NEURAL REGENERATION RESEARCH
Volume 7, Issue 6, February 2012
www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(6):405-412.

Protective effects of curcumin in *APPswe* transfected SH-SY5Y cells★

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Abstract
The *APPswe* plasmid was transfected into the neuroblastoma cell line SH-SY5Y to establish a cell model of Alzheimer's disease. Graded concentration and time course experiments demonstrate that curcumin significantly upregulates phosphatidylinositol 3-kinase (PI3K), Akt, nuclear factor E2-related factor-2 (Nrf2), heme oxygenase 1 and ferritin expression, and that it significantly downregulates heme oxygenase 2, reactive oxygen species and amyloid-beta 40/42 expression. These effects of curcumin on PI3K, Akt and Nrf2 were blocked by LY294002 (PI3k inhibitor) and NF-E2-related factor-2 siRNA. The results indicate that the cytoprotection conferred by curcumin on APPswe transfected SH-SY5Y cells is mediated by its ability to regulate the balance between heme oxygenase 1 and 2 *via* the PI3K/Akt/Nrf2 intracellular signaling pathway.
Key Words: Alzheimer's disease; curcumin; phosphatidylinositol 3-kinase signaling pathway; heme oxygenase-1; heme oxygenase-2; neural regeneration

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Received: 2011-09-24
Accepted: 2011-12-22
(N20111006001/WJ)

Yin WK, Zhang X, Li Y. Protective effects of curcumin in APPswe transfected SH-SY5Y cells. Neural Regen Res. 2012;7(6): 405-412.

www.crter.cn
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doi:10.3969/j.issn.1673-5374.2012.06.001

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized pathologically by the loss of synapses and neurons, the formation of neurofibrillary tangles, and the accumulation of amyloid-beta (Aβ) deposits[1]. A growing number of studies indicate that heme oxygenase (HO) is a critical factor in AD, and oxidative stress, disrupted iron metabolism and Aβ deposition are closely linked to HO homeostasis[2]. Previous studies have shown that HO-1 and HO-2 interact in cells. Reduced HO-2 expression is associated with increased levels of HO-1[3], but the mechanisms regulating their expression remain poorly understood.

Curcumin is the principal constituent of the spice turmeric rhizome[4]. Only very recently have the anti-inflammatory[5-6], antioxidant[7], antiproliferative[8] and other therapeutic properties of curcumin gained the attention of modern pharmacology. Lately, the potential anti-AD effects of curcumin and curcumin derivatives have been reported; these include the ability to induce HO-1[9] and the ability to prevent the formation of Aβ-heme in neurons[10], as demonstrated by various *in vitro* and *in vivo* experiments[11-12]. Insight into the factors that influence HO-1 levels can improve our understanding of the molecular regulation of HO-1 expression and facilitate the design of safe clinical agents that modulate the enzyme's expression.

Curcumin has HO-1 inducing activity, associated with nuclear factor-E2-related factor (Nrf2) binding to the antioxidant response element in the *HO-1* promoter[13-14]. Accumulating evidence indicates that Nrf2 is a key transcription factor, controlling many genes involved in cell cycle progression and protein synthesis.

Based on previous research, curcumin can induce Nrf2 and HO-1 expression in *APPswe* transfected SH-SY5Y cells, but the detailed mechanism remains unclear. To investigate the pharmacological effects of curcumin, we examined potential signaling pathways, and we hypothesized that curcumin's mode of action was related to the inhibition of reactive oxygen species generation, as well as Nrf2 and kinase activation in *APPswe* transfected SH-SY5Y cells. Moreover, we examined the role of the phosphatidylinositol 3-kinase (PI3K)/Akt/Nrf2 intracellular signaling pathway in curcumin mediated cytoprotection using enzyme linked immunosorbent assay, PCR and western blot analysis.

RESULTS

Curcumin protects against H_2O_2-induced toxicity in *APPswe* transfected SH-SY5Y cells

APPswe transfected SH-SY5Y cells were

treated with 50 μM H_2O_2 for 24 hours, followed by curcumin at 0, 1.25, 5.0 or 20.0 μM for 24 hours (as four separate groups) for the concentration gradient assay, or by curcumin at 5.0 μM for 0, 12, 24 or 48 hours (as four separate groups) for the time gradient assay. 0 μM and 0 hour groups were treated with dimethyl sulfoxide as a control. Reactive oxygen species detection results showed that the protective effects of curcumin were achieved at a concentration of 5 and 20 μM (Figure 1A) and at 24 and 48 hours (P < 0.05; Figure 1B).

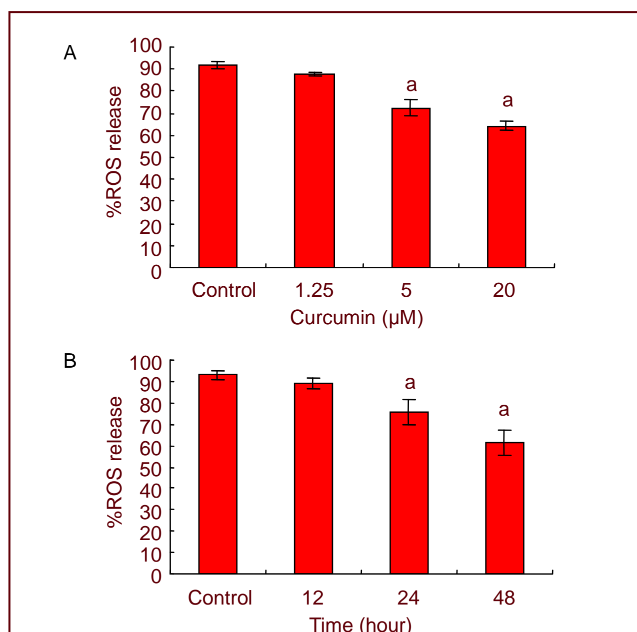

Figure 1 Curcumin provides a concentration and time-dependent protection against H_2O_2-induced toxicity in *APPswe* transfected SH-SY5Y cells.

(A)Transfected cells were treated with increasing concentrations of curcumin for 24 hours, and reactive oxygen species production was measured continuously for 2 hours using H_2DCFDA. Results show that total ROS production was decreased with 5 and 20 μM curcumin.

(B) Cells were treated with 5 μM curcumin for increasing duration and ROS generation was measured continuously for 2 hours using H_2DCFDA. Results show that total ROS generation was also decreased with 5 μM curcumin for 24 and 48 hours. 0 μM and 0 hour groups were treated with dimethyl sulfoxide as a control. Results were expressed as mean ± SD (Student-Newman-Keuls test) of eight wells in each group.

^{a}P < 0.05, *vs*. control group. Absorbance value was determined using a microplate reader under 488 nm wavelength.

Based on the standard curve, the concentration of ROS released in *APPswe* transfected SH-SY5Y cells treated with 50 μM H_2O_2 was calculated.

The percentage of ROS released with 50 μM H_2O_2 treatment was defined as the standard reference value of 100%, and the concentration of ROS released by the different groups is expressed as a percentage of this reference value on the Y axis.

ROS: Reactive oxygen species; H_2DCFDA: dichlorodihydrofluorescein diacetate.

The results demonstrate that curcumin protects *APPswe* transfected SH-SY5Y cells from H_2O_2-induced toxicity in a concentration and time-dependent manner.

Curcumin inhibits Aβ$_{40/42}$ generation in *APPswe* transfected SH-SY5Y cells

To examine the correlation between curcumin and Aβ generation, levels of Aβ$_{40}$ and Aβ$_{42}$ were quantified using enzyme linked immunosorbent assay (ELISA). Aβ$_{40}$ and Aβ$_{42}$ levels were significantly decreased after treatment with curcumin at 5 and 20 μM for 24 hours (Figure 2A) or with curcumin at 5 μM for 24 and 48 hours (Figure 2B; P < 0.05).

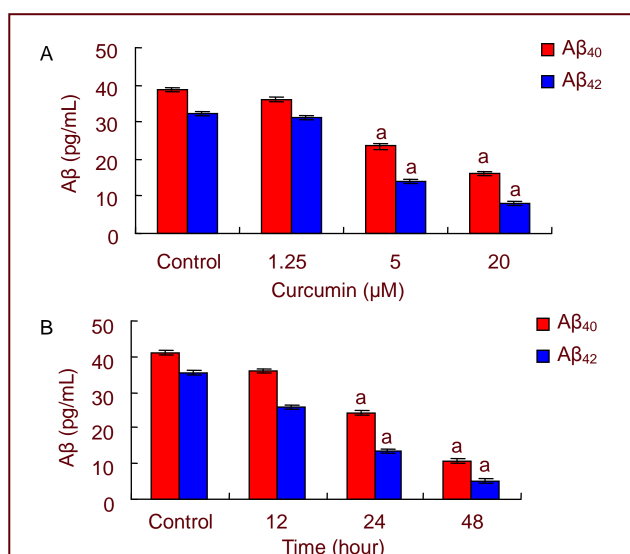

Figure 2 Curcumin significantly reduces generation of Aβ$_{40/42}$ in *APPswe* transfected SH-SY5Y cells (ELISA).

The transfected cells were treated with curcumin at 0, 1.25, 5.0 or 20.0 μM for 24 hours for the concentration-dependent assay (A), or with curcumin at 5.0 μM for 0, 12, 24 or 48 hours for the time course assay (B).

Curcumin reduced the generation of Aβ$_{40}$ and Aβ$_{42}$ in a concentration and time-dependent manner. The reduction in Aβ$_{40}$ and Aβ$_{42}$ levels was both concentration and time-dependent.

The 0 μM and 0 hour groups treated with dimethyl sulfoxide served as controls. Results are expressed as mean ± SD (Student-Newman-Keuls test) of eight wells in each group.

^{a}P < 0.05, *vs*. control group. Absorbance value was determined using a microplate reader under 488 nm wavelength.

Based on the standard curve, the concentration of Aβ$_{40}$ and Aβ$_{42}$ was calculated. Aβ: amyloid-beta; ELISA: enzyme linked immunosorbent assay.

Curcumin induces HO-1 but inhibits HO-2 expression in *APPswe* transfected SH-SY5Y cells

Cell lysates were analyzed by reverse transcription-PCR and western blotting (groupings same as above). Curcumin significantly induced HO-1 expression and inhibited HO-2 expression (P < 0.05). When curcumin was used at 5 μM, or when the duration

of treatment was increased to 24 hours, HO-1 mRNA and protein levels were increased significantly (P < 0.05), and HO-2 mRNA and protein levels were decreased significantly (P < 0.05), compared with the controls. When curcumin was used at 20 μM, or when the duration of treatment was increased to 48 hours, HO-1 mRNA and protein levels reached their maximum and HO-2 mRNA and protein were at their lowest levels (P < 0.05; Figure 3). These results indicate that curcumin increases HO-1 expression and inhibits HO-2 expression in a concentration and time-dependent manner in *APPswe* transfected SH-SY5Y cells.

Curcumin increases ferritin levels in *APPswe* transfected SH-SY5Y cells

APPswe transfected SH-SY5Y cells were treated with curcumin at 0, 1.25, 5.0 or 20.0 μM for 24 hours as for the concentration assay. The 0 μM group was treated with dimethyl sulfoxide as a control. Curcumin significantly increased ferritin levels in *APPswe* transfected SH-SY5Y cells at concentrations of 5 and 20 μM (P < 0.05). This induction was maximal at a curcumin concentration of 20 μM (Figure 4).

Curcumin regulates the PI3K/Akt/Nrf2 signaling pathway in *APPswe* transfected SH-SY5Y cells

To analyze the role of the PI3K/Akt/Nrf2 signaling pathway in the neuroprotective mechanism of curcumin, the *APPswe* transfected SH-SY5Y cells were treated with curcumin (at 5 μM), the PI3K inhibitor LY294002 and Nrf2 siRNA. Curcumin significantly induced the activation of PI3K, Akt and Nrf2, at both the mRNA and protein levels. Nrf2 siRNA silenced Nrf2 expression at both the protein and mRNA levels, and LY294002 inhibited PI3K and Akt expression at both the protein and mRNA levels (P < 0.05; Figures 5–7).

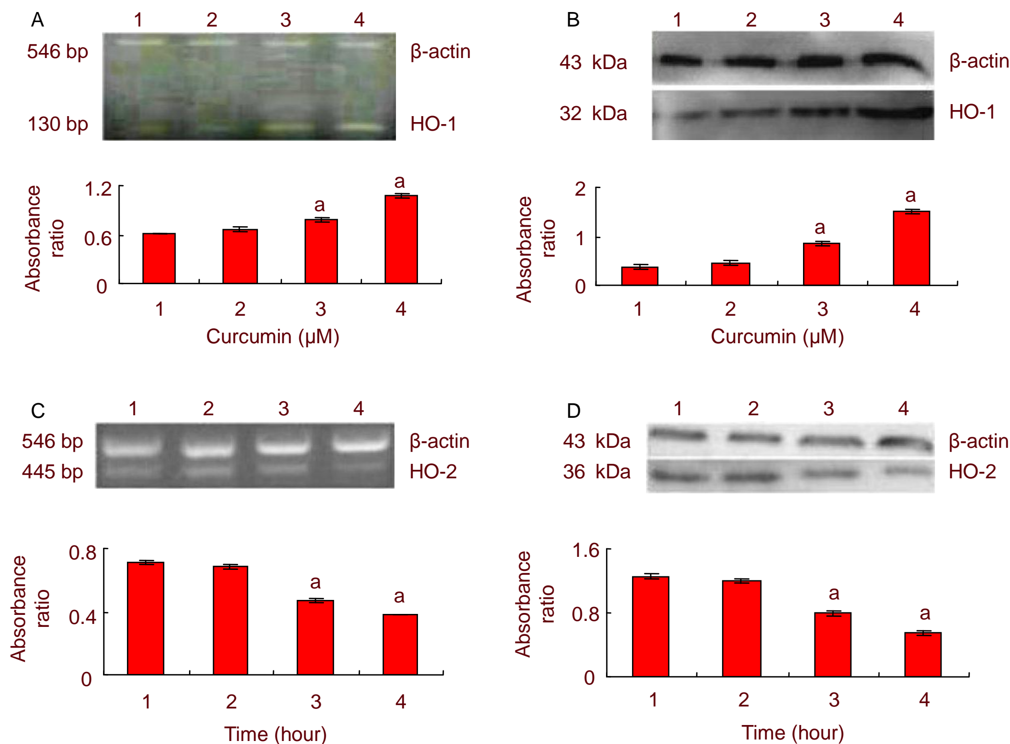

Figure 3 Curcumin induces the expression of heme oxygenase (HO-1), but reduces the expression of HO-2 in *APPswe* transfected SH-SY5Y cells.

Transfected cells were treated with dimethyl sulfoxide or curcumin at 0, 1.25, 5.0 or 20.0 μM for 24 hours or with curcumin at 5.0 μM for 0, 12, 24 or 48 hours.

(A, C) Reverse transcription-PCR analysis demonstrates that curcumin significantly induces HO-1 mRNA expression and reduces HO-2 mRNA expression.

(B, D) Western blot analysis shows that curcumin significantly induces HO-1 protein expression and reduces HO-2 protein expression. All the changes caused by curcumin are in a concentration and time-dependent manner.

Results are expressed as mean ± SD (Student-Newman- Keuls test) of eight wells in each group. ^{a}P < 0.05, *vs*. control group (A and C: 1, control group; 2, 1.25 μM curcumin group; 3, 5 μM curcumin group; 4, 20 μM curcumin group. B and D: 1, control group; 2, 12 hours group; 3, 24 hours group; 4, 48 hours group). The 0 μM and 0 hour groups treated with dimethyl sulfoxide served as controls.

The absorbance of each band in each group was analyzed with Quantity One Image Analysis and defined as the ratio to the corresponding β-actin absorbance, and was plotted as the relative absorbance value on the Y axis.

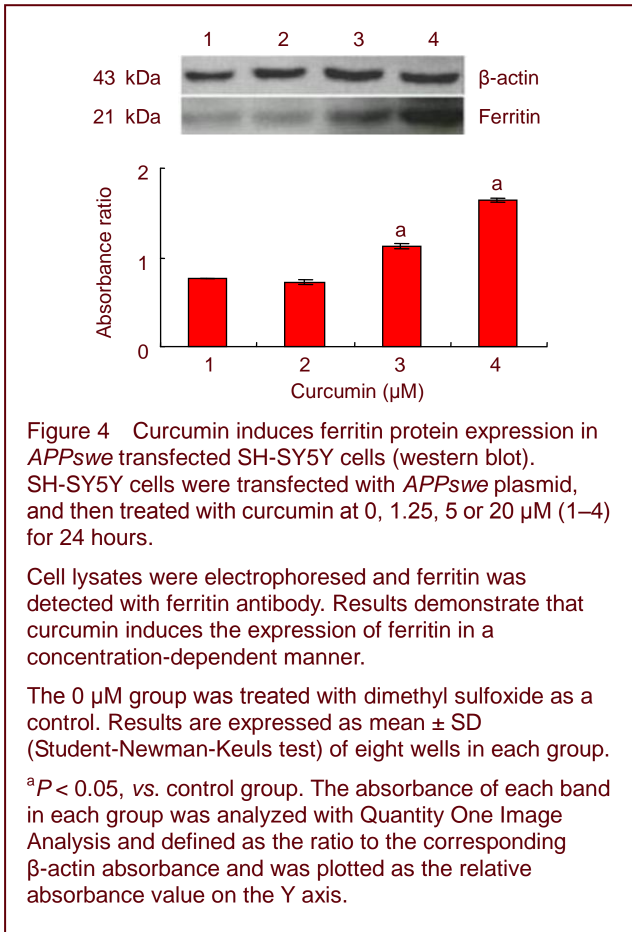

Figure 4 Curcumin induces ferritin protein expression in *APPswe* transfected SH-SY5Y cells (western blot). SH-SY5Y cells were transfected with *APPswe* plasmid, and then treated with curcumin at 0, 1.25, 5 or 20 μM (1–4) for 24 hours.

Cell lysates were electrophoresed and ferritin was detected with ferritin antibody. Results demonstrate that curcumin induces the expression of ferritin in a concentration-dependent manner.

The 0 μM group was treated with dimethyl sulfoxide as a control. Results are expressed as mean ± SD (Student-Newman-Keuls test) of eight wells in each group.

[a]*P* < 0.05, *vs.* control group. The absorbance of each band in each group was analyzed with Quantity One Image Analysis and defined as the ratio to the corresponding β-actin absorbance and was plotted as the relative absorbance value on the Y axis.

DISCUSSION

High levels of reactive oxygen species have been implicated in cell damage and various pathological progresses, including aging, cancer and neurodegenerative disease[15]. In normal cells, including neurons, Aβ is undetectable. Therefore, in the previous study, the *APPswe* plasmids were transfected into SH-SY5Y cells to increase intracellular Aβ and reactive oxygen species levels to establish an *in vitro* AD model. The *APPswe* transfected SH-SY5Y cells were then treated with curcumin.

Our ELISA results demonstrate that curcumin protects cells against the generation of $A\beta_{40}/A\beta_{42}$ in a concentration and time-dependent manner, as reported previously[16-17]. Moreover, curcumin also inhibited the generation of reactive oxygen species in a concentration and time-dependent manner. These findings reveal a relationship between the cytoprotective effect of curcumin and Aβ/reactive oxygen species. Based on these results, we focused on HO-1 and Nrf2, and investigated their potential roles in curcumin mediated cytoprotection.

Based on the previous studies, we hypothesized that HO-1 was associated with the cytoprotective effect of curcumin[18-19]. Our present results indicate that the expression levels of HO-1 and ferritin were low; however, HO-2 was highly expressed in transfected cells not treated with curcumin, consistent with previous studies[19-20].

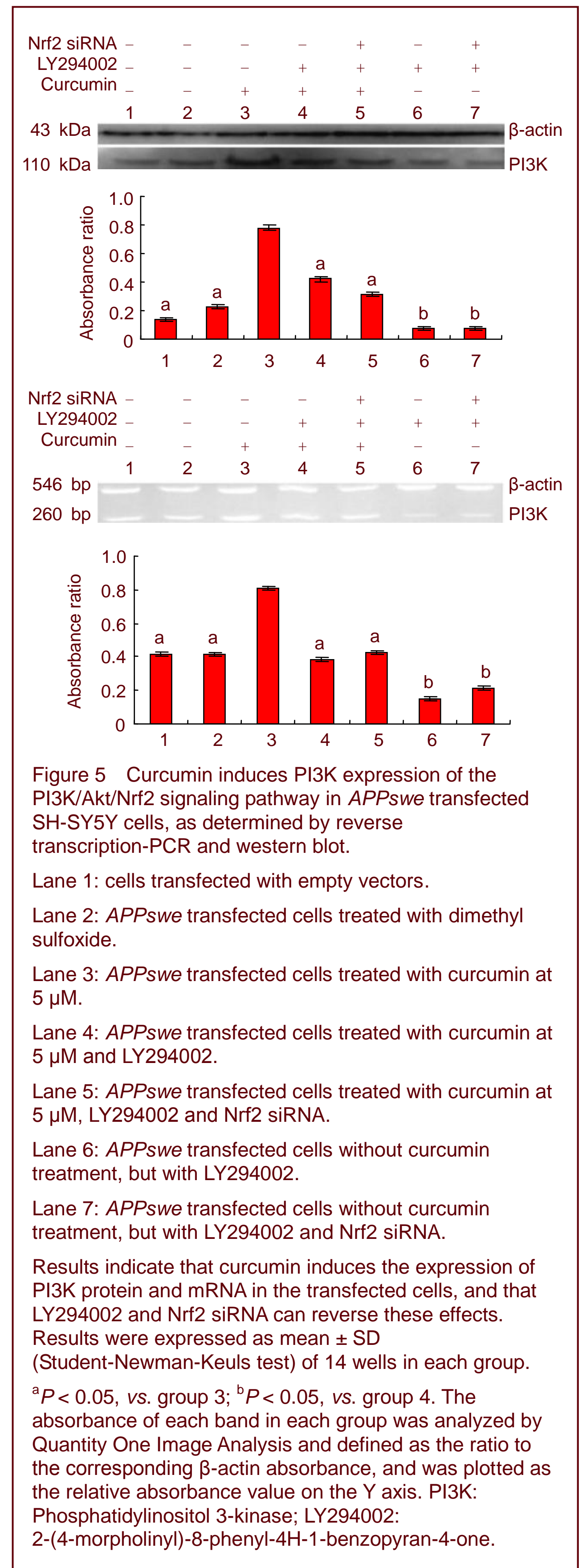

Figure 5 Curcumin induces PI3K expression of the PI3K/Akt/Nrf2 signaling pathway in *APPswe* transfected SH-SY5Y cells, as determined by reverse transcription-PCR and western blot.

Lane 1: cells transfected with empty vectors.

Lane 2: *APPswe* transfected cells treated with dimethyl sulfoxide.

Lane 3: *APPswe* transfected cells treated with curcumin at 5 μM.

Lane 4: *APPswe* transfected cells treated with curcumin at 5 μM and LY294002.

Lane 5: *APPswe* transfected cells treated with curcumin at 5 μM, LY294002 and Nrf2 siRNA.

Lane 6: *APPswe* transfected cells without curcumin treatment, but with LY294002.

Lane 7: *APPswe* transfected cells without curcumin treatment, but with LY294002 and Nrf2 siRNA.

Results indicate that curcumin induces the expression of PI3K protein and mRNA in the transfected cells, and that LY294002 and Nrf2 siRNA can reverse these effects. Results were expressed as mean ± SD (Student-Newman-Keuls test) of 14 wells in each group.

[a]*P* < 0.05, *vs.* group 3; [b]*P* < 0.05, *vs.* group 4. The absorbance of each band in each group was analyzed by Quantity One Image Analysis and defined as the ratio to the corresponding β-actin absorbance, and was plotted as the relative absorbance value on the Y axis. PI3K: Phosphatidylinositol 3-kinase; LY294002: 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one.

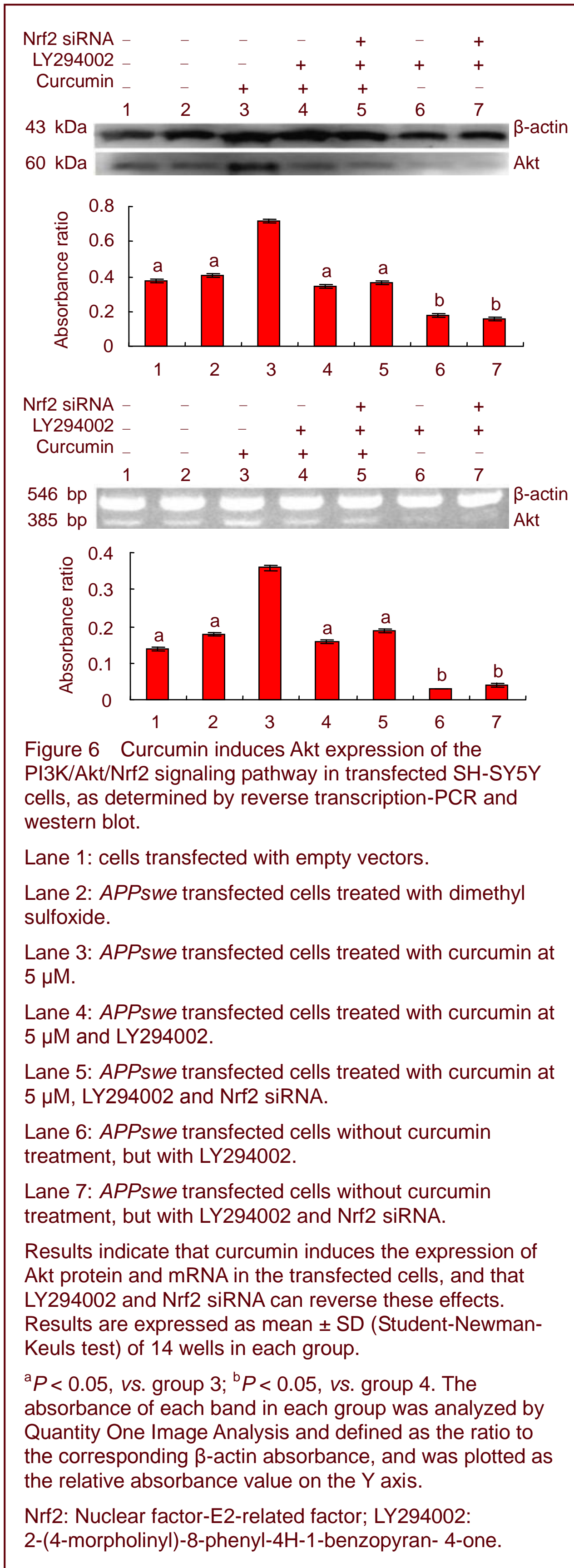

Figure 6 Curcumin induces Akt expression of the PI3K/Akt/Nrf2 signaling pathway in transfected SH-SY5Y cells, as determined by reverse transcription-PCR and western blot.

Lane 1: cells transfected with empty vectors.

Lane 2: *APPswe* transfected cells treated with dimethyl sulfoxide.

Lane 3: *APPswe* transfected cells treated with curcumin at 5 μM.

Lane 4: *APPswe* transfected cells treated with curcumin at 5 μM and LY294002.

Lane 5: *APPswe* transfected cells treated with curcumin at 5 μM, LY294002 and Nrf2 siRNA.

Lane 6: *APPswe* transfected cells without curcumin treatment, but with LY294002.

Lane 7: *APPswe* transfected cells without curcumin treatment, but with LY294002 and Nrf2 siRNA.

Results indicate that curcumin induces the expression of Akt protein and mRNA in the transfected cells, and that LY294002 and Nrf2 siRNA can reverse these effects. Results are expressed as mean ± SD (Student-Newman-Keuls test) of 14 wells in each group.

$^{a}P < 0.05$, *vs.* group 3; $^{b}P < 0.05$, *vs.* group 4. The absorbance of each band in each group was analyzed by Quantity One Image Analysis and defined as the ratio to the corresponding β-actin absorbance, and was plotted as the relative absorbance value on the Y axis.

Nrf2: Nuclear factor-E2-related factor; LY294002: 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran- 4-one.

With increasing concentration and duration of curcumin treatment, the expression of HO-1 and ferritin was significantly increased, but the expression of HO-2 was decreased, suggesting that the inverse regulation of HO-1 and HO-2 expression by curcumin may be beneficial to AD, and that curcumin protects against Aβ-induced oxidative stress by upregulating HO-1 and downregulating HO-2.

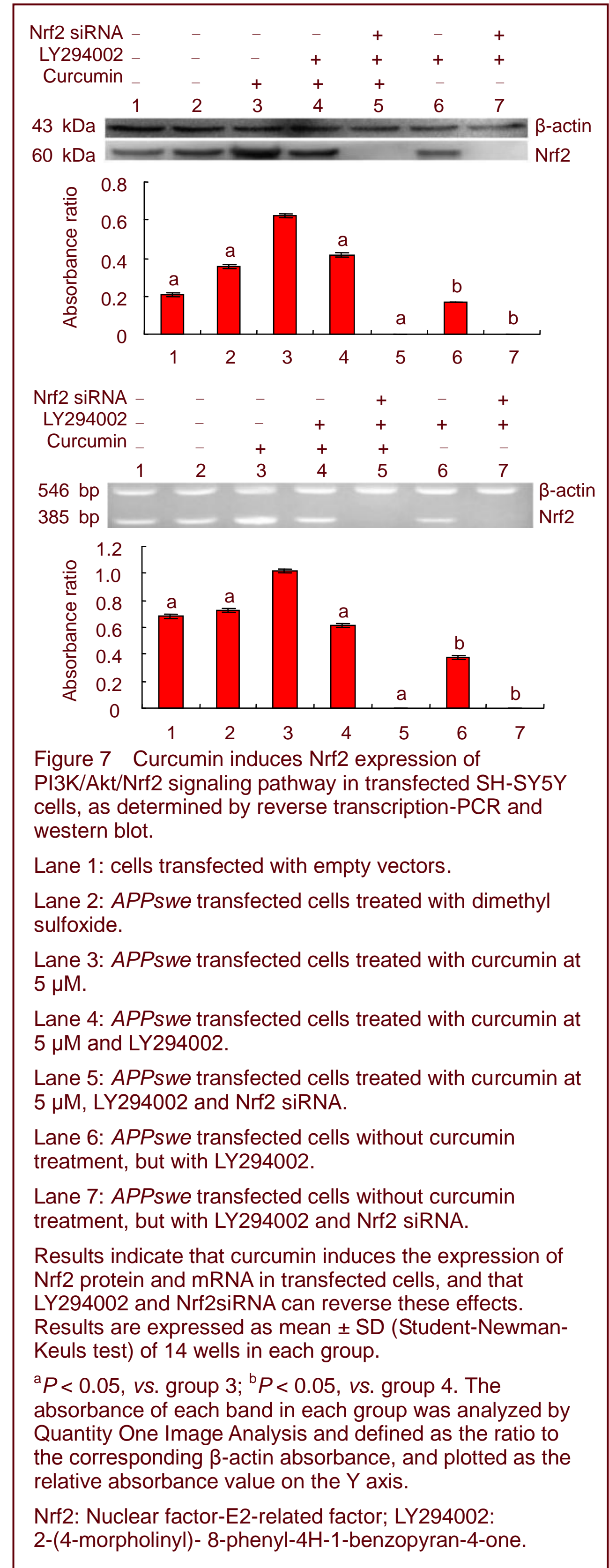

Figure 7 Curcumin induces Nrf2 expression of PI3K/Akt/Nrf2 signaling pathway in transfected SH-SY5Y cells, as determined by reverse transcription-PCR and western blot.

Lane 1: cells transfected with empty vectors.

Lane 2: *APPswe* transfected cells treated with dimethyl sulfoxide.

Lane 3: *APPswe* transfected cells treated with curcumin at 5 μM.

Lane 4: *APPswe* transfected cells treated with curcumin at 5 μM and LY294002.

Lane 5: *APPswe* transfected cells treated with curcumin at 5 μM, LY294002 and Nrf2 siRNA.

Lane 6: *APPswe* transfected cells without curcumin treatment, but with LY294002.

Lane 7: *APPswe* transfected cells without curcumin treatment, but with LY294002 and Nrf2 siRNA.

Results indicate that curcumin induces the expression of Nrf2 protein and mRNA in transfected cells, and that LY294002 and Nrf2siRNA can reverse these effects. Results are expressed as mean ± SD (Student-Newman-Keuls test) of 14 wells in each group.

$^{a}P < 0.05$, *vs.* group 3; $^{b}P < 0.05$, *vs.* group 4. The absorbance of each band in each group was analyzed by Quantity One Image Analysis and defined as the ratio to the corresponding β-actin absorbance, and plotted as the relative absorbance value on the Y axis.

Nrf2: Nuclear factor-E2-related factor; LY294002: 2-(4-morpholinyl)- 8-phenyl-4H-1-benzopyran-4-one.

More interestingly, HO-1 activation by curcumin was blocked by 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), a selective PI3K inhibitor[20], suggesting that PI3K/AKT plays an important role in curcumin mediated cytoprotection. Recent evidence indicates that Nrf2 is a key downstream element of the PI3k/Akt signaling pathway, involved in transduction of various signals from the cell surface to the nucleus. Nrf2 phosphorylation by AKT is a critical event during signal transduction[21], and is associated with curcumin mediated cytoprotection[22] and HO-1 activation[23]. Thus, in this study, we hypothesized that curcumin upregulates HO-1 expression by activating the PI3K/Akt/Nrf2 signaling pathway to mediate cytoprotection. As shown by western blotting and PCR analyses, curcumin significantly induced the expression of PI3K, Akt and Nrf2 in the curcumin group, as well as HO-1 at the mRNA and protein levels. The induction by curcumin was reversed by the PI3K inhibitor LY294002 and by Nrf2 siRNA in the LY294002 + Nrf2 siRNA group, indicating that cytoprotection by curcumin is mediated by the PI3K/AKT/Nrf2 signaling pathway.

In summary, curcumin is neuroprotective against Aβ-induced oxidative stress, as determined by ELISA of SH-SY5Y cells transfected with the *APPswe* plasmid, via activation of the PI3K/Akt/Nrf2 signaling pathway. Curcumin upregulates expression of HO-1 and downregulates expression of HO-2 (Figure 8).

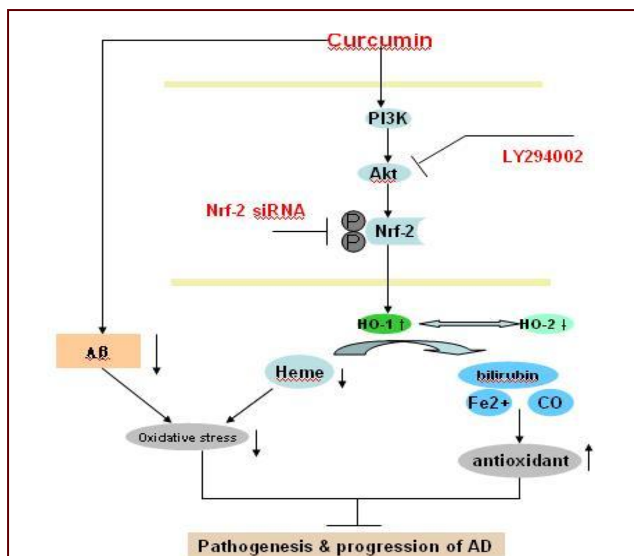

Figure 8 A proposed mechanism for the cytoprotective effects of curcumin against amyloid-beta (Aβ)-induced oxidative stress, involving the upregulation of HO-1 expression *via* activation of the PI3K/Akt/Nrf2 signaling pathway and downregulation of HO-2 expression in *APPswe* transfected SH-SY5Y cells, as a model of Alzheimer's disease.

PI3K: Phosphatidylinositol 3-kinase; Nrf2: nuclear factor-E2-related factor; LY294002: 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one.

These findings provide support for the clinical use of curcumin for AD treatment. Further research is needed to determine whether curcumin may be neuroprotective *in vivo* by upregulating HO-1 and downregulating HO-2 expression. Moreover, future studies should focus on signaling components downstream of HO-1, such as carbon monoxide, bilirubin and ferritin, to fully elucidate the mechanisms behind the pharmacological effects of curcumin, and they should assess the clinical use of curcumin for AD treatment.

MATERIALS AND METHODS

Design

A contrast observation *in vitro* study.

Time and setting

This study was performed at the Laboratory of Neurobiology, Chongqing Medical University, China, between January and July 2010.

Materials

Cells

Human neuroblastoma cell line SH-SY5Y was provided by the Department of Pathophysiology, Chongqing Medical University, China.

Plasmids

p*APPswe* was provided by University of British Columbia, Vancouver, BC, Canada.

Drugs

Curcumin was purchased from Sigma Company (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide at final concentrations of 1.25, 5.0 and 20.0 μM. LY294002 and Nrf2 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and were dissolved in Dulbecco's modified Eagle's medium (DMEM).

Methods

Plasmid APPswe transfection and cell culture

The human neuroblastoma cell line SH-SY5Y (Department of Pathophysiology, Chongqing Medical University, China) was cultured in DMEM containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/mL penicillin G sodium and 50 μg/mL streptomycin sulfate (Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37°C in an incubator containing 5% CO_2. For transfection, cells were cultured in 75 cm^2 flasks to approximately 70% confluence. Each flask was transfected with 8 μg p*APPswe*, graciously provided by Prof. Song, University of British Columbia, Vancouver, BC, Canada, using 30 μL of Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

Curcumin treatment of APPswe transfected SH-SY5Y cells

Curcumin was purchased from Sigma and dissolved in dimethyl sulfoxide. SH-SY5Y cells were treated with curcumin at 0, 1.25, 5.0 or 20.0 μM, as four separate groups, for the gradient concentration assay (24-hour duration), or with curcumin at 5.0 μM for 0, 12, 24 or 48 hours, as four separate groups, for the time course assay[24]. After the LY294002 and Nrf2 siRNA assay, the cells were divided into groups as follows: group 1, the cells were transfected with empty vectors; group 2, the

APPswe transfected cells were treated with dimethyl sulfoxide; group 3, the *APPswe* transfected cells were treated with curcumin at 5 μM; group 4, the *APPswe* transfected cells were treated with curcumin at 5 μM and LY294002; group 5, the *APPswe* transfected cells were treated with curcumin at 5 μM, LY294002 and Nrf2siRNA; group 6, the *APPswe* transfected cells were treated with LY294002 alone; group 7, the *APPswe* transfected cells were treated with LY294002 and Nrf2siRNA.

Nrf2 siRNA and PI3K inhibitor LY294002 treatment of APPswe transfected SH-SY5Y cells

SH-SY5Y cells were cultured in DMEM medium. For transfection, cells were cultured in 75-cm^2 flasks to approximately 80% confluence and each flask was transfected with 15 μL Nrf2 siRNA (Santa Cruz Technology) using 20 μL of Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. The concentration of LY294002 was 5 μM in the culture medium.

Sandwich ELISA assay for $A\beta_{40}/A\beta_{42}$ in APPswe transfected SH-SY5Y cells

Conditioned media was collected from cells. The protease inhibitor AEBSF (Santa Cruz) was added to the media to prevent degradation of Aβ protein. The concentration of $A\beta_{40-42}$ was detected by $A\beta_{1-40}$ or $A\beta_{1-42}$ Colorimetric ELISA kits according to the manufacturer's instructions (Biosource International, Camarillo, CA, USA). The primary antibody *A*β (rabbit monoclonal antibody) was purchased from Santa Cruz Technology and used at a dilution of 1: 200. The secondary peroxidase-conjugated antibodies (anti-rabbit antibodies) were purchased from Zhongshan Goldenbridge Bio-tech Company, Beijing, China and used at 1: 1 000. Absorbance value at 450 nm was measured using a microplate reader. The concentration of $A\beta_{40}/A\beta_{42}$ was calculated based on the standard curve.

Measurement of reactive oxygen species in APPswe transfected SH-SY5Y cells

To measure cellular reactive oxygen species, the molecular probe H_2DCFDA (Invitrogen) was used. SH-SY5Y cells were plated in 96-well plates at a density of 2×10^4 cells per well. On the next day, the cells were washed three times with phosphate-buffered saline (PBS), and then treated with curcumin as required in the presence of H_2DCFDA at a final concentration of 10 μM. H_2DCFDA was diffused through the cell membrane and hydrolyzed by intracellular esterases to the nonfluorescent form dichlorofluorescein. Dichlorofluorescein reacts with intracellular H_2O_2 to form 2', 7'-dichlorodihydrofluorescein, a green fluorescent dye. Fluorescence was measured using a fluorescence microplate reader (Bio-Rad, Hercules, CA, USA). Wavelengths for excitation and emission were 485 and 520 nm, respectively. The microplate reader was used to detect absorbance at 488 nm. Based on the standard curve, the concentration of reactive oxygen species released during the 50 μM H_2O_2 treatment with *APPswe* transfected SH-SY5Y cells was calculated. The amount of reactive oxygen species released with 50 μM H_2O_2 treatment was designated the reference value of 100%, and the various group reactive oxygen species quantities released were expressed as a percentage of this reference.

Semi-quantitative reverse transcription-PCR for gene detection in APPswe transfected SH-SY5Y cells

To evaluate the mRNA expression of HO-1, HO-2, PI3K, Akt, NRf-2 and β-actin after curcumin treatment at 5.0 μM, semi-quantitative reverse transcription PCR was performed. Total RNA was isolated from cells using Biozol (BioFlux, Osaka, Japan). Cellular total RNA (1 μg) was reverse transcribed into single-stranded cDNA (Takara Bio, Dalian, China), then PCR amplification of target cDNAs and an internal control (β-actin) cDNA (Bioer RT-PCR kit, China) were performed using the following primer pairs:

Gene	Sequence (5'–3')
PI3k	Sense: ACC TCG GAC ATG GCG TAT TA Antisense: CAG AAT CCC TGC TCA CTC AG
Akt	Sense: GGG AGG AGT GGA CAA CCG Antisense: CAG GCG ACC GCA CAT CAT C
Nrf2	Sense: TTC AAC CAA AAC CAC CCT Antisense: TGA GAT GAG CCT CCA AGC
HO-1	Sense: CTT GGC TGG CTT CCT TAC C Antisense: CAT TGC CTG GAT GTG CTT T
HO-2	Sense: AGG CTC CGC TTC TCC GAT GG Antisense: ACT TTC CCC GTG GGC CAT GG
β-actin	Sense: CTC GTC ATA CTC CTG CTT GCT G Antisense: CGG GAC CTG ACT GAC TAC CTC

Reaction mixtures were first denatured at 94°C for 3 minutes. PCR conditions were: 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds for 35 cycles in total, followed by 72°C for 5 minutes. The PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide. The lengths of the gene amplification products for PI3K, Nrf2, Akt, HO-1, HO-2 and β-actin were respectively 260, 320, 385, 130, 445 and 546 bp. PCR amplifications were repeated five times. The relative absorbance was expressed as the ratio of the target mRNA absorbance value to the β-actin absorbance value. The absorbance of each band was analyzed with Quantity One Image Analysis (Bio-Rad).

Western blot assay for protein detection in APPswe transfected SH-SY5Y cells

SH-SY5Y cells were washed once with cold PBS and lysed in 500 μL of ice-cold protein lysis liquid (SBS, China). Cell lysates were centrifuged at 13 000 × *g* for 10 minutes at 4°C. Protein concentrations were determined using the Bradford method employing a Universal Microplate Reader (Bio-Rad) at 595 nm. Proteins (30 μg) from the cell lysates were resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (Bio-Rad) membranes (Millipore, Billerica, MA, USA). After blocking, membranes were incubated with the

primary antibodies HO-1 (1: 1 000), HO-2 (1: 1 000), ferritin (1: 1 000), PI3K (1: 1 000), anti-Akt (1: 1 000), Nrf2 (1: 1 000) or β-actin (1: 1 000). All antibodies were rabbit monoclonal antibodies purchased from Santa Cruz, diluted in 0.1% (w/v) fat-free dry milk powder and incubated overnight at 4°C. After washing, the blots were incubated for 1–2 hours at room temperature with secondary peroxidase-conjugated mouse anti-rabbit antibodies (mouse monoclonal antibody; Zhongshan Goldenbridge Bio-tech Company). Membranes were then developed using a commercial enhanced chemiluminescence system (Bio-Rad) and the relative absorbance was expressed as the ratio of the target protein absorbance value to the β-actin absorbance value. The absorbance of each band was analyzed with Quantity One Image Analysis (Bio-Rad).

Statistical analysis

All experimental data were expressed as mean ± SD of three repeated independent experiments. For statistical analysis and homogeneity testing, the statistical software SPSS 11.5 software (SPSS, Chicago, IL, USA) was used for one-way analysis of variance in conjunction with Student-Newman-Keuls test between groups. A value of $P < 0.05$ was considered statistically significant.

Author contributions: Wenke Yin conducted experiments, collected and analyzed data, and wrote the manuscript. Yu Li was in charge of funds, guided the study, provided technical support and approved the final version of the manuscript. Xiong Zhang participated in data analysis and provided technical support.

Conflicts of interest: None declared.

Funding: This work was supported by the National Science Foundation of China, No. 30973154; the Science Foundation of Chongqing, No. 2009BB5270; and the Chongqing Municipal Education Commission Foundation, No. KJ090301.

Ethical approval: The experiment was approved by the Ethics Committee of Chongqing Medical University, China.

Acknowledgments: We thank Weihong Song, the University of British Columbia, Canada, for providing the plasmids.

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(Edited by Zhu FQ, Wang RT/Su LL/Wang L)