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CURCUMIN ALLEVIATES OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION IN ASTROCYTES

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Abstract—Oxidative stress plays a critical role in various neurodegenerative diseases, thus alleviating oxidative stress is a potential strategy for therapeutic intervention and/or prevention of neurodegenerative diseases. In the present study, alleviation of oxidative stress through curcumin is investigated in A172 (human glioblastoma cell line) and HA-sp (human astrocytes cell line derived from the spinal cord) astrocytes. H_2O_2 was used to induce oxidative stress in astrocytes (A172 and HA-sp). Data show that H_2O_2 induces activation of astrocytes in dose- and time-dependent manner as evident by increased expression of GFAP in A172 and HA-sp cells after 24 and 12 h respectively. An upregulation of Prdx6 was also observed in A172 and HA-sp cells after 24 h of H_2O_2 treatment as compared to untreated control. Our data also showed that curcumin inhibits oxidative stress-induced cytoskeleton disarrangement, and impedes the activation of astrocytes by inhibiting upregulation of GFAP, vimentin and Prdx6. In addition, we observed an inhibition of oxidative stress-induced inflammation, apoptosis and mitochondria fragmentation after curcumin treatment. Therefore, our results suggest that curcumin not only protects astrocytes from H_2O_2-induced oxidative stress but also reverses the mitochondrial damage and dysfunction induced by oxidative stress. This study also provides evidence for protective role of curcumin on astrocytes by showing its effects on attenuating reactive astrogliosis and inhibiting apoptosis. Published by Elsevier Ltd on behalf of IBRO.

Key words: oxidative stress, curcumin, astrocytes, GFAP, neurodegenerative diseases.

INTRODUCTION

Oxidative stress is associated with various neurological disorders, including neurodegenerative diseases and traumatic injuries (Chen et al., 2012). Accumulating evidence indicates that oxidative damage (Kim et al., 2015), mitochondria dysfunction (Lin and Beal, 2006) and dysregulation of calcium homeostasis (Bezprozvanny, 2009) contribute to the cascade of events that eventually lead to the degeneration of neurons and astrocytes.

Astrocytes, the most abundant glial cell population in central nervous system (CNS), play an essential role in homeostasis, including glutamate uptake, potassium ion buffering, nutrient support, and antioxidant protection of neurons (Kimelberg and Nedergaard, 2010). The massive activation of astrocytes or astrogliosis is pathological hallmark in several neurodegenerative diseases which is recognized by the upregulation of glial fibrillary acidic protein (GFAP), vimentin and nestin intermediate filament proteins (Phatnani and Maniatis, 2015). Although, activated astrocytes secrete various neurotrophic factors and actively regulate ion homeostasis for neuronal survival, once activated, astrocytes lose their regulation, and initiate inflammatory responses which eventually lead to the neuronal death and other detrimental effects (Ben Haim et al., 2015). Increasing evidence suggests that impairment of astrocyte function or activation of astrocytes may contribute to the pathogenesis of many acute and chronic neurodegenerative disorders (Maragakis and Rothstein, 2006; Sofroniew and Vinters, 2010; Rodriguez-Arellano et al., 2016). Therefore, protection of astrocytes from oxidative stress may provide an alternative approach to delay and/or improve neurodegeneration, and help to identify appropriate molecular targets for therapeutic intervention in various neurodegenerative diseases.

Clinical evidence shows amelioration of neurodegeneration upon dietary intake or supplementary intake of natural antioxidants (Bisht et al., 2010). Curcumin, a phenolic compound extracted from *Curcuma longa* rhizome, is a potent natural antioxidant, shown to have neuroprotective effects (Cole et al., 2007; Gonzalez-Reyes et al., 2013; Monroy et al., 2013). In addition, curcumin has been shown to have bifunctional antioxidant properties, scavenging ROS and inducing an antioxidant response simultaneously. It has been shown that curcumin induces cytoprotective enzymes such as heme oxygenase-1 (HO-1) (Scapagnini et al., 2006), glutathione-S-transferase (GST) (Nishinaka et al., 2007) and γ-glutamyl cysteine

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Abbreviations: γ-GCL, γ-glutamyl cysteine ligase; AMPK, 5′ adenosine monophosphate-activated protein kinase; AP-1, activator protein-1; BSA, bovine serum albumin; Cur, curcumin; CNS, central nervous system; CO_2, carbon dioxide; DMSO, Dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GFAP, glial fibrillary acidic protein; GST, glutathione S transferase; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; MEM, minimum essential medium; MQ, milli-Q; NFkB, nuclear factor kappa B; Nrf2, nuclear respiratory factor 2; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline –Tween 20; PGC-1α, proliferator-activated receptor γ co-activator 1-α; Prdx6, peroxiredoxin 6; RT, room temperature; RIPA, radioimmunoprecipitation assay; SIRT1, NAD^+-dependent protein deacetylase sirtuin 1; TNT, tunneling nanotube.

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ligase (γ-GCL) (Dinkova-Kostova and Talalay, 2008), Moreover, it induces endogenous antioxidant and anti-inflammatory defense mechanisms by modulating transcription factors such as activator protein-1 (AP-1), nuclear respiratory factor 2 (Nrf2), and nuclear factor kappa B (NFκB) (Gonzalez-Reyes et al., 2013). While most of the studies have focused on protective effects of curcumin on neurons, limited studies show effects of curcumin on astrocytes.

In the present study, we aimed to investigate if curcumin protects astrocytes from oxidative stress and mitochondria dysfunction. In addition, we examined if curcumin treatment reverse the mitochondria dysfunction induced by oxidative stress. This study provides evidence for the protective role of curcumin on astrocytes by showing its inhibitory effects on reactive astrogliosis and apoptosis. In addition, this study delineates the role of curcumin on mitochondria recovery after oxidative stress suggesting its possible use in treatment post injury.

EXPERIMENTAL PROCEDURES

Cell culture

A172 was generous gift from Dr. Shilpa Buch at University of Nebraska Medical Center (UNMC), and HA-sp cells were purchased from the ScienCell Research Laboratories. MEM medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technology) and 100 U/mL penicillin/streptomycin (Life Technology) was used to maintain the A172 cells. HA-sp cells were maintained in astrocyte basal medium (Cell Science, Inc., Newburyport, MA, USA) supplemented with growth factors (Cell Science, Inc.). A172 cells were cultured on polystyrene-treated plates, and HA-sp cells were cultured onto poly-L-lysine-coated culture plates. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. When cells reached 80–90% of confluency, they were trypsinized with 0.25% Trypsin and 0.1 mM EDTA solution (HyClone, Logan, UT, USA). All experiments are performed according to local as well as international guidelines on the ethical use of animals and that all efforts were made to minimize the number of animals used and their suffering.

Culture treatment

0.1 M H_2O_2 (Sigma) stock solution was prepared in sterile MQ water (Sigma), and 10 mM curcumin (Enzo Life Sciences, Farmingdale, NY, USA) stock solution was prepared in sterile DMSO (Sigma). Both stock solutions were protected from light and stored at −20 °C until use. All the working solutions were prepared fresh every time in media just before the treatment. For all experiments, cells were allowed to grow for 24 h in CO_2 incubator before treatment. For GFAP and Prdx6 time point study, cells were treated with H_2O_2 (50 μM) for 2 h, 6 h, 12 h and 24 h. For curcumin toxicity study, cells were incubated with different concentrations (1, 5, 10, 15, 20, 25, 30, 40, 50 and 60 μM) of curcumin for 24 h. For all other experiments unless stated otherwise, cells were treated with 50 μM of H_2O_2 or 1 μM of curcumin or combination of 50 μM of H_2O_2 and 1 μM of curcumin for 24 h.

Membrane integrity assay

Cytotoxicity of curcumin was measured with CytoTox-ONE™ homogeneous membrane integrity assay (Promega, Madison, WI, USA) using manufacturer's protocol. Briefly, cells were seeded in a 48-well plate at a density of 2×10^4. After 24 h, cells were incubated with different concentrations of curcumin for 24 h. After that, cells were equilibrated to 22 °C for 15 min, and CytoTox-ONE™ reagent was added followed by 10-min incubation at 22 °C. For maximum LDH release, lysis solution provided in kit was used. 50 μl of Stop Solution per 100 μl CytoTox-ONE™ was added to each well, and fluorescence was recorded with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Percentage of cytotoxicity was measured using following formula:

$$\%\text{Cytotoxicity} = 100 \times \frac{\text{Experimental} - \text{Culture Medium Background}}{\text{Maximum LDH Release} - \text{Culture Medium Background}}$$

Western blotting

Total cell lysate was isolated using Pierce^R RIPA buffer (Thermo Scientific, Waltham, MA, USA), and total protein was quantified with Pierce™ coomassie protein assay kit (Thermo Scientific) using manufacturer's protocol. 15 μg of total protein was resolved on 12% SDS–PAGE, and electroblotted to PVDF membrane (Millipore Crop., Billerica, MA, USA) followed by 2 h incubated with blocking solution (5% skim milk in PBS-Tween20). The membranes were incubated with primary antibodies (Table 1) overnight at 4 °C, and HRP-conjugated secondary antibodies (1:3000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature (RT). Specific protein bands were detected using Pierce™ ECL Western Blotting Substrate (Thermo Scientific) according to the manufacturer's instructions. The membranes were analyzed with Bio-Rad/Chemi Doc Imaging System and Quantity One software.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton-X100 for 5 min at room temperature (RT). After that, samples were incubated with 3% BSA in PBS-Tween20 (PBST) for 1 h

Table 1. Details of antibodies used in the study

Antibody	Catalog No.	Company	Titer
GFAP	sc-9065	Santa Cruz Biotechnology	1:1000
Prdx6	LF-MA0018	Thermo Scientific	1:1000
Vimentin	bs-0756R	Bioss	1:1000
Caspase 1	bs-0169R	Bioss	1:1000
pBax	bs-3010R	Bioss	1:1000
Bax	bs-0127R	Bioss	1:1000
Caspase 8	bs-0052R	Bioss	1:1000
β-actin	sc-69879	Santa Cruz Biotechnology	1:4000