Neuroscience Letters 480 (2010) 21–24

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Potential protection of curcumin against intracellular amyloid β-induced toxicity in cultured rat prefrontal cortical neurons

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ARTICLE INFO

Article history:
Received 4 March 2010
Received in revised form 19 April 2010
Accepted 21 May 2010

Keywords:
Curcumin
Intracellular Aβ
Prefrontal cortical neurons
Caspase-3
AKT
Neuronal protective effect

ABSTRACT

Recently the neuronal toxicity of intracellular amyloid β (iAβ) in Alzheimer's disease is attracting more and more attention. The present study explored the effects of curcumin on the iAβ-induced toxicity in primary cultured rat prefrontal cortical neurons. The cell viability of primary cultured prefrontal cortical neurons decreased significantly after virus driven transfection of Aβ from 1 day to 7 days. Interestingly, administration of 1 μM, 10 μM or 20 μM of curcumin significantly inhibited the iAβ-induced toxicity in primary cultured rat prefrontal cortical neurons tested by MTT and LDH release assays. We further studied the involvements of apoptotic or neuroprotective pathway proteins in curcumin protection against iAβ-induced cytotoxicity in primary cultured rat prefrontal cortical neurons. The results demonstrated that the contents of activated caspase-3 increased significantly by iAβ, while curcumin significantly inhibited the iAβ-induced increases of activated caspase-3 tested by Western blot. And the contents of p-AKT decreased significantly after iAβ treatment, while administration of curcumin significantly inhibited the iAβ-induced decreases in the contents of p-AKT. The results suggest that curcumin may play a protective effect in primary cultured rat prefrontal cortical neurons against iAβ-induced cytotoxicity, and both AKT and caspase-3 are involved in the curcumin-induced protective effects.

It is well known that Alzheimer's disease (AD) may be related to amyloid beta peptide (Aβ)-induced neuronal death [4,6,8] and extracellular Aβ toxicity is thought to contribute to neuronal loss in AD [20,21]. However, intracellular Aβ (iAβ) accumulation and toxicity have attracted more attention recently [6,8]. The accumulation of iAβ is observed in brains from patients with AD and in neurons of a transgenic mouse model [3,10]. Besides, iAβ accumulation correlates with synaptic dysfunction and long term potentiation deficits [2,6,8,11]. Moreover, direct injection of low amount of $iA\beta_{1-42}$ (1500 molecules/cell) causes cell death in primary cultured human neurons [22] and virus driven expression of $iA\beta_{1-42}$ induces cytotoxicity in primary cultured neurons [9].

Curcumin (a ginger family member) is extracted from plant *Curcuma longa* Linn and its chemical structure was identified in 1815 [16]. *C. longa* Linn has a long history as dye, food additive and medicine in South and Southeast Asia [13,16,19], and curcumin has shown its anti-inflammatory effects [1,15]. Moreover, curcumin has been demonstrated to prevent the formation of Aβ aggregates at sub-micromolar concentration range [12] and we have previously shown that curcumin protects against extracellular Aβ-induced neurotoxicity in primary cultured prefrontal cortical neurons [14]. The present study explores the possible protective effects of curcumin on iAβ-induced cytotoxicity in primary cultured prefrontal cortical neurons.

Primary cultured prefrontal cortical neurons of rats were prepared as previous described [14]. Every measure was taken to minimize pain of animals and experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Newborn (postnatal day 0) Sprague–Dawley rat pups were provided by the Experimental Animal Center of Peking University Health Science Center (Beijing, China). Brain tissues were dissected in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA). The prefrontal cortical tissues was dissociated by mechanical chopping 10–20 times, and then dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA) for 30 min at 37 °C and filtered through nylon meshes to obtain a single-cell suspension. Cells were sedimented and resuspended in DMEM containing 10% fetal bovine serum, 2 g/L HEPES, 2 g/L $NaHCO_3$ penicillin (0.1 g/L), and streptomycin (0.1 g/L, all from Invitrogen, Carlsbad, CA). Cells were seeded in poly-L-lysine-coated Petri dishes or plates and maintained in an atmosphere of 5% CO_2 and 95% O_2 humidified air at 37 °C. Neurons were treated at 5–7 days in culture. Cytosine arabinoside (10 μM; Sigma, St. Louis, Missouri) was supplemented after plating for 3

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0304-3940/$ – see front matter © 2010 Published by Elsevier Ireland Ltd.
doi:10.1016/j.neulet.2010.05.062

days to inhibit glia cell growth. For immunocytochemistry, cells were plated on coverslips coated overnight with 50 μg/ml poly-L-lysine (Sigma, St. Louis, Missouri). Curcumin (Sigma, MO) dissolved in 100% DMSO at a concentration of 100 mM was added freshly into culture medium during treatments.

Intracellular $A\beta_{1-42}$ cDNA was subcloned from pEGFP-N3 into pAdTrack with BglII and XhoI digestion. The adenovirus was packaged in HEK293 cells and the infectious particle was measured as 2×10^6/ml (MOI = 1.33). The purified virus supernatant was added to cell culture medium at different time points.

The viability of cells after various treatments was estimated in terms of their ability to reduce the dye methyl thiazolyl tetrazolium (MTT, Sigma, MO) to blue formazan crystal. Primary rat prefrontal cortical neurons cultured in 96-well plate were gently washed with 0.01 M PBS. After washing, 90 μl of medium with 10 μl of MTT-PBS solution (5 mg/ml) was added to each well and the plate was maintained at 37 °C for 2–4 h. Then the products were dissolved in DMSO for quantification by measuring the absorption at 570 nm using a micro-plate spectrophotometer (Bio-Rad CA), representing relative cell viability.

Cell cytotoxicity after various treatments was evaluated by LDH release. This was achieved with a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit according to the manufacturer's instructions (Promega, Madison, WI).

Protein lysates of prefrontal cortex neurons were extracted after treatments. Neurons cultured in 6-well plates were washed three times with 0.01 M PBS, after which, 100 μl of lysis buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF) was added into each well and cells were harvested with cell scrapers. The lysates were iced for 30 min and centrifuged at 14,800 × *g* for 15 min, and protein in the supernatant was harvested. Denatured protein samples diluted with loading buffer were loaded equally to each lane and separated by 10% SDS-PAGE and then blotted onto a polyvinylidene fluoride (PVDF, Millipore, MA) membrane. The membrane was then incubated for 1 h in blocking buffer (tris-buffered saline containing 5% defatted milk powder) at room temperature. The membrane was incubated at 4 °C with the primary antibodies, washed with tris-buffered saline Tween-20 (TBST) and incubated again with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., PA) followed by another washing. The primary antibodies used include: purified polyclonal rabbit anti-β-actin antibody (Santa Cruz, CA), monoclonal rabbit anti-activated caspase-3 antibody (Cell Signaling Technology, Beverly, MA) and polyclonal rabbit anti p-Akt antibody (Santa Cruz, CA). Immunoblots were developed in the presence of enhanced chemiluminescence reagents, and the images detected on X-ray films were quantified by densitometric scanning using Gel Imaging Analysis System Gel-Pro 4400 (Media Cybernetics, MD).

All data are presented as means ± S.E.M. Statistical significance ($^*p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$) between groups was determined by two-tailed Student's *t*-test.

Previous study in our laboratory shows that virus driven Aβ expresses intracellularly in neurons, and the iAβ expression caused impairments of neuronal electrophysiological properties [3]. In the present study the Aβ virus construct or EGFP alone virus construct was applied to primary cultured prefrontal cortex neurons and subsequently the intracellular neurotoxicity of Aβ or EGFP was assessed by MTT assay. As shown in Fig. 1A, the cell viability of primary cultured prefrontal cortical neurons decreased significantly after one ($n = 10$, $t = 2.66$, $p < 0.05$), three ($n = 10$, $t = 6.01$, $p < 0.001$), five ($n = 10$, $t = 7.95$, $p < 0.001$), and 7 days ($n = 10$, $t = 9.92$, $p < 0.001$) of Aβ transfection compared to the control group. However, as shown in Fig. 1B, EGFP transfection alone had no significant influence on cell viability of cultured prefrontal cortical neurons compared to the control group.

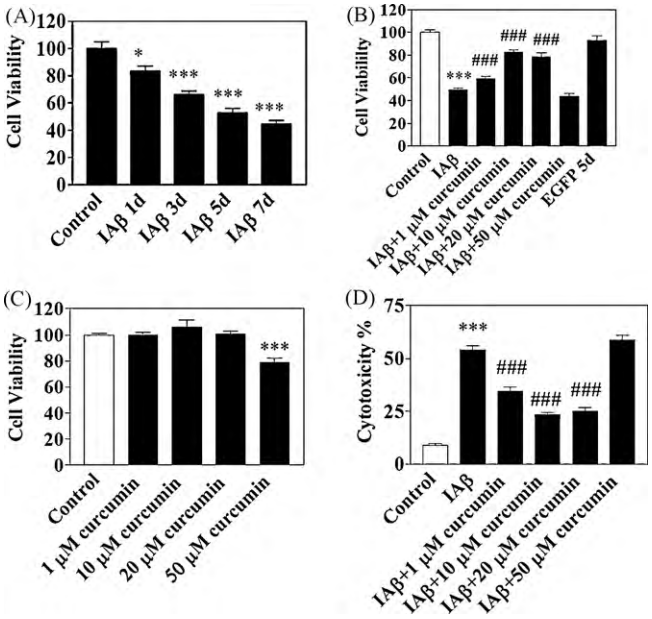

Fig. 1. Influence of curcumin on iAβ-induced neurotoxicity in primary cultured prefrontal cortical neurons. (A) $iA\beta_{1-42}$ induces neurotoxicity in primary cultured prefrontal cortical neurons. (B) Influence of curcumin on iAβ-induced neurotoxicity in primary cultured prefrontal cortical neurons tested by MTT assay. (C) Influence of curcumin alone on cell viability in primary cultured prefrontal cortical neurons tested by MTT assay. (D) 1 μM, 10 μM or 20 μM of curcumin reduces $iA\beta_{1-42}$ induced cytotoxicity in the neurons assessed by LDH release assay.

To explore the effect of curcumin in iAβ-induced neurotoxicity in primary cultured prefrontal cortex neurons, cultured neurons with 5 days of Aβ transfection were treated with 1, 10, 20 and 50 μM of curcumin, and with 0.01% DMSO as a control. As shown in Fig. 1B, the cell viability of primary cultured prefrontal cortical neurons increased significantly after treatments with 1 μM ($n = 10$, $t = 4.00$, $p < 0.001$), 10 μM ($n = 10$, $t = 12.48$, $p < 0.001$) or 20 μM ($n = 10$, $t = 7.50$, $p < 0.001$) of curcumin compared to the control group tested by MTT assay. We further demonstrated that 1, 10, 20 μM of curcumin alone had no significant influence on cell viability of cultured prefrontal cortical neurons compared to the control group, as shown in Fig. 1C. However, the cell viability of primary cultured prefrontal cortical neurons decreased significantly after 50 μM of curcumin alone treatment compared to the control group, as shown in Fig. 1C. The results indicate that curcumin may play potential protective effects against iAβ-induced toxicity in cultured rat prefrontal cortical neurons.

LDH release assay was used to further confirm the possible protective effects of curcumin against iAβ-induced cytotoxicity in cultured rat prefrontal cortical neurons. As shown in Fig. 1D, LDH release increased significantly after 5 days transfection of Aβ in the neurons ($n = 8$, $t = 21.42$, $p < 0.001$) compared to the control group. Application of 1 μM ($n = 8$, $t = 7.22$, $p < 0.001$), 10 μM ($n = 8$, $t = 14.27$, $p < 0.001$) or 20 μM ($n = 8$, $t = 11.99$, $p < 0.001$) of curcumin showed significant protective effects against iAβ-induced cytotoxicity on the neurons compared to the group without curcumin treatment tested by LDH release assay.

To understand the mechanism of the protective effects of curcumin against iAβ-induced cytotoxicity in the cultured prefrontal cortical neurons, the involvement of apoptotic protein caspase-3 and neuroprotective pathway protein AKT were further determined. As shown in Fig. 2A, the contents of activated caspase-3 increased significantly ($n = 3$, $t = 5.75$, $p < 0.01$) after Aβ transfection into the neurons compared to the control group. The results also demonstrated that application of 10 μM of curcumin significantly inhibited iAβ-induced increases in the contents of activated

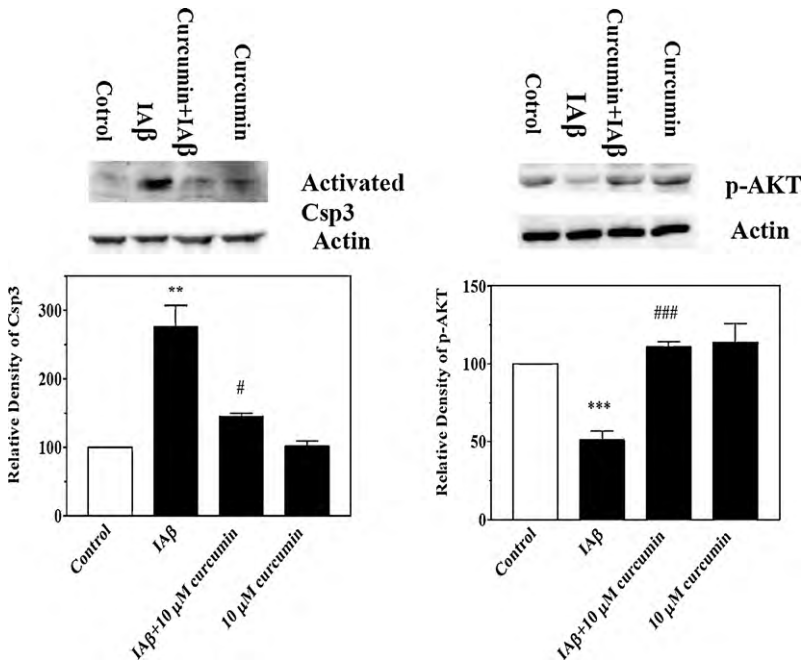

Fig. 2. Influence of curcumin on the contents of caspase-3 and AKT in primary cultured prefrontal cortical neurons. (A) Curcumin inhibited iAβ-induced increases in the contents of activated caspase-3. Top panel, representative Western blot probed with an antibody specific against activated caspase-3 after various treatments in the neurons. Bottom panel, summary of optical density of activated caspase-3. (B) Curcumin inhibited iAβ-induced increases in the contents of p-Akt in the cultured neurons. Top panel, representative Western blot probed with an antibody specific against p-Akt after various treatments in the neurons. Bottom panel, summary of optical density of p-Akt.

caspase-3 in the cultured prefrontal cortical neurons ($n = 3$, $t = 4.22$, $p < 0.05$) compared to the iAβ treated group.

As shown in Fig. 2B, the contents of p-AKT decreased significantly after iAβ treatment ($n = 3$, $t = 8.66$, $p < 0.001$) compared to the control group. Interestingly, administration of 10 μM of curcumin significantly inhibited iAβ-induced decreases in the contents of p-AKT ($n = 3$, $t = 9.20$, $p < 0.001$) compared to the iAβ treated group. These results suggest that both caspase-3 activation and phosphorylation of AKT are involved in curcumin-induced protective effects against iAβ-induced neurotoxicity in the primary cultured prefrontal cortical neurons.

The present study demonstrates the potential protection by curcumin against iAβ-induced toxicity in cultured rat prefrontal cortical neurons. The important role of extracellular Aβ has been widely proposed [6]. However, several evidences suggested that extracellular Aβ deposition alone is inadequate to account for the AD pathology. In cultures of rat PC12 cells, human IMR32 cells and monkey cerebral cortical neurons, extracellular Aβ failed to demonstrate consistent cytotoxicity [8]. Some studies demonstrate that the accumulation of intra-neuronal Aβ is an early event in the progression of AD, preceding the formation of extracellular Aβ deposits [6], and iAβ causes cell death in primary cultured neurons [9,22]. These results strongly suggest that iAβ is a viable alternative candidate for explaining defective neuronal physiology and neuronal cell loss in the development of AD.

Both *in vivo* and *in vitro* studies have been demonstrated the protective effects of curcumin against insults associating with AD induced toxicity. *In vivo*, curcumin inhibits aluminium-induced spatial learning deficits in rats [5,18]. Moreover, in animal model with human Aβ infused with lipoprotein chaperone into the cerebral ventricles of rats, curcumim reverses changes in synaptophysin and post-synaptic density 95 (PSD-95), as well as length and latency in finding the hidden platform in the water maze test [16]. *In vitro*, curcumin protects against extracellular Aβ-induced neurotoxicity in prefrontal cortical neurons of rats and caspase activation is involved in the protective effects [14]. Here, we show that transfection of $A\beta_{1-42}$ driven by virus infection caused significant cytotoxicity in primary cultured prefrontal cortical neurons as tested by MTT assay and LDH release assay and curcumin inhibited this cytotoxicity. These data are consistent with the hypothesis that curcumin may be beneficial according to the amyloid hypothesis of Alzheimer's disease.

Moreover, our results demonstrate that both the neuroprotective pathway protein AKT and the apoptotic protein caspase-3 are involved in the protective effects of curcumin. It has been shown that p-Akt levels are decreased in brains from patients with AD and the Tg2576 AD mouse model [7,9]. Moreover, induction of intra-neuronal $A\beta_{1-42}$ expression leads to a decrease in levels of phospho-Akt in primary cultured neurons and overexpression of active Akt protects against iAβ induced neurotoxicity [9]. Our results support the hypothesis that Akt is involved in iAβ induced neurotoxicity in AD. More importantly, our data demonstrated that curcumin restored the decreased level of p-Akt caused by iAβ, possibly explaining the protective effects of curcumin in primary cultured neurons. Caspase-3 plays a central role in the execution of apoptosis and caspase-3 is activated in response to Aβ exposure *in vitro* and in brains from patients with AD [17]. Our results first showed that caspase-3 was activated in response to iAβ treatment and curcumin prevented this activation.

The data support the hypothesis that curcumin has an intracellular action mediating a protective effect against iAβ-induced neurotoxicity. Though the relationship between curcumin and AD needs further investigation, our data provides a new perspective for potential therapeutic intervention according the amyloid hypothesis of Alzheimer's disease.

Acknowledgements

This work was supported by the National Program of Basic Research sponsored by the Ministry of Science and Technology of China (2006CB500706), National Science Foundation of China (NSFC, 30670658). Also, the authors thank the supports from Minzu University 985 fund (MUC985) and the 111 project (BO8044).

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