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Original Article

Curcumin protects against intracellular amyloid toxicity in rat primary neurons

Jelina Ye[2], Yan Zhang[1]

[1]State Key Laboratory of Biomembrane and Membrane Biotechnology, College of Life Sciences, Peking University, Beijing, 100871, China; [2]UMC, St. Radboud, Postbus 9101, 6500 HB Nijmegen, the Netherland

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Abstract: To investigate whether curcumin is protective against intracellular amyloid β (Aβ) toxicity, different concentrations of curcumin were applied to with intracellular Aβ in rat primary hippocampal neurons in culture. We find that at low dosages, curcumin effectively inhibits intracellular Aβ toxicity. Reactive oxidative species (ROS) is involved in mediating intracellular Aβ toxicity and possibly curcumin protection. Our results indicate that oxidative stress may mediate cell death induced by intracellular Aβ in neurons.

Keywords: Amyloid, curcumin, ROS, toxicity, Alzheimer's disease

Introduction

Alzheimer's disease (AD) features with neuronal/synaptic loss, extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs). The components of senile plaques are identified as amyloid β (Aβ) peptides. Although at the late stage, Aβ depositions are observed mainly located extracellularly, the accumulation of iAβ has been observed in various systems. $IAβ_{1-42}$ significantly accumulates in the pyramidal neurons of the hippocampus and the entorhinal cortex in mild cognitive impairment and AD patients at early stage [1-7]. $IAβ_{1-42}$ deposition appears earlier than SP formation [1, 2, 4, 5, 8]. In addition, accumulation of $iAβ_{1-42}$ is reported in several cell culture systems [9, 10]. IAβ is also observed in the APP mutant mice where synaptic loss happens before the presence of extracellular Aβ (eAβ) [11, 12]. Microinjection of intracellular $Aβ_{1-42}$ into neurons induces remarkable cell death mediated by the activation of p53, Bax and caspase-6 [13, 14]. Intracellular $Aβ_{1-42}$ also causes electrophysiological property changes in human primary neurons [15]. Several reagents, such as androgen [16], estrogen [16], galanin [17] and morphine [18], can protect against iAβ toxicity in human and rodent neurons.

Curcumin, the yellow pigment of turmeric, a phenolic compound, acts as a chain-breaking molecule by scavenging nitrogen oxide and superoxide anion [12-14]. It has been shown to protect against extracellular $Aβ_{25-35}$ toxicity in rat primary neurons from the frontal cortex [19]. Here, we first delivered intracellular $Aβ_{1-42}$ into the cultured rat primary hippocampal neurons by virus-mediated manner. We find that curcumin can protect against $iAβ_{1-42}$ toxicity. Reactive oxidative species (ROS) is involved in $iAβ_{1-42}$ toxicity and curcumin protection.

Methods and materials

Cell culture

Rat primary neurons were cultured from new born Sprague-Dawley rat hippocampus, following the regulations of Peking University Animal Care and Use Committee. In brief, fresh rat hippocampal tissues were dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA), which was then inactivated by 10% decomplemented fetal bovine serum (FBS, HyClone, Logan, UT). The mixture was triturated through pipette to make a homogenous mixture. After filtering the mixture through 70 μm sterilized filters, the flow-through was centrifuged. The pellet was then

washed once by phosphate buffered saline (PBS) and once by Dulbecco's modified Eagle's medium (DMEM) containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose, 1× antibiotic Pen-Strep (all from Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS). Cells were then plated on poly-L-lysine (Sigma, St. Louis, MO) coated plates or glass coverslips at the density of 1×10^5 cells/ml. Neurons were incubated at 37°C in DMEM without phenol red with 5% FBS and with 5% circulating CO_2. Cytarabine was added to culture media 24 hours after plating at 10 μM to inhibit dividing cell growth. Medium was changed every 48 hours. Cells were treated for experiments at 7 days in culture.

Adeno-virus infection

I$A\beta_{1-42}$ [20] was subcloned from pEGFP-N3 into pAdTrack with BglII and XhoI digestions. Adeno-virus was packaged in HEK293 cells and the infectious particle was measured as 2×10^6 particles/ml (MOI=1.33). To infection of cell cultures, the purified virus supernatant was added to cell culture medium at the dilution of 1:500 for 24 hours.

Chemical treatments

Curcumin (Sigma, MO) was added freshly into culture medium during treatments. MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes, M36008) was used to measure ROS as described by the manufacturer.

Measurement of neuronal cell death and viability

Cells were fixed in fresh 4% paraformaldehyde, 4% sucrose in PBS for 20 minutes at room temperature and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate in PBS for 2 minutes on ice. Terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining was performed using the in situ cell death detection kit I as described by the manufacturer (Roche, Quebec, Canada). The coverslips were then washed once in distilled water for 5 minutes and mounted on glass slides to be observed under a fluorescence microscope.

The cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which correlates cell number with mitochondrial reduction of MTT to a blue formazan precipitate [21]. In brief, cells were plated in 96-well plates beforehand. The medium was then replaced with fresh medium containing 1 mg/ml MTT. Following incubation at 37°C for 4 h, the wells were aspirated, the dye was solubilized in DMSO and the absorbency was measured at 595 nm. The viability of cells was compared with that of control cells. Lactate dehydrogenase (LDH) release was measured using the LDH Cytotoxicity Detection Kit (Roche, Quebec, Canada) according to the manufacturer's instructions.

Statistical evaluation

Statistical significance was assessed by one-way analysis of variances (ANOVA). The Sheffé's test was applied as a post hoc for the significant difference shown by ANOVAs. A p value of less than 0.05 or 0.01 was used as an indicative of statistical significance.

Results

Curcumin protected against intracellular Aβ toxicity

Rat primary hippocampal neurons in culture were confirmed by staining with β-tubulin-III, a neuronal marker (**Figure 1A** and **1B**). The infection of by adeno-5 virus packaged with i$A\beta_{1-42}$ achieved around 20%-30% efficiency in these cultures (**Figure 1C**). When expressed intracellularly [15], $A\beta_{1-42}$ induced around 50% decrease in cell viability shown by MTT assay compared with empty vector infection alone (**Figure 1D**). Curcumin was applied to the culture medium with i$A\beta_{1-42}$ infection. After 24 hours of incubation, curcumin at various concentrations had either protective or destructive effects on neurons (**Figure 1E**). At low dosages (1-50 μM), curcumin increased cell viability while at dosages higher than 80 μM, curcumin decreased cell viability, suggesting that curcumin at certain concentrations, can be protective against i$A\beta_{1-42}$ toxicity in rat primary neurons. The protection of curcumin was further confirmed by application of 30 μM of curcumin to the culture medium. Curcumin significantly increased cell viability indicated by LDH release assay (**Figure 2A**) and decreased cell death measured by TUNEL assay (**Figure 2B**).

ROS was involved in intracellular Aβ toxicity

To further investigate the possible mechanism

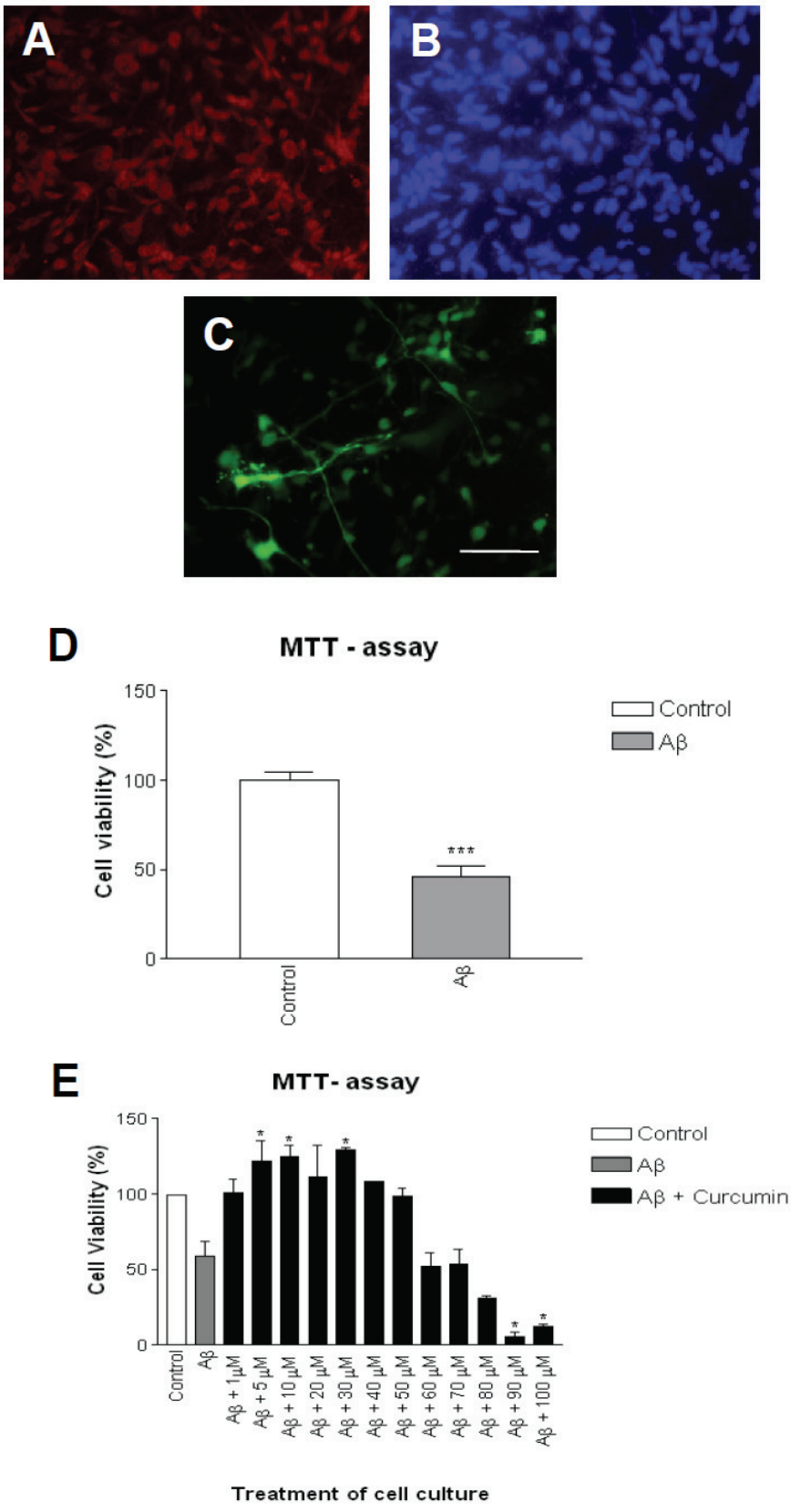

Figure 1. Intracellular $A\beta_{1-42}$ induced toxicity in rat primary neurons. A. Rat hippocampal neurons in culture were stained with neuronal marker b-tubulin -III. B. Neurons were stained with DAPI to show the whole population. C. Around 20-30% neurons were infected indicated by EGFP positive. Scale bar: 100 μm. D. Cell viability was indicated by MTT assay at 24 hours after infection. E. Cell viability was indicated by MTT assay at 24 hours after infection and treatment. Data represented mean ± SE (n=200 cells/preparation, each experiment was repeated in 3 preparations). ***: $p < 0.001$ compared with control group; *: $p < 0.05$ compared with control group.

of intracellular Aβ toxicity, we examined if ROS was involved since evidence showed Aβ interacted with mitochondria function [22]. Relative ROS levels were measured in the control (**Figure 3A** and **3B**), $iA\beta_{1-42}$ infected (**Figure 3C** and **3D**) and $iA\beta_{1-42}$ with curcumin treatment (**Figure 3E** and **3F**) groups. ROS levels increased remarkably with $iA\beta_{1-42}$ infection, whereas curcumin treatment dramatically decreased ROS level (**Figure 3G**), suggesting that ROS played an important role mediating intracellular Aβ toxicity in rat primary neurons and

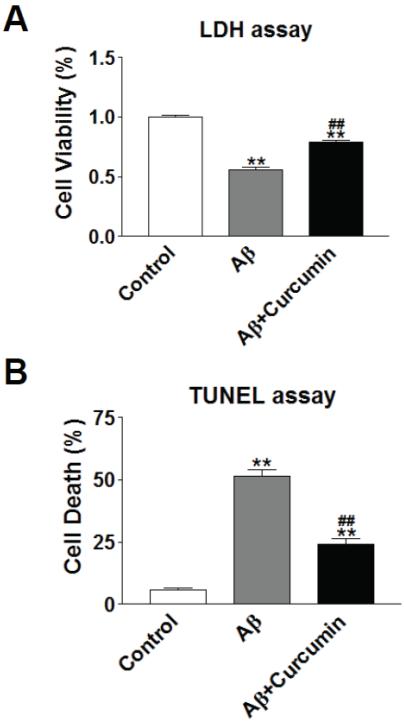

Figure 2. Curcumin protected against intracellular $A\beta_{1-42}$ toxicity. A. Cell viability was indicated by LDH release assay at 24 hours after infection and treatment. B. Cell death was indicated by TUNEL assay at 24 hours after infection and treatment. **: $p<0.01$ compared with control group. ##: $p<0.01$ compared with Aβ group.

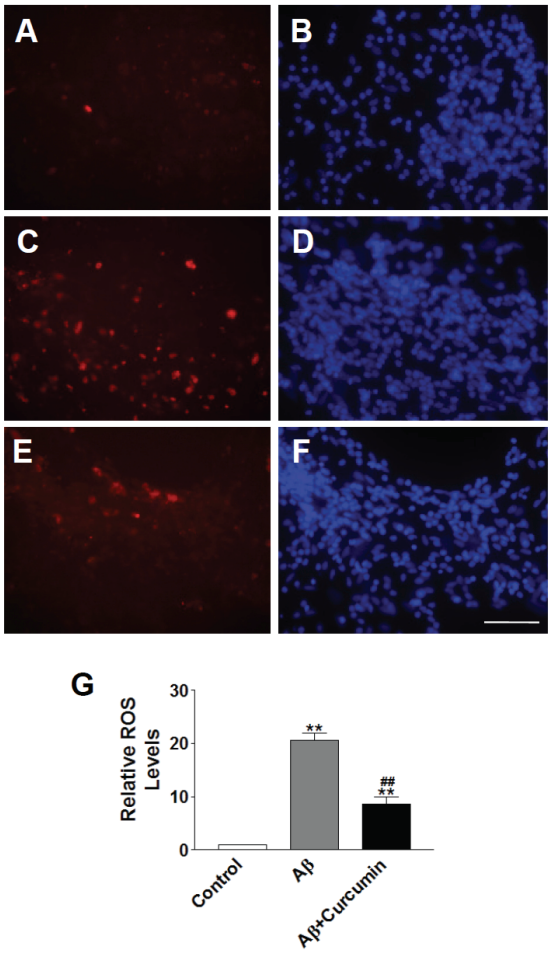

Figure 3. ROS was involved in intracellular $A\beta_{1-42}$ toxicity. A. ROS level stained with MitoSOX™ Red mitochondrial superoxide indicator in control neurons. B. DAPI staining indicated cell population in control neurons. C. ROS level stained with MitoSOX™ Red mitochondrial superoxide indicator in Aβ-infected neurons. D. DAPI staining indicated cell population in Aβ-infected neurons. E. ROS level stained with MitoSOX™ Red mitochondrial superoxide indicator in Aβ-infected neurons in the presence of curcumin. F. DAPI staining indicated cell population in Aβ-infected neurons in the presence of curcumin. Scale bar: 100 μm. G. Quantification of relative ROS levels in neurons. Data represented mean±SE (n=500 cells/preparation, each experiment was repeated in 3 preparations). **: $p<0.01$ compared with control group. ##: $p<0.01$ compared with Aβ group.

curcumin may play protective role through lowering down ROS levels.

Discussion

Our results of the present study indicate that curcumin can protect against intracellular $A\beta_{1-42}$-induced cytotoxicity in rat primary hippocampal neurons, which is consistent with our previous data that curcumin inhibits $A\beta_{25-35}$-induced cytotoxicity in rat primary prefrontal cortex neurons [19]. Other studies also support the beneficial role of curcumin. Curcumin decreases the levels of soluble and insoluble Aβ in transgenic APP_{swe} mice [23]. Similar protection of curcumin is also reported in animal model with human Aβ infused with lipoprotein chaperone into the cerebral ventricles [24]. Curcumin treatment reverses the change of synaptophysin and postsynaptic density 95 (PSD-95) as well as impaired performance in water maze test [25]. Curcumin binds to SPs in the brain tissues when fed or injected in the carotid artery in Tg2576 mice [25]. These data suggest that curcumin

crosses the blood-brain barrier and plays anti-amyloid roles, which makes curcumin a potential drug candidate for amyloid hypothesis-based therapy. A phase II, double-blind, placebo -controlled study of curcumin safety and tolerability in human AD patients is undergoing [25]. Our data from this study confirm that curcumin is protective against intracellular Aβ toxicity in neuronal cultures.

Evidence supports that neurotoxicity induced by intracellular Aβ is partially caused by the formation of ROS, leading to increase of oxidative stress. It is showed that endocytosed Aβ, through binding with specific potential receptors, such as the receptor for advanced glycation end products (RAGE) and the class A scavenger-receptor, leads to increased reactive oxygen production [26-28]. In the other hand, ROS may trigger a positive feedback mechanism for intracellular Aβ toxicity. Oxidative stress can influence Aβ production by interacting with amyloid precursor protein [29]. Indirectly, oxidative stress can also influence Aβ processing by modulating the activity and levels of β-secretase and γ-secretase [26, 30, 31]. Curcumin is suggested to be a more potent anti-oxidant than vitamin E α-tocopherol [32]. Our data suggest that ROS is involved in intracellular Aβ toxicity and curcumin may play its protective role through decreasing ROS levels in neurons. Taken together, our study indicates that curcumin may be beneficial to AD pathology.

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Address correspondence to: Dr. Yan Zhang, College of Life Sciences, Room 219, Peking University, Beijing, 100871, China Tel: 86-10-62754880; Fax: 86-10-62751526; E-mail: yanzhang@pku.edu.cn

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