The Protection of EGCG Against 6-OHDA-Induced Oxidative Damage by Regulating PPARγ and Nrf2/HO-1 Signaling

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Qi Xu[1*], Yujie Chen[1*], Dan Chen[2] and Manju B Reddy[2]

[1]School of Public Health, Shanghai University of Traditional Chinese Medicine, Shanghai, China.
[2]Iowa State University, Ames, IA, USA.

ABSTRACT: 6-Hydroxydopamine (6-OHDA) is a classic neurotoxin that has been widely used in Parkinson's disease research. 6-OHDA can increase intracellular reactive oxygen species (ROS) and can cause cell damage, which can be attenuated with (-)-Epigallocatechin-3-gallate (EGCG) treatment. However, the mechanism by which EGCG alters the 6-OHDA toxicity remains unclear; In this study, we found 6-OHDA (25 μM) alone increased intracellular ROS concentration in N27 cells, which was attenuated by pretreating with EGCG (100 μM). We evaluated the intracellular oxidative damage by determining the level of thiobarbituric acid reactive substances (TBARS) and protein carbonyl content. 6-OHDA significantly increased TBARS by 82.7% ($P < .05$) and protein carbonyl content by 47.8 ($P < .05$), compared to the control. Pretreatment of EGCG decreased TBARS and protein carbonyls by 36.4% ($P < .001$) and 27.7% ($P < .05$), respectively, compared to 6-OHDA alone treatment. Antioxidant effect was tested with E2-related factor 2 (Nrf2), heme oxygenase-1(HO-1) and peroxisome-proliferator activator receptor γ (PPARγ) expression. 6-OHDA increased Nrf2 expression by 69.6% ($P < .001$), HO-1 by 173.3% ($P < .001$), and PPARγ by 122.7% ($P < .001$), compared with untreatment. EGCG pretreatment stabilized these alterations induced by 6-OHDA. Our results suggested that the neurotoxicity of 6-OHDA in N27 cells was associated with ROS pathway, whereas pretreatment of EGCG suppressed the ROS generation and deactivated the Nrf2/HO-1 and PPARγ expression.

KEYWORDS: 6-OHDA, EGCG, Nrf2, PPARγ

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CORRESPONDING AUTHOR: Manju B Reddy, Iowa State University, Ames, IA 50010, USA. Email: mbreddy@iastate.edu

Introduction

Parkinson's disease (PD) a progressive neurodegenerative disorder characterized by motor dysfunctions including abnormal involuntary movements, bradykinesia, rigidity and tremor, as well as non-motor symptoms such as cognitive impairment and mood disorders.[1] 6-hydroxydopamine (6-OHDA) is a selective catecholaminergic neurotoxin that can induce damage in the nigrostriatal dopaminergic neurons and has been widely used for in vitro and in vivo experimental models of PD to study neurodegeneration and potential therapeutic approaches.[2] One of the main features of the neurotoxicity of 6-OHDA is associated with its ability to generate reactive oxygen species (ROS).[3] Increased intracellular ROS would disrupt redox homeostasis, causing oxidative stress and resulting into irreversible oxidative modification on DNA, lipids and proteins.[4] The presence of excess intracellular Fe (II) and Fe (III) may exacerbate the ROS generation by producing hydroperoxyl radical (HO·) through the Fenton reaction.[5] Our previous in vitro study showed 6-OHDA induced iron burden in the dopaminergic mesencephalon-derived N27 cell line.[6] Therefore, the excess intracellular iron is associated with ROS generation and PD progression. Another hallmark of PD pathogenesis is microglia mediated neuroinflammation.[7,8] Studies have shown that neuroinflammation can release neurotoxic factors such as tumor necrosis factor (TNFα) and nitric oxide, and accelerate the progression of dopaminergic neurodegeneration.[9,10] The rapid microglia activation after injection of 6-OHDA also indicates the critical role of inflammation in neurotoxin induced PD models.[11]

The transcription factor nuclear factor E2-related factor 2 (Nrf2) is a key transcriptional factor that maintains cellular homeostasis through regulating a battery of antioxidant and cellular protective genes, primarily in response to oxidative stress. Under normal or unstressed conditions, Nrf2 is anchored in the cytoplasm by a cluster of proteins that degrade it quickly. On exposure to oxidative stress, Nrf2 binds to antioxidant response element (ARE) present in the promoters of multitude of antioxidant enzymes and phase II detoxifying genes such as heme oxygenase-1(HO-1), NAD(P)H quinone oxidoreductase-1 and glutathione-S-transferases.[12,13]

Nrf2 also regulates inflammation and promotes resolution via cross talk with nuclear factor kappa B (NF-κB) or reducing lipopolysaccharide (LPS) mediated induction of cyclooxygenase 2, TNFα and interleukin-1 β (IL-1β) expression.[14,15] Recent study shows that Nrf2 interacts with the intracellular anti-inflammatory system, such as the peroxisome-proliferator activator receptor γ (PPARγ) to suppress inflammation reaction and maintain normal redox status.[10,16] Although the detailed mechanisms of the interaction between Nrf2 and PPARγ remain unclear at this time, the relationship between Nrf2 and PPARγ suggests the connection between oxidative stress and inflammation.[17,18]

* These authors contributed equally to this work.

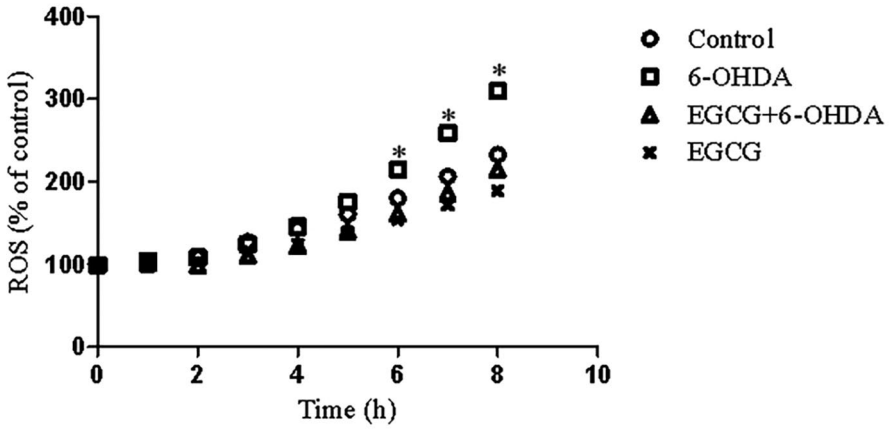

Figure 1. EGCG attenuated 6-OHDA-induced intracellular ROS generation in N27 cells. The fluorescence density showed in the time course of ROS levels in control, 6-OHDA alone, EGCG and 6-OHDA and EGCG alone. The values are expressed as mean ± SEM, n = 3. Pretreatment with 100 μM EGCG for 2 hours, followed by 25 μM 6-OHDA for 8 hours. $P < .001$.

Nrf2 might be a key signaling molecule in the neuroprotective strategies against PD pathogenesis. It is found that Nrf2-mediated transcriptional deletion aggravates vulnerability to 6-OHDA both in vitro and in vivo, and Nrf2-ARE activation provided significant protection against 6-OHDA in vivo,[19,20] Moreover, a greater loss of dopaminergic neurons were found in Nrf2 knockout mice compared to 1-methyl-4-pheynl-1,2,3,6-tetrahydropyridine (MPTP) treated wild-type mice.[21]

Based on the involvement of iron loading and ROS in the damage of neurons, use of iron chelation and antioxidants are important strategies to prevent neurodegeneration. (-)-Epigallocatechin-3-gallate (EGCG) is an antioxidant that can induce the expression of a variety of antioxidant enzymes and alleviate the generation of ROS and electrophilic reagents during the progression of neurodegeneration. Furthermore, EGCG can also relieve the inflammatory reactions in neurodegenerative diseases.[22,23] It has been shown that EGCG treatment can prevent against 6-OHDA induced neurotoxicity in in vitro model of PD.[6,24] However, the mechanism by which EGCG exerts its neuroprotection remains unclear. In this study, we hypothesized that 6-OHDA increases ROS levels, resulting oxidative damage in N27 cells and EGCG treatment reduces the adverse effects by reducing oxidative damage on lipids and proteins through stabilizing the Nrf2 and PPARγ system.

Results

EGCG attenuated ROS generation induced by 6-OHDA

Intracellular ROS concentrations with 6-OHDA and EGCG are shown in Figure 1. Compared with the control (no treatment), 6-OHDA increased intracellular ROS by 19% ($P < .001$) after 6 hours treatment, and by 33.1% ($P < .001$) after 8 hours. EGCG pretreatment for 2 hours prior to 6-OHDA treatment reduced ROS by 24.9% ($P < .001$) at 6 hours, and by 31% ($P < .001$) at 8 hours that was induced by 6-OHDA treatment; and reduced by 10.6% ($P < .05$) at 6 hours, and by 8.1% ($P > .05$) at 8 hours, compared to the control. There was a decrease in ROS (14.3%, $P < .01$) at 6 hours, and (18.7%, $P < .001$) at 8 hours with EGCG alone compared with control group. Overall, EGCG pretreatment attenuated 6-OHDA induced ROS generation.

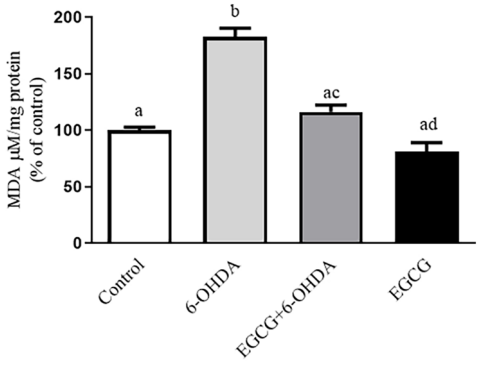

Figure 2. Neuroprotective effect of EGCG against 6-OHDA -induced lipid peroxidation. The values are expressed as mean ± SEM, n = 2 from 3 individual measurements. Means without a common letter significantly differ, $P < .05$.

EGCG counteracted 6-OHDA-induced lipid peroxidation

6-OHDA markedly increased lipid peroxidation, measured as MDA concnetation, by 82.7% ($P < .001$) after 24 hours treatment (Figure 2). Pretreatment of EGCG for 2 hours decreased lipid peroxidation by 36.4% ($P < .001$) when compared to the 6-OHDA treatment. Although EGCG alone had lower (30.3%, $P < .01$) MDA value than 6-OHDA + EGCG group, the values were not significantly different compared to control.

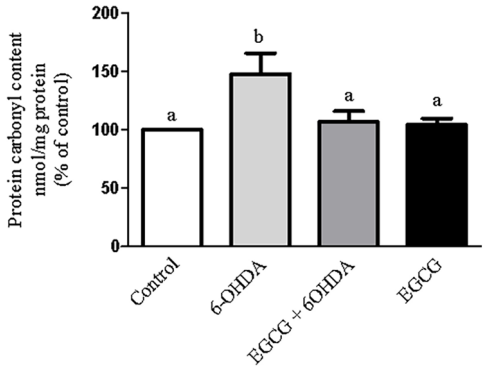

Figure 3. Neuroprotective effect of EGCG against 6-OHDA-induced PC accumulation. Values are mean ± SEM (n = 3). Means without a common letter significantly differ, $P < .05$.

EGCG counteracted 6-OHDA-induced protein carbonyls (PC)

Protein carbonyls were increased by 47.8% ($P < .05$) after 24 hours of 6-OHDA treatment compared with the control (Figure 3). EGCG pretreatment reduced 6-OHDA-induced increase in PC content by 27.7% ($P < .05$). No difference between control and EGCG alone suggests there was no effect of EGCG alone on PC.

EGCG counteracted the increased antioxidant (Nrf2 and HO-1) and anti-inflammatory (PPARγ) system altered by 6-OHDA

6-OHDA increased Nrf2 by 69.6% ($P < .001$), HO-1 by 173.3% ($P < .001$) and PPARγ by 122.7% ($P < .001$), compared to the control (Figure 4). Higher concentration of EGCG (100 uM) pretreatment significantly reduced protein expression of Nrf2 (by 36.7%, $P < .001$), HO-1 (by 49.3%, $P < .001$) and PPARγ (by 41.1%, $P < .01$), altered by 6-OHDA group. Pretreatment of lower concentration of EGCG (10 μM) didn't decrease HO-1 and PPARγ expression, but only significantly decreased Nrf2 (18.9%, $P < .05$), compared to 6-OHDA alone treatment. Therefore, these results suggested high concentration of EGCG exerted antioxidant effect.

Discussion

There are several proposed mechanisms of the 6-OHDA neurotoxicity, including free radicals damage, mitochondrial dysfunction, and oxidative stress.[25,26] 6-OHDA can alter the expression of iron-related proteins, resulting into perturbed iron homeostasis.[27,28] 6-OHDA can also cause iron release from cytosolic ferritin and increase intracellular free iron concentration.[29] Since iron is involved in PD progression due to its pro-oxidant nature, the interruption of iron homeostasis by 6-OHDA could cause neuronal damage, especially in the substantia nigra (SN).[30,31] Our previous study found that 6-OHDA regulated the expression of iron-related proteins, increased cellular iron uptake and retention in N27 cells.[6] Here, we further confirmed the involvement of oxidative stress with 6-OHDA showing the increased intracellular ROS as a result of exposure to 6-OHDA for 6 hours in N27 cells.. Similar to our results, another study using rat pheochromocytoma PC12 cells found that exposure to 100 μM 6-OHDA for 30 minutes could lead to intracellular ROS overproduction[32] Therefore, maintaining normal iron homeostasis and reducing oxidative stress are important strategies to prevent progression of PD.

In addition to oxidative stress, inflammation is also attributed to the PD progression. It had been shown that neuroinflammation was significantly higher in PD brains, including elevated levels of numerous inflammation-related enzymes and cytokines within the substantia nigra, striatum, and cerebral spinal fluid. For example, TNFα and nitric oxide synthase (iNOS) were significantly elevated in PD patients, compared to age-matched controls.[33] One of the proposed mechanisms of 6-OHDA toxicity is its ability to induce inflammation.[34,35] Inflammatory factors, such as TNFα could directly activate cell apoptotic pathway or indirectly suppress the mitochondrial function. Considerable evidence from in vitro and in vivo studies suggests that inflammation is related to ROS generation in PD,[36,37] and increased ROS level could augment the inflammation process,[38,39] thereby exacerbating neuronal damage.

Since oxidative stress links to the progression of neurodegeneration, reducing oxidative stress would be useful neuroprotective strategie to prevent the progression of PD. Nrf2/ARE system is considered as a self-defense antioxidant system and it controls various of genes including glutathione-produced and glutathione-regenerated enzymes n maintaining redox homeostasis.[40] The increased ROS or oxidative stress can trigger Nrf2 expression and Nrf2-associated antioxidant enzymes expression.[40,41] In the present study, we showed that 6-OHDA not only increased ROS level, but also elevated Nrf2 (by ~70%) and HO-1 expression (by ~173%). However, the increased Nrf2/ARE system might not be enough to counteract 6-OHDA induced toxicity, since oxidative damage to lipids and proteins was still high. PPARγ is associated with the anti-inflammatory system to inhibit inflammatory reaction, increase dopamine, alter mitochondrial bioenergetics, reduce oxidative stress and impede PD progression.[42,43] In our study, we found the 6-OHDA also increased PPARγ expression, which may indicate the increased inflammatory activity along with the increased ROS level and impaired mitochonrial biogenesis. Similar to Nrf2, the increased PPARγ could not completely counteract against 6-OHDA induced toxicity, which may indicate the inadequate anti-inflammatory effect from PPARγ. Recent research found that PPARγ may activate PPARγ coactivator 1 alpha (PGC1α) which can further activate Nrf2 leading to mitochondrial biogenesis and maintaining cellular redox homeostasis.[16] Future research is needed to explore the relationship among PPARγ, Nrf2, mitochondrial biogenesis and antioxidant enzymes such as glutathione level in 6-OHDA induced cytotoxicity.

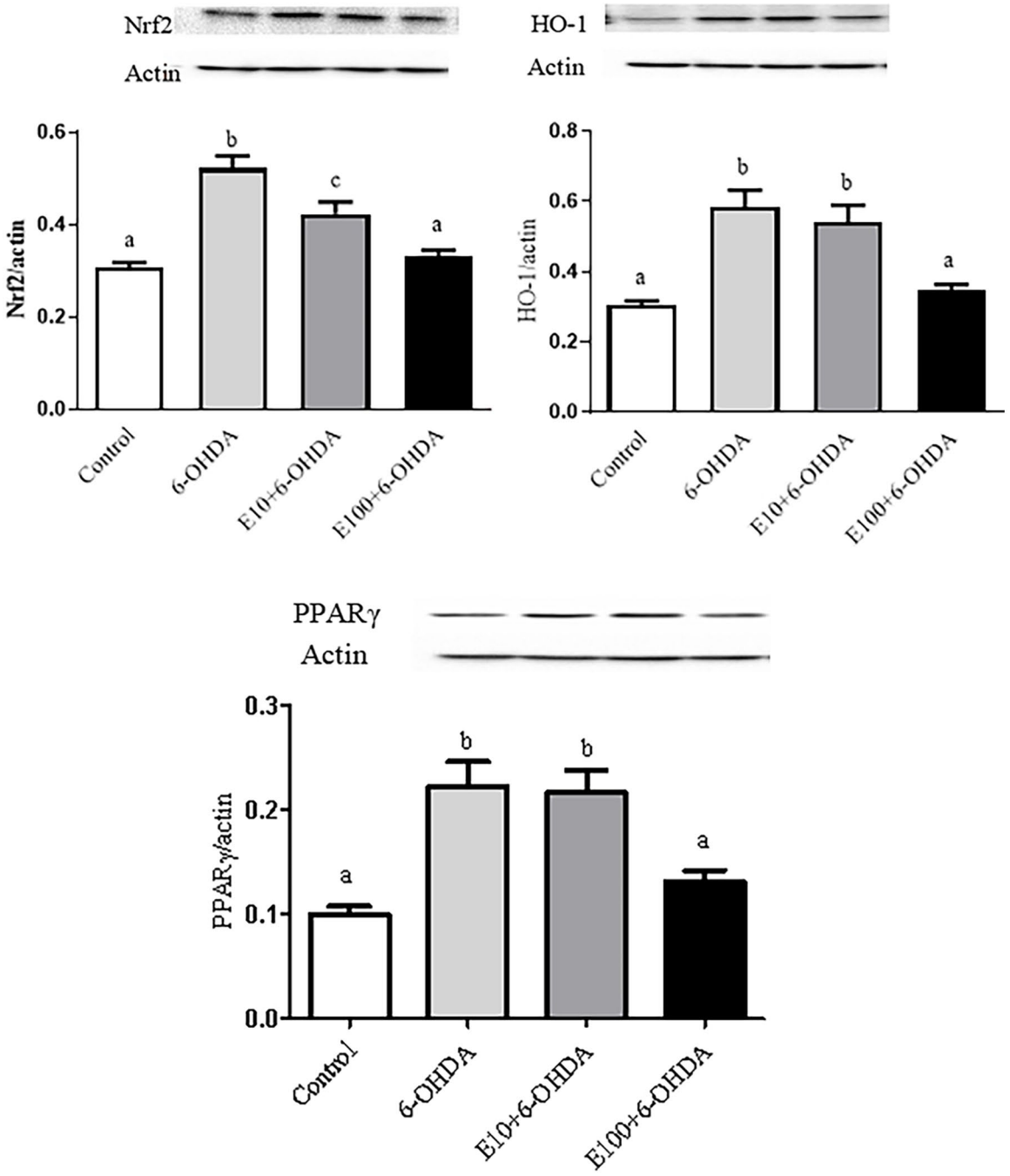

Figure 4. Neuroprotective effect of EGCG (10 and 100 μM) against 6-OHDA -induced Nrf2, HO-1 and PPARγ protein expression. Western blot analysis for Nrf2, HO-1 and PPARγ protein expression (normalized with β-actin in N27 cells), and quantification analysis. Values are mean ± SEM (n = 4-8). Means without a common letter significantly differ, $P < .05$.

EGCG has been considered as an antioxidant to decrease oxidative stress and maintain the redox balance due to its radical scavenging ability. EGCG is also a strong iron chelator and may reduce ROS by binding free iron. Several in vitro and in vivo studies have shown that EGCG treatment can prevent cancer, vascular diseases and neurological disorders[44-46] that are associated with iron dysregulation. In our previous study, EGCG decreased iron uptake and retention in N27 cells and prevented against 6-OHDA induced neurotoxicity.[6] In this study, we further showed that that EGCG reduced intracellular ROS, lipid peroxidation and protein carbonyls, which may be attributed to reduced iron accumulation in N27 cells. In addition, we found that higher concentrations of EGCG (100 μM) counteracted the effects of 6-OHDA on the increased expression of Nrf2, HO-1, and PPARγ. This is inconsistent with the results of other studies showing that EGCG acted as a promoter of antioxidant and anti-inflammatory properties by activating Nrf2 and HO-1 exression.[44,46,47] One reason for this may be that 6-OHDA-induced oxidative stress and inflammatory response could further activate the

protein expression of Nrf2, HO-1, and PPARγ, whereas EGCG treatment restored the cells to a level of low oxidative stress and inflammation. Another reason for the discrepancy of our results might be related to the difference of cell types. We used N27 cells which were much more sensitive to oxidative stress and complicated regulation pathways compared to non-neuronal cells. In addition, PPARγ and Nrf2/HO-1 system were associated with antioxidant system and mitochondrial biogenesis, future study is also needed to investigate whether EGCG reversed 6-OHDA induced neurotoxicity by altering mitochondrial biogenesis and antioxidant enzymes such as glutathione through targeting PPARγ and Nrf2/HO-1

Materials and Methods

Reagents

EGCG, 6-OHDA, Hanks' Balanced Salts (HBSS) used for cell culture were purchased from Sigma (St. Louis, MO). RPMI-1640 medium, penicillin, streptomycin fetal bovine serum, L-glutamine, and CM-H_2DCFDA oxidative indicator probe were obtained from Invitrogen (Carlsbad, CA). Trichloroacetic Acid (TCA) was purchased from Fisher Scientific (Pittsburgh, PA). 1, 1, 3, 3-Tetramethoxypropane, 2-Thiobarbituric Acid (TBA) were purchased from Sigma (St. Louis, MO). Protein carbonyl was assessed by commercial kit from Cayman Chemical Company (Ann Arbor, MI). Protein concentration was determined by Pierce BCA protein assay kit (Rockford, IL). Rabbit anti-rat-Nrf2 polyclonal antibody, rabbit anti-rat-HO-1 polyclonal antibody and rabbit anti-rat-PPARγ antibody, secondary antibody goat- anti-rabbit -IgG-HRP and goat- anti-mouse-IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin mouse-anti-mouse-monoclonal antibody was obtained from Sigma (St. Louis, MO).

Cell culture

N27 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1%P/S, and 2 mmol/L l-glutamine and were placed in a constant temperature incubator containing 5% CO_2 at 37°C for growth as previously described.[48]

Treatments

N27 dopaminergic cells were treated with EGCG (100 μM) for 2 hours, prior to treatment with 6-OHDA (25 μM) for 8 hours to measure intracellular ROS generation or 24 hours to measure lipid proxidation, protein carbonyls and protein expression of Nrf2, HO-1, and PPARγ. The cells without treatment were used as a controls. After treatments, cell lysate were collected for the following assays.

Measurement of intracellular ROS generation

The formation of intracellular ROS were measured by using a fluorescent probe, 2, 7-dichlorofluorescein diacetate as described previously.[49,50] The N27 cells were plated as the density of 2×10^4 cells/well on the 96-well plate. Medium was changed to HBSS before each treatment, and CM-H_2DCFDA was added to a final concentration of 10 μM to each well. The fluorescence intensity of dichlorofluorescein (DCF) was continuously measured using fluorescence spectrophotometer with excitation wavelength of 485 nm and emission wavelength of 535 nm with 30 minutes interval for 8 hours.

TBARS assay

The TBARS assay was used to determine the lipid peroxidation and performed fluorometrically with a modified protocol[51] and measured as MDA. Briefly, cell lysates (200 μl) from different treatments were combined with ice cold 20% TCA (200 μl), and then incubated on ice for 5 minutes. After centrifuging, 200 μl supernatant was mixed with 200 μl 0.67% (w/v) TBA reagent, and incubated at 95°C for 60 minutes. Then 400 μl butanol/pyridine (15:1) was added to each sample. The fluorescence intensity of MDA in the upper butanol/pyridine phase was quantified with an excitation wavelength of 520 nm and emission wavelength of 550 nm on a black 96-well plate. 1, 1, 3, 3-Tetramethoxypropane was used as a standard to calculate the final sample concentration.

Protein carbonyl (PC) assay

The protein oxidation in the cell lysate samples were determined by measuring the PC with the DNPH (2,4-dinitrophenylhydrazine). According to the protocol provided by Cayman's protein carbonyl assay kit (Ann Arbor, MI), the amount of protein-hydrozone product was quantified spectrophotometrically at wavelength of 363 nm on using a microplate reader. Total protein content of cell lysates was determined by Pierce BCA protein assay kit (Rockford, IL). The PC concentrations was calculated and normalized to the total cell protein content.

Western blot analyses

After treatments described above, N27 cells were rinsed with PBS and lysed with RIPA buffer supplemented with protease and phosphatase inhibitors. After centrifuging, aliquots of supernatants were used to measure the protein concentrations. Protein samples were loaded onto a 12% or 15% SDS-polyacrylamide gel. The blot was later transferred onto the nitrocellulose membrane (Bio-Rad). After blocking, the proteins on the membrane were incubated with rabbit anti-rat antibodies against Nrf2, HO-1, and PPARγ (1:500) separately (4°C, 12 hours). To ensure even loading of the protein samples, β-actin (1:1000) was used as an internal control, with the incubation of the corresponding goat-anti-mouse-IgG-HRP secondary antibody (1:5000). All protein blotting assays were repeated 3 times.

Statistical analyses

One-way ANOVA was used to detect differences between groups by Tukey's multiple comparisons test. All values were normalized according to their respective controls. Data analysis was performed using Prism 4.0 software (Graphics Software, San Diego, CA). All values were expressed as mean ± SEM as percentage of the controls. Differences were considered significant at $P < .05$.

Conclusions

In conclusion, our in vitro data suggested that the neurotoxicity of 6-OHDA was closely related to ROS, oxidative stress and inflammation. Pretreatment of EGCG protected against 6-OHDA toxicity by inhibiting the ROS, consequently inactivating Nrf2, HO-1, and PPARγ expression. To our knowledge, this is the first in vitro study of EGCG on Nrf2 regulation to prevent 6-OHDA induced neurotoxicity. Further studies in vivo are needed to clarify the antioxidant and anti-inflammatory protective mechanisms of EGCG in PD.

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Author Contributions

Conceptualization, M.B.R.; methodology, D.C. and Y.C.; validation, Q.X.; formal analysis, D.C. and Y.C.; investigation, M.B.R.; writing—original draft preparation, Q.X. and D.C.; writing—review and editing, M.B.R.; supervision, M.B.R.; funding acquisition, M.B.R.; All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Data Availability Statement

All data used to support the findings of this study are included within the article and they are also available from the corresponding author upon request.

Sample Availability

Not applicable.

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