Activation of Nuclear Factor Erythroid 2-Related Factor 2 Cytoprotective Signaling by Curcumin Protect Primary Spinal Cord Astrocytes against Oxidative Toxicity

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Abstract
Oxidative stress, a deleterious processes resulting from an imbalance between formation of ROS and antioxidative defense, is considered to play a critical role in the pathogenesis of various neurodegenerative diseases that are characterized by selective neuronal death. Astrocytes support neuronal survival through inactivation of ROS by the activity of antioxidant and glutathione (GSH). Deficiency of antioxidant function in astrocytes has been implicated in many neurodegenerative processes. Neuronal loss occurred in spinal cord, such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and spinal muscular atrophy (SMA), has been attributed to the deterioration of the ability of spinal cord astrocytes to support neurons. On the other hand, compromised astrocytes function may increase the neuron's susceptibility to oxidative stress. The involvement of ROS in neuronal toxicity and the important role of astrocytes in neurodegenerative processes make it important to determine the detoxification pathways and regulation of antioxidants in astrocytes. An increasing number of antioxidants and phase II enzymes are found to be essential in detoxification of reactive oxygen species.

Antioxidant response element (ARE) is an enhancer of many phase II antioxidant enzymes genes, such as heme oxygenase 1 (HO1), glutamate cysteine ligase (GCL) and reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H): quinine oxidoreductase 1 (NQO1). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a master transcriptional regulator of phase II antioxidant genes. We report herein that curcumin significantly activates Nrf2 target genes in primary spinal cord astrocytes, decreases the level of intracellular reactive oxygen species (ROS), and attenuates oxidative damage and mitochondrial dysfunction.

Key words nuclear factor erythroid 2-related factor 2; curcumin; astrocyte; oxidative stress; reactive oxygen species

MATERIALS AND METHODS

Animals and Genotyping Genotypes (Nrf2+/+ and Nrf2−/−) of the animals were determined by polymerase chain reaction (PCR) amplification of genomic DNA from tails. PCR amplification was carried out using three different primers, 5′-TGGACGG-GACTATTGAAAGCTG-3′ (sense for Nrf2+/+ and Nrf2−/−), 5′-GCCGCCCTTTCATGATG-GAGG-3′ (antisense for Nrf2+/+) and 5′-GGGATTGAC-CGTAATGGGATAG-3′ (antisense for Nrf2−/−). The genotypes of mice were verified by examining the sizes of the PCR products: Nrf2+/+ (700 bp) and Nrf2−/− (400 bp).

Primary Cell Culture Primary astrocytes were prepared from spinal cords of postnatal day 1 to 3 Nrf2+/+ male mice and Nrf2−/− male mice. In brief, animals were killed. Spinal cords were removed and placed in Ca2+/Mg2+-free phosphate buffered saline pH 7.4. Spinal cord pieces were trypsinized for 15 min and mechanically dissociated. After centrifugation at 300 g for 5 min, the pellets were re-suspended in Dulbecco’s modified Eagle’s medium (DMEM) (pH 7.4) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin. The cells were seeded (at a density of 2×105 cells per cm2) into cell culture flasks.

Curcumin and H2O2 Treatment In the solvent group, cells were incubated in serum-free DMEM with 0.1% dimethylsulfoxide (DMSO) for 24 h. In the curcumin groups, cells were pre-incubated in serum-free DMEM with different concentrations of curcumin for 24 h. In the H2O2 plus curcumin group, cells were first pretreated with curcumin for 24 h, followed by washing and then treatment with H2O2 in serum-free DMEM for 30 min.

Evaluation of Intracellular ROS Production and Viability Assay Intracellular ROS production was detected

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using the nonfluorescent cell permeating compound, 2′,7′-dichlorofluorescein diacetate (DCF-DA). Astrocytes were treated with DCF-DA (10 μM) for 30 min at 37 °C and rinsed with serum-free DMEM. The cell viability assay involves the use of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), which forms a formazan product in cells.

Detection of Cytotoxicity by Measuring Lactate Dehydrogenase (LDH) Release and Flow Cytometry Cytotoxicity was evaluated by measuring LDH release from the cytosol of damaged cells into the medium using a standard colormetric assay procedures. Mitochondrial transmembrane potential were measured using flow cytometry. One milliliter cell suspension (10^6 cells) was incubated with rhodamin 123 at 37 °C for 30 min.

Statistical Analysis Numerical data are expressed as mean±S.E.M. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple range test. Statistical significance was considered at p<0.05.

RESULTS

Curcumin Activates Nrf2 and Nrf2 Target Genes in Astrocytes As shown in Fig. 1A, curcumin at 5, 10, 15 μM caused significant increase in expression of three Nrf2 target genes in primary Nrf2^+/+ astrocytes. In contrast, curcumin treatment did not result in significant increases in Nrf2 target genes in Nrf2^−/− astrocytes in Fig. 1B. Curcumin caused significant increase in nuclear Nrf2 in primary Nrf2^+/+ astrocytes, while the total Nrf2 was not significantly altered as shown in Fig. 1C. Nrf2^+/+ cells in the control group showed the cytosolic distribution pattern of Nrf2, as the green fluorescence of Nrf2 was mainly confined in the cytoplasm. Curcumin treatment converted the distribution pattern, as Nrf2 accumulated in the nucleus as shown in Fig. 2. These results show that not only is the Nrf2-ARE signaling pathway responsive in astrocytes but also it can be significantly activated by curcumin.

Curcumin Inhibited Intracellular ROS Production Induced by H_2O_2 in Nrf2^+/+ Astrocytes, while Its Activity Was Abolished in Nrf2^−/− Astrocytes Thirty minutes after the insult with 200 μM H_2O_2, we observed an increase in ROS production in both Nrf2^+/+ astrocytes and Nrf2^−/− astrocytes. But ROS production in Nrf2^−/− astrocytes was higher than in Nrf2^+/+ astrocytes. Addition of 10 μM curcumin prevented this alteration in Nrf2^+/+ astrocytes, as ROS production decreased significantly. In contrast, no significant alterations were observed in Nrf2^−/− cells, as shown in Fig. 3. Drug treatment as described (see Fig. 3C for treatment schedules).

Decreased Oxidative Damage in Curcumin-Treated Astrocytes Is Accompanied by Activation of Nrf2 Signaling and Protection of Mitochondrial Damage As shown in Fig. 4A, exposure of Nrf2^+/+ astrocytes and Nrf2^−/− astrocytes to H_2O_2 resulted in a substantial loss in the protein levels of HO1 and NQO1. But pretreatment of these cells with curcumin significantly attenuated this decrease. In contrast, curcumin pretreatment was ineffective in Nrf2^−/− astrocytes. To gain further insight into the protective activity of curcumin, we measured mitochondrial integrity in Nrf2^+/+ astrocytes and Nrf2^−/− astrocytes. H_2O_2 treatment caused significant loss of mitochondrial trans-membrane potential in both Nrf2^−/− astrocytes and Nrf2^+/+ astrocytes. Cells were first treated with curcumin at 10 μM for 24 h before combined treatment with H_2O_2. We found that curcumin efficiently prevented the loss of mitochondrial trans-membrane potential in Nrf2^+/+ astrocytes, while failed to do so in Nrf2^−/− astrocytes, as shown in Fig. 4B.

Nrf2 Is a Crucial Determinant of Susceptibility of Astrocytes to Oxidative Stress, and Curcumin Decreases the Susceptibility to Oxidative Stress and Protects against H_2O_2-Induced Oxidative Damage As shown in Fig. 5, H_2O_2 treatment markedly reduced the viability and increased LDH release from Nrf2^+/+ astrocytes and Nrf2^−/− astrocytes,
but Nrf2−/− astrocytes were more susceptible than Nrf2+/+ astrocytes. Thus, Nrf2−/− cells exhibited a significantly increased sensitivity to H2O2-induced oxidative damage. Pretreatment of Nrf2−/− astrocytes with 10 μM curcumin for 24 h led to a significant protection against and decreased the sensitivity to H2O2-induced oxidative stress. In contrast, the same curcumin pretreatment in Nrf2−/− cells resulted in either none or only a slight cytoprotection against the cell injury and led to no significant alteration in the sensitivity to H2O2-induced oxidative stress.

DISCUSSION

Phase II enzymes play an important role in protecting cells against stress through the removal of free radicals and detoxification of toxins. HO1 has been recognized as an important cytoprotective enzyme in addition to its metabolic role in the breakdown of heme. NQO1, an enzyme that detoxifies quinones, is reported to have antiapoptotic effects. GSH is the most abundant low-molecular-weight thiol that regulates the redox state of the cell. The synthesis of GSH involves GCLC.

Our study demonstrated that incubation of Nrf2+/+ astrocytes with curcumin led to a significant induction of phase II enzymes. The up-regulation of phase II enzymes was abolished in Nrf2−/− cells. This observation suggested that Nrf2 cytoprotective signaling can be significantly activated by curcumin in astrocytes. Curcumin effectively intercepts and neutralizes ROS induced by H2O2, which is accompanied by activation of Nrf2 signaling.

In normally situation, Nrf2 resides in the cytoplasm as part of a complex with the redox-sensitive protein Keap-1. After a short period, this Nrf2 is ubiquitinated and released from Keap-1 for proteasomal degradation. After the curcumin treatment, Keap-1 releases Nrf2, allowing it to translocate to the nucleus. In the nucleus, Nrf2 binds to ARE containing promoter of many phase II antioxidant enzymes genes. These enzymes and molecules act together to reduce the ROS load and to restore and maintain redox balance.

Many observations suggest that the lack of Nrf2 in astrocytes may be a common contributing factor to neurodegenerative disorders and the activation of Nrf2 in astrocytes confer...
significant protection to neurons in vivo. Chemical/drugs that activate this pathway may have efficacy in blocking neuronal cell death. A focused effort to find those that cross the blood–brain barrier efficiently, activate the pathway in astrocytes, and have efficacy against neurotoxicity is underway.

Activation of phase II enzymes by curcumin offer a great advantage for therapeutic purposes, as curcumin could become part of the human diet and be consumed daily as herbal supplements. It was low toxicity and able to enter the central nervous system. Curcumin activates of Nrf2-ARE pathway in spinal cord astrocytes may serve as a therapeutic strategy for neurodegenerative diseases.

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