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Curcumin attenuates peroxynitrite-induced neurotoxicity in spiral ganglion neurons

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ABSTRACT

The present study was designed to investigate the effect of curcumin on peroxynitrite ($ONOO^-$)-induced damage in rat spiral ganglion neurons (SGNs). The primary cultured rat SGNs were exposed to $ONOO^-$ with or without curcumin pretreatment. Cell viability was measured by MTT assay. Apoptosis was determined by Ho.33342 and propidium iodide (PI) double staining and flow cytometry. The cellular glutathione (GSH) content, superoxide dismutase (SOD) activity and malonaldehyde (MDA) levels were evaluated by spectrophotometer. The mRNA expressions of Apaf-1, Caspase-9, Caspase-3, Bcl-2, and Bax were examined by RT-PCR, while, the protein expressions of mitochondrial and cytosolic cytochrome *c*, Caspase-9, Caspase-3, Bcl-2 and Bax proteins were determined by Western blot respectively. The cell viability was markedly reduced, while, the apoptotic rate increased significantly after exposure of $ONOO^-$ (100 μM) to SGNs. The activity of SOD and level of GSH were notably reduced, whereas, the MDA level was significantly increased. Pretreatment with curcumin protected SGNs against $ONOO^-$-induced cell damage, declined the apoptotic rate, and improved the levels of SOD and GSH, decreased the elevation of MDA. $ONOO^-$ induced cytochrome *c* release from the mitochondria of SGNs and subsequently activated Caspase-9, Caspase-3 and cell apoptosis. Meanwhile, pretreatment with curcumin abrogated cytochrome *c* release, blocked activation of Caspase-3, and altered the expression of Bcl-2 family triggered by $ONOO^-$. Our data indicate that curcumin can attenuate $ONOO^-$-induced damage in SGNs by the anti-oxidative activity as well as protect mitochondria from oxidative stress.

1. Introduction

Curcumin is a natural product derived from turmeric of the herb *Curcuma longa*, which has been used historically as a component in ancient medicine of China and India to treat a broad variety of diseases (Aggarwal et al., 2007). Modern researches have identified curcumin as the major active constituent of turmeric (Bar-Sela et al., 2009). Mounting evidence from *in vitro* and *in vivo* studies reveals that curcumin possesses various biological functions such as anti-inflammation, antitumor, immunomodulation, enhancement of apoptosis, etc. Curcumin exerts these different effects via specific mechanisms. For instance, influences on transcription and cell signaling pathways may be the possible mechanisms underlying the actions of curcumin (Beevers et al., 2009). Notably, curcumin has been classically characterized as an antioxidant (DuVoix et al., 2005), which effectively detoxifies superoxide either by superoxide dismutation or by directly reacting with it (Mishra et al., 2004; Toniolo et al., 2002). Furthermore, the phenolic hydrogens and the enol structure of curcumin strongly enhance its free radical scavenging activity (Barclay et al., 2000; Priyadarsini et al., 2003).

It is well established that free radicals, which consist of two major types, i.e., reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as $OH^•$, $O_2^•$, H_2O_2, $NO^•$, and $ONOO^-$, are produced in the course of normal metabolic processes in biological systems. Of note, $ONOO^-$, a product of a reaction between nitric oxide and superoxide, is a potent and versatile oxidant, which can oxidize biomolecules either by direct reactions or by $ONOO^-$-derived radicals (Augusto et al., 2002; Schopfer et al., 2003; Radi, 2004). Also, $ONOO^-$ affects protein activity, as illustrated by the inhibition of mitochondrial manganese superoxide dismutase (SOD) and tyrosine phosphatases (Takakura et al., 1999; Crow et al., 1995; MacMillan-Crow et al., 1996). In addition, $ONOO^-$ is able to influence cell

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metabolism by inducing lipid peroxidation (Radi et al., 1991), interfering with mitochondrial function (Radi et al., 2002), damaging DNA (Salgo et al., 1995a,b), and mediating necrosis and apoptosis in different cell types (Bonfoco et al., 1995).

Hearing loss is one of the most common diseases that can impose a heavy social and economic burden on individuals and families. This disorder is associated, in large part, with the impairment of SGNs. SGNs are bipolar neurons that transmit auditory information from the ear to the brain. The survival of SGNs depends on genetic and environmental interactions, which is indispensible for the preservation of hearing and, in contrast, the impairment of SGNs contributes to the loss of hearing, which is irreversible (Diaz, 2009). While the causes of certain types of auditory diseases have been established, the causation of the majority of hearing losses, especially, the sensorineural disorder, is still unclear. It has been documented that oxidative stress has been identified as a basic mechanism involved in SGNs death caused by ROS and RNS, including $ONOO^-$ (Klein et al., 2008).

In *in vitro* studies, exposure of cells to $ONOO^-$ evokes responses that depend on the environment and cell types. To date, a series of publications on $ONOO^-$-induced cell death in different cell types have appeared, but, the effects of $ONOO^-$ on SGNs have been conducted only sparingly (Lee et al., 2003). Although the antioxidative activity of curcumin involves direct detoxification of $ONOO^-$ via the two phenoxyl groups (Iwunze and McEwan, 2004), no study relevant to interaction between curcumin and $ONOO^-$ on primary cultured SGNs has been reported in literature. Thus, the present study was designed to determine whether curcumin possesses a neuroprotective action against $ONOO^-$ on primary cultured SGNs and, if so, the possible mechanism underlying the action of such an oxidative stress.

2. Materials and methods

2.1. Materials

The Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and fetal calf serum was purchased from GIBCO (USA). $ONOO^-$, curcumin, and MTT were obtained from Sigma (St. Louis, MO, USA). Primary antibodies used in this work included anti-neuron specific enolase (NSE) antibody, Caspase-9 (Abcam, Cambridge, MA, USA), cytochrome *c*, Caspase-3, Bcl-2, Bax and β-actin (Santa Cruz Biotechnology, CA, USA). Glutathione (GSH), superoxide dismutase (SOD) and malonaldehyde (MDA) assay kits were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA protein assay kit was a product from Shenergy Biocolor Bioscience & Technology Company (Shanghai, China). Cell Mitochondria Isolation Kit was obtained from Beyotime Co. (Nantong, China). RevertAid First Strand cDNA Synthesis Kit was obtained from Fermentas (Burlington, Ontario, Canada). All other agents used in this experiment were purchased from Sigma (St. Louis, MO, USA).

2.2. Primary cultures of rat SGNs

The experiments were performed on rats at age less than 5 postnatal days. After anesthesia with pentobarbital sodium, the rats were decapitated at the base of the foramen magnum, the epidermis was removed, cranium was opened along the sagittal suture and the brain halves were removed. The following steps were carried out under the microscope and in phosphate buffered solution (PBS). The bulla of the temporal bone was opened, the capsule of the inner ear as well as the stria vascularis and the organ of corti were removed. Rosenthal's canal was isolated and placed into Ca^{2+}–Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 0.125% trypsinase. After digestion for 15 min at 37 °C, the tissue was eluted with the plating medium DMEM supplemented with 10% fetal bovine serum, then cells were collected by centrifugation at 1000 rpm for 8 min, re-suspended and plated in poly-L-lysine-coated 50-cm^2 flask, 24 or 96-well culture plates (Corning, USA) at a density of 1.0×10^5 cells/ml at 37 °C in a humid atmosphere of 5% CO_2.

2.3. The identification of SGNs by immunocytochemistry

Primary SGNs were identified by immunocytochemistry with anti-NSE antibody. Briefly, SGNs (1.0×10^5/ml) were inoculated in poly-L-lysine-coated 24-well plate, fixed in 4% paraformaldehyde for 15 min, and permeabilized and blocked in PBS containing 0.3% Triton X-100 and 10% goat serum 30 min at 37 °C in a humid atmosphere. Subsequently, the cells were incubated for 16–18 h at 4 °C with primary anti-NSE antibody at a concentration of 1:400 diluted, and then stained with secondary goat-anti-rabbit Cy3 antibody. Finally, the cells were visualized under inverted phase contrast and fluorescence microscope for image acquisition.

2.4. Drug treatment

Curcumin was dissolved in dimethyl sulfoxide (DMSO) and its concentration did not exceed 0.1% of the total volume in the cell culture well. Routinely, on day 1 *in vitro*, SGNs were pretreated with curcumin (doses and treatment schedules were presented in the methods and results of each experiment) and $ONOO^-$ (100 μM) was then added to the culture medium. Dose and administration schedules of $ONOO^-$ and agents used here were chosen on the basis of our previous results (data not shown).

2.5. Assessment for cell viability by MTT

SGNs (1.0×10^5/ml) sub-cultured in 96-well plate were treated with $ONOO^-$ (100 μM) for 24 h incubation with or without curcumin pretreatment. In the dose-dependent study, neurons were pretreated with 5, 10, 15, 20, 25, 30, 50 μM curcumin for 12 h respectively. In the time-dependent study, cells were pretreated with 15 μM curcumin for 1, 3, 6, 12, 18, 24 h respectively. Cell viability was quantified by MTT assay. MTT (5 mg/ml, 20 μl) was added to each well before 4 h of the indicated time points. After 4 h of incubation at 37 °C, the medium was removed and the pellet was dissolved in DMSO. Then, the photo density (OD) values were measured at 570 nm using an ELISA reader (Multiskan MK3). Cell relative viability was calculated according to the following formula:

$$\text{Cell relative viability } (\%) = \frac{\text{OD}_{\text{experiment}}}{\text{OD}_{\text{control}}} \times 100\%$$

OD_{blank} was used to zero.

2.6. Morphological measurement by Ho.33342 and PI double staining

SGNs (1.0×10^5/ml) were incubated with $ONOO^-$ (100 μM) for 24 h with the pretreatment of curcumin (15 μM) for 12 h, control cells were without any treatment. Then cells were washed twice by PBS, fixed with 95% alcohol for 10 min, and then stained by Ho.33342 (10 μg/ml) and PI (50 μg/ml) at 37 °C for 30 min. Morphological changes were examined by fluorescence microscope under green light (515–560 nm) and ultraviolet (UV) light (340–380 nm) respectively.

2.7. Determination of apoptotic rate by flow cytometry

For this purpose, apoptotic cells were determined by flow cytometry using Annexin V-FITC and PI. In brief, SGNs (1.0×10^5/ml) were incubated with $ONOO^-$ (100 μM) for 24 h with or