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·Original Article·

Curcumin protects against staurosporine toxicity in rat neurons

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Abstract: Objective Curcumin is extracted from the turmeric plant (*Curcuma longa* Linn.) and is widely used as a food additive and traditional medicine. The present study investigated the activity of curcumin against staurosporine (STS) toxicity in cell culture. **Methods** Rat hippocampal neurons in primary culture were exposed to STS (20 μmol/L) and treated with curcumin (20 μmol/L). Cell viability was tested by MTT assay and reactive oxygen species (ROS) were measured using the MitoSOX™ red mitochondrial superoxide indicator. Western blot was used to assess changes in the levels of caspase-3 (Csp3), heat shock protein 70 (Hsp70) and Akt. **Results** The results showed that curcumin protects against STS-induced cytotoxicity in rat hippocampal neurons. Csp3, Hsp70, Akt and ROS activation may be involved in this protection. **Conclusion** Curcumin could be a potential drug for combination with STS in cancer treatment to reduce the unwanted cytotoxicity of STS.

Keywords: curcumin; staurosporine-induced cytotoxicity; hippocampal neurons; caspase-3; Akt; Hsp70; reactive oxygen species; cancer treatment

1 Introduction

Staurosporine (STS) is a cell-permeable, prototypical alkaloid of the indolocarbazole family and was first discovered in 1977 in a screen for microbial alkaloids[1]. It was isolated from the bacterium *Streptomyces staurosporeus*, and then from other actinomycetes[2]. It is stable in serum[1] and its antifungal and hypotensive activities were described first. Later, it was reported that STS is a potent inhibitor of protein kinase C and therefore toxic to various cells, including cancer cells[3]. Since then, several STS analogs have been discovered in marine invertebrates, such as sponges, tunicates, bryozoans and mollusks[4], and have been synthesized[4]. Clinical trials have been carried out for analogs aiming at targeted cytotoxicity to cancer cells and relatively low toxicity to other cells[4].

Curcumin is extracted from *Curcuma longa* Linn. (turmeric), a member of the ginger family with a long history as a spice, medicine, food additive and dye in south and southeast Asia, especially India[5]. Its chemical structure was reported in 1815[5]. Curcumin is suggested to be a more potent anti-oxidant than vitamin E α-tocopherol[6]. It has also been reported to have anti-inflammatory effects through inhibiting transcription related to cytokine regulation[7] and suppressing inducible nitric oxide synthase in

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activated macrophages[8]. In addition, curcumin prevents the formation of amyloid β (Aβ) aggregates in the sub-micromolar range[9] and protects against Aβ toxicity in primary cultures of rat neurons[10]. Because of its protective property, we hypothesized that curcumin may protect against STS cytotoxicity in neurons and therefore may be a potential drug to combine with STS in cancer treatment to reduce the unwanted cytotoxicity.

Therefore, we set out to determine whether curcumin is protective against STS toxicity and, if so, the likely mechanism of action.

2 Materials and methods

2.1 Primary cell culture and treatments Newborn Sprague-Dawley rat pups were provided by the Experimental Animal Center of Peking University Health Science Center (Beijing, China). Animal experiments were approved by Peking University Animal Care and Use Committee. The animals were rapidly decapitated and the brain was dissected in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA). Hippocampal tissue was macerated by chopping 10–20 times, and then dissociated by incubation with 0.25% trypsin (Invitrogen) for 30 min at 37°C. The mixture was then filtered through nylon mesh to obtain a single-cell suspension. After filtering through 70-μm sterile filters, the flow-through was centrifuged to pellet the cells, which were then centrifuged and resuspended in DMEM containing 10% fetal bovine serum, 2 g/L HEPES, 100 U/mL penicillin G, and 100 μg/mL streptomycin (all from Invitrogen). Cells were then plated in poly-*L*-lysine-coated (Sigma, St. Louis, MO) Petri dishes or plates and maintained in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. The cultured neurons were treated at 7 days *in vitro* (DIV). Cytosine arabinoside (10 μmol/L; Sigma) was added on DIVs 1 and 2 to inhibit the growth of glial cells. Fresh solutions of curcumin (Sigma) were added to the culture medium during treatment.

2.2 Cell viability and death assays The viability of cells after various treatments was measured by their ability to reduce the dye methyl thiazolyl tetrazolium (MTT; Sigma) to formazan crystals. Cultured neurons were gently washed with 0.01 mol/L phosphate-buffered saline (PBS). After washing, 90 μL medium with 10 μL MTT-PBS (5 mg/mL) was added to each well and the plate was maintained at 37°C for 2 h. Then the absorption of each well at 570 nm was measured using a micro-plate spectrophotometer (Bio-Rad, Hercules, CA). Reactive oxygen species (ROS) was measured by the MitoSOX™ Red Mitochondrial Superoxide Indicator for Live-cell Imaging as described by the manufacturer (Invitrogen).

2.3 Western blots Protein lysates of the hippocampal neurons were extracted after treatment. The neurons cultured in 6-well plates were washed three times with 0.01 mol/L PBS, after which 100 μL of lysis buffer with 1% phenylmethanesulfonyl fluoride was added to each well and the cells were harvested with scrapers. The lysates were kept on ice for 30 min, 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, separated by 10% SDS-PAGE, and then blotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA). 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 and incubated again with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by another wash. The primary antibodies used were: purified polyclonal anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-activated caspase-3 (CSP3) (Santa Cruz), polyclonal anti-heat shock protein 70 (Hsp70) (Stressgen, Farmingdale, NY) and polyclonal anti-Akt (Santa Cruz). Immunoblots were developed with enhanced chemiluminescence reagents, and the images detected in ChemiDox (Bio-Rad). The relative density was calculated by the total absolute density of (active Csp3/GAPDH)*100, (Akt/GAPDH)*100 or (Hsp70/GAPDH)*100.

2.4 Statistical evaluation All data are presented as mean ± SEM. The statistical significance of differences (*P <0.05 or **P <0.01) among groups was determined by two-way

analysis of variance and two-tailed Student's *t*-test. Sheffé's test was applied as a *post hoc* test for the significant differences.

3 Results

3.1 Curcumin protects against STS-induced cytotoxicity MTT assays demonstrated that STS (20 μmol/L) induced cytotoxicity in primary cultures of rat hippocampal neurons after 24-h exposure (Fig. 1A) and curcumin (20 μmol/L) effectively protected against STS toxicity by increasing the viability by ~20% compared with STS alone (Fig. 1A). ROS was greatly increased by STS compared to control (Fig. 1B, C), and curcumin reduced the numbers of ROS-positive cells (Fig. 1E). Taken together, our data suggested that curcumin at 20 μmol/L significantly decreased STS-induced cytotoxicity in primary cultures of rat hippocampal neurons.

3.2 Csp3, Hsp70 and Akt activation may be involved in protection by curcumin Since it is commonly accepted that MTT is not a specific measure of apoptosis, we investigated whether apoptotic or anti-apoptotic proteins are involved in the protection. Activation of the apoptotic protein Csp3 was examined first. Curcumin (20 μmol/L) remarkably blocked the increase of activated Csp3 induced by STS (Fig. 2A). Hsp70 is reported to protect against various insults[11-14], so we measured its levels. Curcumin significantly increased the Hsp70 level when applied with STS, suggesting that Hsp70 activation may be involved in the protection (Fig. 2B). Since the Akt pathway is also implicated in cancer therapy[15], its relative levels were determined, and the data showed that STS increased the Akt level and curcumin effectively blocked this (Fig. 1C).

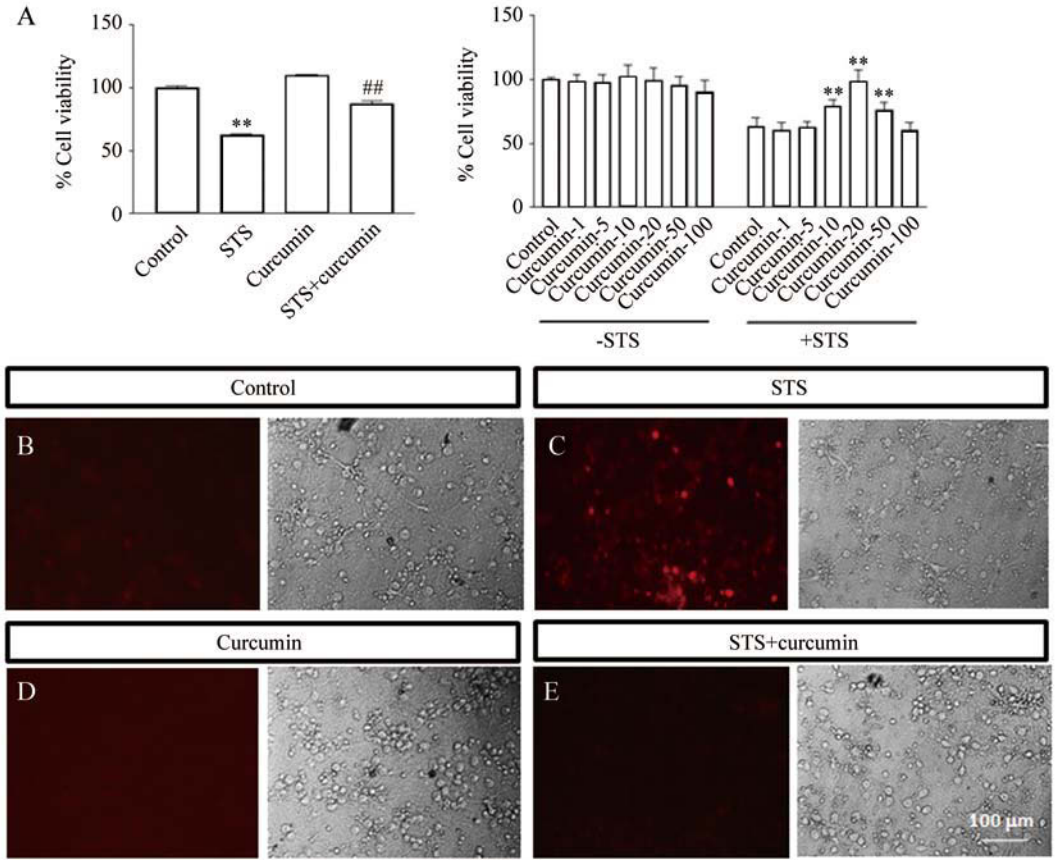

Fig. 1. Curcumin protects against staurosprine (STS) toxicity in primary clutures of rat hippocampal neurons. A: MTT data show that curcumin (left panel: 20 μmol/L; right panel: concentrations ranging from 1 to 100 μmol/L) increases cell viability compared with STS alone (20 μmol/L) at 24 h. B–E: Photomicrographs showing that curcumin decreases the number of ROS-positive cells (red) induced by STS. Phase-contrast images shown on right. Data are mean ± SEM (n = 3). $^{**}P$ <0.01 compared to control; $^{\#\#}P$ <0.01 compared with STS alone. Scale bar, 100 μm.

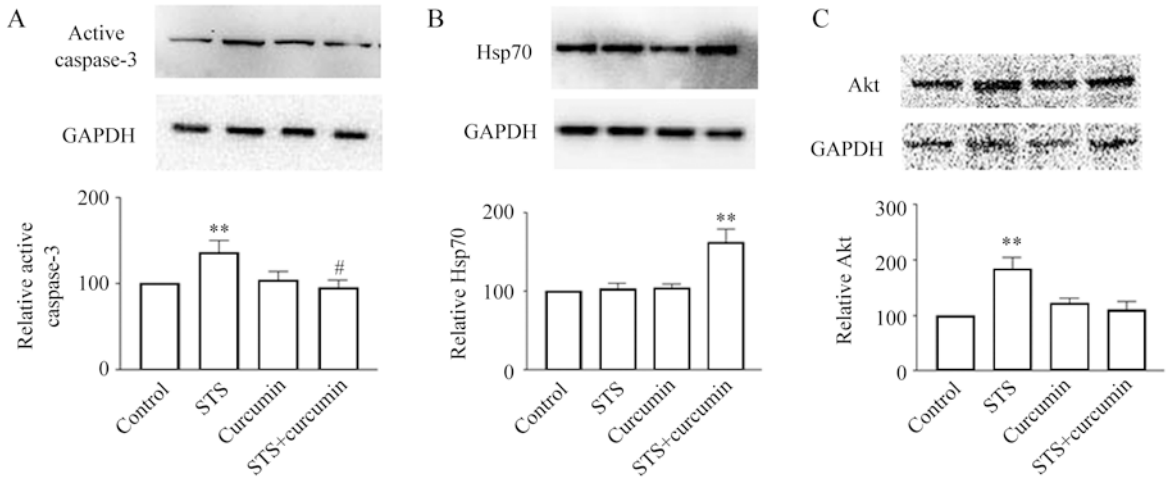

Fig. 2. Curcumin downregulates activated caspase-3 and Akt and upregulates heat shock protein 70 (Hsp70). A: Activated caspase-3 was measured by western blot. GAPDH served as a loading control. Our data indicated that curcumin (20 μmol/L) decreased the level of activated caspase-3. B: Hsp70 levels were measured by western blot. Curcumin increased Hsp70 levels. C: Total Akt was measured by western blot. Curcumin decreased Akt levels. Data are mean ± SEM (n = 3). **P <0.01 compared to control, #P <0.05 compared with staurosprine (STS) group.

4 Discussion

The results of the present study showed that curcumin can protect against STS-induced cytotoxicity in primary cultures of rat hippocampal neurons. Recently, it was reported that curcumin protects against STS-induced cell death in retinal ganglion cells, possibly by regulating NFκB[16]. Here, we showed that ROS, Csp3, Hsp70 and Akt activation may be involved in the protection by curcumin. Curcumin is relatively insoluble in water, but readily dissolves in organic solvents[17]. It is cell-membrane permeable[17] and is relatively stable at physiological pH in the presence of fetal bovine serum or human blood[17]. Its ability to penetrate the blood-brain barrier is poor and up to 0.5% of intravenous curcumin, which is equivalent to 25 μg/kg, is found in the brain[18]. The beneficial effects of curcumin are consistent with other reports that it is protective in an animal model in which human Aβ is infused with a lipoprotein chaperone into the cerebral ventricles[19]. Curcumin treatment reverses the change of synaptophysin and post-synaptic density 95 as well as the distance and latency in finding the hidden platform in the water maze test[5]. Synaptic loss and degeneration cause neuronal dysfunction and cell death. Neuronal loss in the cerebral cortex and the hippocampus is a hallmark of neurodegenerative diseases like Alzheimer's disease[20,21]. A phase II, double-blind, placebo-controlled study of curcumin safety and tolerability in Alzheimers patients is ongoing[5]. These data suggest that curcumin crosses the blood-brain barrier and plays beneficial roles, which makes it a potential candidate for neuroprotection. Our data showed that at high concentrations, curcumin was not protective against STS-induced toxicity. We think this may be explained by the possibility that at high concentrations, it may be cytotoxic. Although the mechanisms of curcumin protection are not fully understood, in the present study, it increased Hsp70 and decreased activated Csp3, Akt and ROS, suggesting that these factors may be involved in STS toxicity and the protection by curcumin.

Derivatives of STS, such as CGP41251, PKC412, Ro31-8220, CEP-701, CP-751, edotecarin and becatecarin are being investigated as potential anti-cancer drugs in phase II or III studies[4]. Protein kinase inhibition by STS is broadly potent across the kinome, so STS itself is cytotoxic when used alone as an anti-cancer drug[4]. One possible way to solve this problem is to look for or generate STS cognates with a more selective kinase inhibition profile[4]. On the other hand, application of combinations of STS with other protective agents, such as curcumin, may help to reduce the cytotoxicity. Further studies need to be done

to test this hypothesis.

In conclusion, curcumin can prevent cytotoxicity induced by STS in rat hippocampal neurons in culture, probably through the regulation of ROS, csp3, Hsp70 and Akt levels. Our data indicate that curcumin may counteract STS toxicity and can be combined with it during cancer treatment.

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