (China). The scramble RNA or si p65 RNA (5'-GGAGUACCCUGAGGCUAUATT-3') were transfected into SH-SY5Y cells using Lipofectamine 2000 (Invitrogen, USA).

Western blot

After indicated treatments, cells were collected and prepared in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 1 mM EDTA, 1 mM Na₃VO₄, 0.5 μ g/ml leupeptin). The protein content of cell lysates was determined by BCA assay

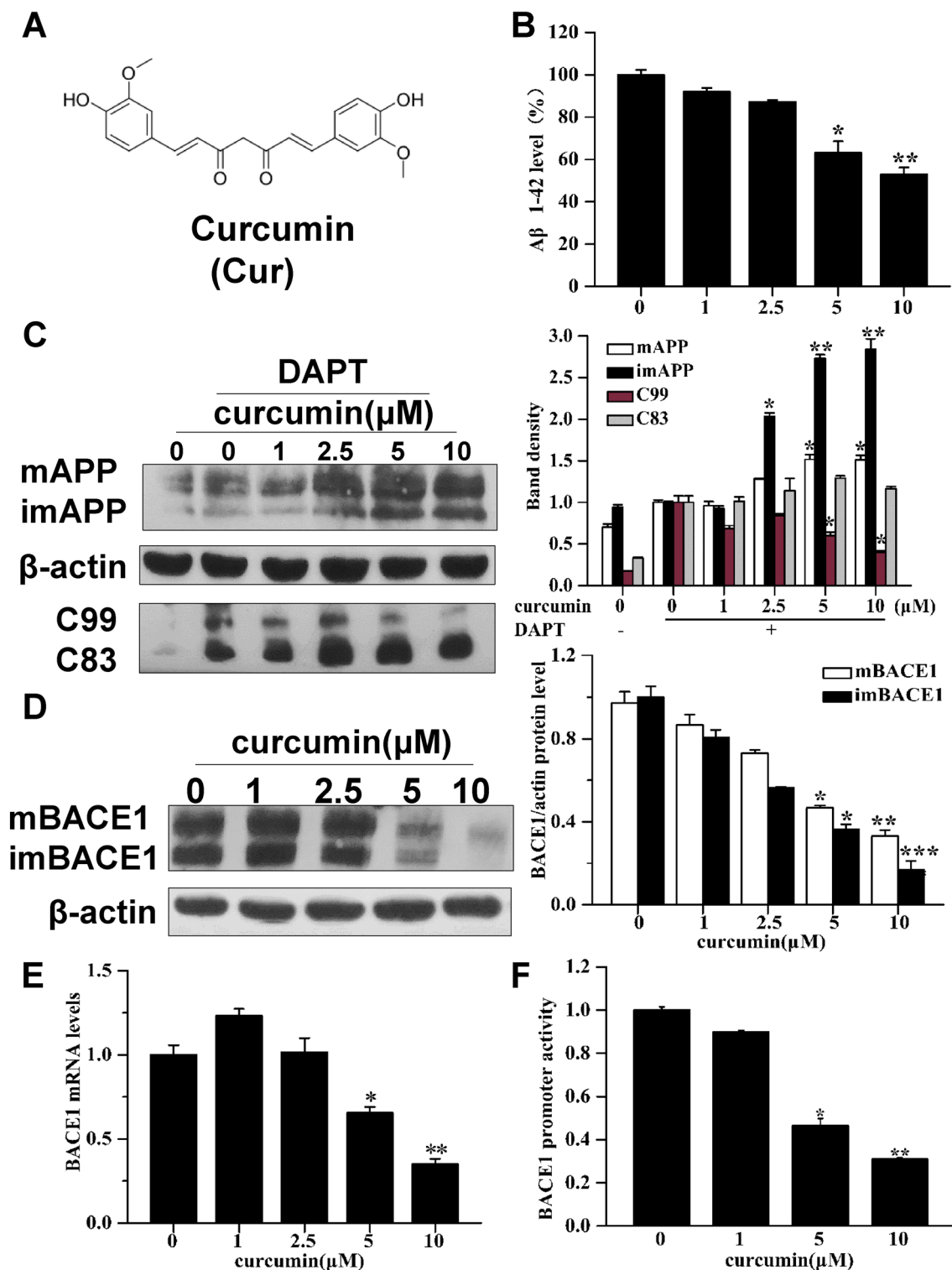


Fig. 1 Effects of curcumin on A β generation, APP processing, and BACE1 expression in H293-APP_{swe} and SH-SY5Y cells. **a** The chemical structure of curcumin. **b** Curcumin reduces A β ₄₂ levels in a dose manner by ELISA. * p < 0.05, ** p < 0.01 versus cont group. **c** The effects of curcumin on protein mAPP, imAPP, β -CTF(C99), and α -CTF(C83) were detected by western blot, with β -actin as an internal control. Protein levels were quantified by densitometry analysis (right panel). * p < 0.05, ** p < 0.01 versus DAPT only group. **d**

Western blot showing that curcumin greatly reduces BACE1 protein levels. * p < 0.05, ** p < 0.01 and *** p < 0.001 versus cont group. **e** QPCR showing a decrease of BACE1 mRNA expression after curcumin treatment. * p < 0.05, ** p < 0.01 versus cont group. **f** Luciferase reporter system shows that curcumin markedly inhibits BACE1 promoter activity. The data were expressed as mean \pm SEM. n = 3. * p < 0.05, ** p < 0.01 versus cont group

(Thermo, USA). Equivalent amounts of protein were separated by 10–15% SDS-PAGE gels and then transferred to PVDF membranes. After incubation with blocking buffer, membranes were incubated with primary antibodies overnight. The following antibodies were used: APP (1:6000, C-terminus, Abcam), BACE1(1:1000, Abcam), Akt (1:1000, CST), phospho-Akt (1:1000, CST), NF κ B (1:10,000, Abcam), I κ B α (1:1000, CST), phospho-I κ B α (1:1000, CST), phospho-NF κ B (1:1000, CST), β -actin (1:10,000, ABclonal), ER α (1:1000, CST), and ER β (1:1000, Sigma). Blots were detected using HRP-conjugated secondary antibodies (Pierce) and the ECL chemiluminescence kit (Millipore, USA). The bands were calculated via quantity one software (Bio-rad, USA).

Nuclear-cytoplasmic fraction

SH-SY5Y cells were pretreated with vehicle or curcumin for 1 h, followed by 10 ng/ml TNF α stimulation for the indicated times, and then harvested and spun down in cold PBS. Approximately 10 volumes of buffer A (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 1 mM DTT, plus proteinase inhibitors; compared with the volume of the cell pellet) was added to the cells, which were then incubated on ice for 15 min. A certain volume of buffer B (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10% NP40, plus proteinase inhibitors) was then added to ensure the final concentration of NP40 is 0.5%. After agitation and incubation on ice for 1 min, the mixture was centrifuged at 500 g for 10 min. The obtained supernatant was centrifuged at 16,000 \times g for another 30 min and the new supernatant was taken as the cytoplasmic fraction. The left pellet after the first centrifuge was washed by buffer A for several times before being re-suspended by buffer C (20 mM Hepes pH 7.9, 0.5 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, plus proteinase inhibitors). The mixture after re-suspension was then incubated on ice for 30 min and centrifuged at 16,000 \times g for 30 min. The resulting supernatant was taken as the nucleus fraction.

Immunocytochemistry staining

SH-SY5Y cells were cultured on a glass coverslip and incubated for 24 h. Cells were treated with 10 ng/ml TNF α or curcumin, or were pretreated with curcumin for 1 h and then co-treated with 10 ng/ml TNF α for 40 min in serum-free medium. Cells were rinsed with PBS before 4% paraformaldehyde fixation for 15 min at ambient temperature. Then cells were permeabilized with 0.1% TritonX-100 for 10 min. Fixed cells were blocked in 2% BSA for 30 min and

incubated with primary antibodies at 4 °C overnight. They were gently washed three times with PBS before incubation with fluorescence-conjugated secondary antibody for 1 h at dark situation. The antibody was used: Rabbit anti-NF κ B (1:100, CST), Alexa Fluor 594 secondary antibody (1:500, Invitrogen). Counterstaining was performed with DAPI to visualize the cell nuclei. Samples were evaluated by light microscopy (Olympus, Japan) and photomicrographs were digitally captured and stored.

Competitive fluorometric receptor-binding assay

The relative binding affinities of curcumin for estrogen receptors were identified according to the fluorescence polarization method as described previously [16]. Briefly, 40 nM coumestrol and 0.8 μ M human GST-ER α or GST-ER β were added into 384 plates at the indicated dose. Fluorescence polarization was measured after incubating at room temperature for 2 h.

Statistical analysis

All results were repeated at least three duplicates and all values were represented as mean \pm SEM. Statistical analysis was performed by ANOVA or student's *t* test. Statistical significance is accepted when $p < 0.05$. All statistical analysis and calculations were done using Origin 8 (Origin Lab, UK).

Results

Curcumin effects on β APP processing through β -secretase inhibition

To measure cell viability under the treatment of curcumin (0, 1, 2.5, 5, 10 μ M), MTT assay was first conducted. We found that curcumin had no detectable cytotoxic effects on cells at the concentration used (Supplementary Fig. S1). We then investigated the effect of curcumin on A β accumulation in HEK293-APP_{swe} cells. ELISA analysis was performed which showed that 10 μ M curcumin significantly reduced A β levels to $52.98 \pm 3.11\%$ ($p < 0.01$) compared to the untreated group (Fig. 1b). Next, we measured the levels of β -C-Terminal fragment (CTF, C99) and α -C-Terminal fragment (CTF, C83), two major APP C-terminal metabolites. To inhibit γ -secretase activity and prevent subsequent proteolysis cleavage, DAPT was added during the measurement thus making CTFs detectable. As shown in Fig. 1c, curcumin treatment attenuated the amount of C99 fragment but not C83 fragment, and yet augmented mature and immature

APP (mAPP and imAPP) levels. These results indicated that curcumin affects the amyloidogenic pathway of β APP and reduces A β levels in H293-APP_{swe} cells.

Since BACE1 is the rate-limiting enzyme initiating the production of A β in the amyloidogenic pathway, we next checked whether curcumin could decrease its expression. Our results showed that curcumin significantly reduced mature and immature BACE1 (mBACE1 and imBACE1) protein levels in a dose-dependent manner, with a maximum reduction ($33.08 \pm 2.89\%$ for mBACE1 and $16.87 \pm 4.22\%$ for imBACE1) at the concentration of 10 μ M (Fig. 1d), respectively. Curcumin treatment was also found to attenuate BACE1 mRNA levels dose-dependently (Fig. 1e). To further test the transcriptional activity of BACE1 promoter under curcumin treatment, we constructed a vector with BACE1 promoter ranging from -2464 to -1 bp and found that BACE1 promoter activity was also decreased (Fig. 1f). These findings demonstrated that curcumin might inhibit the amyloidogenic processing of β APP through inhibiting BACE1 gene expression.

NF κ B mediates the suppressive effect of curcumin on BACE1 expression

NF κ B is closely correlated with BACE1 levels in AD. In the study, exogenously expressed p65 enhanced BACE1 levels by almost threefold, which was markedly decreased by curcumin exposure (Fig. 2a). To further examine the effect of p65 on APP processing, A β generation was measured. Our results showed that p65 expression increased A β levels by $171.38 \pm 1.18\%$ in H293-APP_{swe} cells, and this increase was reversed after treating with curcumin (Fig. 2b). Meanwhile, disruption of p65 gene by siRNA results in BACE1 levels decreasing to $51.01 \pm 1.18\%$ (Fig. 2c), implying that curcumin might affect BACE1 expression through NF κ B pathway.

To assess the potential role of NF κ B in curcumin-regulated BACE1 gene transcription, HEK293 cells were transfected with pBACE1-NF κ B or pNF κ B followed by TNF α for the activation of NF κ B signaling. Dual luciferase revealed that TNF α induced pBACE1-NF κ B and pNF κ B transcription activity to $181.37 \pm 3.79\%$ and $432.78 \pm 3.49\%$ relative to control, respectively. Contrarily, curcumin treatment elicits a reduction to $34.23 \pm 1.89\%$ and $25.08 \pm 1.72\%$ (Fig. 2d). Similar results were obtained by transfecting p65 expression plasmid to HEK293 cells. P65 overexpression dramatically enhanced pBACE1-NF κ B activity to 10.73 ± 1.04 -fold ($p < 0.001$) compared to control. This increase was then significantly restored to 2.64 ± 0.25 -fold ($p < 0.001$)

by curcumin. Notably, pNF κ B activity showed a higher upregulation level (61.64 ± 0.59 -fold, $p < 0.001$) which is also inhibited by curcumin treatment (19.27 ± 1.69 -fold, $p < 0.001$) (Fig. 2e). These aforementioned results unveiled that NF κ B mediates the downregulation of curcumin on BACE1 transcriptional levels.

Curcumin inhibits inflammatory NF κ B activation

NF κ B activation comprises numerous steps and integrates with PKB/Akt signaling pathway, which is known to be aberrantly regulated in AD pathology. Accordingly, we examined several key factors to test how NF κ B signaling cascade was modulated upon exposure to curcumin.

In our cell line models, we first confirmed that TNF α induced the activation of Akt in time-dependent manner; however, curcumin clearly inhibited the phosphorylation of Akt in co-treating cells (Supplementary Fig. S2). Then, Cell fraction analysis was performed, revealing that TNF α induced rapid phosphorylation and degradation of I κ B α in a short time. This response was abolished in the presence of curcumin, accompanied by the inhibition of the ser536 phosphorylation of NF κ B p65 subunit in the cytoplasm (Fig. 3a). In addition, immunoblot analysis exhibited the enormous difference of the amount of NF κ B p65 in the nucleus between curcumin-treated group and curcumin-untreated group (Fig. 3b), indicating TNF α -induced p65 translocation from the cytoplasm to the nucleus was also blocked by curcumin. And this phenomenon was further proved by immunocytochemical analysis, with no observable stained p65 (red) merging with DAPI-stained nucleus (blue) in curcumin-treated group vs TNF α group (Fig. 3c). Taken together, these results suggest that curcumin inhibits TNF α -induced NF κ B activation.

ER β mediates curcumin's inhibition on BACE1 expression

Curcumin is reported to display estrogenicity and antiestrogenicity in various studies. We then investigated whether curcumin regulated BACE1 through estrogen receptor (ER).

The relative binding affinities [17] of curcumin for ER subtypes were first determined by a competitive fluorometric receptor-binding assay. The IC₅₀ values of 17 β -estradiol (E2) for ER α and ER β were 1.09×10^{-8} M and 1.27×10^{-8} M (Fig. 4a), approximating to the theoretical value of 10^{-8} M, confirming the validity of the assay. The RBA values of curcumin to ER α and ER β were 0.27% and 1.18% compared to that of E2, and its IC₅₀ values are 4.09×10^{-6} M and

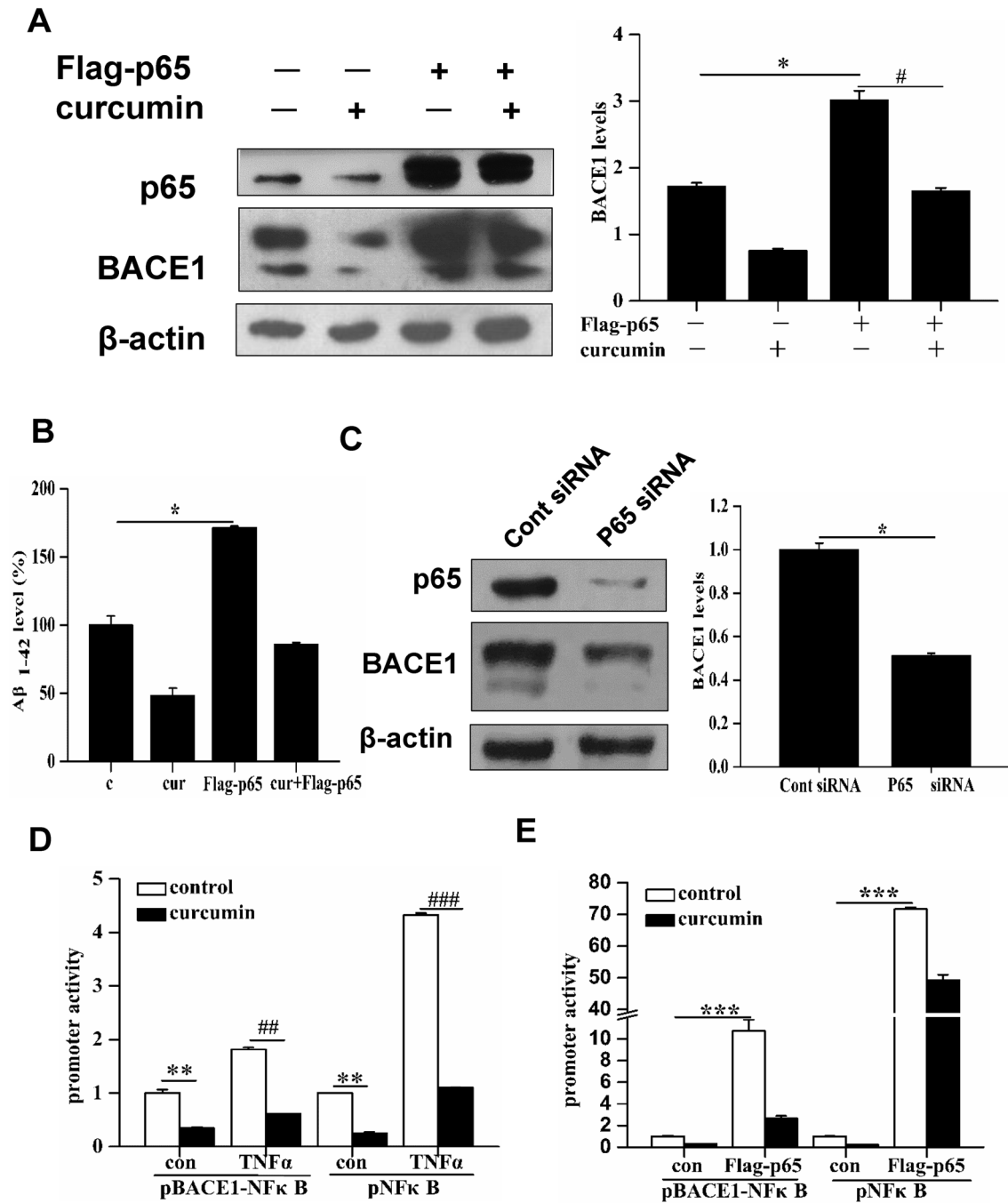


Fig. 2 The effect of NF κ B on BACE1 expression and A β generation, and the effect of curcumin on NF κ B transcriptional activity. **a** Western blot shows that p65 and BACE1 protein levels were both increased in H293 cells transfected with pcDNA-FLAG-p65. * p < 0.05 versus cont group, # p < 0.05 versus Flag-p65 only group. **b** ELISA showing that the levels of A β ₄₂ were enhanced by transfecting pcDNA-FLAG-p65. * p < 0.05 versus cont group. **c** SY5Y cells were transfected with cont si RNA or p65 si RNA. P65 and BACE1 expression were evaluated by western blot and quantified based on

densitometry. * p < 0.05 versus cont group. **d** Luciferase activities of pBACE1-NF κ B/pNF κ B were performed in H293 cells treated with curcumin and TNF α , respectively. ** p < 0.01 versus cont group, ## p < 0.01 and ### p < 0.001 versus TNF α only group. **e** Luciferase activities of pBACE1-NF κ B/pNF κ B were performed in H293 cells transfected with pcDNA5-FLAG-P65 and with/without curcumin, respectively. The data were represented as mean \pm SEM. n = 3 or 4. *** p < 0.001 versus cont group

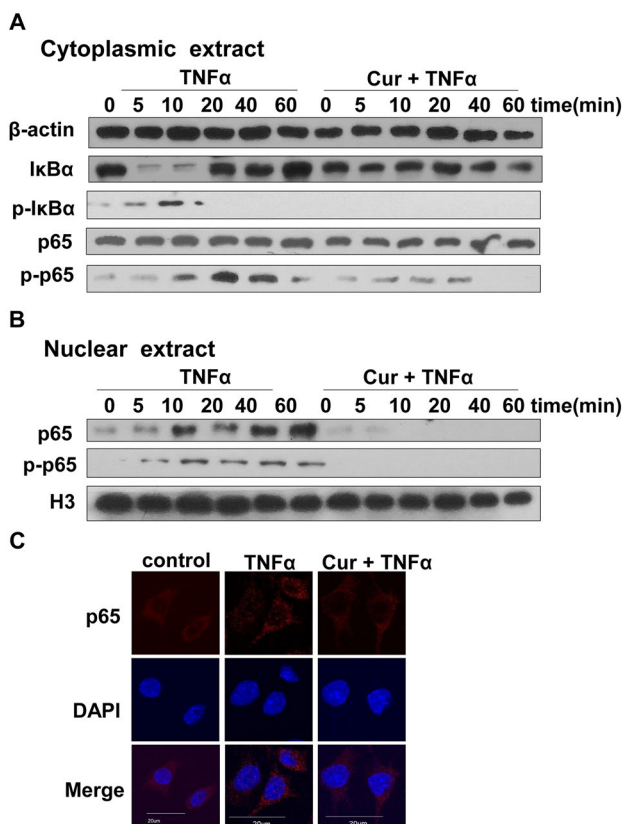


Fig. 3 Curcumin inhibits NF κ B signaling in SY5Y cells. Cytoplasmic (a) and nuclear (b) extracts were prepared. The expressions of I κ B α , p-I κ B α , p65, and p-p65 were determined by western blot after curcumin treatment. β -actin and H3 were used as cytoplasmic and nuclear control, respectively. c Immunofluorescence showed that p65 subunit was stained in red, and nuclear was counterstained by DAPI. Scale bar = 20 μ m. The results shown are representative of three independent experiments

1.02E-6 M (Fig. 4b). These results showed that curcumin has a higher selectivity for ER β than ER α .

Meanwhile, we performed transactivation assay to assess the agonist/antagonist profile of curcumin. Agonist was first tested. As shown in Fig. 4c, E2 induced ER α -mediated transactivation in a dose-dependent manner with significant induction at two concentrations, 10⁻⁹ M (2.08 \pm 0.20-fold) and 10⁻⁸ M (2.83 \pm 0.44-fold). A similar result was seen for ER β (2.69 \pm 0.20-fold at 10⁻⁹ M and 3.06 \pm 0.5-fold at 10⁻⁸ M). By contrast, curcumin activates transcription through ER β (2.54 \pm 0.14-fold) at the maximum concentration, but no estrogenic activity was displayed at 1 and 5 μ M. While curcumin exhibits no activity through ER α . In parallel, antagonism was conducted in the presence of E2. Curcumin had an additive effect on E2-induced activation in the ER β group (1.52 \pm 0.16-fold) (Fig. 4d), confirming its

agonism through ER β . However, curcumin did not antagonize E2-induced activation via ER α . Therefore, curcumin has been identified as an ER β agonist.

We then speculated if curcumin could exert its estrogenic effect by influencing ER expression. We found that curcumin treatment decreases ER α levels, whereas it has an upregulation effect on ER β expression (Fig. 4e). Consequently, regulation of two ER isoforms in opposite directions leads to increase in ER β /ER α ratio. Regarding the previous results, we speculated that ER β may be involved in the regulation of BACE1 by curcumin. Truly, we found that curcumin and ER antagonist ICI 182,780 co-treatment restore the inhibitory effect of BACE1 caused by curcumin alone (Fig. 4f). It might occur as a consequence of inhibition of estrogen receptors. Unexpectedly, ER β overexpression fails to inhibit BACE1 expression, but exhibits a more pronounced inhibition of BACE1 levels when co-acting with curcumin (data not shown).

The activation of ERs is shown to involve in the modulation of multiple signaling pathways. We then tested how ER β acts on NF κ B signaling pathway in our system. Our data showed that both ER β agonist (DPN) and ER β overexpression treatment reverse the degradation of I κ B α , compared to the TNF α -treated group alone (Fig. 4g, h). In contrast, pretreatment with ER α agonist (PPT) or ER α overexpression did not alter the inhibitory effect of TNF α on I κ B α protein levels. These results suggest that ER β inhibits NF κ B signaling by inhibiting I κ B α degradation.

Discussion

Though previous researches discovered curcumin's characteristic for preventing anti-amyloid β in AD, the mechanism underlying this beneficial effect remains to be clarified [18]. In this study, we confirmed curcumin's capability to reduce A β ₁₋₄₂ levels and its upstream intermediate product β -CTF(C99) at the concentration with no obvious negative effects on cell viability in HEK293-APPsw cells. This cell line harbors human APP Swedish mutation (K595N/M596L), enhancing the generation of A β , which is a well-established AD cell model [19, 20] (Figs. 1b and S1). β -secretase cleavage is the primary step in the APP processing [21], thus, these data point to BACE1 as the main cause for its anti-amyloidogenesis effect. Given that γ -secretase was blocked by its specific inhibitor DAPT in the intermediate product analysis, we did not inspect curcumin's role in γ -secretase [22]. Nevertheless, several studies hold that curcumin can act as a dual inhibitor for BACE1 and GSK-3 β

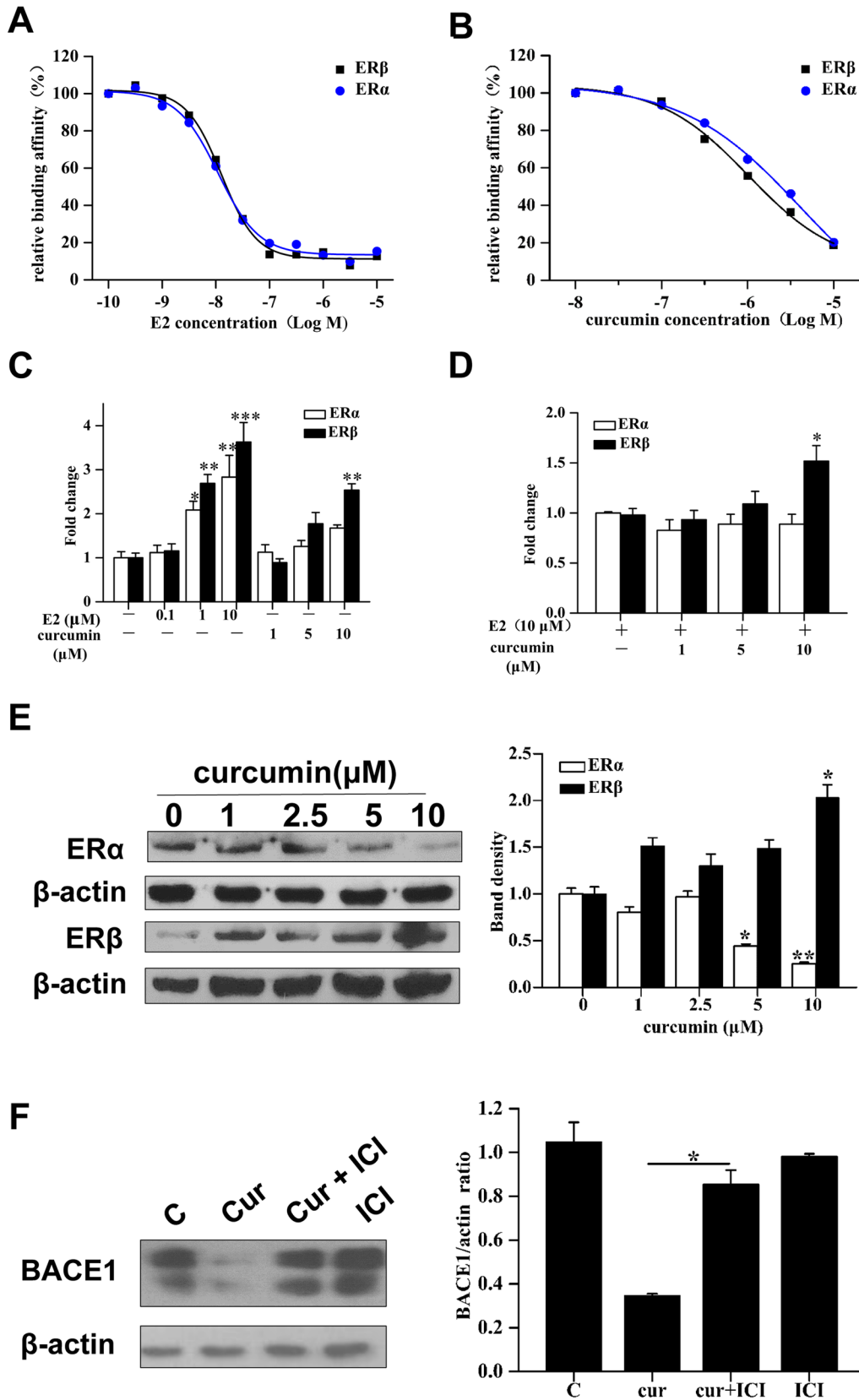


Fig. 4 ER β participates in the regulation of curcumin on BACE1 expression. Competitive binding curves of E2 (**a**) and curcumin (**b**) to ER α and ER β . H293 cells were treated with E2 and curcumin after transfection of pEGFP-ER β /phage-ER α with pERE luc. **c** Luciferase assay showing that curcumin was ER α and ER β agonist like E2. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus cont group. **d** Luciferase assay showed that curcumin was an ER β antagonist. * $p < 0.05$ versus cont group. **e** Representative western blots of ER α and ER β after curcumin treatments. * $p < 0.05$, ** $p < 0.01$ versus cont group. **f** Western blot shows that ICI 182,780 treatment reverses curcumin effect on BACE1 levels. * $p < 0.05$ versus cont group. **g** SY5Y cells were treated with DPN, PPT, or vehicle, then with TNF α for additional 15 min. The effect of curcumin on I κ B α protein level was detected using western blot. * $p < 0.05$ versus cont group, # $p < 0.05$ versus TNF α only group. **h** SY5Y cells were transfected with pEGFP-ER β /phage-ER α then with TNF α for additional 15 min. The effect of curcumin on I κ B α protein level was detected using western blot. Bar graph shows the densitometric analysis of relative I κ B α expression normalized to β -actin levels. Results were mean \pm SEM. $n = 3$ or 4. * $p < 0.05$ versus cont group, # $p < 0.05$ versus TNF α only group

[23], and GSK-3 β is the mediator for controlling γ -secretase activity [24]. Additionally, in SY5Y-APPswe cells, curcumin was shown to inhibit PS1, the major unit responsible for γ -secretase's cleavage role [25]. Although both BACE1 and γ -secretase are indispensable for A β generation, results from Tg2576 mouse model found presenilin-1 could be

compensated by other types of γ -secretase, making it a more complex enzyme for such study [26]. Interestingly, also in the intermediate product study, we observed a significant increase of mature and immature APP, a result contrasting with those from Zhang's group and Ronia's group, who reported that curcumin would stabilize the immature form of APP by altering its trafficking [27, 28]. The reason for our discovery could be attributed to the APP accumulation triggered by downstream β -secretase inhibition. Based on the above results, we reason that the anti-amyloid effect of curcumin is achieved through regulation of BACE1 expression. In line with this hypothesis, our data show that curcumin is able to suppress BACE1 at the promoter, mRNA, and protein levels. These results were also in agreement with the in vivo study using a 5 \times AD mouse model by Zheng's group [29].

We further discovered that curcumin exerts inhibitory effect on BACE1's transcriptional activity by NF κ B signaling. NF κ B is reported to link to BACE1 activation [30]. Therefore, we first proved the activity of NF κ B and BACE1 is positively correlated. NF κ B activation, whether induced by TNF α or p65 transfection, could enhance BACE1 promoter activity and expression, as well as promote A β production, while p65 siRNA knockdown would give rise to BACE1 inhibition. On the other hand, we proved that

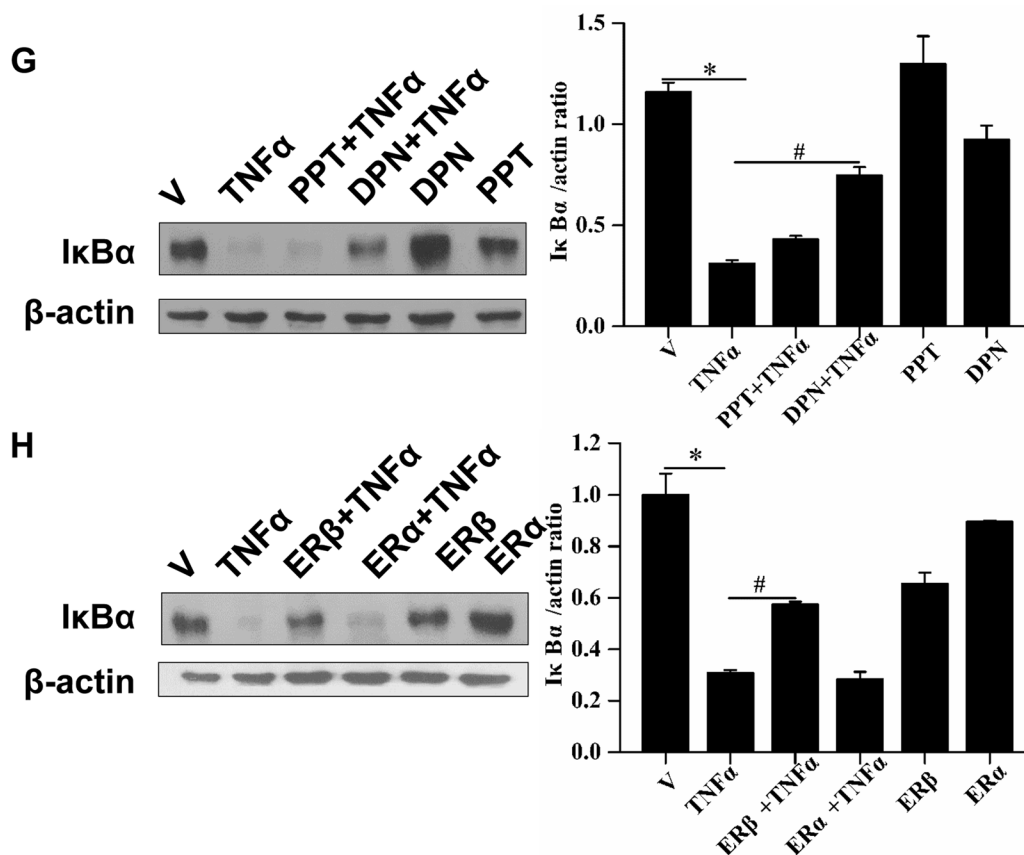


Fig. 4 (continued)

curcumin interferes with NF κ B activity in promoter level. According to Chen's group, human BACE1 promoter region includes four putative NF κ B binding sites. Our results confirmed that they are effective, which is also consistent with former results [31]. Here, we provide evidence that targeting NF κ B signaling by curcumin confers anti-inflammatory changes, and support the notion that inhibiting the pathway might be therapeutically beneficial in AD.

A broad spectrum of molecular processes has been elucidated as to modulation of NF κ B activity [32]. Based on biochemical and cytochemical methods, we discovered that curcumin imposes multiple effects on different steps of this signaling pathway: by reducing its phosphorylation status, it activated and stabilized I κ B α . These data can be repeated in other cell lines, as Buharmann found that curcumin inhibits I κ B α phosphorylation and degradation in tenocytes [33]. However, other groups found no difference in I κ B α levels in chondrocytes under the treatment of curcumin [34]. By inhibiting phosphorylation of p65, it disturbs the shuttle of p65 from the cytoplasm to nuclear. Previous studies show that the phosphorylation of p65 on ser536 determines the amount of p65 remaining in the nucleus by attenuating its association with newly I κ B α [35]. In addition, p65 is phosphorylated at ser536 requiring PI3 K/Akt activity to promote cell proliferation and apoptosis [36]. As our data show, TNF α -induced phosphorylation of Akt was decreased by curcumin, in consistent with the modulatory trend of curcumin on the phosphorylation status of p65. Our results revealed that curcumin might exploit multiple strategies as to suppressing NF κ B signaling, which explained its potency in BACE1 inhibition.

Estrogen is restricted in clinical use due to its side effects, such as risks for breast cancer [37]. In spite of its side effects, it is still considered plausible to use estrogen-like substances for AD treatment. Curcumin, generally belonging to plant polyphenols, is deemed to possess the estrogen-like effects [38]. However, Kelly et al. found that curcumin has anti-estrogenic activity in T47D cells [39]. Herein, we provide a thorough analysis of its estrogenic and anti-estrogenic properties. Our results show that curcumin binds to both receptors within the micromolar range and displays a preferential affinity to ER β over ER α (Fig. 4b). The subsequent reporter assay further demonstrated that curcumin selectively activates ER β other than ER α . These results suggest that curcumin is an ER β agonist and could directly associate with the ER. Like most of the phytoestrogens previously reported, curcumin has weaker estrogenic activity than estradiol. Our discovery is supported by Liu's group whose result using computational molecular docking also noted curcumin as an ER binder [40]. Estrogen deficiency is related to the occurrence of AD [41]. Our lab and Zhao's group suggest that estrogen relieves A β burden by transactivating neprilysin and insulin-degrading enzyme in an ER-dependent manner

[42, 43]. Both endogenous and exogenous estrogen were shown to reduce A β levels by activating receptors. As our results show, curcumin alters the ratio of two ER subtypes. Both receptors are reported to operate in a complex manner. The disparity of the expression between two receptors might affect their neuronal response. Moreover, ER β seems to play a more significant role in neuroprotection in the brain than ER α [44]. Previous study showed that estrogen deficiency mice display elevated BACE1 expression and enzyme activity [45], though the detail is unclear. In our data, overexpression and pharmacology inhibition experiments have shown the importance of ER β in the protective effect of curcumin.

The multiple crosstalks between NF κ B and ER signaling pathway have been documented [46]. In vitro, ER α directly binds to Rel A, p50, and c-Rel [47]. ER β inhibits the DNA binding ability of NF κ B p50 dimers [48]. Here, we found that ER β dampened NF κ B activity by heightened I κ B α levels, not ER α . This result prompts us that ER β exerts functions through non-genomic pathway. This is corroborated by Xing's work that ER β activation by E2 enhanced I κ B α mRNA levels and restored I κ B α protein [49]. In addition to this non-genomic signaling, ER and NF κ B subunits are reported to form a complex at the gene promoter to facilitate transcription [50]. However, whether this direct genomic activation is implicated in curcumin's effect needs further study.

In conclusion, we proposed curcumin as a multiple-target reagent for AD in vitro level. However, a clinical study initiated by Baum showed no difference in cognitive impairment among AD patients administered with oral curcumin for 6 months [51]. This may be due to different bioavailability and absorption in human test. Interestingly, one study revealed the benefits of curcumin using solid lipid formulation on cognition and mood in older population [52], suggesting its neuroprotection capacity on clinical level. In our study, we found that the multiple-target effect of curcumin may help increase the impairment of BACE1 transcriptional activity through two separate ways, which is likely to converge at the promoter level. This signaling crosstalk between NF κ B and ER β might also be indispensable for the ultimate BACE1 protein suppression. Therefore, we hold that this multitarget strategy may represent a promising approach to slow AD progression.

Acknowledgements This work was supported by the National Natural Science Foundation of China (31870786, 81573279, and 31371331) and Guidance Project of Science and Technology Research Project of HuBei Provincial Education Department (Category B Project:B2018456). We are grateful to HuZhiYe and NinWenTao (Wuhan university, school of pharmaceutical science, China) for their assistance provided in the receptor-binding experiments.

Author contributions PH: Conceived and designed the experiments; PH: performed the data; PH: analyzed the data; PH and NZ: wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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