European Review for Medical and Pharmacological Sciences 2013; 17: 1360-1368

Ameliorating effects of curcumin on 6-OHDA-induced dopaminergic denervation, glial response, and SOD1 reduction in the striatum of hemiparkinsonian mice

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Abstract. – BACKGROUND: Inflammation and oxidative stress are believed to contribute to neuronal degeneration of the nigrostriatal dopaminergic (DA) pathway in Parkinson's disease. Curcumin, a component of the yellow curry spice, has been reported possessing anti-inflammatory and anti-oxidative effects.

AIM: The present study investigated the effects of curcumin on the extent of DA innervation, glial response, and Cu/Zn superoxide dismutase (SOD1) expression in the striatum of 6-hydroxydopamine (6-OHDA)-lesioned mice.

MATERIALS AND METHODS: 6-OHDA was unilaterally injected into the right striatum of ICR male mice. Curcumin (200 mg/kg) was administered daily for 7 days starting instantaneously after 6-OHDA injection. Seven days after 6-OHDA insult, mice were euthanized and striatal sections were collected, immunohistochemically stained, and quantitated for tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), ionized calcium binding adapter molecule 1 (Iba1), and SOD1 immunoreactivity.

RESULTS: 6-OHDA injection triggered a significant loss of TH-immunoreactive (-IR) axons, induced reaction of GFAP-IR astrocytes and Iba1-IR microglia, and decreased SOD1 expression in the 6-OHDA-lesioned striatum. Curcumin attenuated loss of TH-IR fibers, diminished activation of astrocytes and microglia, and sustained SOD1 level in the lesioned striatum.

CONCLUSIONS: These results suggest that curcumin counteracts the neurotoxicity of 6-OHDA through its anti-inflammatory properties (inhibition of glial response) and preservation of SOD1 expression.

Key Words:
Curcumin, 6-OHDA, Nigrostriatal dopaminergic axon, Astrocyte, Microglia, SOD1.

Introduction

Parkinson's disease (PD) is primarily due to the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and their projections in the striatum. Although the pathogenesis of PD remains obscure, studies in PD brains and animal models of PD suggest that neuroinflammation characterized by neuroglial activation might contribute to pathophysiological processes underlying PD[1-3], provided that activated astrocytes and microglia express broad array of neurotoxic molecules, including pro-inflammatory cytokines, pro-inflammatory prostaglandins, reactive oxygen species (ROS), and reactive nitrogen species[1,4,5]. In addition, impairment of glial response improves the deficit outcome[3,6]. Thus, inhibition of reactive glia is considered as a target for alleviation of neuronal damage caused by neuroinflammation.

Oxidative stress is believed to be another underlying cause of neurodegeneration in PD[7-10]. Normally, cells are protected from free radical-induced injury by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase. The first essential stage of the endogenous antioxidant defense mechanism involves SOD activity that regulates superoxide radical concentration by catalyzing the dismutation of superoxide to hydrogen peroxide, which is then converted to water by glutathione peroxidase and/or catalase[11]. Intracellular Cu/Zn SOD (SOD1) is one of the three major endogenous SODs presently known. Within cells, SOD1 is primarily localized in the cytoplasm[12] and also in the intermembrane space of mitochondria[13]. Consistent with the antioxidant role for SOD, mice with overexpression of SOD1 show attenuated pathology to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine-(6-OHDA)-induced neurotoxicity[14,15], while SOD1 deficient mice are more vulnerable to MPTP[16]. It is, therefore, proposed that SOD1 is necessary to confer resistance against MPTP and 6-OHDA insult.

Curcumin, a yellow pigment isolated from the rhizome of Curcuma Longa Linn (Tumeric), is one of the primary ingredients in curry powders

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that are used as spices and also as herbal medicine in India. Curcumin exhibits anti-inflammatory and anti-oxidant properties[17] and has been shown exerting neuroprotective effects against cerebral ischemia or traumatic brain injury[18,19]. In previous qualitative study we observed that curcumin exerted neuroprotection in the 6-OHDA-lesioned striatum[20]. The aim of the present work was to further quantify the ameliorating effects of curcumin against 6-OHDA-induced DA denervation, glial response, and SOD1 depletion in the 6-OHDA-injected striatum.

Materials and Methods

Animals

Twenty-eight male mice of the ICR strain (National Laboratory Animal Center, Thailand) were 7 weeks old at the start of experiment. They were housed individually per cage with a 12-h light-dark cycle at a room temperature of 23°C and had access to food and water ad libitum. The animals were acclimatized to the laboratory environment for 7-10 days before the experiments. All experiments were performed using protocols approved by the Committee of Animal Used for Research at Faculty of Medicine, Srinakharinwirot University, Thailand.

6-OHDA Lesion Model and Curcumin Administration

Animals were divided into four groups (n=7) (sham control group, 6-hydroxydopamine (6-OHDA) group, 6-OHDA/dimethylsulfoxide (DMSO) group, and 6-OHDA/curcumin group). Mice anesthetized intraperitoneally with pentobarbital sodium (60 mg/kg) received two stereotaxic injections of 6-OHDA hydrobromide (Sigma, 5.5 μg/μl), (Sigma Chemical Co, St Louis, MO, USA) 3 μl/injection site, or an equivalent volume of 0.9% saline containing 0.2% ascorbic acid, into the right striatum, at a rate of 1 μl/min (coordinates: point I: AP + 0.7, ML + 2.0, DV - 3.4 mm and point II: AP - 0.1, ML + 2.7, DV - 3.4 mm from bregma and dura according to the atlas of Franklin and Paxinos[21]). At the completion of each injection, the needle was left *in situ* for a further 3 min to allow for diffusion and then withdrawn at 1 mm/min. The wound was sutured and applied with antiseptic (1% w/w iodine, betadine). During the experiment the 6-OHDA solution was kept on ice and light protected to minimize oxidation. Curcumin (Sigma, 200 mg/kg) dissolved in DMSO (Sigma) or an equivalent volume of DMSO (1 ml/kg) was injected intraperitoneally for 7 days starting immediately after the surgery and was repeated every 24 hours. Animals were euthanized on day 7 after 6-OHDA injection. Only those animals in which the needle tracts were placed at appropriate coordinates were included in the study.

Immunohistochemistry

All mice were sacrificed by an overdose of pentobarbital sodium (350 mg/kg, intraperitoneal, Sanofi, Thailand) and perfused transcardially with 30 ml of ice-cold heparinized 0.1 M phosphate buffer saline (PBS), pH 7.4, followed by 20 ml of chilled 4% paraformaldehyde (Sigma) in 0.1M phosphate buffer, pH 7.4. The brains were post-fixed in the same fixative for 18 hr at 4°C and then cryoprotected with 30% sucrose in PBS at 4°C until sunk. A 1:10 series of thirty five-micrometer coronal sections were cut through the striatum, using a cryostat (Leica CM 1850, Wetzlar, Germany). The sections were stored free-floating at -20°C in cryoprotectant until used.

For tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), ionized calcium binding adapter molecule 1 (Iba1), and SOD1 immunohistochemistry, sections were rinsed in PBS to remove any cryoprotectant, then incubated for 15 min in 0.3% H_2O_2 in 50% methanol to block endogenous peroxidase activity. After 30 min incubation in blocking buffer (PBS containing 0.3% triton X-100 and 3% normal goat serum for GFAP and SOD1 staining or 10% normal goat serum for TH and Iba1 staining) to reduce non-specific binding, sections were rinsed in PBS, then incubated overnight at room temperature with a polyclonal rabbit anti-TH antibody (a marker for DA neurons, Chemicon International, Temecula, CA, USA, 1:3000 dilution), a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (a marker of activated astrocytes, Dako, Glostrup, Denmark, 1:3000 dilution), a polyclonal rabbit anti-Iba1 antibody (a marker of microglia and macrophage, Wako Pure Chemical Industries, Tokyo, Japan, 1:1500 dilution), or a polyclonal rabbit anti-SOD1 antibody (Stressgen, Ann Arbor, MI, USA, 1:1000 dilution). This was followed by incubation in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA, 1:500 for TH and GFAP staining, and 1:400 for Iba1 and SOD1 staining) at room temperature and subsequently in avidin peroxidase (Sigma, 1:5000 dilution).

Immunoreactivity was visualized with cobalt and nickel-intensified 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma)/H_2O_2. PBS rinses (5 min × 3) were performed between each step. Finally, sections were mounted onto slides, dehydrated, and coverslipped. Negative control study was performed by omission of the primary antibody.

Quantification of TH-Immunoractive (-IR) Fibers, GFAP-IR Cells, IBA1-IR Cells, and SOD1 Expression in the Striatum

The extent of DA innervation, the degree of reactive astrocytosis and microgliosis, and the expression of SOD1 in the ipsilateral striatum were assessed by measuring the optical density (OD) of TH, GFAP, Iba1, and SOD1 immunoreactivity, respectively, with Leica image processing and analysis software (Leica Image Processing Solution Ltd., Cambridge, UK). Images of the striatum of each section were captured at 4 × magnification using an Olympus DP-70 digital color camera (Olympus America Inc., Corporate Parkway Center Valley, PA, USA) mounted to an Olympus BX50 microscope. To correct for variability in lighting conditions, all images were photographed under identical conditions. After converting images to an 8-bit file, pictures were calibrated to OD values. Background levels were captured from the cerebral cortex (for TH, GFAP, and Iba1 quantification) or corpus callosum (for SOD1 quantification) in each section and subtracted from the total OD measurement. The OD value of each animal was the average OD from each of the nine to twelve measured sections. All analyses were performed in a blinded fashion.

Statistical Analysis

All values were expressed as mean ± SEM. Statistical analysis was carried out using one-way ANOVA with Student-Newman-Keuls *post hoc* tests. $p < 0.05$ was considered statistically significant.

Results

Curcumin Significantly Reduces 6-OHDA-Induced Loss of Striatal TH-IR Fibers

In the saline-injected striatum, minimal disruption of TH immunoreactivity associated with the needle track was evident (Figure 1B′). Seven days after 6-OHDA injection, TH-IR fibers in the ipsilateral striatum decreased significantly and the OD of TH-immunoreactivity was 22.38 ± 3.95% of the saline-injected striatum ($p < 0.05$ vs. the saline-injected striatum) (Figures 1A and 1C′). Treatment with DMSO had no effect on 6-OHDA-induced loss of striatal TH-IR fibers (Figures 1A and 1D′). However, curcumin partially protected against 6-OHDA-triggered loss of striatal DA fibers and the OD of TH immunostaining was 49.48 ± 6.39% of the saline-injected striatum ($p < 0.05$ vs. the ipsilateral striatum of sham control, 6-OHDA-, and 6-OHDA/DMSO-injected mice) (Figures 1A and 1E′). There was no significant difference in TH immunoreactivity in the contralateral striatum among all animal groups (Figures 1B-1E).

Curcumin Attenuates 6-OHDA-Triggered Activation of Striatal Astrocytes and Microglia

There were a small number of GFAP-IR astrocytes in the contralateral striatum of sham controls (Figure 2B). Injection of normal saline induced an increase in GFAP immunolabeling in the vicinity of the needle track (Figure 2B′). A greater astroglial reaction was evident in the 6-OHDA-injected striatum ($p < 0.05$ vs. the saline-injected striatum) (Figures 2A and 2C′). This was significantly diminished by curcumin ($p < 0.05$ vs. the ipsilateral striatum of sham control, 6-OHDA-, and 6-OHDA/DMSO-injected mice), but not by DMSO (Figures 2A, 2D′, and 2E′). In the unlesioned striatum, there was no significant difference in the GFAP immunostaining among the animal groups (Figures 2B-2E).

Saline injection triggered reactivity of striatal microglia nearby the needle track (Figure 3B′). A greater extent of microgliosis was evident in the 6-OHDA-lesioned striatum ($p < 0.05$ vs. the saline-injected striatum) (Figures 3A and 3C′), and this response was not affected by DMSO (Figures 3A and 3D′). Curcumin markedly suppressed microgliosis in the 6-OHDA-injected striatum ($p < 0.05$ vs. the lesioned striatum of 6-OHDA- and 6-OHDA/DMSO-injected mice) (Figures 3A and 3E′).

In the saline-injected striatum, GFAP-IR astrocytes and Iba1-IR microglia in the vicinity of the needle track transformed morphologically into reactive state (Figures 2G and 3G). In the 6-OHDA-injected striatum, similar morphological change of striatal glia was evident in greater area beyond the vicinity of the needle track (Figures 2H and 3H). DMSO had no effect on 6-OHDA-

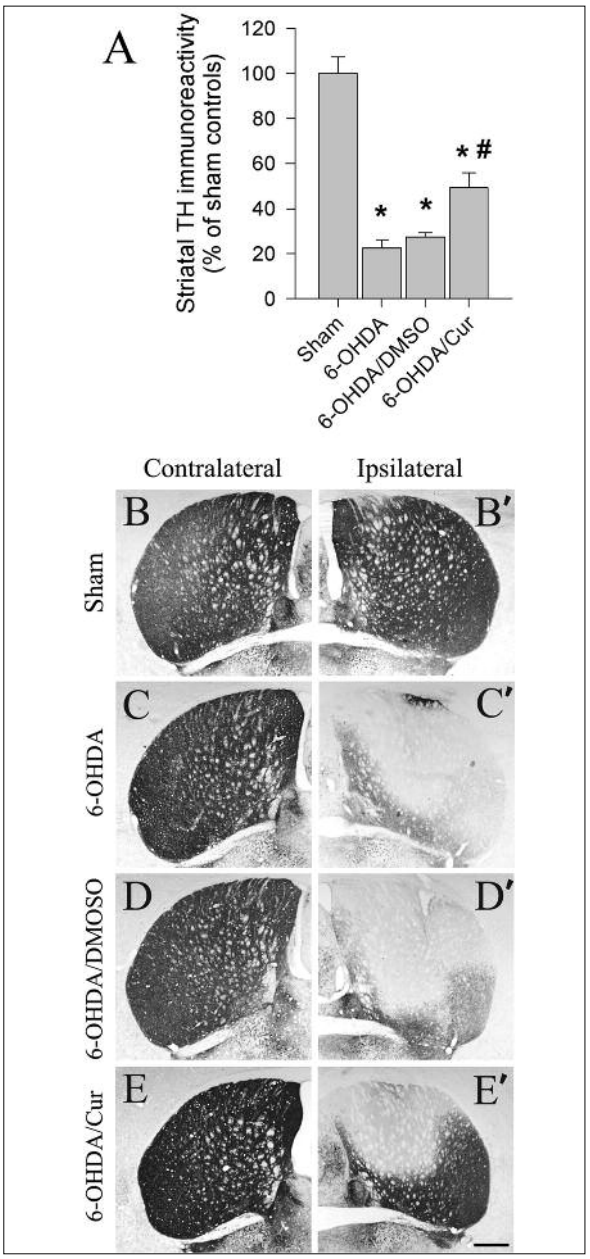

Figure 1. Neuroprotective effects of curcumin on nigrostriatal DA axons. ***(A)*** Histogram showing the OD of TH immunoreactivity in the ipsilateral striatum of sham control, 6-OHDA-, 6-OHDA/DMSO- and 6-OHDA/curcumin-injected mice. Values are mean ± SEM (n = 7). *Indicates significant difference from sham mice, $p < 0.05$. #Indicates significant difference from 6-OHDA- and 6-OHDA/DMSO-treated animals, $p < 0.05$. ***(B-E** and **B′-E′)***: Representative photomicrographs depicting TH immunoreactivity in the contralateral (left panels) and the ipsilateral striatum (right panels) of sham control ***(B** and **B′)***, 6-OHDA- ***(C** and **C′)***, 6-OHDA/DMSO- ***(D** and **D′)***, and 6-OHDA/curcumin-injected mice ***(E** and **E′)***. Note the more numerous TH-IR axons in the lesioned striatum of 6-OHDA/curcumin-injected mice compared with that of 6-OHDA- and 6-OHDA/DMSO mice. Scale bar in ***(E′)*** represents a length of 500 μm for all photomicrographs.

induced morphological change into reactive astrocytes and microglia (data not shown). Likewise, curcumin did not impede 6-OHDA-triggered transformation into reactive glia (Figures 2I and 3I).

Diminished SOD1 Immunoreactivity in the 6-OHDA-Lesioned Striatum is Restored by Curcumin

Majority of SOD1-IR cells in the striatum of all animal groups were striatal neurons (Figures 4B-4E). 6-OHDA significantly decreased SOD1 expression in the ipsilateral striatum ($p < 0.05$ vs. the saline-injected striatum) (Figures 4A and 4C). This was impeded by curcumin ($p < 0.05$ vs. the ipsilateral striatum of 6-OHDA- and 6-OHDA/DMSO-treated mice), but not DMSO vehicle (Figures 4A, 4D, and 4E).

Discussion

The present study shows that curcumin attenuates loss of striatal DA axons triggered by 6-OHDA. The curry spice also reduces glial response and maintains SOD1 level in the 6-OHDA-lesioned striatum.

Previous study has shown that curcumin reverses the 6-OHDA-induced depletion of striatal dopamine[22]. From this data, it is not exactly known what the underlying tissue changes that associate with such benefit effect. Two possible mechanisms may responsible for this; (1) curcumin enhances striatal dopamine levels through inhibition of monoamine oxidase (MAO)-A and MAO-B[23], leading to an increase in the amount of monoamines stored and released from DA nerve terminals; and (2) curcumin protects nigrostriatal DA axons against 6-OHDA toxin (the present study), resulting in greater striatal dopamine levels than that in lesion-only animals.

Recent data indicate direct neuroprotective effects of curcumin on cultured DA neurons against 6-OHDA or 1-methyl-4-phenylpyridnium ion[24,25]. Several neuronal effects that may contribute to the neuroprotective properties of curcumin have been suggested, including inhibition of the c-Jun N-terminal kinase pathway, restoring the mitochondrial membrane potential, increasing the level of SOD1, suppressing an increase in intracellular reactive oxygen species, reducing an overexpression of inducible nitric oxide synthase, and inhibition of 6-OHDA-induced nuclear factor-kappaB translocation[24-26]. Thus, in this study

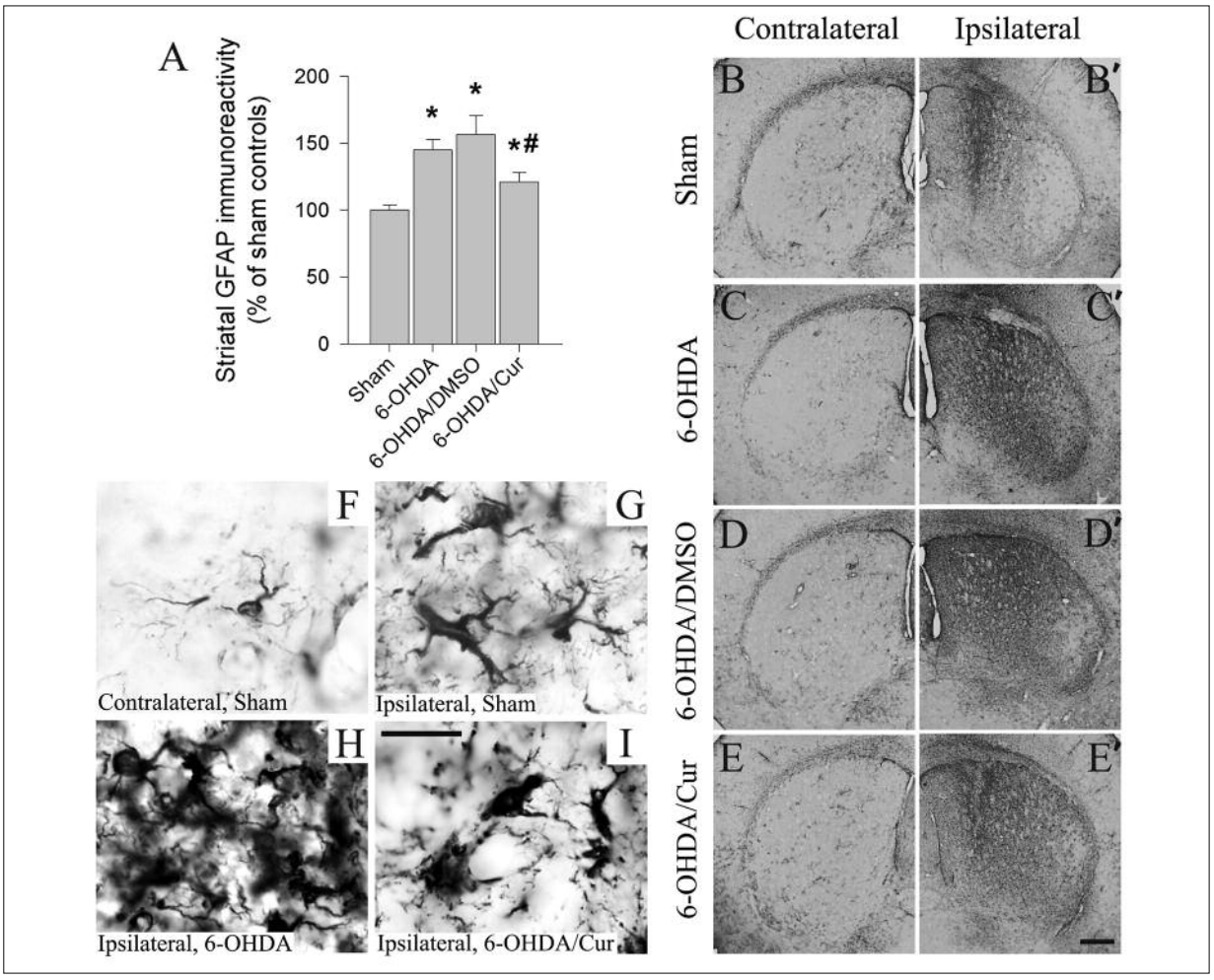

Figure 2. Curcumin reduces 6-OHDA-induced astroglial response. ***(A)*** Histogram illustrating the OD of GFAP immunoreactivity in the ipsilateral striatum of sham control, 6-OHDA-, 6-OHDA/DMSO- and 6-OHDA/curcumin-injected mice. Data represent mean ± SEM (n = 7). *Indicates significant difference from sham mice, $p < 0.05$. #Indicates significant difference from 6-OHDA- and 6-OHDA/DMSO-treated animals, $p < 0.05$. ***(B-E*** and ***B′-E′)***: Representative photomicrographs showing GFAP immunoreactivity in the contralateral ***(B-E)*** and the ipsilateral striatum ***(B′-E′)*** at day 7 following unilateral stereotaxic injection of saline or 6-OHDA into the right striatum of sham control ***(B*** and ***B′)***, 6-OHDA- ***(C*** and ***C′)***, 6-OHDA/DMSO- ***(D*** and ***D′)***, and 6-OHDA/curcumin-injected mice ***(E*** and ***E′)***. ***(F-I)*** High magnification of GFAP-IR astrocytes in the contralateral striatum of sham controls ***(F)*** and the ipsilateral striatum of sham controls ***(G)***, 6-OHDA mice ***(H)***, and 6-OHDA/curcumin mice ***(I)***. Scale bar in panel ***E′*** represents a length of 500 μm for panels ***B-E*** and ***B′-E′*** and scale bar in panel ***I*** represents a length of 25 μm for panels ***F-I***.

curcumin may provide neuroprotection by acting on nigrostriatal DA neurons and axons directly, resulting in more striatal TH-IR axons surviving 6-OHDA toxin. Since products (as yet identified) released from damaged axon terminals may activate astrocytes and microglia[27-30], the decreased glial activation in 6-OHDA/curcumin-treated mice may be resulting from reduced loss of DA axons compared to 6-OHDA-treated mice.

However, inhibition of glial activation may be an alternative mechanism by which curcumin exerts its neuroprotection. The function of early glial response following brain injury may be part of repair and protective response, but the presence of glial reaction in the SNpc of PD brains[4] raises the question of whether this response could contribute to the neuronal injury. Indeed, inhibition of microglial reaction decreases TH-IR neuronal loss induced by 6-OHDA[31] or MPTP[3]. In addition, suppression of astroglial activation reduces the number of apoptotic cells in the peri-infarct area following permanent middle cerebral artery occlusion[6]. In line with this, body of evidence has shown that activated glia can confer neuronal destruction via secreting of pro-inflammatory cytokines[5,32-35].

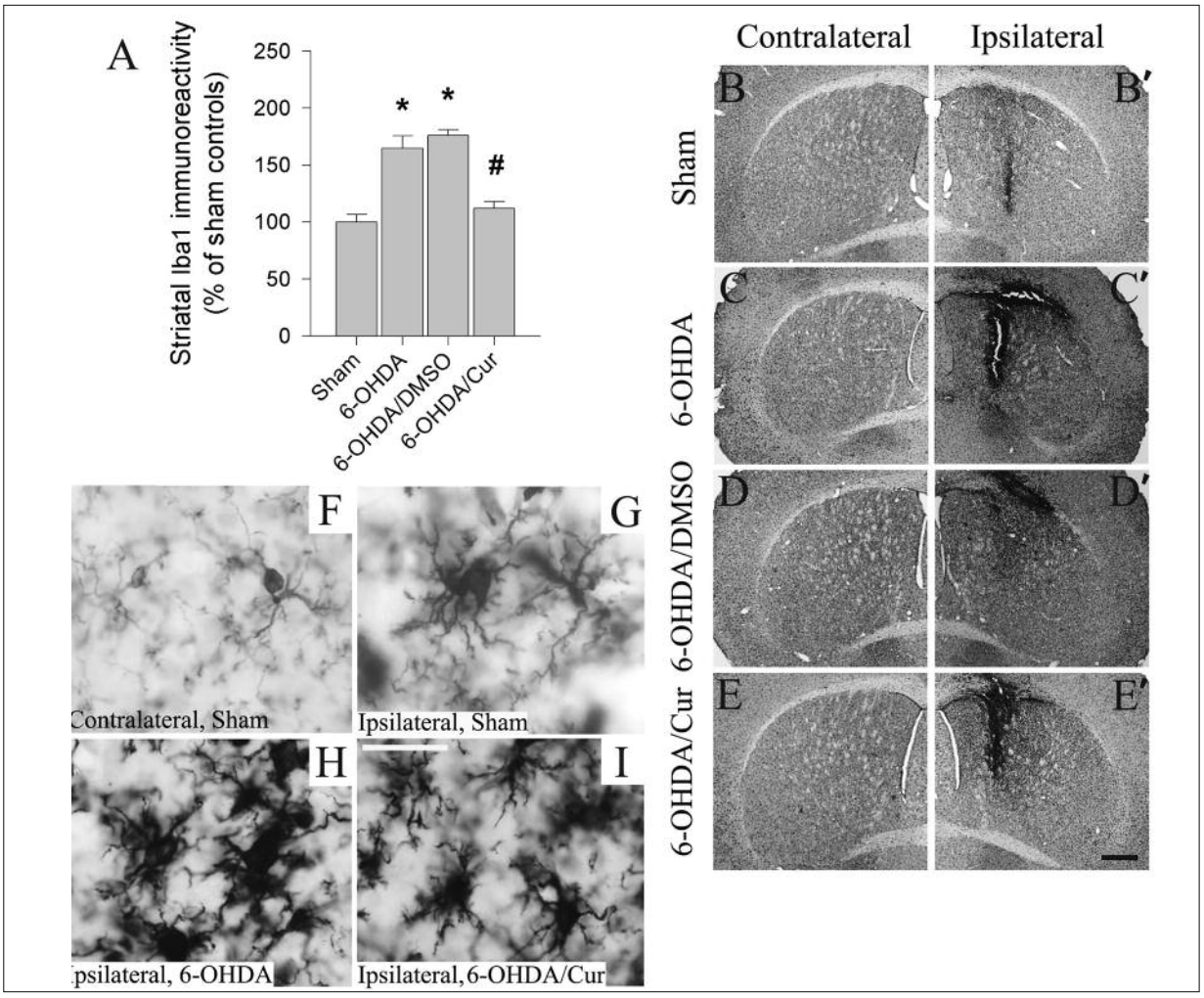

Figure 3. Curcumin diminishes microglial response activated by 6-OHDA. ***(A)*** Histogram illustrating the OD of Iba1 immunoreactivity in the ipsilateral striatum of sham control, 6-OHDA-, 6-OHDA/DMSO- and 6-OHDA/curcumin-injected mice. Data represent mean ± SEM (n = 7). *Indicates significant difference from sham mice, $p < 0.05$. #Indicates significant difference from 6-OHDA- and 6-OHDA/DMSO-treated animals, $p < 0.05$. ***(B-E** and **B′-E′)***: Representative photomicrographs showing Iba1 immunoreactivity in the contralateral striatum ***(B-E)*** and the ipsilateral striatum ***(B′-E′)*** 7 days after unilateral injection of saline or 6-OHDA into the right striatum of sham control ***(B** and **B′)***, 6-OHDA- ***(C** and **C′)***, 6-OHDA/DMSO- ***(D** and **D′)***, and 6-OHDA/curcumin-injected mice ***(E** and **E′)***. ***(F-I)*** High magnification of Iba1-IR microglia in the contralateral striatum of sham controls ***(F)*** and the ipsilateral striatum of sham controls ***(G)***, 6-OHDA mice ***(H)***, and 6-OHDA/curcumin mice ***(I)***. Scale bar in panel ***E′*** represents a length of 500 μm for panels ***B-E*** and ***B′-E′*** and scale bar in panel ***I*** represents a length of 25 μm for panels ***F-I***.

Curcumin reduced GFAP and Iba1 immunoreactivity in the 6-OHDA-injected striatum. This is consistent with previous data showing that curcumin inhibits proliferation of C-6 rat glioma 2B-clone cells, a mixed colony of astroglia and oligodendrocytes[36], impedes astrocytic reactivation[37], and attenuates lipopolysaccharide-induced microglial activation[38]. Also, curcumin deters the production of factors, which are pro-inflammatory and potentially cytotoxic, including tumor necrosis factor alpha, nitric oxide, interleukin-1, and prostaglandin E_2 from primary microglia induced by lipopolysaccharide[38,39]. Hence, inhibition of microglial and astroglial reaction observed in this study could be one of the mechanisms underlying the neuroprotective effects of curcumin on nigrostriatal DA axons against 6-OHDA neurotoxin.

Decreased SOD1 mRNA, SOD1 immunoreactivity, and SOD activity are observed in the ipsilateral striatum following 6-OHDA injection into the medial forebrain bundle or the striatum[40-44] . Since 6-OHDA induces production of both H_2O_2 and superoxide radicals[45], decreasing of striatal

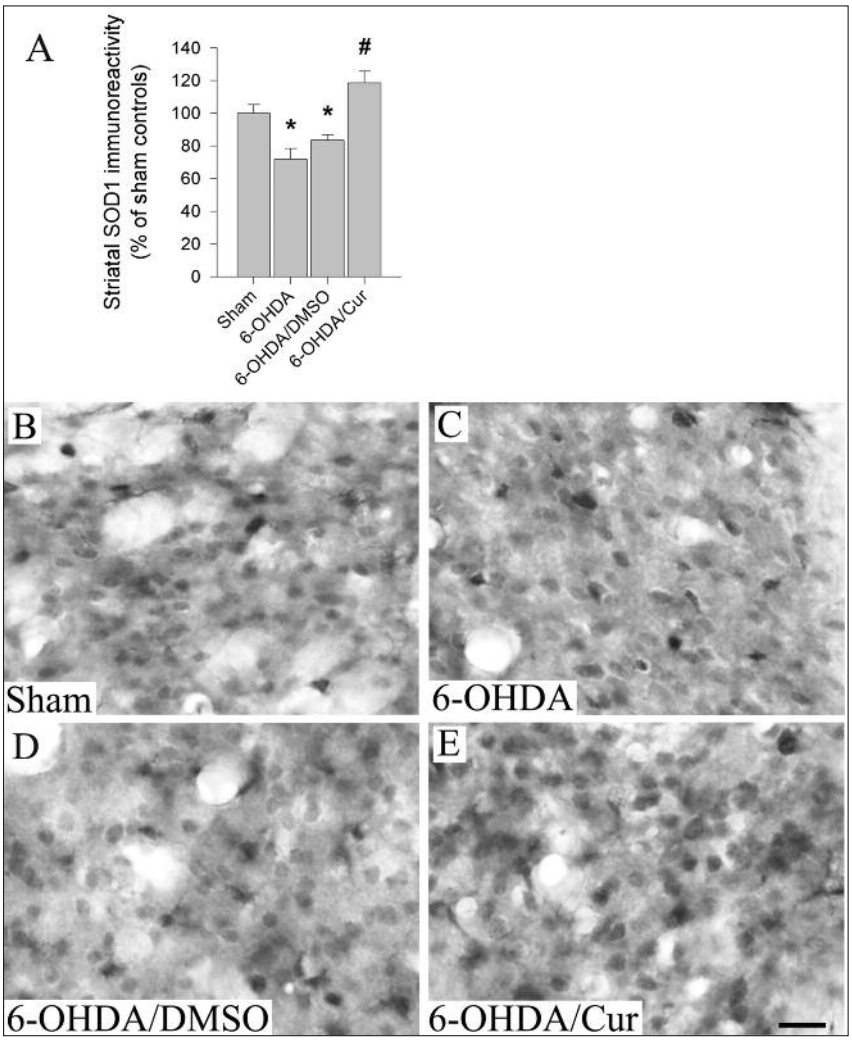

Figure 4. Curcumin maintains SOD1 immunoreactivity in the 6-OHDA-injected striatum. ***A,*** Histogram showing the OD of SOD1 immunoreactivity in the ipsilateral striatum of sham control, 6-OHDA-, 6-OHDA/DMSO- and 6-OHDA/curcumin-injected mice. Data represent mean ± SEM (n = 7). *Indicates significant difference from sham mice, $p < 0.05$. #Indicates significant difference from 6-OHDA- and 6-OHDA/DMSO-treated animals, $p < 0.05$. ***B-E,*** Photomicrographs of SOD1 immunostaining in the ipsilateral striatum 7 days after unilateral injection of saline or 6-OHDA into the right striatum of sham control ***(B)***, 6-OHDA- ***(C)***, 6-OHDA/DMSO- ***(D)***, and 6-OHDA/curcumin-injected mice ***(E)***. Scale bar in panel ***E*** represents a length of 20 μm for panels ***B-E***.

SOD1 and SOD activity can lead to 6-OHDA-provoked degeneration of striatal DA terminals as indicated by decreased levels of dopamine and its metabolites[40], diminished RTI-121-labelled dopamine transporters[14], and reduced TH immunoreactivity (our study) in the ipsilateral striatum of 6-OHDA-lesioned animals. Data from SOD1 transgenic mice lend further support to the neuroprotective effect of SOD1 on striatal DA axons. Overexpression of SOD1 activity attenuates reduction of RTI-121-labelled dopamine transporters and preserves levels of dopamine and its metabolites in the striatum of 6-OHDA-, MPTP-, and paraquat-treated mice[14,15,46].

Antioxidant activity of curcumin is well-documented. The spice significantly reduces level of oxidized proteins in several brain regions of Alzheimer transgenic mouse[47], diminishes the oxidative damage in the hippocampus of rats exposed to the parathion[48], decreases the elevated levels of malondialdehyde in an experimental model of subarachnoid hemorrhage[49] and forebrain ischemia[50], and inhibits decline of antioxidant enzymes in experimental models of neuronal injury[41,49,50]. Given the protective effect of SOD1 on striatal DA terminals observed in this investigation, curcumin may protect striatal TH-IR axons against 6-OHDA, at least in part, via preservation of striatal SOD1.

Conclusions

Curcumin protects nigrostriatal DA axons, decreases astrocytic and microglial reactivity, and restores SOD1 content in the 6-OHDA–injected striatum. Inhibition of striatal glial response and SOD1 reduction may, in part, underlie the neuroprotective effects of curcumin in unilaterally-lesioned 6-OHDA mouse model of PD.

Acknowledgements

This study was funded in full by research fund from Faculty of Medicine, Srinakharinwirot University, Thailand.

Conflict of Interest

None.

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