Curcumin Treatment is Associated with Increased Expression of the N-Methyl-D-Aspartate Receptor (NMDAR) Subunit, NR2A, in a Rat PC12 Cell Line Model of Alzheimer’s Disease Treated with the Acetyl Amyloid-β Peptide, Aβ(25–35)

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Background: The aim of this study was to investigate the effect of curcumin treatment on the expression of the N-methyl-D-aspartate receptor (NMDAR) subunit, NR2A, in a rat PC12 cell line treated with the acetyl amyloid-β peptide, Aβ(25–35), in an in vitro model of Alzheimer’s disease.

Material/Methods: PC12 cells, derived from rat phaeochromocytoma, were treated for 24 hours with increasing concentrations of curcumin (5, 10, 20, 30 μM/L) in the presence of the acetyl amyloid-β peptide, Aβ(25–35). A Cell Counting Kit-8 (CCK-8) assay was used to determine cell viability, and flow cytometry was used to measure cell apoptosis. In the supernatant of the treated PC12 cells, Western blotting was used to measure the cell injury biomarker, lactate dehydrogenase (LDH), and the biomarker for oxidative stress, malondialdehyde (MDA). Expression of the N-methyl-D-aspartate receptor (NMDAR) subunit, NR2A, was analyzed by Western blotting and quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Results: Curcumin treatment protected the rat PC12 cells from Aβ(25–35)-induced reduction in cell viability, apoptosis, release of LDH, and MDA production. Curcumin treatment of PC12 cells was associated with increased expression of the NMDAR subunit, NR2A.

Conclusions: The findings of this study showed a neuroprotective effect of curcumin treatment in an in vitro model of Alzheimer’s disease that was associated with the increased expression of the NMDAR subunit, NR2A.

MeSH Keywords: Alzheimer Disease • Apoptosis • Cell Survival • Curcumin • Receptors, N-Methyl-D-Aspartate

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Background

Alzheimer’s disease is the most common type of dementia in elderly people and is a progressive neurodegenerative disease [1,2]. Progressive cognitive impairment and memory loss are the major features of Alzheimer’s disease [3]. The accumulation of beta-amyloid (Aβ) has been shown to induce neurotoxicity and oxidative stress, which are involved in the pathogenesis of Alzheimer’s disease [4].

Knowledge of Aβ-induced neuronal cell injury in Alzheimer’s disease has resulted in the widespread use of in vitro cell models to study this disease [5]. Previously, in vitro studies have been an important component of preclinical studies on the effects of some approved drugs for Alzheimer’s disease, such as rivastigmine, galantamine, and donepezil [4,5]. Although there have been an increasing number of preclinical and clinical studies on the pathogenesis and treatment of Alzheimer’s disease, there are still no effective treatments. Therefore, there remains an urgent need to find novel approaches for the treatment of Alzheimer’s disease.

Curcumin, or 1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a natural active ingredient extracted from the rhizomes of turmeric plants. Several studies have now shown that curcumin has a variety of pharmacological properties that can affect cancer, inflammation, and result in neuroprotection [6–9]. Previously published studies have been undertaken to determine the potential therapeutic effects of curcumin in Alzheimer’s disease [10]. Curcumin has been shown to reduce Aβ oligomer and fibril formation [11]. Previous studies also have shown that curcumin inhibited Aβ-induced neurotoxicity in the brain [12,13]. Studies have also found that curcumin suppressed Aβ-induced inflammation and reduced the levels of inducible nitric oxide synthase (iNOS) and interleukin (IL)-1β in the brain of transgenic mouse models [14–16]. Also, in 2008, the findings of a pilot randomized, controlled, clinical trial showed beneficial effects of curcumin in patients with Alzheimer’s disease [17]. However, the mechanism of neuroprotection exerted by curcumin remains unknown.

The N-methyl-D-aspartate receptor (NMDAR) is a glutamate receptor and ion channel protein found in nerve cells. NMDAR contains the NR1 and NR2 receptor sub-units; the NR2 sub-unit of NMDAR is a key factor the organization and function of neuronal synapses and in NMDAR function. Previously published studies have shown that NR2A and NR2B were abundant in brain tissues, with the highest levels in the hippocampus and cerebral cortex, with NR2A and NR2B regulating the NMDA receptor-mediated excitatory postsynaptic potential (EPSP), and inducing long-term potentiation (LTP) formation, all of which are factors that affect the synaptic plasticity of the central nervous system [18]. NR2A has been shown to promote neuronal survival and protect against neuronal damage [19].

The aim of this study was to investigate the effect of curcumin treatment on the expression of the NMDAR subunit, NR2A, in a rat PC12 cell line treated with the acetyl amyloid-β peptide, Aβ(25–35), in an in vitro model of Alzheimer’s disease.

Material and Methods

Preparation of the acetyl amyloid-β peptide, Aβ(25–35)

Acetyl amyloid-β peptide, Aβ(25–35), aggregation was prepared as previously described [20]. Briefly, lyophilized Aβ(25–35) was dissolved in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37°C with constant mixing for three days to induce aggregation. The aggregated Aβ(25–35) was then diluted to 100 µg/mL (100 µM) and stored at −20°C until required.

PC12 cell culture and the development of the in vitro Alzheimer’s disease model

The PC12 rat pheochromocytoma cell line was obtained from American Type Culture Collection (ATCC). PC12 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Sangon Biotech Co. Ltd., Shanghai, China), including 1% penicillin and streptomycin solution, at 37°C with 5% CO₂. Cells were passaged when 80% confluence was reached.

For the in vitro cell model, PC12 cells were seeded in 96-well plates, at 3×10⁴ cells in 100 µl per well. After incubation for 24 h, 20 µM of aggregated Aβ(25–35) dissolved in serum-free DMEM was added, followed by incubation for 24 h at 37°C. PC12 cells (3×10⁴ cells per well) were treated with for 24 h with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM of Aβ(25–35).

Cell viability assay

PC12 cells were plated into 96-well plates, at 3×10⁴ cells per well, and cultured in DMEM medium at 37°C for 24 h. The cells were treated with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM of Aβ(25–35) for 24 h. Cells in the control group were treated with the same volume of DMEM medium. At the end of the experiment, cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay kit, according to the manufacturer’s instructions. The assays were performed in triplicate.
Detection of cell apoptosis

PC12 cells, at 3×10⁴ cells per well, were treated with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM of Aβ(25–35) for 24 h. PC12 cells were then harvested and washed with cold PBS. PC12 cell apoptosis was determined using a cell apoptosis assay. Briefly, PC12 cells (1×10⁶) from each group were re-suspended in binding buffer and then labeled with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) using an Annexin-V FITC/PI kit (BD Pharmingen, San Diego, CA, USA), according to the manufacturer’s instructions. Flow cytometry analysis was performed using the BD FACS Aria (BD Biosciences, Franklin Lakes, NJ, USA) to analyze and quantify the cells. The assays were performed in triplicate.

Lactate dehydrogenase (LDH) and malondialdehyde (MDA) measurements

PC12 cells (3×10⁴ cells per well) were treated with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM of Aβ(25–35) for 24 h. Cell injury was assessed by measuring the LDH activity in the supernatant of PC12 cells, using an LDH kit, according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Measurement of the oxidative stress biomarker, malondialdehyde (MDA) in the supernatant of the PC12 cells was detected using a commercial kit (Nanjing Jiancheng Bioengineering Institute) following the manufacturer’s instructions. The results were compared with the non-treated control. The assays were performed in triplicate.

Western blot analysis

PC12 cells, at 3×10⁴ cells per well, were treated with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM of Aβ(25–35) for 24 h. Then log-phase PC12 cells were collected and RIPA buffer (Auragene, Changsha, China) was used to extract the total cellular proteins. A BCA protein quantitation kit (Thermo, USA) was used to detect the concentration of protein samples. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed to separate the protein samples. The separated proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk powder at room temperature for 1 h, and incubated at 4°C overnight with primary polyclonal rabbit antibodies that included anti-β-actin (1: 1000), anti-NR2A (1: 1000), anti-caspase-3 (1: 1000), anti-pAkt (1: 1000) (Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were washed and then incubated with a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG (1: 5,000) at room temperature for 1 h. To visualize the protein bands, an enhanced chemiluminescence (ECL) kit (Applygen Technologies Inc., Beijing, China) was used, according to the manufacturer’s instructions.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To extract the total RNA from the PC12 cells, TRIzol reagent (Takara, Japan) was used, according to the manufacturer’s instructions. The A260/A280 ratio was used to evaluate the quality and integrity of the RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The PrimeScript™ RT reagent kit (Takara, Japan) was used to generate cDNAs. SYBR Premix Ex Taq (Takara, Japan) was used for RT-PCR analysis, according to the manufacturer’s instructions. Primer sequences used for real-time PCR were obtained from Gen Script Co., Ltd., Nanjing, China, as required.

Statistical analysis

All data were presented as the mean ± standard deviation (SD). The SPSS 17.0 statistical software (SPSS, Chicago, IL, USA) was used for statistical analysis. The Student’s t-test or analysis of variance (ANOVA) were used for statistical comparison between groups. Statistical significance was assumed to be p<0.05.

Results

Effects of curcumin on the cell viability of Aβ(25–35)-treated PC12 cells

Acetyl amyloid-β peptide, Aβ(25–35)-treated PC12 cells were used as an in vitro cell model for Alzheimer’s disease. The effect of curcumin on PC12 cell viability was determined by performing a Cell Counting Kit-8 (CCK-8) assay. As shown in Figure 1, Aβ(25–35)-treated PC12 cells were compared with the non-treated control. Curcumin treatment significantly reduced Aβ(25–35)-treated PC12 cell viability in a dose-dependent manner (p<0.01).

Effects of curcumin on Aβ(25–35)-induced apoptosis in PC12 cells

The effect of curcumin on cell apoptosis in Aβ(25–35)-treated PC12 cells was analyzed by flow cytometry. The findings were that PC12 cell apoptosis was significantly induced by Aβ(25–35) treatment. Curcumin treatment significantly reduced Aβ(25–35)-induced PC12 cell apoptosis, in a dose-dependent manner (Figure 2).
Effects of curcumin on the release of lactate dehydrogenase (LDH) in Aβ(25–35)-treated PC12 cells

The release of cellular lactate dehydrogenase (LDH) was considered to be an indicator for Aβ(25–35)-induced cytotoxicity. As shown in Figure 3, Aβ(25–35) treatment increased the release of LDH from PC12 cells when compared with the control group. Curcumin treatment significantly reduced Aβ(25–35)-induced LDH release by PC12 cells, in a dose-dependent manner.

Effects of curcumin on malondialdehyde (MDA) production in Aβ(25–35)-treated PC12 cells

Levels of the oxidative stress biomarker, malondialdehyde (MDA), were determined in this study. The findings were that Aβ(25–35) significantly increased the production of MDA in the supernatant of PC12 cells, and this increase was significantly inhibited by curcumin treatment, in a dose-dependent manner (Figure 4).
Effects of curcumin on the expression of NR2A in Aβ(25–35)-treated PC12 cells.

Because the expression of the N-methyl-D-aspartate receptor (NMDAR) subunit, NR2A, has previously been demonstrated to be neuroprotective, the effects of curcumin on NR2A expression were in this in vitro cell model. When compared with the control group, the expression of NR2A was significantly decreased by treatment with Aβ(25–35). Compared with the group of cells treated with Aβ(25–35) alone, curcumin treatment promoted NR2A expression in Aβ(25–35)-treated PC12 cells, in a dose-dependent manner (Figure 5).

Figure 3. Effects of curcumin on the release of lactate dehydrogenase (LDH) in Aβ(25–35)-treated PC12 cells. PC12 cells were treated with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM Aβ(25–35) for 24 h. The release of LDH was measured by using a LDH Cytotoxicity Assay kit, and the result is expressed as the fold increase of the control (non-treated) group. Data are shown as mean ±SD. ** p<0.01 vs. non-treated group; * p<0.05, 0.01 vs. the Aβ(25–35) treatment alone group. Cur – curcumin.

Figure 4. Effects of curcumin on the malondialdehyde (MDA) content of Aβ(25–35)-treated PC12 cells. PC12 cells were treated with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM Aβ(25–35) for 24 h. The levels of MDA level was measured by using an MDA detection kit, and the result is expressed as the fold increase of the control (non-treated) group. Data are shown as mean ±SD. ** p<0.01 vs. non-treated group; ## p<0.01 vs. Aβ(25–35) treatment alone group. Cur – curcumin.

Figure 5. Effects of curcumin on the expression of NR2A expression in Aβ(25–35)-treated PC12 cells. PC12 cells were treated with increasing concentrations of curcumin (5, 10, 20, 30 µM/L) in the presence of 20 µM Aβ(25–35) for 24 h. The protein (A) and mRNA (B) expression levels of NR2A were detected by Western blotting and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Data are shown as mean ±SD. ** p<0.01 vs. non-treated group; * p<0.05, 0.01 vs. Aβ(25–35) treatment alone group. Cur – curcumin.
Several studies have reported to have a low prevalence of Alzheimer's disease [27].

In previously published studies, NR2A expression has been shown to have neuroprotective effects [19,29]. In the present study, the effect of curcumin on NR2A expression in PC12 cells, when compared with the non-treated control, showed that NR2A expression was inhibited by αβ(25–35) treatment alone group. Cur – curcumin.

The acetyl amyloid-β peptide, αβ(25–35) has been commonly used in models of Alzheimer’s disease [28]. In this study, an in vitro model of Alzheimer’s disease was constructed that included αβ(25–35)-induced PC12 cell injury. In this study, pre-treatment with curcumin protected PC12 cells from αβ(25–35)-induced loss of cell viability and apoptosis. Because the release of lactate dehydrogenase (LDH from cells is a sign of cells damage, and the presence of reactive oxygen species (ROS) due to oxidative stress results in the production of malondialdehyde (MDA), LDH and MDA levels were measured in this study to determine the extent of the oxidative damage. The findings were that αβ(25–35) treatment enhanced the production of lactate dehydrogenase (LDH) for 24 h. (A) The protein expression levels of caspase-3 and p-AKT were detected by Western blotting. (B, C) Protein expression of caspase-3 and p-AKT are presented as the fold increase of the control (non-treated) group. Data are shown as mean ±SD. ** p<0.01 vs. non-treated group; ## p<0.01 vs. αβ(25–35) treatment alone group. Cur – curcumin.

**Effects of curcumin on the expression of caspase-3 and p-AKT (p-AKT) in αβ(25–35)-treated PC12 cells**

The activation of caspase-3 is a marker of cell apoptosis. The findings of this study showed that αβ(25–35) treatment significantly increased caspase-3 expression in PC12 cells when compared with the control group. Curcumin treatment significantly reduced the expression of caspase-3 expression in PC12 cells, in a dose-dependent manner (Figure 6). The phosphorylation of AKT (p-AKT) was analyzed in the present study, and the results showed that αβ(25–35) treatment significantly decreased the expression levels of p-AKT in PC12 cells, and this reduction was markedly inhibited by curcumin treatment, in a dose-dependent manner (Figure 6).

**Discussion**

As the most common cause of dementia in the elderly, Alzheimer’s disease, seriously affects the quality of life for patients. However, at this time, there is no effective treatment, and the underlying pathogenesis of Alzheimer’s disease has not been fully elucidated. Recently published research findings showed that immunity and inflammation were involved in the occurrence of Alzheimer’s disease [21–23]. Furthermore, studies have shown that oxidative stress was the main mechanism underlying β-amyloid (Aβ)-mediated neurotoxicity in Alzheimer’s disease [24–26].

Curcumin, the yellow pigment and active constituent of turmeric, is commonly used in Indian foods, and parts of India have been reported to have a low prevalence of Alzheimer’s disease [27]. Several studies have determined the effects of curcumin on the progression of Alzheimer’s disease, and several mechanisms have been proposed for the effects of curcumin on the pathogenesis of Alzheimer’s disease, including anti-oligomerization, anti-phosphorylation, and anti-inflammatory effects. This study investigated the effect of curcumin on Alzheimer’s disease in an in vitro model, to explore whether the N-methyl-D-aspartate receptor (NMDAR) subunit, NR2A, had a role in the effects of curcumin in Alzheimer’s disease prevention or treatment.

In previously published studies, NR2A expression has been shown to have neuroprotective effects [19,29]. In the present study, the effect of curcumin on NR2A expression in PC12 cells, when compared with the non-treated control, showed that NR2A expression was inhibited by αβ(25–35) treatment, and these effects were inhibited by curcumin treatment, in a dose-dependent manner.
In this study, the effects of curcumin on the expression of caspase-3 and p-AKT in Aβ(25–35)-treated PC12 cells were also studied. The results showed that Aβ(25–35) treatment significantly increased caspase-3 expression in PC12 cells when compared with the control group, and this increase was prevented by curcumin treatment. The findings of this study also showed that the phosphorylation of AKT in PC12 cells was decreased by Aβ(25–35) treatment, and this reduction was inhibited by curcumin treatment.

The findings of the present study showed that curcumin could protect PC12 cells from Aβ(25–35)-induced inhibition of cell viability, apoptosis, and release of cellular LDH, as well as the production of MDA. The expression of NR2A was involved in this cellular protection, supporting the potential neuroprotective role of curcumin in Alzheimer’s disease.

Conclusions

The findings of this study showed a neuroprotective effect of curcumin treatment in an in vitro model of Alzheimer’s disease that was associated with the increased expression of the NMDAR subunit, NR2A. For the first time, these findings have shown that the neuroprotective role that curcumin may have in Alzheimer’s disease could occur via the regulation of oxidative stress and NR2A expression. Further studies, including future controlled clinical studies, are recommended to determine whether curcumin is a potential treatment for Alzheimer’s disease.

Conflict of interest

None.

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