OXIDATIVE STRESS: HOW HAS IT BEEN CONSIDERED IN THE DESIGN OF NEW DRUG CANDIDATES FOR NEURODEGENERATIVE DISEASES?

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EDITORIAL
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Editorial: Oxidative Stress: How Has It Been Considered in the Design of New Drug Candidates for Neurodegenerative Diseases?

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Editorial on the Research Topic

Oxidative Stress: How Has It Been Considered in the Design of New Drug Candidates for Neurodegenerative Diseases?

Central Nervous System (CNS) disorders affect millions of people worldwide (Feigin et al., 2019). Despite their growing impact on health systems and the urgent need for new, safer, and more effective drugs, the Pharmaceutical Industries have avoided efforts to develop new candidates due to high costs and low success rates in the clinical phase (Seyhan, 2019), mainly because of the insufficient knowledge of the mechanisms underlying CNS dysfunctions (Gribkoff and Kaczmarek, 2017).

In this scenario, neurodegenerative diseases (NDs), i.e., those that result from loss of function and death of nerve cells in the brain or peripheral nervous system, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS), deserve to be highlighted due to their increasing prevalence, most of the time dependent on age, and the absence of treatments capable of controlling and reversing the processes associated with their pathogenesis (Martin, 1999). Therefore, the need for a better understanding of the complex mechanisms associated with the genesis of NDs and the development of new therapeutically effective approaches become essential (Golde, 2009; Santiago et al., 2017).

Although the clinical and neuropathological aspects of these disorders are distinct and most have the formation of abnormal protein deposits as a crucial factor associated with their onset, all have a common and characteristic pattern of neuronal degeneration in anatomically or functionally related regions (Vadakkan, 2016). Moreover, oxidative stress (OS) is considered as a common key player in the etiology and progression of these NDs and, for this reason, it could be observed a significant increase of interest in searching antioxidant and also anti-inflammatory effects of diverse classes of natural and synthetic compounds as promising drug candidates for the treatment of NDs (Chen et al., 2012).

Abundant literature data suggest that OS may induce not only cellular and membrane damage, but also DNA repair system breakdown or mitochondrial disfunction, contributing to a complex network of events related to energy supply, neurodegeneration and aging, a phenomena observed in AD or PD, for example (Schieber and Chandel, 2014). Increased reactive oxygen species (ROS) levels is a prompt consequence of OS and it is usually related to down-regulation in several defense systems including antioxidant enzymes or endogenous small-molecule antioxidants (Sayre et al., 2001). In

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this context, several hypotheses have been recently proposed to explain the complexity and multifactorial pathogenesis of AD, including OS as not only a central player, but, perhaps, one of the main causative factors of NDs, unifying a series of other sequential or individual pathophysiological events (Singh et al., 2019). According to this consensus, oxidative damage in the brain of patients is resultant from excessive production of free radicals induced by fragments of insoluble and/or overproduced proteins, such as β-amyloid peptide, α-synuclein, tau and huntingtin, with functional alteration in the mitochondria, inadequate energy supply, production of inflammatory mediators and alteration of antioxidant defenses (Islam, 2016; Liu et al., 2017). Thus, the modulation of the cellular oxidative process should lead to a new concept in the design of drugs and, possibly, a new way of searching for more effective disease-modifying chemical entities, reinforcing the hope of at least real clinical relief, if healing is not yet possible (Ghosh et al., 2011; Rekatsina et al., 2020).

The collection of research articles and reviews that composed this Research Topic clearly demonstrate some advances to better understand the role of OS in the mechanisms associated with the genesis of NDs and the ability of some natural products to modulate these phenomena. In this context, the paper by Huang et al. (2020) describes the neuroprotective effects of α-cyperone, a terpene isolated from *Cyperus rotundus*, against hydrogen peroxide (H_2O_2)-induced OS and apoptosis in dopaminergic neuronal SH-SY5Y cells, used as models to study PD. Their findings indicated that α-cyperone, which is well-known to inhibit neuroinflammation, was able to decrease H_2O_2-induced death and production of ROS in SH-SY5Y cells, through a mechanism dependent on the activation of nuclear factor erythroid 2-related factor 2 (Nrf-2), which is a key transcription factor for the regulation of antioxidant proteins expression. This behavior makes this class of natural products attractive in the search for newer therapies for treatment of PD.

Mhillaj et al. (2019) described in their review the effects of the natural antioxidant products ferulic acid, resveratrol and *Ginkgo biloba* extracts on heme oxygenase/biliverdin reductase system, which could be responsible for producing OS in neurons under unbalanced redox conditions. Despite countless preclinical studies with these herbal products demonstrating their neuroprotective effects, the compiled results were not sufficiently indicative of the efficacy of these natural products for NDs treatment, mainly because most of them showed poor bioavailability in humans. On the contrary, the results highlight the importance of taking care with the use of these herbal-derived products due to their ability to promote drug interactions resulting from their inhibitory or up-regulatory actions on different CYP isoforms.

On the other hand, the paper of Zhou et al. (2019) described the anti-inflammatory effects of cryptotanshinone, a diterpene extracted from *Salvia miltiorrhiza*, in neuroinflammation models induced by lipopolysaccharide in BV-2 microglial cells. By modulating Nrf2/heme-oxygenase 1 signaling pathway cryptotanshinone attenuates the upregulated expression of several proinflammatory proteins, such as inducible nitric oxide synthase, cyclooxygenase 2, NOD-like receptor pyrin domain-containing-3 (NLRP3), and it also reduces the increased release of pro-inflammatory cytokines, such as interleukin-1β, interleukin-6 and tumor necrosis factor-α. These results were important to elucidate the mechanisms associated with the anti-inflammatory effects of cryptotanshinone, opening new horizons for the possibility of research and development of new analogues for the therapy of NDs related with microglial cell activation.

Moreover, the actions promoted by the commonly found natural product luteolin on Nrf-2 translocation to the nucleus and the improvement of the expression of antioxidant proteins were the subject of the paper by Tan et al. (2020). Their findings indicated the beneficial neuroprotective effects of this flavonoid in intracerebral hemorrhage induced secondary brain injury, through the enhancement of autophagy and anti-oxidative processes dependent on activation of the p62-Keap1-Nrf2 pathway.

Some endogenous thiols, such as the redox couples glutathione/glutathione disulfide, cysteine/cystine and thioredoxin-reduced/thioredoxin-oxidized, are well known as markers of OS because of their antioxidant and cellular protective roles. Therefore, the paper by Paul and Snyder reviewed the role of the cysteamine, an aminothiol resultant from decarboxylation of amino acid cysteine, and its oxidized disulfide form cystamine in the progression of NDs through the modulation of different multiple targets, such as those from brain-derived neurotrophic factors and Nrf2 signaling pathways. The authors reported many evidences of the participation of cysteamine/cystamine and/or their metabolites in the reduction of OS and, consequently, promoting upregulation of cytoprotective effects in HD and other NDs.

The search for new small molecule bioactive compounds able to modulate receptors stimulated by lipid mediator sphingosine-1-phosphate (S1P) represents a great opportunity to discover drug candidates for NDs. One example is the agonist S1P receptor fingolimod, approved by FDA in 2010 as the first oral treatment for multiple sclerosis, but that also demonstrated ability to promote neuroprotection in animal models of PD. Stimulated by these previous results, Pépin et al. (2020) investigated the profile of compound SEW2871, a selective agonist of S1P receptors in animal models of PD. The obtained results clearly indicated that SEW2871 presented a neuroprotective activity similar to that displayed by fingolimod in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of PD, being a promising candidate for treatment of this ND.

Last, but not least, the paper by Cassano et al. (2020) reviewed the therapeutic effects of cannabidiol, a non-psychoactive component of *Cannabis sativa*, highlighting not only its profile as a cannabinoid receptors antagonist, but also as a modulator of multiple signaling pathways and receptors, such as the *N*-methyl-D-aspartate. Thus, its ability to stimulate the production of important anti-inflammatory and antioxidant endogenous molecules makes this natural product an important drug candidate for treatment of different NDs.

For our concluding remarks, the contributions of this Research Topic indicated that, despite the morphological and biochemical differences among NDs, OS is recognized as a common component of these pathological conditions. In addition, many natural products belonging to different chemical classes and synthetic derivatives have been shown to

be able to modulate OS by controlling ROS levels directly or indirectly by increasing the expression of antioxidant proteins, being important therapeutic alternatives for the treatment of NDs, possibly in combination with other therapies.

AUTHOR CONTRIBUTIONS

CF, MS, AT, and CV discussed and revised the contents of the paper. CF drafted the manuscript.

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ORIGINAL RESEARCH
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Cryptotanshinone Attenuates Inflammatory Response of Microglial Cells via the Nrf2/HO-1 Pathway

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Cryptotanshinone (CTN), a monomer compound extracted from the dried roots and rhizomes of *Salvia miltiorrhiza* Bge, has a variety of pharmacological effects. However, little research has been done on the mechanism of CTN in attenuating neuroinflammation. The present study aimed to investigate whether CTN can ameliorate neuroinflammation induced by lipopolysaccharide (LPS) through the Nrf2/heme-oxygenase 1 (HO-1) signaling pathway in BV-2 microglial cells. We found that CTN attenuated the upregulated expression of inducible nitric oxide synthase, cyclooxygenase 2, NOD-like receptor pyrin domains-3, and nitric oxide induced by LPS in microglial cells. In addition, it curtailed the increased release of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α in LPS-activated microglial cells. Furthermore, CTN significantly increased the levels of NF-κB, Nrf2, HO-1, and Akt proteins. We demonstrated that the anti-inflammatory action of CTN in BV-2 microglial cells was partially through the activation of the Nrf2/HO-1 pathway, which was regulated by the PI3K/Akt signaling pathway. Taken together, our results indicated that CTN downregulated the production and release of proinflammatory mediators in BV-2 microglial cells through activating the Nrf2/HO-1 pathway and subsequently protected neurons from inflammatory injury.

Keywords: cryptotanshinone, neuroinflammation, microglial, Nrf2, HO-1, PI3K

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INTRODUCTION

Neuroinflammation and oxidative stress play an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's disease (PD). Neuroinflammatory response leads to production of proinflammatory and anti-inflammatory mediators. Regulating the balance between these two types of mediators may avoid or delay the onset of such diseases (Fullerton and Gilroy, 2016; Fülöp et al., 2019). Microglial cells, activated by lipopolysaccharide (LPS), are commonly used for *in vitro* studies on neuroinflammation-related diseases (Velagapudi et al., 2014; Nam et al., 2018). When activated, microglial cells increase the expression of nuclear factor-kappaB (NF-κB) to induce the activity of proinflammatory proteases such as inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2). As a result, proinflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 were released. Overproduction of these proinflammatory mediators results in neurodegeneration or even

neuronal death, accelerating the onset and progression of neurodegenerative diseases (Block et al., 2007). Thus, regulating the activated microglial cells may be a potential therapeutic target for neuroinflammation-related neurodegenerative diseases.

Under the normal condition, multiple anti-oxidative systems counteract oxidative stress and inflammation. NF-E2 p45-related factor 2 (Nrf2, coded by NFE2L2), a member of the human Cap "n" Collar (CNC) basic leucine zipper transcription factor family (Cuadrado et al., 2019) and discovered in 1994, is the key player in defending cells from multiple stress-related injuries. Under the physiological condition, Nrf-2 binds to Kelch-like ECH-associated protein 1 (Keap1) and is degraded continuously. However, under stress conditions, it dissociates from Keap-1 and translocates to the nucleus (Loboda et al., 2016), increasing the gene expression of anti-inflammatory mediators. Apart from this, Nrf-2 induces the expression of heme-oxygenase 1 (HO-1) (Na and Surh, 2014). HO-1, a type of heat shock protein, has the anti-oxidative and anti-inflammatory potential. It has been proven that increasing the activity of HO-1 can ameliorate inflammatory response and inhibiting its activity leads to aggravated inflammatory injury (Wu et al., 2014; Yin et al., 2015). Therefore, the Nrf-2/HO-1 signaling pathway is an important therapeutic target for effectively managing neuroinflammation-related neurodegenerative diseases.

Cryptotanshinone (CTN), a monomer compound extracted from the dried roots and rhizomes of *Salvia miltiorrhiza* Bge, exhibits anti-oxidative (Wang W. et al., 2018) and anti-inflammatory effects (Feng et al., 2017). It inhibits the activation of NF-κB induced by LPS in macrophages (Tang et al., 2011) as well as the activity of COX2. In addition, it decreases the expression of endothelin-1, leading to ameliorated inflammatory response (Jin et al., 2006). In Caco-2 cells, CTN exerts anti-inflammatory effects through the toll-like receptor 4 (TLR4)/NF-κB pathway (Cao et al., 2018). It has been shown that CTN attenuates the increased production of NO induced by LPS in BV-2 microglial cells (Lee et al., 2006). Little research has been done on the underlying mechanisms other than this. Therefore, the present study aimed to investigate whether CTN can inhibit inflammatory response of BV-2 microglial cells induced by LPS through the Nrf-2/HO-1 signaling pathway.

MATERIALS AND METHODS

Materials

Cryptotanshinone (C5624) and LPS (L2880) were purchased from Sigma–Aldrich (St. Louis, MO, United States). LY294002 (HY-10108) was purchased from MedChemExpress (Shanghai, China). LY294002 is a PI3K/Akt pathway inhibitor CTN and LY294002 were dissolved in dimethyl sulfoxide to a stock concentration of 10 mM/l and LPS was dissolved in the phosphate buffer saline (PBS) to a stock concentration of 1 mg/ml. All of them were frozen in a −20°C freezer before use. Nrf2 siRNA, HO-1 siRNA, and the control siRNA were purchased from GenePharma (Shanghai, China).

Cells Culture

Murine BV-2 microglial cells were obtained from Institute of Neurology, Ruijin Hospital (Shanghai, China) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Logan, UT, United States) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, United States). 100 U/ml penicillin and 100 μg/ml streptomycin were added into the culture medium in order to prevent bacterial infection. Cells were maintained in a 37°C incubator supplied with 5% CO_2 and 95% air.

Intracellular Lactate Dehydrogenase (LDH) Assay

The intracellular LDH assay was conducted as previously described (Ma et al., 2014) in order to test the viability of cells. Briefly, cells were lysed in a lysis buffer containing 0.04% Triton X-100, 2 mM HEPES, 0.01% bovine serum albumin (pH 7.5) for 20 min after washing with PBS. 40 μl cell lysate was mixed with 200 μl potassium phosphate buffer (500 mM, pH 7.5) containing 0.3 mM NADH and 2.5 mM sodium pyruvate before reading the absorbance at 340 nm on a microplate photometer.

Extracellular LDH Assay

The extracellular LDH assay was conducted as previously described (Ma et al., 2014) to determine the level of cell death. Briefly, 50 μl cell culture supernatant was mixed with 160 μl potassium phosphate buffer (500 mM, pH 7.5) containing 0.3 mM β-Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH) and 2.5 mM sodium pyruvate, followed by reading the absorbance at 340 nm using a microplate photometer.

Morphological Analysis of BV-2 Microglial Cells

BV-2 microglial cells were pretreated with CTN for 1 h, followed by being stimulated with LPS for 18 h. The control group was treated with the phosphate buffer for 19 h. Then the cell morphology was observed using a light microscope.

Nitric Oxide Assay

The level of NO was determined using a NO Test Kit (Beyotime, Jiangsu, China) by following the manufacturer's instructions. Equal volumes of cell culture medium, Griess reagent I, and Griess reagent II were mixed before reading the absorbance at 540 nm using a microplate photometer. Concentrations of nitrite in the samples were calculated by normalizing them to concentrations of the standards and concentrations of proteins were tested using the bicinchoninic acid (BCA) assay.

Real-Time PCR Assay

Total RNA of BV-2 microglial cells was extracted with a total RNA extraction kit (Bioteck Pharma, Beijing, China) by following the manufacturer's instructions after washing cells with PBS. Concentrations and purity of total RNA were measured using a spectrophotometer at 260 and 280 nm. One microgram of total RNA was reverse transcribed into cDNA using a Prime-Script RT kit (Takara Bio, Dalian, China). cDNA was then mixed with

reagents of a SYBR Premix Ex Taq kit (Takara Bio, Dalian, China) before running real-time PCR on an ABI 7900HT machine. The sequences of primers were:

IL-1β (sense 5′-AAGGGCTGCTTCCAAACCTTTGAC-3′, anti-sense 5′-ATACTGCCTGCCTGAAGCTCTTGT-3′);
IL-6 (sense 5′-TCCATCCAGTTGCCTTCTTG-3′, anti-sense 5′-AAGCCTCCGACTTGTGAAGTG-3′);
TNF-α (sense 5′-CCCTCACACTCAGATCATCTTCT-3′, anti-sense 5′-GCTACGACGTGGGCTACAG-3′);
GAPDH (sense 5′-CCTGCACCACCAACTGCTTA-3′, anti-sense 5′-GGCCATCCACAGTCTTCTGA-3′).

Data were analyzed by comparing the threshold cycles (Ct) of target genes which were normalized to their corresponding Ct of GAPDH.

Western Blot

Cells were washed with PBS and lysed in the radio immunoprecipitation assay (RIPA) buffer (Millipore, Temecula, CA, United States) containing 1% Complete Protease Inhibitor (CWBIO, Shanghai, China) and 1 mM phenylmethanesulfonyl fluoride (PMSF). The lysates were centrifuged at 15,000 rpm for 10 min at 4°C before testing their concentrations of proteins using the BCA assay kit (Thermo Scientific, Waltham, MA, United States). Thirty micrograms of total protein was separated using SDS–PAGE (10% gel) and the proteins were transferred to a nitrocellulose membrane (0.45 μm, Millipore, Temecula, CA, United States). After blocking with 5% skimmed milk for 1 h, the membrane was sequentially incubated in the primary and the corresponding HRP conjugated secondary antibody solutions (1:4000, Epitomics, Zhejiang, China) for overnight at 4°C and 1 h at room temperature, respectively. Finally, target proteins were visualized using the ECL detection reagents and photographs were taken. The primary antibodies were: Akt (1:1000, Cell Signaling Technology, Danvers, MA, United States), phospho-Akt (1:1000, Cell Signaling Technology, Danvers, MA, United States), Nrf2 (1:1000, Cell Signaling Technology, Danvers, MA, United States), NOD-like receptor pyrin domains-3 (NLRP3) (1:1000, Cell Signaling Technology, Danvers, MA, United States), iNOS (1:1000, Abcam), COX2 (1:1000, Abcam), tubulin (1:2000, Abcam), Lamin A/C (1:5000, Abcam), NF-κB p65 (1:20,000, Abcam), and HO-1(1:5000, Abcam).

Cytoplasmic and Nuclear Protein Extraction

Cells were washed with PBS and centrifuged at 15,000 rpm for 10 min at 4°C. Nuclear and cytoplasmic proteins were extracted separately using a commercial kit (Beyotime, Jiangsu, China) by following the manufacturer's instructions. Concentrations of cytoplasmic and nuclear proteins were quantified using the BCA assay kit.

Immunofluorescence Assay

BV-2 microglial cells were seeded onto sterile glass coverslips in 24-well culture plates. After attaching to the coverslips, cells were pretreated with CTN (5 and 10 μM) for 1 h prior to incubation with LPS (1 μg/ml) for 30 min, 1 h, or 18 h. Cells were fixed with 4% paraformaldehyde for 20 min and blocked with 10% normal goat serum in PBS for 30 min at room temperature after being permeabilized with 0.2% Triton X-100 for 20 min at room temperature. Cells were sequentially incubated in the primary antibody (NF-κB 1:100, Nrf2 1:200, HO-1 1:250, overnight at 4°C) and the appropriate fluorophore-conjugated secondary antibody solutions (Alexa Fluor 488 conjugated, 1:500, Invitrogen, Carlsbad, CA, United States, 1 h at room temperature) before being counterstained with DAPI (1:300, Beyotime, Jiangsu, China). Finally, the cells were washed with PBS and coverslipped with a fluorescent mounting medium containing the anti-fading reagent. Images were then captured using a confocal laser scanning microscope (Leica TCS SP5 II, Germany).

Nrf2 and HO-1 siRNA Transient Transfections

BV-2 microglial cells were cultured in 24-well culture plates. When cell confluence reached 40–60%, cells were transfected with Nrf2 siRNA, HO-1 siRNA, and control siRNA, respectively, using the siRNA transfection reagent Lipofectamine 3000 (Invitrogen, Carlsbad, CA, United States) by following the manufacturer's instructions. Six hours later, the transfected cells were stimulated with LPS as described above after pretreatment with or without CTN for 1 h, respectively.

Statistical Analyses

All experiments were repeated at least three times. Data were expressed as mean ± SEM. Data were analyzed using one-way ANOVA followed by Student–Newman–Keuls *post hoc* test. It was considered statistically significant when p-value was <0.05.

RESULTS

Effect of CTN on the Viability of BV-2 Microglial Cells

In the present study, different concentrations of CTN (**Supplementary Figure S1A**) were tested using the intracellular or extracellular LDH assay in order to find the optimal concentration of CTN. BV-2 microglial cells were pretreated with CTN for 1 h, followed by being stimulated with LPS for 24 h with CTN being present or absent. It was found that CTN in the range of 1–10 μM was innocuous to BV-2 microglial cells, but toxic at the concentration of 20 μM or over (**Supplementary Figure S1B**). Therefore, 5 and 10 μM were used in the following experiments.

Effect of CTN on the Morphology of LPS-Activated BV-2 Microglial Cells

Morphological analysis was performed to assess changes of BV-2 microglial cells before and after drug treatment. Microglial cells in the normal condition were round in shape with few branches, whereas microglial cells stimulated with LPS had significantly larger and longer cell body areas with longer perimeters. When BV2 microglial cells were pretreated with CTN for 1 h and then

treated with LPS for 18 h, the perimeter of BV2 microglia was significantly shortened compared with that of microglial cells stimulated with LPS, but the area did not significantly decrease (**Supplementary Figure S2**).

Influence of CTN on LPS-Induced NO Production by BV-2 Microglial Cells

To determine the influence of CTN on LPS-induced NO production, BV2 microglial cells were pretreated with CTN for 1 h and then co-treated with LPS for 24 h. It was found that LPS significantly increased the level of NO, which was attenuated by CTN in a dose-dependent manner (**Figure 1**).

Effect of CTN on LPS-Induced Expression of iNOS and COX2 in BV-2 Microglial Cells

Inducible nitric oxide synthase and COX2 are two pro-inflammatory proteases produced by activated microglial cells and they play an important role in neuroinflammation as inflammatory mediators. To examine the effect of CTN on LPS-induced expression of iNOS and COX2, BV-2 microglial cells were pretreated with CTN (5 and 10 μM) for 1 h and then co-treated with LPS for 18 h. Western blot results showed that LPS significantly increased the levels of iNOS (**Figure 2A**) and COX2 (**Figure 2B**) in BV-2 microglial cells, which were significantly downregulated by CTN in a dose-dependent manner.

Effect of CTN on LPS-Induced Expression of IL-1β, IL-6, and TNF-α in BV-2 Microglial Cells

Cytokines such as IL-1β, IL-6, and TNF-α play an important role in inflammation. To investigate whether CTN can prevent or delay the onset of neurodegenerative diseases as well as their progression by decreasing the production and release of inflammatory cytokines, RT-PCR was performed to test the levels of genes of inflammatory cytokines. It was observed that levels of IL-1β, IL-6, and TNF-α mRNA were significantly increased by LPS. CTN significantly downregulated levels of IL-1β (**Figure 3A**), IL-6 (**Figure 3B**), and TNF-α (**Figure 3C**) in BV-2 microglial cells induced by LPS in a dose-dependent manner.

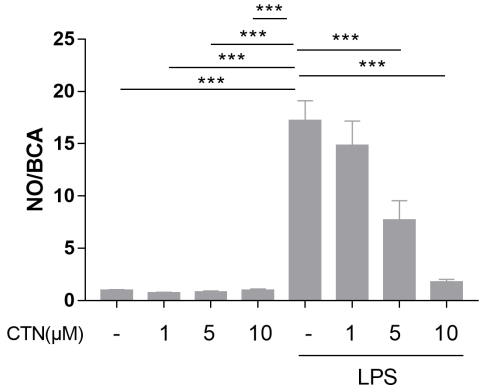

FIGURE 1 | Effect of CTN on LPS-induced NO production in BV-2 microglial cells. BV-2 microglial cells were pretreated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 24 h. CTN at concentrations of 1, 5, and 10 μM did not influence the amount of NO produced by BV-2 microglial cells. LPS significantly increased the amount of NO, which was significantly attenuated by CTN in a dose-dependent manner. Data were presented as mean ± SEM. All experiments were repeated at least three times. ***$P < 0.001$.

Effects of CTN on LPS-Induced Activation of NF-κB in BV-2 Microglial Cells

It is well known that induction of many proinflammatory cytokines is primarily relying on the activation of NF-κB. In the present study, the level of NF-κB in BV-2 microglial cells was tested after pretreating these cells with CTN for 1 h and then co-treating with LPS for 30 min. Western blot showed that LPS significantly increased the level of NF-κB, indicating that LPS induced the activation of NF-κB. CTN significantly downregulated the level of NF-κB, suggesting an inhibitory effect on the activity of NF-κB (**Figure 4A**). Immunofluorescence staining further confirmed the result from Western blot (**Figure 4B**).

Effect of CTN on LPS-Induced Expression of Nrf2 and HO-1 in BV-2 Microglial Cells

To investigate the underlying anti-inflammatory mechanism of CTN, we further examined the effect of CTN on LPS-induced levels of Nrf2 and HO-1 proteins. Western blot showed that CTN significantly increased the levels of Nrf2 (**Figure 5B**) and HO-1 in a dose-dependent manner (**Figure 5A**), LPS did not significantly increase the expression of Nrf2 and HO-1, but augmented the increase induced by 5 or 10 μM CTN. This was confirmed by immunofluorescence analysis (**Figures 5C,D**).

The Anti-inflammatory Effect of CTN Is Dependent on Nrf2

To determine whether the anti-inflammatory effect of CTN is dependent on the activation of Nrf2, the level of Nrf2 was decreased by knocking down Nrf2 using Nrf2 siRNA. Western blot showed that Nrf2 siRNA significantly inhibited the expression of Nrf2 (**Figure 6A**). The NO assay showed that Nrf2 siRNA significantly reversed the inhibitory effect of CTN on NO production (**Figure 6B**). Similarly, the level of IL-1β mRNA (**Figure 6E**) and levels of iNOS (**Figure 6C**) and COX2 (**Figure 6D**) were reversed by Nrf2 siRNA. However, levels of IL6 (**Figure 6F**) and TNF-α mRNA (**Figure 6G**) were not significantly reversed by Nrf2 siRNA. Taken together, the anti-inflammatory effect of CTN was partially dependent on the activation of Nrf2.

The Anti-inflammatory Effect of CTN Is Dependent on HO-1

To determine whether the anti-inflammatory effect of CTN is dependent on the activation of HO-1, the level of HO-1

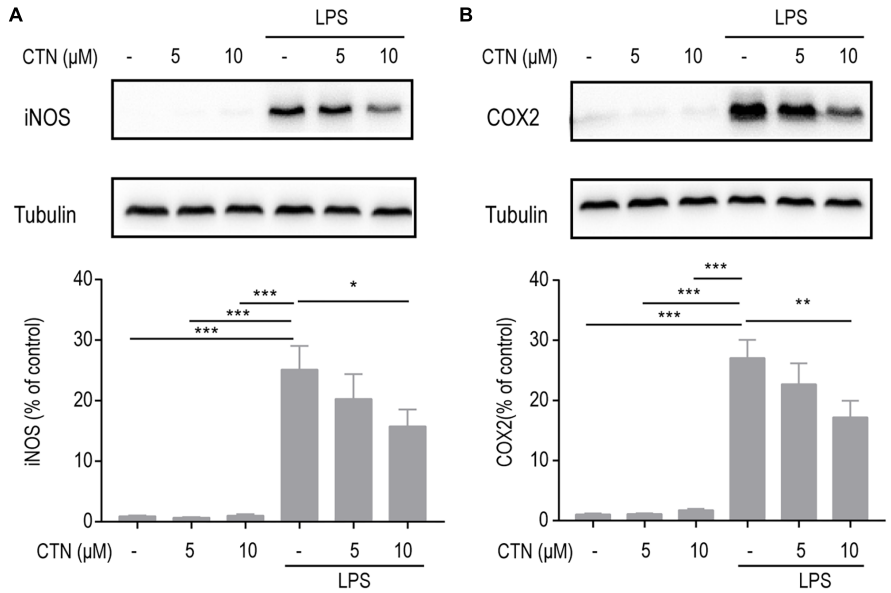

FIGURE 2 | Effect of CTN on LPS-induced expression of iNOS and COX2 in BV2 microglial cells. BV-2 microglial cells were pretreated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 18 h. CTN at concentrations of 5 and 10 μM did not influence the expression of iNOS **(A)** or COX2 **(B)** produced by BV-2 microglial cells. LPS significantly increased the levels of iNOS and COX2, which were significantly attenuated by CTN in a dose-dependent manner. Data were presented as mean ± SEM. All experiments were repeated at least three times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

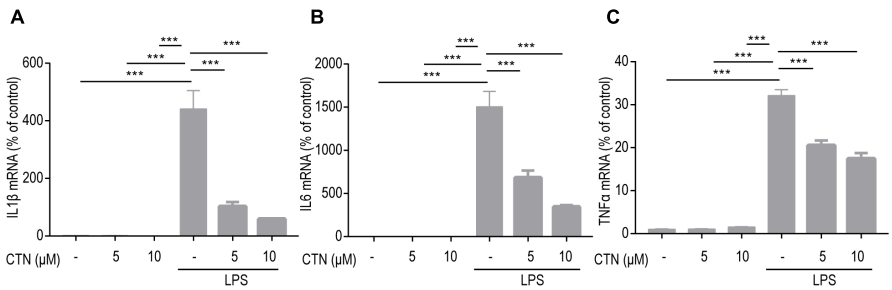

FIGURE 3 | Effect of CTN on LPS-induced expression of IL-1β, IL-6, and TNF-α in BV2 microglial cells. BV-2 microglial cells were pretreated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 6 h. Five or 10 μM CTN alone did not influence the expression of IL-1β **(A)**, IL-6 **(B)**, and TNF-α **(C)** in BV-2 microglial cells. LPS significantly increased the expression of these three genes, which was significantly attenuated by 5 or 10 μM CTN. Data were presented as mean ± SEM. All experiments were repeated at least three times. $^{***}P < 0.001$.

was decreased by knocking down HO-1 using HO-1 siRNA. Western blot showed that HO-1 siRNA significantly inhibited the expression of HO-1 (**Figure 7A**). The NO assay showed that HO-1 siRNA partially reversed the inhibitory effect of CTN on NO production, but this effect was non-significant (**Figure 7B**). Western blot showed the reversal of iNOS (**Figure 7C**) and COX2 (**Figure 7D**) expression downregulated by CTN. Taken together, the anti-inflammatory effect of CTN was partially dependent on HO-1.

CTN-Induced Upregulation of Nrf2 and HO-1 Depends on the PI3K/Akt Pathway

To investigate the effect of CTN on Akt phosphorylation, BV-2 microglial cells were treated with CTN at multiple time points. Western blot showed that CTN increased the phosphorylation of Akt with time (**Figure 8A**). To determine whether upregulation of Nrf2 and HO-1 by CTN is dependent on the PI3K/Akt pathway, LY294002, a PI3K/Akt pathway inhibitor, was added to the culture medium. It was found that LY294002 downregulated CTN-induced expression of Nrf2 (**Figure 8B**) and HO-1 (**Figure 8C**). Therefore, CTN-induced expression of Nrf2 and HO-1 was partially dependent on the activation of the PI3K/Akt pathway.

The Inhibitory Effect of CTN on NLRP3 Inflammasomes Is Dependent on the Activation of Nrf2

NOD-like receptor pyrin domains-3 inflammasomes are important players in the development of inflammation. To investigate the effect of CTN on LPS-induced expression of

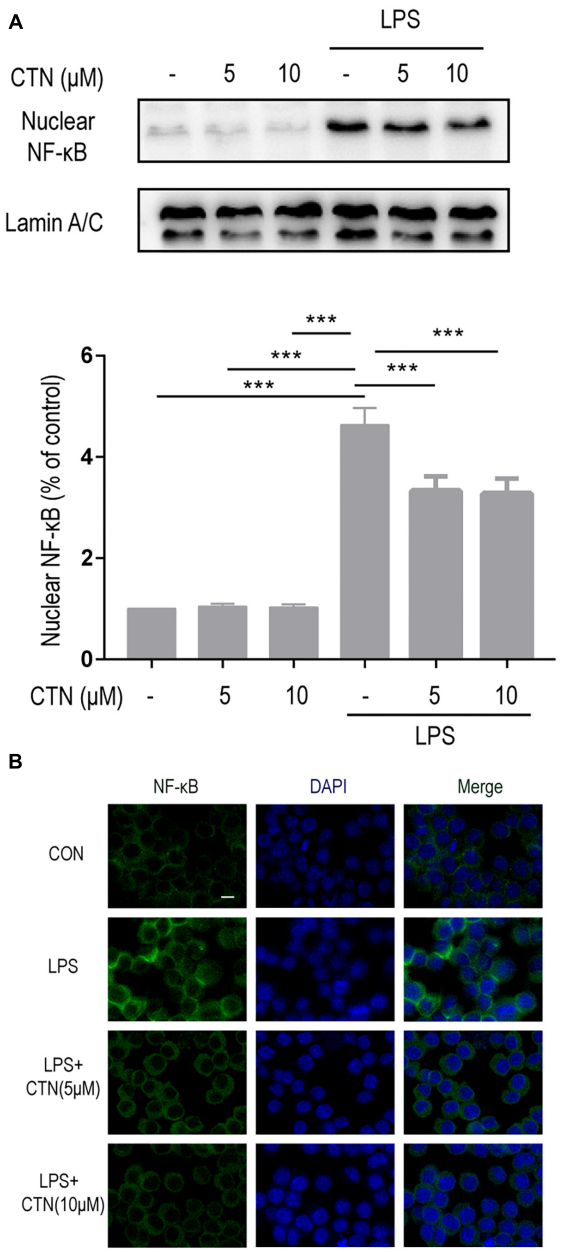

FIGURE 4 | Effect of CTN on LPS-induced activation of NF-κB in BV2 microglial cells. BV-2 microglial cells were pretreated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 30 min. **(A)** Five or 10 μM CTN alone did not influence the expression of NF-κB in BV-2 microglial cells. LPS significantly increased the expression of NF-κB, which was significantly attenuated by 5 or 10 μM CTN. Data were presented as mean ± SEM. All experiments were repeated at least three times. ***$P < 0.001$. **(B)** Immunofluorescence staining against NF-κB showing similar results to those in **(A)**. Green is NF-κB staining, blue is DAPI counterstain (magnification, 40×, scale bar: 20 μm). All experiments were repeated at least three times.

NLRP3 in BV-2 microglial cells, Western blot was performed after pretreating BV-2 microglial cells with CTN (5 and 10 μM) for 1 h and then co-treating with LPS for 18 h. It was shown that LPS significantly increased the level of NLRP3 protein, which was attenuated by CTN (**Figure 9A**). When the level of Nrf2 was downregulated by Nrf2 siRNA, the inhibitory effect of CTN on NLRP3 was partially reversed (**Figure 9B**).

DISCUSSION

Cryptotanshinone is an important component of the extract from the dry root and rhizomes of *S. miltiorrhiza* Bge. It is a small lipophilic molecule that easily passes the blood–brain barrier. It has shown multiple pharmacological effects such as anti-inflammatory (Feng et al., 2017) and anti-oxidative properties (Wang W. et al., 2018). However, its potential in anti-inflammatory response has not been comprehensively studied. The present study found that CTN effectively ameliorated LPS-induced inflammatory response of BV-2 microglial cells. We are the first to reveal the signaling pathways involved in its anti-inflammatory effect. We found that CTN downregulated LPS-induced expression of proinflammatory mediators such as iNOS and COX-2, as well as the production and release of NO, IL-1β, IL-6, and TNF-α. Apart from these, we found that CTN inhibited the NLRP3 inflammasome and its anti-inflammatory effect was related to the expression and activation of NF-κB, Nrf2, and HO-1. The other key finding is that CTN-induced upregulation of Nrf2 and HO-1 was dependent on the activation of the PI3K/Akt signaling pathway.

Microglial cells are the key inflammatory cells in the brain. Under the normal condition, microglial cells are in a resting state with apparent ramifications. When stimulated, these cells are converted to an amoeboid shape with few ramifications and larger cell bodies, indicating an activated state. Endotoxins-activated microglial cells can release diverse proinflammatory mediators, which contribute to the onset and progression of neurodegenerative diseases (Lull and Block, 2010). LPS-activated microglial cells have been widely used as an *in vitro* model of neuroinflammatory diseases due to their release of multiple inflammatory mediators (Velagapudi et al., 2014; Nam et al., 2018). In the present study, we first observed that CTN suppressed LPS-induced morphological changes in BV2 microglial cells, indicating that CTN inhibited microglial activation induced by LPS. We also demonstrated that CTN inhibited the upregulation of NO, TNF-α, IL-1β, and IL-6, which might attribute to the inhibited expression of their enzymes such as iNOS and COX2 in BV-2 microglial cells.

NF-κB is widely expressed in the central nervous system and is a key regulator of inflammatory and immune responses as well as expression of multiple genes such as iNOS and COX2. It also promotes the secretion of a variety of proinflammatory cytokines, such as TNF-α and IL-1β (Yamamoto and Gaynor, 2001; Li and Verma, 2002). Under the physiological condition, NF-κB binds to its inhibitor I-κB and stays dormant in the cytoplasm. Once activated, it dissociates from I-κB and translocates to the nucleus where it initiates the transcription of downstream genes. It is our finding that LPS increased the activity of NF-κB and subsequently increased the expression of

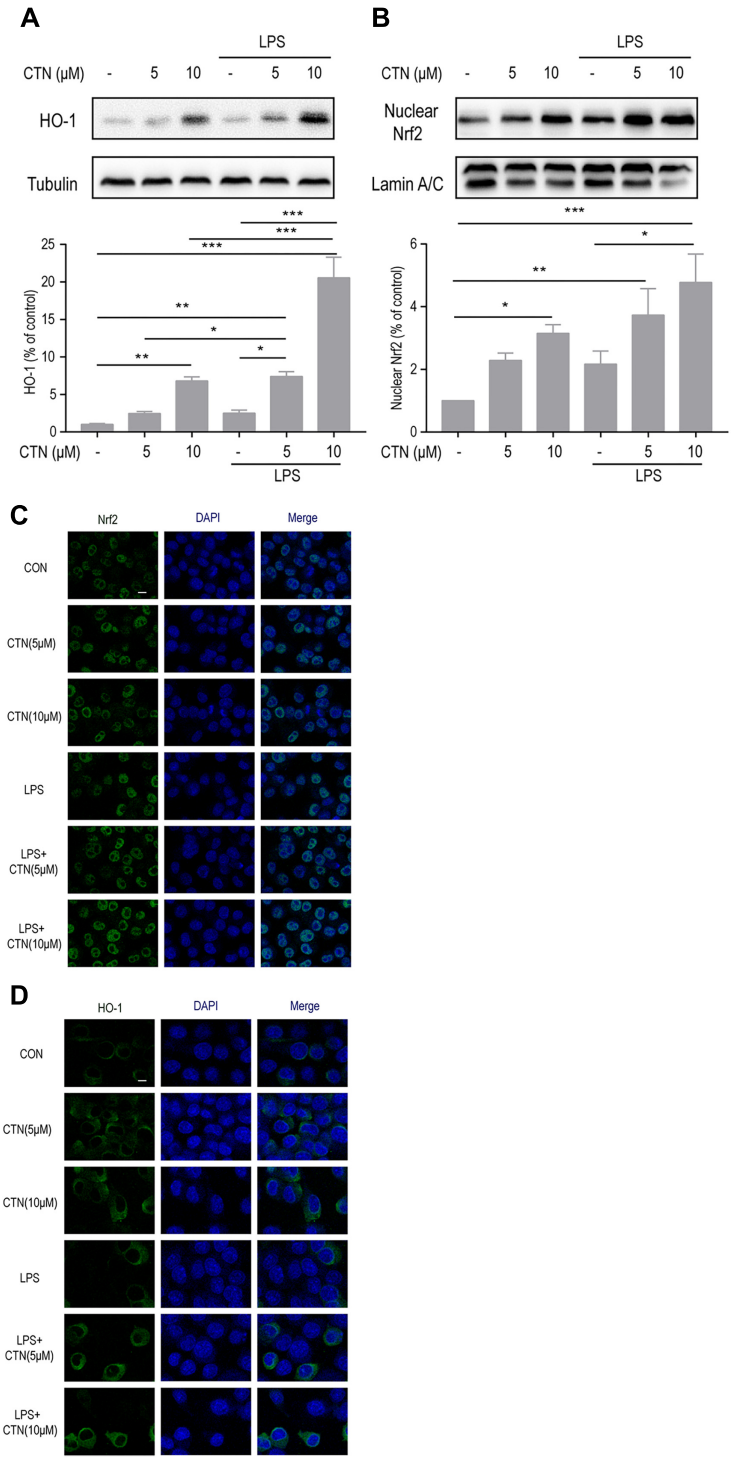

FIGURE 5 | Effect of CTN on LPS-induced expression of Nrf2 and HO-1 in BV2 microglial cells. **(A)** BV-2 microglial cells were pretreated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 18 h. Ten micromolars of CTN significantly increased the expression of HO-1 in BV-2 microglial cells. LPS did not significantly increased the expression of HO-1, but augmented the increase induced by 5 or 10 μM CTN. Data were presented as mean ± SEM. All experiments were repeated at least three times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(B)** BV-2 microglial cells were pretreated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 1 h. Five micromolars of CTN alone did not influence the expression of Nrf2 in BV-2 microglial cells, but 10 μM CTN significantly increased the expression of Nrf2. LPS did not significantly increase the expression of Nrf2, but augmented the increase induced by 5 or 10 μM CTN. **(C)** Immunofluorescence staining against Nrf2 showing similar results to those in **(B)**. Green is Nrf2 staining, blue is DAPI counterstain (magnification, 40×, scale bar: 20 μm). All experiments were repeated at least three times. **(D)** Immunofluorescence staining against HO-1 showing similar results to those in **(A)**. Green is HO-1 staining, blue is DAPI counterstain (magnification, 40×, scale bar: 20 μm). All experiments were repeated at least three times.

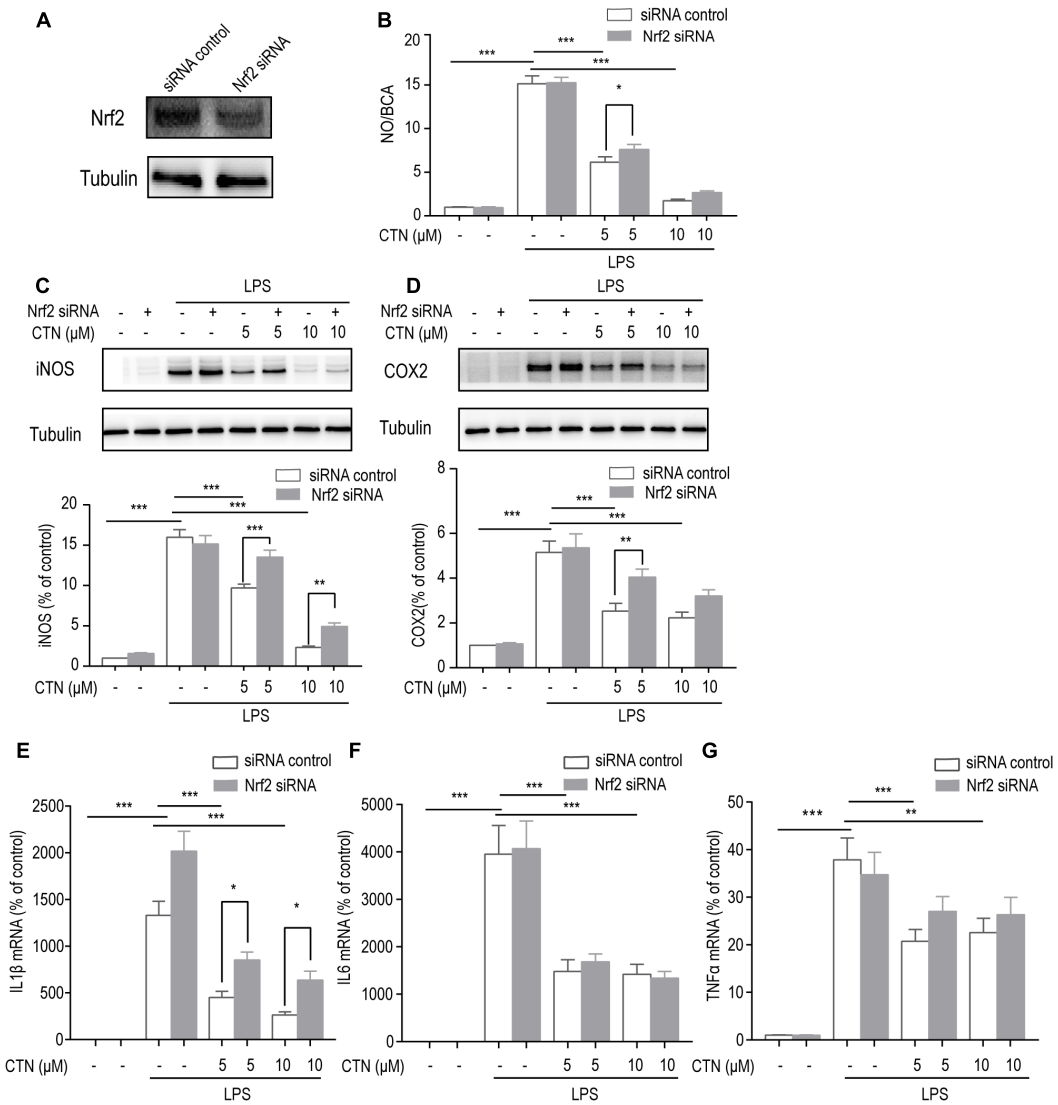

FIGURE 6 | The anti-inflammatory effect of CTN is partially through Nrf2. **(A)** Expression of Nrf2 was significantly decreased after transfecting cells with Nrf2 siRNA. **(B)** BV2 microglial cells were treated with CTN at various concentrations for 1 h in the presence or absence of Nrf2 siRNA and subsequently co-treated with 1 μg/ml LPS for 24 h. LPS significantly increased the amount of NO in the presence or absence of Nrf2 siRNA, which was significantly attenuated by CTN at 5 μM. Transfection with Nrf2 siRNA partially reversed CTN's inhibitory effect. **(C,D)** BV2 microglia cells were treated with CTN at various concentrations for 1 h in the presence or absence of Nrf2 siRNA and subsequently co-treated with 1 μg/ml LPS for 18 h. LPS significantly increased the expression of iNOS **(C)** and COX2 **(D)** in the presence or absence of Nrf2 siRNA, which was significantly attenuated by CTN. Transfection with Nrf2 siRNA partially reversed CTN's inhibitory effect. **(E–G)** BV2 microglia cells were treated with CTN at various concentrations for 1 h in the presence or absence of Nrf2 siRNA and subsequently co-treated with 1 μg/ml LPS for 6 h. LPS significantly increased the expression of IL-1β **(E)**, IL-6 **(F)**, and TNF-α **(G)** in the presence or absence of Nrf2 siRNA, which was significantly attenuated by CTN at 5 or 10 μM. Transfection with Nrf2 siRNA partially reversed CTN's inhibitory effect on the expression of IL-1β. Data were presented as mean ± SEM. All experiments were repeated at least three times. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

iNOS and COX2. CTN downregulated the increased expression of these two proinflammatory mediators by inhibiting the activity of NF-κB. As a result, the secretion of proinflammatory mediators was decreased.

We are the first to demonstrate that the anti-inflammatory effect of CTN was partially through the activation of Nrf2/HO-1. It is known that Nrf2-ARE is one of the protecting mechanisms of neurodegenerative diseases by attenuating neuroinflammation (Buendia et al., 2016; Draheim et al., 2016). It has been shown that multiple drugs decrease the secretion of proinflammatory mediators by increasing the level of Nrf2 and thereby ameliorate neuroinflammation (Wang X. et al., 2018; Xu et al., 2019). We found that CTN increased the level of Nrf2, suggesting that CTN had anti-inflammatory effect on activated microglial cells. In addition, knocking down Nrf2 led to reversal of CTN's inhibitory effect on LPS-induced production of NO, iNOS, COX2, and IL-1β, indicating that the anti-inflammatory effect of CTN is partially through the activation of Nrf2 in BV-2 microglial

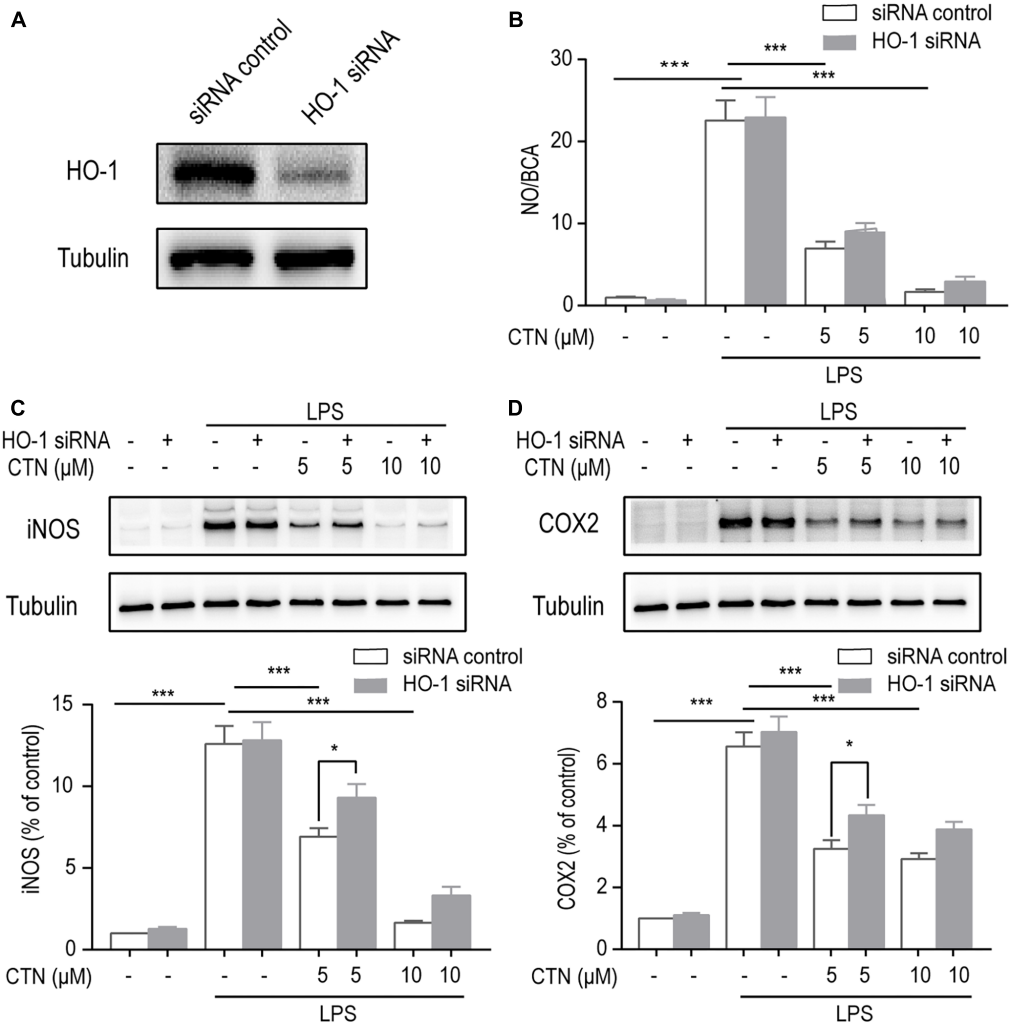

FIGURE 7 | The anti-inflammatory effect of CTN is partially through HO-1. **(A)** Expression of HO-1 was significantly decreased after transfecting cells with HO-1 siRNA. **(B)** BV2 microglial cells were treated with CTN at various concentrations for 1 h in the presence or absence of HO-1 siRNA and subsequently co-treated with 1 μg/ml LPS for 24 h. LPS significantly increased the level of NO in the presence or absence of HO-1 siRNA, which was significantly attenuated by CTN at 5 or 10 μM. Transfection with HO-1 siRNA did not significantly reverse CTN's inhibitory effect. **(C,D)** BV2 microglia cells were treated with CTN at various concentrations for 1 h in the presence or absence of HO-1 siRNA and subsequently co-treated with 1 μg/ml LPS for 18 h. LPS significantly increased the expression of iNOS **(C)** and COX2 **(D)** in the presence or absence of HO-1 siRNA, which was significantly attenuated by CTN at 5 and 10 μM. Transfection with HO-1 siRNA partially reversed CTN's inhibitory effect. Data were presented as mean ± SEM. All experiments were repeated at least three times. $^{*}P < 0.05$, $^{***}P < 0.001$.

cells. One of the downstream molecules of Nrf2 is HO-1. The latter is a stress-induced antioxidative enzyme catalyzing the degradation of heme into carbon monoxide, biliverdin, and free ions in mammalian cells. Many studies have confirmed that HO-1 and metabolites from the enzymatic reaction have anti-inflammatory and neuroprotective effects (Li et al., 2014; Lv et al., 2018). For example, increased expression of HO-1 in dendritic cells can inhibit the secretion of pro-inflammatory cytokines induced by LPS (Chauveau et al., 2005). In the present study, CTN significantly increased the expression of HO-1 and knocking down HO-1 led to reversal of CTN's inhibitory effect on LPS-induced production of iNOS and COX2. Therefore, it can be concluded that the anti-inflammatory effect of CTN is partially through the activation of HO-1.

We are the first to show that CTN increased the expression of Nrf2 and HO-1, which was regulated by the PI3K/Akt signaling pathway. Akt, also named protein kinase B, is one of the downstream molecules of PI3K. Under physiological and pathological conditions, the PI3K/Akt signaling pathway regulates cell proliferation, apoptosis, autophagy, and differentiation. The PI3K/Akt pathway also promotes cell survival by facilitating the translocation of Nrf2 to the nucleus (Lee et al., 2001; Nakaso et al., 2003). It also regulates the expression of HO-1 (Lee and Jeong, 2016). Our study found that CTN increased the level of Akt phosphorylation with time in BV-2 microglial cells. Inhibiting the PI3K/Akt pathway with LY294002 downregulated the expression of Nrf2 and HO-1 induced by CTN. These indicate that CTN induces

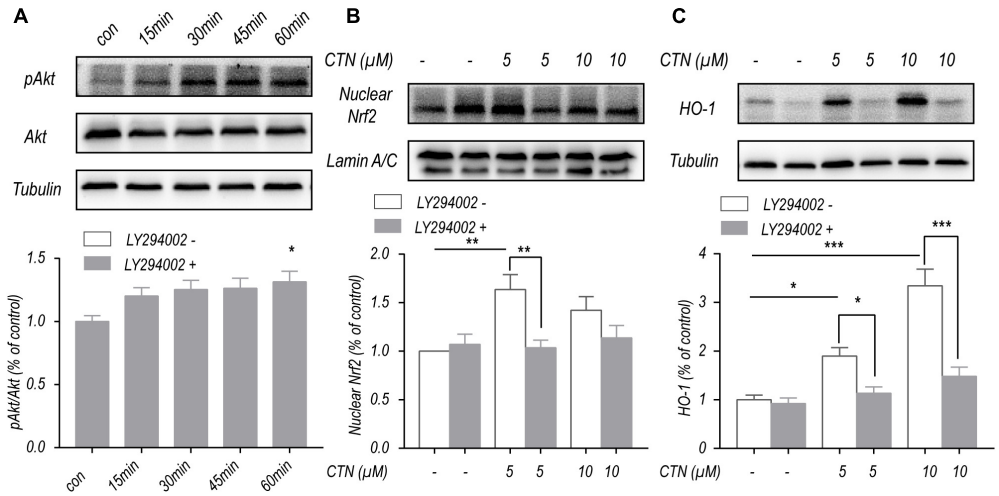

FIGURE 8 | CTN increases the expression of Nrf2 and HO-1 partially through the PI3K/Akt signaling pathway. **(A)** Five micromolars of CTN increased the level of pAkt/Akt with time. **(B)** BV2 microglial cells were treated with CTN at various concentrations for 1 h in the presence or absence of LY294002 (20 μM) for 3 h and subsequently co-treated with 1 μg/ml LPS for 1 h. Five micromolars of CTN increased the level of nuclear Nrf2 in BV-2 microglial cells, which was significantly attenuated by LY294002 in the 5 μM group, but not in the 10 μM group. **(C)** BV2 microglia cells were treated with CTN at various concentrations for 1 h in the presence or absence of LY294002 (20 μM) for 3 h and subsequently co-treated with 1 μg/ml LPS for 18 h. Five or 10 μM CTN significantly increased the expression of HO-1 in BV-2 microglial cells, which was significantly attenuated by LY294002. Data were presented as mean ± SEM. All experiments were repeated at least three times.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

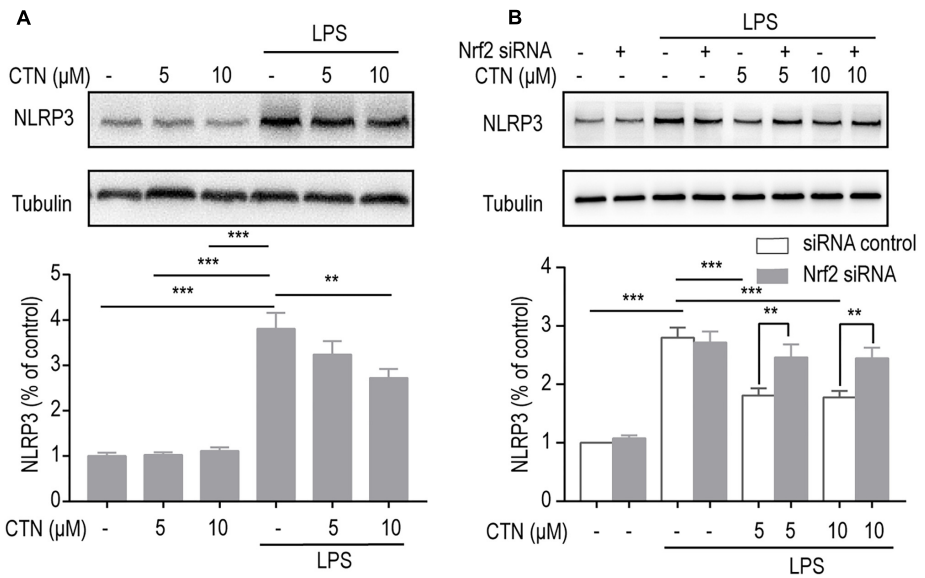

FIGURE 9 | The inhibitory effect of CTN on NLRP3 inflammasomes is partially through Nrf2. **(A)** BV2 microglia cells were treated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 18 h. CTN at concentrations of 5 or 10 μM did not influence the expression of NLRP3, whereas LPS significantly increased the expression of NLRP3, which was attenuated by 10 μM CTN, but not by 5 μM CTN. **(B)** BV2 microglia cells were treated with CTN at various concentrations for 1 h in the presence or absence of Nrf2 siRNA and subsequently co-treated with 1 μg/ml LPS for 18 h. After transfecting cells with Nrf2 siRNA, the inhibitory effect of 5 μM CTN was partially reversed. Data were presented as mean ± SEM. All experiments were repeated at least three times. **$P < 0.01$, ***$P < 0.001$.

activation of Nrf2 and HO-1 by activating the PI3K/Akt signaling pathway.

One of the interesting findings of our study is that downregulated expression of NLRP3 by CTN was related to the activation of Nrf2. This has not been reported by others. Inflammasomes are a type of cytosolic sensors detecting microbial infection, tissue injury, and metabolic imbalance. They stimulate the maturation and release of proinflammatory mediators including IL-1β and IL-18. A typical example is NLRP3 which plays a key role in the pathogenesis of multiple

neurodegenerative diseases such as PD and AD (Zhou et al., 2016; Li et al., 2019). The present study is the first to report that CTN inhibits LPS-induced expression of NLRP3 and this is partially reversed when Nrf2 was knocked down in BV-2 microglial cells. It is, therefore, concluded that CTN inhibited LPS-induced NLRP3 expression partially through Nrf2.

CONCLUSION

In conclusion, our *in vitro* results show that CTN has anti-inflammatory effect at concentrations non-toxic to cultured cells. This effect is mediated by activating NF-κB and the Nrf2/HO-1 signaling pathway. The latter is regulated by the PI3K/Akt signaling pathway. Due to the complexity of live tissues, the anti-inflammatory mechanism of CTN *in vivo* might be different from that of *in vitro* experiments. Future studies can test this anti-inflammatory mechanism of CTN *in vivo*. Our findings provide new insights into the anti-inflammatory mechanism of CTN and lay the theoretical foundation of preventing or attenuating neuroinflammation-related diseases, especially neurodegenerative diseases with activated microglial cells.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

YZ and PZ conceived and designed the study. DW and XW contributed to the data analysis. YZ drafted the manuscript. PZ, DW, and WY critically revised the manuscript. All authors read and approved the final manuscript.

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The authors appreciate the valuable suggestions from other members of their team.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00852/full#supplementary-material

FIGURE S1 | Effect of CTN on the viability of BV-2 microglial cells. **(A)** Chemical structure of CTN. **(B)** BV-2 microglial cells were pretreated with CTN at various concentrations for 1 h and subsequently co-treated with 1 μg/ml LPS for 24 h. Cell viability was assessed using both intracellular and extracellular LDH assay. CTN at concentrations of 1, 5, and 10 μM did not influence the viability of cells, whereas CTN at 20 and 40 μM decreased the viability of cells. Data were presented as mean ± SEM. All experiments were repeated at least three times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

FIGURE S2 | Effect of CTN on the morphology of LPS-activated BV2 microglial cells. BV2 microglial cells were pretreated with CTN at different concentrations for 1 h and then treated with LPS for 18 h. LPS significantly increased the area of microglial cells and their perimeters, whereas CTN can partially attenuate this increase. Data were presented as mean ± SEM. All experiments were repeated at least three times. $^{*}P < 0.05$, $^{***}P < 0.001$.

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REVIEW
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The Heme Oxygenase/Biliverdin Reductase System as Effector of the Neuroprotective Outcomes of Herb-Based Nutritional Supplements

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Over the last few years, several preclinical studies have shown that some herbal products, such as ferulic acid, *Ginkgo biloba*, and resveratrol, exert neuroprotective effects through the modulation of the heme oxygenase/biliverdin reductase system. Unfortunately, sufficient data supporting the shift of knowledge from preclinical studies to humans, particularly in neurodegenerative diseases, are not yet available in the literature. The purpose of this review is to summarize the studies and the main results achieved on the potential therapeutic role of the interaction between the heme oxygenase/biliverdin reductase system with ferulic acid, *G. biloba*, and resveratrol. Some critical issues have also been reported, mainly concerning the safety profile and the toxicological *sequelae* associated to the supplementation with the herbs mentioned above, based on both current literature and specific reports issued by the competent Regulatory Authorities.

Keywords: ferulic acid, *Ginkgo biloba*, heme oxygenase, neuroprotection, resveratrol

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BACKGROUND

At the end of Sixties, three skilled scientists from the University of California San Francisco Medical Center first described and characterized the enzymatic activity of heme oxygenase (HO), a microsomal enzyme catalyzing the oxidative cleavage of hemoproteins' prosthetic group in equimolar amounts of ferrous iron, carbon monoxide (CO) and biliverdin (BV) (Tenhunen et al., 1968; Tenhunen et al., 1969). Following this initial description of the enzymatic activity, there were many other findings, including the inducible and constitutive nature of HO (HO-1 and HO-2, respectively), as well as the full characterization of biliverdin reductase (BVR), a cytosolic enzyme that works in combination with HO and reduces BV into bilirubin (BR). A significant contribution to these discoveries was provided by Mahin Maines and her research group [see (Maines, 1988; Maines, 1997; Maines, 2005) and references therein] who, recently, have deepened the field by describing pleiotropic effects of BVR in terms of modulation of numerous cytoprotective signaling pathways [see (Maines and Gibbs, 2005; Kapitulnik and Maines, 2009; Gibbs et al., 2015) and references therein].

Following these observations, the HO/BVR system has been studied worldwide by scientists who have gradually discovered its enormous potential. Just to give an idea of the attention aroused, suffice it to say that since 1969, more than 15300 papers have been published containing the keyword "heme oxygenase" (source: PubMed, accessed on August 8, 2019), while, concerning "biliverdin reductase," the first studies appeared in 1965 reaching, to date, more than 450 papers (source: PubMed, accessed

on August 8, 2019). The numbers mentioned so far have been achieved thanks to the many studies that have described the involvement of the HO/BVR system in the pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD), atherosclerosis and other cardiovascular disorders, kidney diseases, diabetes, etc. The molecular mechanisms through which the HO-1/BVR system exerts neuroprotective effects mainly depends on the down-stream effectors CO and BR. In the nervous system, CO has been shown to modulate synaptic plasticity, neuropeptide secretion, and neurogenesis (Verma et al., 1993; Mancuso et al., 1998; Mancuso et al., 2010; Choi, 2018), whereas BR interacts with reactive oxygen species (ROS), reactive nitrogen species (RNS) and nitric oxide (NO) to prevent neurotoxicity due to free radical injury (Mancuso et al., 2006a; Mancuso et al., 2008; Barone et al., 2009b; Mancuso et al., 2012a). While the antioxidant and cytoprotective effects of the HO/BVR system and its by-products have been extensively described in literature, scarce attention has been dedicated to a critical examination of their potentially toxic effects. Our research group has contributed to this field by describing the cytotoxic effects of CO either through the inhibition of the stress axis under pro-inflammatory conditions or production of pro-inflammatory prostaglandins (PG) (e.g., PGE2) in rat hypothalamus [see (Mancuso et al., 1997; Mancuso et al., 2006c; Mancuso et al., 2010) and references therein]. Moreover, we have also explored the cytoprotective *vs* cytotoxic effects of the interaction between BR and NO [see (Mancuso et al., 2006b; Mancuso, 2017) and references therein; (Barone et al., 2009b)]. Hyman Schipper's group, instead, decisively contributed to the discovery of the neurotoxic role of HO-derived iron in mitochondrial dysfunctions and in the genesis of oxidative stress-induced damage in neurons and glial cells [see (Schipper, 2004; Schipper et al., 2009; Schipper et al., 2019) and references therein]. Finally, Shigeki Shibahara and Kazuhiko Igarashi, with collaborators, discovered the importance of HO-1 gene repression, through the transcription factor Bach1, to reduce cellular toxic effects due to iron and CO accumulation as in the case of strong and long-lasting pro-oxidant conditions (Shibahara, 2003; Shibahara et al., 2003; Igarashi and Sun, 2006). These findings contributed to increase the awareness of the risks associated with the uncontrolled activation of the HO/BVR system.

A particularly detailed aspect, starting from the early 2000s, was the interaction between HO-1 and products of herbal origin, and caffeic acid derivatives were the first to be studied (Scapagnini et al., 2002); only later, many studies have appeared in the literature concerning the ability of several other herbal products to induce HO-1 (Mancuso et al., 2012b; Mancuso and Santangelo, 2014; Fetoni et al., 2015; Mancuso and Santangelo, 2017). However, as detailed below, the up-regulation of HO-1 by herb-derived nutritional supplements is claimed as a beneficial mechanism through which they exert neuroprotective outcomes. In this regard, however, it is worth underlining how not always the fine involvement of HO-1 in the claimed neuroprotective effects of herbal products has been studied, but frequently HO-1 induction has been considered rather as a mere biomarker of activation of the cell stress response. In our opinion, the aspects that are still weak and are worth investigating, with regard to the effects of herbal products through HO-1 up-regulation, are the following: (i) the correlation between the dose/concentration of herbal product capable of inducing HO-1 *in vitro* and its effective concentration in the target organ within *in vivo* studies; (ii) the extent and duration of HO-1 induction, considering that, as previously mentioned, the accumulation of products, such as iron and CO can become toxic to cells, (iii) loss of specificity of the obtained results, as nearly all the studied herbal products induced HO-1 and (iv) the reduced number of studies that evaluated the BVR modulation, through herbal products.

The purpose of this review is to provide a critical overview of the potential therapeutic role of ferulic acid, *Ginkgo biloba*, and resveratrol *via* the modulation of the HO/BVR system; the reason why the attention has been focused on these herbal products depends on the consistent number of articles published on this topic through the years strong enough to substantiate a potential interest towards clinics. The safety profile of ferulic acid, *G. biloba*, and resveratrol has been also evaluated with the purpose to provide a complete overview of the risk/benefit balance of a chronic supplementation with these agents.

FERULIC ACID

Ferulic acid {[(E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid)], FA, **Figure 1**} belongs to the family of phenolic acids and is highly abundant in fruits and vegetables. Furthermore, FA is also a component of Chinese medicinal herbs, such as *Angelica sinensis*, *Cimicifuga racemosa*, and *Ligusticum chuangxiong*. The main pharmacokinetic parameters of FA are shown in **Table 1**.

Over the past years, several studies have shown that FA acts as a strong antioxidant not only by a direct free radical-scavenging mechanism, but largely by enhancing the cell stress response through the up-regulation of the HO/BVR system (Barone et al., 2009a; Mancuso and Santangelo, 2014). On the other hand, literature data have highlighted the contribution of FA-induced HO-1 up-regulation to numerous biological effects in several preclinical models (see **Table 2**).

Ferulic acid and its ethyl ester [5–50 μM or 150 mg/kg intraperitoneal (i.p.)] were reported to over-express HO-1 in rat neurons and gerbil synaptosomes resulting in a significant neuroprotective effect on ROS- and glucose oxidase- related oxidative damage (Kanski et al., 2002; Scapagnini et al., 2004;

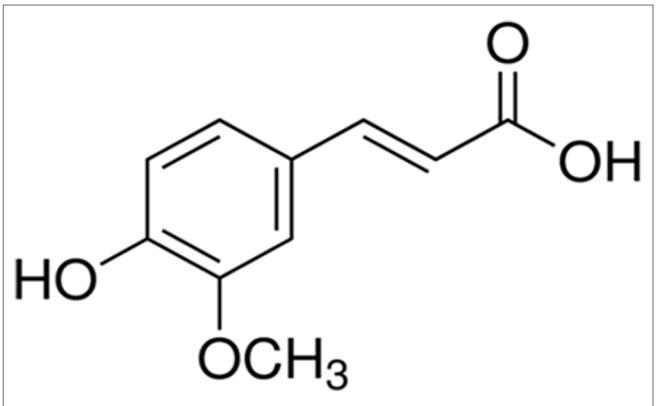

FIGURE 1 | The chemical structure of ferulic acid.

TABLE 1 | Main pharmacokinetic parameters for ferulic acid, *Ginkgo biloba*, and resveratrol in humans.

	Bioavail. (%)	T_{max} (h)	$T_{1/2}$ (h)	Excretion	References
Ferulic acid *per os*	20%	0.4–3	0.7–5	Urine (glucuronide, sulfoglucuronide, and glycine metabolites)	(Mancuso and Santangelo, 2014)
G. biloba ginkgolides	> 80%	0.5–3	4–10	Urine (40–70% unchanged)	(Kleijnen and Knipschild, 1992; Ude et al., 2013)
bilobalide *per os*	~ 70%	0.5–3	3–5	Urine (30% unchanged)	
Resveratrol *per os*		1–1.5 6*	9–11	Urine (monoglucuronide and sulfate metabolites)	(Wang and Sang, 2018)

**Second peak probably due to the enteric recirculation of conjugate metabolites.*
Bioavail., biovailability; T_{max}, time to reach peak plasma concentration; $T_{1/2}$, half-life.

TABLE 2 | Contribution of HO-1 up-regulation to the biological effects of ferulic acid (FA) in preclinical *in vitro* and *in vivo* models.

Preclinical model	Ferulic acid* (concentration or dose)	Effect(s)	Reference(s)
Radiation-induced damage in mice	50 mg/kg *per os* for 5 days	Prevention of radiation-induced oxidative damage in the duodenum	(Das et al., 2017)
Cisplatin-induced nephrotoxicity in rats	50 mg/kg *per os* for 5 days	Prevention of drug-induced injury and improvement of renal function	(Bami et al., 2017)
Pre-adipocytes	100 μM	Reduction of adipocyte tissue mass	(Koh et al., 2017)
Lymphocytes	0.001–0.1 μM	Inhibition of oxidative damage.	(Ma et al., 2011)
Endothelial cells	0.2–5 μM	Prevention of radiation-induced oxidative damage	(Ma et al., 2010)
Melanocytes	1–50 μM†	Prevention of UVB-induced skin oxidative damage	(Di Domenico et al., 2009)
Rat heart	100 mg/kg *per os* for 14 days	Increase of the antioxidant defense in cardiac tissue	(Yeh et al., 2009)
Dermal fibroblasts	25 μM†	Prevention of hydrogen peroxide-induced oxidative damage.	(Calabrese et al., 2008)

**Only studies carried out with purified FA or congeners have been included in this table.*
†Ferulic acid ethyl ester.
UVB, ultraviolet B.

Joshi et al., 2006). In the neuronal cell line SH-SY5Y, FA (1–10 μM) exerted marked neuroprotective effects against trimethyltin-induced damage by increasing the expression of HO-1; the translocation of the transcriptional inducer nuclear factor erythroid 2-related factor 2 (Nrf2) from cytosol to the nucleus has been described as the molecular mechanism underlining the FA-induced HO-1 up-regulation (Catino et al., 2016). Moreover, CO and BR have been identified as the by-products of HO and BVR activities responsible for the antioxidant and neuroprotective effects of FA (Catino et al., 2016). Through the specific up-regulation of the Nrf2/HO-1 system, FA (3–30 μM) counteracted lead-induced inhibition of neurite outgrow in PC12 cells (Yu et al., 2016). As shown by Ma et al. (Ma et al., 2011), the extracellular signal-regulated kinase (ERK) has a role in mediating the FA-activation of Nrf2/HO-1 since its blockade counteracts the nuclear translocation and transcriptional activity of Nrf2 on the HO-1 gene. Quite recently, our research group has demonstrated how FA exerts neuroprotective effects not only under pro-oxidant conditions, but also during psychosocial stress. As shown by Mhillaj et al. (Mhillaj et al., 2018), FA (150 mg/kg i.p.) enhances long-term memory in rats exposed to novelty-induced emotional arousal through the up-regulation of HO-1 in the hippocampus and frontal cortex; it is worth mentioning the finding that FA also over-expressed HO-2 in the frontal cortex. CO is responsible for this nootropic effect of FA, whereas BV does not have any significant effect (Mhillaj et al., 2018).

Ferulic acid (150 mg/kg i.p. for 4 days) up-regulated HO-1 in the organ of Corti of guinea pigs exposed to acoustic trauma; FA-induced improvement of the auditory function was counteracted by the HO inhibitor zinc-protoporphyrin-IX and paralleled the time-course of FA-induced HO-1 overexpression, thus supporting the hypothesis that the neuroprotective effect of this phenolic acid was due to the induction of cytoprotective HO-1 (Fetoni et al., 2010). Interestingly, FA (21.61 mg/kg for 3 weeks intragastric) increased the HO-1 expression and counteracted visible light-induced retinal degeneration in pigmented rabbits (Wang et al., 2016b).

Ferulic acid has also been complexed with tacrine, one of the earliest drugs developed for AD therapy and later discarded for severe hepatotoxicity. Tacrine-FA (2–100 μM) has been shown to prevent β-amyloid (Aβ) aggregation, ROS formation, and apoptosis in PC12 cells; in addition, tacrine-FA (2–20 mg/kg intragastric) improved cognitive skills in a mouse model of AD (Pi et al., 2012). In a subsequent paper, Huang et al. (Huang et al., 2012) showed how tacrine-FA inhibits oxidative stress-induced damage by over-expressing HO-1, through Nrf2 translocation, in HT22 cells.

Ferulic acid has an enviable safety profile, since no significant toxicities have been reported in humans; furthermore, FA's

modulation of drug metabolizing enzymes is negligible (see https://toxnet.nlm.nih.gov. Accessed on June 27, 2019). However, female rats treated with the highest tolerated dose of tacrine-FA have shown both glycogen depletion and HO-1 induction and cytochrome-P450 (CYP) CYP1A1, 2B1, and 3A2 up-regulation in the liver, mainly due to the tacrine moiety (Lupp et al., 2010). A careful risk/benefit analysis of the pharmacological and safety profiles of tacrine-FA is required.

GINKGO BILOBA

Gingko biloba is a plant growing in the mountainous valleys of Eastern China. *G. biloba* extracts contain several active compounds, including flavonoids, terpenes, organic acids, and polyphenols, the most important being flavonol-glycosides (primarily quercetin, kaempferol, and isorhamnetin) and terpene-lactones; the latter are further divided into diterpenes (ginkgolides) and sesquiterpenes (bilobalide) (**Figure 2**). For a summary of the pharmacokinetic parameters and biological effects of *G. biloba*, see **Tables 1** and **3**, respectively.

Through the induction of HO-1, ginkgolides A, B, and C [1, 3, and 10 mg/kg by intravenous route (i.v.)] decreased neurological deficits and brain infarct volume in rats exposed to ischemia/reperfusion damage (Zhang et al., 2018). In a similar experimental setting the *G. biloba* extract EGb 761 [containing flavonol-glycosides (24%), ginkgolides A, B, and C (2.8–3.4%), and bilobalide (2.6–3.2%)] at the dosage of 100 mg/kg *per os* for 7 days before or 4-24 h after damage reduced cortical infarct volume and stimulated the proliferation of neuronal stem/progenitor cells *via* HO-1 overexpression in mice with permanent middle cerebral artery occlusion (Shah et al., 2011; Nada and Shah, 2012; Nada et al., 2014). The same research group confirmed the neuroprotective effects of *G. biloba* in the hippocampus of mice pre-treated with EGb 761 (100 mg/kg *per os* for 7 days) and then susceptible to bilateral common carotid artery occlusion (Tulsulkar and Shah, 2013). The contribution of HO-1 to the neuroprotective effects of EGb 761 (100 mg/kg *per os*) was also demonstrated in HO-1 knockout male mice with middle cerebral artery occlusion (Saleem et al., 2008). Ginkgolide B (1–50 μM or 10 mg/kg i.p.) prevented cisplatin-induced damage in HEI-OC1 auditory cells and rats through HO-1 induction (Ma et al., 2015). Interestingly, in order to improve brain penetration, *G. biloba* has been complexed with phosphatidylcholine (Carini et al., 2001); this novel formulation exhibited neuroprotective effects by increasing catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase activities in rat brain (Naik et al., 2006). Regrettably, there are no studies addressing the potential role of this novel formulation on HO-1. Worth mentioning is the nootropic effects of a novel formulation of *G. biloba* (120 mg/

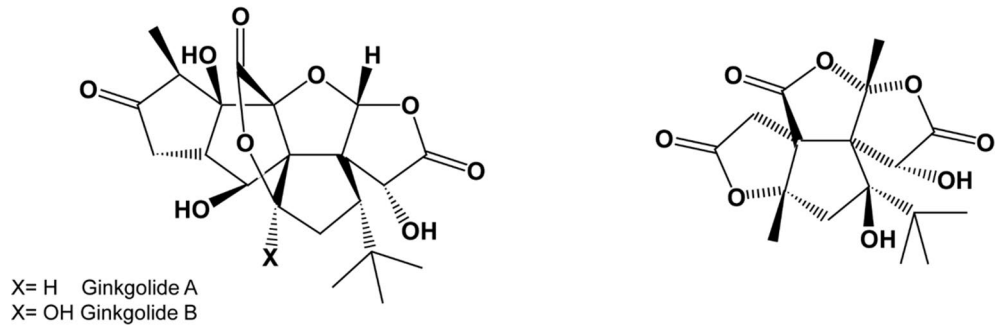

FIGURE 2 | The chemical structure of ginkgolides and bilobalide.

TABLE 3 | Contribution of HO-1 up-regulation to the biological effects of *Ginkgo biloba* in preclinical *in vitro* and *in vivo* models.

Preclinical model	*G. biloba* (concentration or dose)	Effect(s)	Reference(s)
Myoblasts	25–100 μg/mL	Cytoprotection from alcohol-induced oxidative damage	(Wang et al., 2015)
Endothelial cells	50–200 μg/mL	Endothelial protection from high-glucose- or TNF-α–induced vascular oxidative damage; cytoprotection against cigarette smoke-induced apoptosis in lungs	(Hsu et al., 2009; Chen et al., 2011; Tsai et al., 2013)
Macrophages	1–100 μg/mL	Inhibition of inflammatory damage in LPS-treated cells; regulation of cholesterol homeostasis and reduction in atherosclerosis lesion size	(Tsai et al., 2010; Ryu et al., 2012)
Ethanol-induced liver damage in rats	48 or 96 mg/kg intragastric for 90 days	Reduction of oxidative damage and improvement of ethanol-induced microvesicular steatosis and parenchimatous degeneration in hepatocytes	(Yao et al., 2007)

LPS, lipopolysaccharide; TNF, tumor necrosis factor.

day *per os*) complexed with phosphatidylserine administered to healthy volunteers for 7 days (Kennedy et al., 2007).

As far as neurodegenerative disorders, no evidence has been found in literature dealing with the involvement of HO-1 and/or BVR in *G. biloba*-related neuroprotection. Indeed, the neuroprotective effects of EGb 761 have been extensively reported in both AD (Augustin et al., 2009; Liu et al., 2015; Wan et al., 2016) or PD rodent models (Kim et al., 2004; Rojas et al., 2012; El-Ghazaly et al., 2015) and in humans. Over the last 2–3 years, some retrospective analyses, meta-analyses, and systematic reviews on the neurotherapeutic effects of *G. biloba* extracts in subjects with dementia, including AD, have been published and the conclusions are the following: (i) over 12 months treatment, EGb 761 and donepezil showed similar effects in cognitive decline in patients aged 80 years or older affected by AD (Rapp et al., 2018); (ii) EGb 761 (240 mg/day *per os* for 22–24 weeks) had a better performance than placebo in 1,598 patients with dementia (probable AD with or without cerebrovascular disease and probable vascular dementia) (Savaskan et al., 2018); (iii) doses lower than 200 mg/day did not have any remarkable clinical effects in demented people (Yuan et al., 2017) and (iv) *G. biloba* extract (240 mg/day *per os*) also showed neuroprotective effects in subjects with mild cognitive impairment (Zhang et al., 2016; Kandiah et al., 2019). Unfortunately, there are no clinical studies on the neuroprotective effects of *G. biloba* in PD patients, therefore no conclusions can be drawn.

Although the safety profile of *G. biloba* extracts is acceptable, few mild adverse effects, including mild gastrointestinal complaints, headaches, and allergic reactions have been reported (Kleijnen and Knipschild, 1992). However, important interactions between *G. biloba* and common drugs should be underlined. *G. biloba* is an inducer of CYP2C19 and, through this mechanism, it has been shown to reduce omeprazole plasma levels in individuals sharing the poor metabolizer phenotype (Yin et al., 2004) and to increase metabolism and reduce plasma concentrations of the antiepileptic drugs, valproic acid, and phenytoin, thus increasing the risk of fatal seizures (Kupiec and Raj, 2005). A greater risk of bleeding has been reported in people taking *G. biloba* and aspirin or warfarin (Agbabiaka et al., 2017). *G. biloba* has been discouraged in people taking selective serotonin-reuptake inhibitors for the increased risk of developing serotonin syndrome (Kreijkamp-Kaspers et al., 2015). Lastly, ginkgo flavonol-glycosides caused a coma in an 80-year-old AD patient taking trazodone, probably by stimulating the CYP3A4 activity which increases the transformation of trazodone into the active metabolite 1-(m-chlorophenyl) piperazine and up-regulates GABAergic activity in the brain (Galluzzi et al., 2000).

RESVERATROL

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, (**Figure 3**) is a phytoalexin found in grapes, cranberries, peanuts, and some beverages (Mancuso et al., 2007). However, wine is considered the main source of resveratrol since the solubility of the latter in ethanol is about 1,667-times higher than that in water (Weiskirchen and Weiskirchen, 2016). Also based on the interest aroused by the so-called "French paradox" over the last few years, the interaction resveratrol-HO-1 has been extensively studied for its beneficial effects in several preclinical models of disease (**Table 4**).

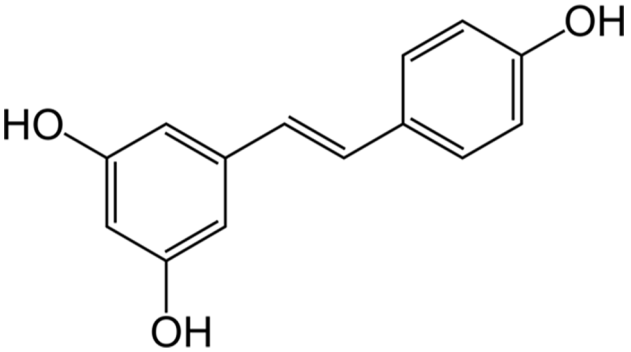

FIGURE 3 | The chemical structure of resveratrol.

With regard to the neuroprotective effects, earlier studies showed that resveratrol (5–100 μM) up-regulates HO-1 in primary cultures of mouse neurons (Zhuang et al., 2003). Resveratrol (15 μM), was also found to increase HO-1 expression in neuron-like PC12 cells through both phosphoinositide 3-kinase and MEK1/2 activities (Chen et al., 2005). Quite recently, resveratrol (10 μM) has exhibited a marked protective effect on primary rat oligodendrocyte progenitor cells exposed to lipopolysaccharide through the modulation of Nrf2/HO-1 pathway and the enhancement of cell stress response (Rosa et al., 2018). Neuroprotective effects, due to HO-1 up-regulation, have also been shown in C6 astroglial cells and in hippocampal primary rat astrocytes treated with 100 μM resveratrol and then exposed to neurotoxicants, such as buthionine sulfoximine, azide, or ammonia, for 3–24 h (Bobermin et al., 2015; Bellaver et al., 2016; Arus et al., 2017).

Resveratrol (20–40 mg/kg i.p. for 7 days) significantly enhanced HO-1 expression and prevented both cerebral edema and infarction in seven-day-old rat pups exposed to hypoxic/ischemic injury secondary to unilateral carotid artery ligation (Gao et al., 2018). Similar results have been obtained in adult male rats treated with resveratrol (15–30 mg/kg i.p. for 7 days) and undergoing middle cerebral artery occlusion; in these animals, HO-1 induction was paralleled by down-regulation of caspase-3 and improvement of neuronal viability (Ren et al., 2011). Resveratrol (1–100 μM) increased HO-1 expression in both rat neural stem cells and primary rat cortical neurons exposed to glucose deprivation/reoxygenation injury (an *in vitro* experimental model mimicking cerebral artery occlusion and reperfusion injury) along with a strong enhancement of cell stress response and a marked reduction of apoptotic cell death (Shen et al., 2016a; Yang et al., 2018). As far as AD is concerned, resveratrol (10–40 μM) has been shown to counteract Aβ-induced oxidative stress in PC12 cells through the overexpression of Nrf2/HO-1 system (Hui et al., 2018). In Aβ-treated rats, resveratrol [100 μM/5 μL by intracerebroventricular route (i.c.v.)] up-regulated hippocampal HO-1, reduced neuronal death in the same area, and improved spatial memory (Huang et al., 2011). In SH-SY5Y

TABLE 4 | Contribution of HO-1 up-regulation to the biological effects of resveratrol in preclinical *in vitro* and *in vivo* models.

Preclinical model	Resveratrol (concentration or dose)	Effect(s)	Reference(s)
Kidney injury in rats	30 mg/kg i.p.	Amelioration of sepsis-induced kidney injury	(Wang et al., 2018a)
Renal cells	20 μM	Cytoprotection from nicotine-induced oxidative damage.	(Arany et al., 2017)
Lung injury in rodents	30 mg/kg i.p.	Improvement of sepsis- or paraquat-induced lung injury in rats	(Li et al., 2016; Wang et al., 2018b)
	1–3 mg/kg *per os* for 3 days	Enhancement of cell stress response and attenuation of cigarette smoke-induced damage in mice	(Liu et al., 2014)
Renal carcinoma in rats	30 mg/kg *per os* for 24 weeks	Inhibition of proliferation and improvement of renal function; increase in the antioxidant system	(Kabel et al., 2018)
Membranous nephropathy in mice	30 mg/kg s.c. every other day for 6 weeks	Reduction of apoptosis and complement-induced damage; amelioration of renal function	(Wu et al., 2015)
Endothelial cells	0.01–10 μM	Reduction of oxidative stress-induced damage and inhibition of senescence in progenitor cells	(Shen et al., 2016b)
Smooth muscle cells	1–10 μM	Inhibition of oxidative damage and inflammation; vascular protection	(Juan et al., 2005)
Obstructive jaundice in rats	10–20 mg/kg *per os*	Restoration of intestinal permeability and improvement of gut barrier function	(Wang et al., 2016a)
Gastric inflammation in mice	100 mg/kg *per os* for 6 weeks	Reduction of oxidative damage and inflammation in *Helicobacter pylori*–infected gastric mucosa	(Zhang et al., 2015)
Myocardial damage in rats	100 μM i.v.	Reduction of oxidative damage and improvement of cardiac function following ischemia/reperfusion injury	(Cheng et al., 2015)
Hepatoma cells	1 μM	Stimulation of mitochondrial biogenesis and reduction of inflammatory damage	(Kim et al., 2014)
Macrophages	1–10 μM	Inhibition of inflammatory damage	(Son et al., 2014)

i.p., intraperitoneal route of administration; i.v., intravenous route of administration; s.c., subcutaneous route of administration.

cells exposed to rotenone, resveratrol (10–20 μM) induced HO-1 expression and prevented dopaminergic cell death by autophagy (Lin et al., 2014).

Studies in humans have found that resveratrol (25 mg *per os*) has a low bioavailability and reaches plasma concentrations of about 40 nM within 2 h from administration (Goldberg et al., 2003; Walle et al., 2004); plasma concentrations up to 2.4 μM are reached if the dose of resveratrol increases up to 1 g (Almeida et al., 2009). For a summary of resveratrol pharmacokinetics see **Table 1**. In order to improve bioavailability, resveratrol has been complexed with solid lipid nanoparticles (SLN) or gold-conjugated nanoparticles (Park et al., 2016; Yadav et al., 2018). Among these formulations, SLN increased by about 4-times the brain levels of resveratrol, which was able to up-regulate HO-1 and improve cognitive decline in rats with permanent bilateral common carotid artery occlusion (Yadav et al., 2018). Unfortunately, there are no studies in humans on the effective concentrations of resveratrol in the brain and a brain concentration greater than 2.4 μM is unlikely, by considering the plasma concentrations reached in the studies mentioned above and the presence of the blood-brain barrier. This suggests that most of the preclinical studies on the neuroprotective effects of resveratrol cannot be applied to humans. As far as the role of wine as the source of resveratrol is concerned, considering both the annual consumption of red and white wines in France (31.7 vs 11.7 L, respectively) and the amount of resveratrol contained in red and white wines (2 and 0.5 mg/L, respectively), it is possible to calculate an amount of resveratrol "drunk" of about 0.2 mg/day, 5000-times less than the highest daily dosage (1 g/day) claimed to give rise to pharmacological effects (Weiskirchen and Weiskirchen, 2016).

It is worth mentioning the document released by the EFSA regarding the request by the European Commission on the safety of trans-resveratrol as a novel food (Efsa, 2016). In this document, the EFSA established that the 38 clinical studies provided in support of the claim do not lead to any conclusion about the efficacy of resveratrol, at doses up to 5 g/day for either acute (4 days) or chronic administration (4–12 weeks), in the treatment of metabolic diseases or cancer. The EFSA pointed out the heterogeneity of the resveratrol doses, the low number of individuals recruited, the uncontrolled experimental design, and the exploratory character of several studies.

In some clinical studies, patients supplemented with resveratrol reported mild adverse effects, such as diarrhea and hot flushes. As far as the interaction with drugs-metabolizing enzymes is concerned, resveratrol has been shown to inhibit CYP3A4, CYP2D6, and CYP2C9 and to induce CYP1A2 *in vitro*. No significant changes in plasma levels of common drugs have been reported so far (Efsa, 2016).

CONCLUSIONS

In illustrating the interactions between the HO/BVR system with FA, *G. biloba*, and resveratrol, we have attempted to address some critical points presented in the Introduction, discussing each issue to the best of our knowledge.

An initial consideration concerns the janus face of the HO-1/BVR by-products. As mentioned in the Introduction, both CO and BR have important pleiotropic and neuroprotective effects in the nervous system, but they may become toxic in the case of a disproportionate production or under conditions of redox imbalance (Mancuso et al., 1997; Mancuso et al., 2010; Mancuso, 2017). The potential toxic effect due to an over activation of the HO/BVR system has also been supported by the discovery of the importance of HO-1 gene repression in order to preserve

cell homeostasis and integrity (Shibahara, 2003; Shibahara et al., 2003; Palozza et al., 2006). For this reason, the long-lasting induction of HO-1 due to chronic supplementation with herbal products may be a double-edged sword and the possibility of neurotoxicity must be carefully considered.

Regarding the effective dose correlation *in vitro* and blood or tissue concentrations found *in vivo*, resveratrol is an indisputable example. Both the bioavailability data through wine consumption and the EFSA opinion on anti-inflammatory efficacy do not justify the turmoil often generated by the media regarding the beneficial properties of resveratrol in red wine. In this regard, the antioxidant, antithrombotic, and metabolic effects of ethanol *per se* often are not mentioned; moreover, red wines usually contain ethanol in greater concentration than white wines and the beneficial effects claimed for the former can be due, once again, to ethanol (Goldberg et al., 1995; Criqui, 1998; Belleville, 2002). Focusing on the beneficial effects of moderate ethanol consumption would also allow a greater responsibility on the consumers' part towards their own health, because of the pathological effects of uncontrolled alcohol intake are well known. On the contrary, if attention is focused on resveratrol, of which only the beneficial effects are artificially advertised, the need for a controlled intake of red wine is lost since the attention is driven towards a natural compound.

The risk linked to the effects on drug metabolizing enzymes and the health consequences in case of concomitant drug intake, concepts that are very often neglected also by health professionals, should be emphasized. Particularly dangerous are the interactions between *G. biloba* and CYP or other phase II enzymes, which could increase the risk of toxicity in patients treated with drugs, such as valproic acid, trazodone, talinolol, warfarin, etc. It is worth mentioning that the lack of reliable data on the neuroprotective effects of resveratrol and *G. biloba* challenges their potential beneficial effects in the treatment of neurodegenerative diseases. In this regard, more attention is required for FA for which, to our knowledge, there are no clinical studies confirming the promising neuroprotective results obtained on preclinical models. The few clinical studies available, addressing the kinetics in most cases, have been performed using foods rich in FA and this does not always allow to ascertain the actual dose administered and any confounding effects related to other active compounds present. Actually, only one randomized and double-blind study on hyperlipidemic patients (Bumrungpert et al., 2018) has appeared in the literature and shows the hypolipemic, anti-inflammatory, and antioxidant effects of FA (1 g/day *per os* for 6 weeks).

These considerations lead to the conclusion that there is not sufficient evidence on the efficacy of these herbal products in neurodegenerative diseases and that further efforts and many attempts are recommended and requested by doctors, researchers, and other health professionals to bridge this gap for the benefit of patients and their families.

AUTHOR CONTRIBUTIONS

Conceptualization: CM, VC, LT. Data curation: CM, EM. Writing–original draft preparation: CM, EM. Writing–review and editing: CM, EM, VC, LT.

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MINI REVIEW
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Therapeutic Applications of Cysteamine and Cystamine in Neurodegenerative and Neuropsychiatric Diseases

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Current medications for neurodegenerative and neuropsychiatric diseases such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and Schizophrenia mainly target disease symptoms. Thus, there is an urgent need to develop novel therapeutics that can delay, halt or reverse disease progression. AD, HD, PD, and schizophrenia are characterized by elevated oxidative and nitrosative stress, which play a central role in pathogenesis. Clinical trials utilizing antioxidants to counter disease progression have largely been unsuccessful. Most antioxidants are relatively non-specific and do not adequately target neuroprotective pathways. Accordingly, a search for agents that restore redox balance as well as halt or reverse neuronal loss is underway. The small molecules, cysteamine, the decarboxylated derivative of the amino acid cysteine, and cystamine, the oxidized form of cysteamine, respectively, mitigate oxidative stress and inflammation and upregulate neuroprotective pathways involving brain-derived neurotrophic factor (BDNF) and Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling. Cysteamine can traverse the blood brain barrier, a desirable characteristic of drugs targeting neurodegeneration. This review addresses recent developments in the use of these aminothiols to counter neurodegeneration and neuropsychiatric deficits.

Keywords: BDNF, brain, cystamine, cysteamine, cysteine, neurodegeneration, neuropsychiatric disorder, redox

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INTRODUCTION

Cysteamine, also known as 2-mercaptoethylamine or aminoethanethiol, is the decarboxylated derivative of the amino acid cysteine. It exerts radioprotective effects and is more effective than cysteine alone, although a combination of cysteamine and cysteine display synergistic effects (1, 2). Although cysteamine reduced mortality in irradiated *Drosophila* and mice, mutagenic effects of radiation were not prevented (3, 4). Cysteamine has been utilized for the treatment of cystinosis, a lysosomal disorder, and, more recently, has been evaluated for the treatment of neurodegenerative disorders. This review will summarize the current understanding of cysteamine and cystamine, its oxidized derivative.

In cells, the amino thiol is generated by the degradation of coenzyme A, which in turn, is generated from pantothenate (vitamin B5) and cysteine (**Figure 1A**) (5). Coenzyme A degradation yields pantetheine, which is hydrolyzed by pantetheinase or vanin, generating cysteamine

and pantothenic acid. Cysteamine is then oxidized to hypotaurine by cysteamine dioxygenase (6). Hypotaurine can be converted into taurine by hypotaurine dehydrogenase. Taurine is eliminated in the form of bile salts such as taurocholate, either via the urine or feces (7). Levels of cysteamine has been reported to be in the low micromolar range in tissues such as the liver, kidney and brain, which were measured after treating lysates with DTT to liberate free cysteamine (6), indicating association with proteins via disulfide bonding. Similarly, another study measured cysteamine after reducing perchloric acid treated kidney and liver lysates with mercaptopropionic acid (8). The presence of disulfide-bonded cysteamine with proteins was subsequently shown by Duffel and associates (9), which could account for the effects of cysteamine and cystamine on the activity of several proteins.

The metabolism of cysteamine, cystamine and cysteine are linked in cells. Both cysteamine and cystamine increase cysteine levels intracellularly in a temporal and dose-dependent manner (10). As cysteine is a component of glutathione and a potent antioxidant itself, treatment of cells with these aminothiols can mitigate oxidative stress. Treatment of SN56 cholinergic cells causes an increase in cysteine levels in 30 min. Cystamine is first converted to cysteamine in the reducing atmosphere of cells, and treating cells with cystamine elicits an increase of cysteine in 3 h. N-acetylcysteine (NAC), 2-mercaptoethanesulfonic acid (MESNA) and mercaptopropionylglycine (MPG), on the other hand, elevate cysteine levels to a lesser extent (2-fold as compared to 6-fold in the case of cysteamine). The study also revealed the importance of these thiols in sequestering reactive aldehyde species in cells and bolstering the antioxidant capacity of cells. Thus, cystamine and cysteamine also act as antioxidants themselves. Consistent with these observations, cysteamine affords protection against acetaminophen- mediated liver damage, where the highly toxic unsaturated aldehyde acrolein, is produced (11, 12). Cysteamine has also been proposed to replace homocysteine as the substrate for cystathionine β-synthase (CBS) in a reaction with serine to generate thialysine or (S-(2-aminoethyl)-L-cysteine) (13). Consistent with these studies, thialysine levels increase in the brain after feeding cysteamine to rats (14).

PROTECTIVE EFFECTS OF CYSTEAMINE AND CYSTAMINE

Therapeutic Applications of Cysteamine and Cystamine in Peripheral Tissues

Both cysteamine and cystamine, have been used for the treatment of several conditions (**Figure 1B**). These compounds possess radioprotective properties and were initially used to treat radiation sickness that arises in cancer patients after radiotherapy, but subsequently discontinued after unsuccessful clinical trials (1, 15). One of the earliest uses of cysteamine in medicine, which is FDA-approved, is the treatment of cystinosis, an inherited autosomal recessive disorder in which the body accumulates cystine due a defect in the lysosomal cysteine transporter, cystinosin (16, 17). Cystine crystals build up in many tissues and damage organs such as the kidney and the eye. One of the initial manifestations of juvenile cystinosis is renal Fanconi syndrome which manifests as dysfunction of the renal proximal tubule leading to polyuria, phosphaturia, glycosuria, proteinuria, acidosis, growth retardation, and rickets (18). Cysteamine participates in disulfide exchange reactions to form cysteine and mixed disulfides of cysteine and cysteamine, which can then exit the lysosome.

Cysteamine also has anti-malarial effects preventing the replication of the parasite, *Plasmodium falciparum in vivo* and also potentiates the action of the anti-malarial artemisin (19, 20). Cysteamine has also been reported to have anti-HIV-1 effects (21, 22). Cysteamine elicits both beneficial and harmful effects, some of which included ulcer formation and anti-angiogenic effects (23). Cystamine, the oxidized form of cysteamine, inhibits erythrocyte sickling in sickle cell anemia (24). Incubating sickle cells with cystamine leads to the formation of an S-ethylamine derivative and a decrease in sickling under hypoxic conditions. Several other beneficial effects of the two cysteine derivatives are summarized in **Table 1**.

Therapeutic Applications of Cysteamine and Cystamine in Brain Diseases

Cysteamine and cystamine appear to be promising in the treatment of certain mouse models of neurodegenerative diseases, such as Parkinson's disease (PD) and Huntington's disease (HD) (47). Cysteamine can cross the blood-brain barrier, which makes it an attractive candidate for therapeutic applications (48).

Huntington's Disease

Huntington's disease is a neurodegenerative disorder caused by expansion of polyglutamine repeats in the protein huntingtin, Htt, which causes it to aggregate and cause widespread damage in almost all tissues expressing it (49). Initial studies on cystamine and its therapeutic effects on disease progression in HD focused on its inhibitory effects on the enzyme transglutaminase (37, 40). Transglutaminases catalyze the formation of ε-N-(γ-glutamyl)-lysyl crosslinks between proteins and were proposed to contribute to neuropathology of HD (50–52). However, later studies revealed that ablation of the transglutaminase gene did not prevent neurodegeneration in HD (53). Cystamine has also been beneficial in a fly model of HD, where photoreceptor degeneration was rescued in adult flies (54). Cystamine treatment in mouse models of HD lead to increased cysteine levels, which was proposed to be neuroprotective (35, 55). Cysteine is a potent antioxidant and dysregulated cysteine metabolism mediates neurodegeneration in HD (56–58). Cysteine is also the precursor of the gaseous signaling molecule, hydrogen

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; APP, amyloid precursor protein; ALS, Amyotrophic lateral sclerosis; ASD, Autism spectrum disorders; BDNF, brain-derived neurotrophic factor; BECN1, beclin 1; CBS, cystathionine β-synthase; HD, Huntington's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MESNA, 2-mercaptoethanesulfonic acid; MPG, mercaptopropionylglycine; MeCp2, methyl-CpG binding protein 2; MTDL, multi-target-directed ligand; PD, Parkinson's disease; Nrf2, Nuclear factor erythroid 2-related factor 2; PSEN1, presenilin 1; SOD1, superoxide dismutase 1; TG2, transglutaminase 2.

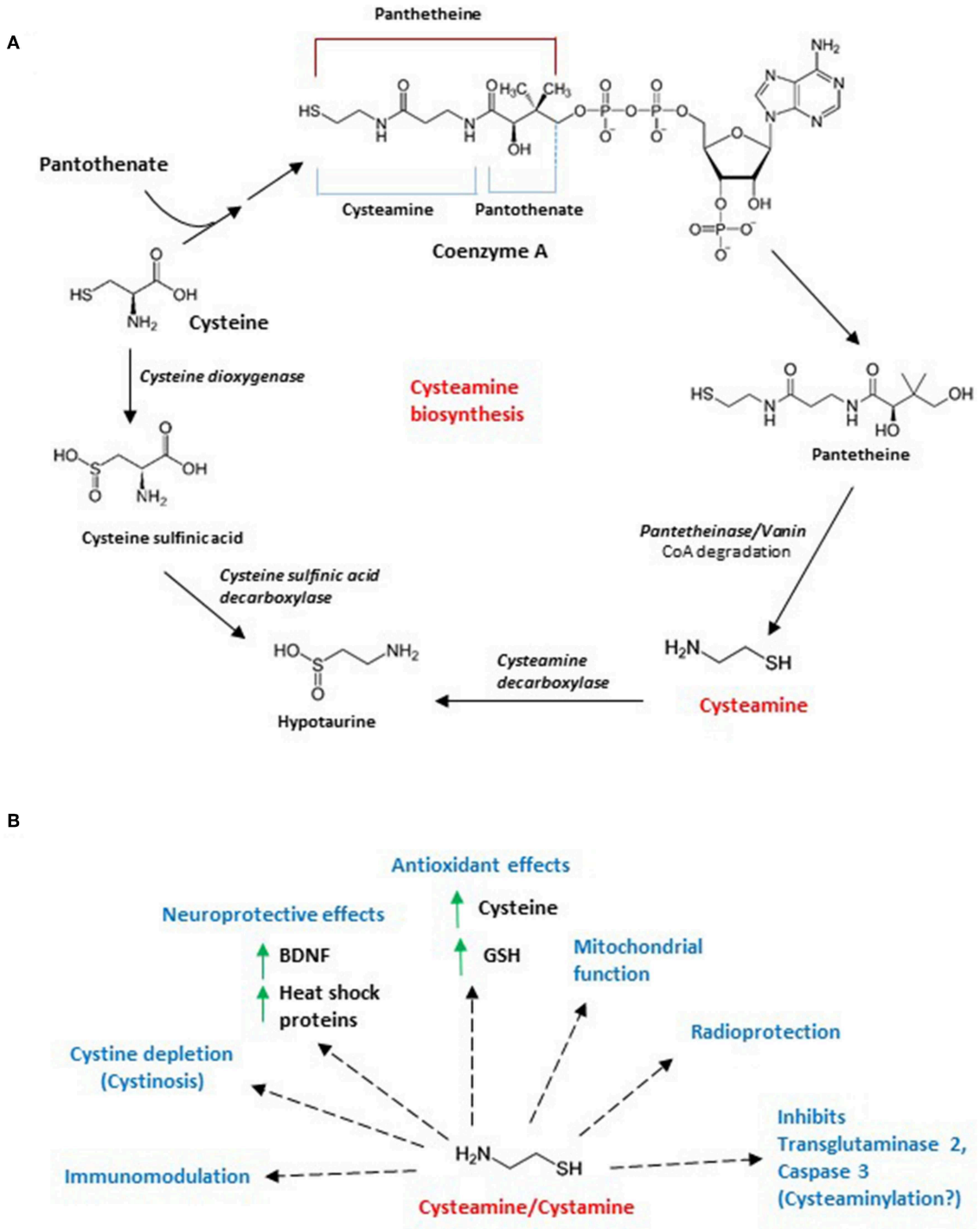

FIGURE 1 | (A) Biosynthesis of cysteamine and intersection with cysteine catabolism. Cysteamine is generated in mammals by the degradation of coenzyme A, which is required for the metabolism of fatty acids, carbohydrates, amino acids and ketone bodies. When coenzyme A is cleaved (cleavage at the dotted line), pantetheine is generated, which is acted on by pantetheinase or vanin to form cysteamine. Cysteamine is converted to hypotaurine by cysteamine decarboxylase. Cysteine, a component of coenzyme A, is acted on by cysteine dioxygenase to form cysteine sulfonate which is decarboxylated by cysteine sulfonate decarboxylase to form hypotaurine. Hypotaurine generated is further metabolized to taurine by hypotaurine decarboxylase. **(B)** Effects of cysteamine/cystamine. Both cysteamine and its oxidized form cystamine have protective effects in cells and tissues. Originally identified as radioprotective molecules, subsequently these aminothiols have been reported to mitigate cystinosis, a condition characterized by accumulation of cystine crystals in the body. Cystamine and cysteamine have a variety of other effects which include antioxidant effects (by increasing cysteine and glutathione levels), inhibition of transglutaminase 2 and caspase 3 (possibly by modifying reactive cysteine residues or cysteaminylation), modulation of mitochondrial function, immunomodulation. These molecules have also been reported to increase levels of brain derived neurotrophic factor (BDNF) and heat shock proteins, which affords neuroprotective benefits.

TABLE 1 | Neuroprotective actions of cysteamine/cystamine.

Cytoprotective effects of Cysteamine/Cystamine	System	References
Protection against glutamate-induced toxicity	Primary glial cells	(25)
Scavenges acrolein, a toxic metabolite generated during lipid peroxidation. Drug detoxification and polyamine oxidation	Cysteamine: Acetaminophen-induced hepatic injury in mice	(26)
Reduces oxidative stress and antioxidant balance in regulatory T cells	Cystamine: systemic lupus erythematosus (SLE)-prone mice	(27)
Improved membrane functionality, reduced lipid peroxidation and improved viability of sperm	Cysteamine: Cyropreserved Ram semen	(28)
Intraperitoneal injection of cystamine mediates neuroprotection by enhancing neuronal progenitor cell proliferation and proliferation through the BDNF pathway	Cystamine: mouse model of stroke	(29)
Dopaminergic neurodegeneration induced by MPTP is prevented by cysteamine and cystamine	MPTP model of neurodegeneration	(30, 31)
Neuroprotection from 3-nitropropionic acid (3NP) toxicity by cystamine	Stimulates NF-E2 related factor 2 (Nrf2) signaling in cell culture and the 3-NP model of neurodegeneration in mice	(32)
Administration of cystamine confers protection against haloperidol-induced toxicity and ischemic brain injury	Mouse model	(33)
Aggregation of amyloid β_{1-42} (Aβ) in astrocyte cultures reduced by cystamine	Cultured astrocystes	(34)
Cystamine elevated L-cysteine levels in HD	R6/2 mouse model of HD and PC12 model of polyglutamine aggregation	(35)
Transglutaminase-induced aggregation of alpha-synuclein decreased by cystamine	*in vitro* and in COS-7 cells	(36)
Cystamine significantly extended survival, improved body weight and motor performance, and delayed the neuropathological sequela	R6/2 mouse model of HD	(37)
Cystamine increased viability of striatal progenitor cells harboring mutant huntingtin and prevented ROS formation in HD cells subjected to H2O2 and STS	$STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ striatal progenitor cell lines	(38)
Cysteamine and cystamine prevented the 3-NP-mediated decrease in cellular and mitochondrial GSH levels as well as mitochondrial depolarization	$STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ striatal progenitor cell lines	(39)
Cystamine extended survival, reduced associated tremor and abnormal movements and ameliorated weight loss. Increased the transcription of the chaperone HDJ1/Hsp40	R6/2 mouse model of HD	(40)
Cystamine significantly delayed the progression of ALS symptoms and reduced SOD1 oligomers and microglial activation	G93A mouse model of ALS, cell culture models	(41)
Cystamine prevents toxicity induced by aggregation of polyadenylate-binding protein nuclear 1	Mouse model of Oculopharyngeal muscular dystrophy (OPMD)	(42)
Cystamine modulates blood pressure and reduces hypertension	Spontaneously hypertensive rats	(43)
Cysteamine alleviates fibrosis and symptoms associated with chronic kidney disease (CKD)	Mouse models of CKD	(44)
Cysteamine suppresses cataract formation induced by selenite	Rats	(45)
Cystamine rescued behavioral deficits induced by 2,5-hexanedione by increasing BDNF and hsp70 expression	Rats	(46)

sulfide, which participates in a myriad of physiological processes (59–61). Cystamine, in combination with mithramycin, was also shown to be protective in the R6/2 model of HD (62). The beneficial effects of cysteamine led to clinical trials in HD (63). In addition, cystamine can augment levels of brain derived neurotrophic factor, BDNF, in mouse models of HD (64). More recently cysteamine was shown to counteract toxicity mediated by mutant huntingtin *in vitro* in primary neuron and iPSC models of HD although the exact molecular mechanism by which cytoprotection is conferred is still unknown (65).

Alzheimer's Disease

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and the most common form of dementia (66, 67). The molecular hallmarks of AD include increased load of amyloid plaques and neurofibrillary tangles, which affect multiple cellular processes. Numerous reports describe links between dementia and AD with amyloid deposits or tangles. Postmortem analysis of cognitively normal subjects have revealed increased amyloid plaques, a pathogenic signature of AD, but no dementia (68). Conversely, several diagnosed AD patients have no signs of neuritic plaques (69). Thus, the correlation between amyloid plaques and AD awaits further study (70). Regardless of these inconsistencies, it is clear that the brain has corrective mechanisms that delay cognitive decline and if harnessed, may stall neurodegeneration. The search for small molecules that stimulate neuroprotective signaling cascades may be beneficial. Cystamine and its derivatives are being evaluated as possible therapies for the disease. Chronic cysteamine treatment (daily injections for a period of 4 months) resulted in improvements in habituation and spatial learning deficits in the APP-Psen1 mouse model of AD (71). The APP-Psen1 model harbors the human transgenes for the Swedish mutation of the

amyloid precursor protein (APP) and presenilin-1 (PSEN1) containing an L166P mutation, regulated by the Thy-1 promoter (72). AD patients have elevated transglutaminase levels, which colocalize with the amyloid plaques (34). Transglutaminases accelerate amyloid beta aggregation and toxicity. Accordingly, cystamine therapy is being considered for lowering the amyloid plaque burden in AD patients. In particular, Multi-Target Directed Ligands (MTDLs) or single compounds which may simultaneously act on different targets are being explored. Along these lines, a cysamine-tacrine dimer has been developed, which decreased acetylcholinesterase (AChE)-induced beta-amyloid aggregation (73).

Parkinson's Disease

Aggregation of alpha-synuclein, leading to the formation of Lewy bodies, is a hallmark of Parkinson's disease (PD), which affects the substantia nigra of the brain causing motor deficits and multiple abnormalities. Existing therapies for PD largely target symptoms and do not mitigate neuronal loss observed. Several lines of evidence suggest the therapeutic potential of the aminothiol in PD (71). Cystamine ameliorated mitochondrial dysfunction and oxidative stress associated with 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced models of PD (74). In the MPTP-induced neurotoxicity model of PD in mice, independent studies revealed various effects of cystamine such as elevation in the levels of tyrosine hydroxylase and BDNF (30, 75). Similarly, cysteamine, the reduced form of cystamine, also afforded neuroprotection. Similar to AD, elevated transglutaminase activity caused an increase in the formation of cross-linked alpha-synuclein and insoluble aggregates, which could be abrogated by cystamine (36).

Amyotrophic Lateral Sclerosis

ALS, also known as Lou Gehrig's disease, is a neurodegenerative disease where selective degeneration of motor neurons in the brain and spinal cord occurs leading to paralysis of skeletal muscles and progressive weakness and atrophy of limbs (76). Difficulties in speech and movement follow and patients are typically wheelchair-bound. Causes of ALS can be either genetic or sporadic (refers to patients without a family history). Among the best studied genetic mutations in familial ALS include mutations in superoxide dismutase 1 (SOD1), which misfolds, aggregates, and elicit toxicity by multiple mechanisms (77, 78). Proposed reasons for SOD1 aggregation include crosslinking mediated by transglutaminase 2 (TG2). Studies with cell culture models of ALS reveal that cystamine prevents aggregation of SOD1 and improved cell survival (79). Furthermore, inhibiting spinal TG2 by cystamine reduces SOD1 oligomers, microglial activation and delayed progression in the G93A SOD1 mouse model of ALS (41). Thus, cystamine treatment may be beneficial in treating ALS.

Neurological Complications of Cystinosis

Although cystinosis was not considered to affect brain function, it is now known that cystinosis can result in neurocognitive deficits in adults as well as children. These include impaired visual spatial, visual memory, language problems, academic impairment, seizures, memory impairment, motor incoordination, and neuromuscular dysfunction and is often accompanied by structural abnormalities in the brain (80–82). Early treatment with cysteamine orally prevents several of these neurocognitive deficits. Patients with cystinosis treated at or after age 2 years (late-treatment group) score poorer than the early treatment group (before 2 years) on verbal, performance, and full-scale IQ tests and tests rating visual-spatial skills (83). Similarly, adults with cystinosis who receive consistent chronic treatment with cysteamine fare better on visual learning and memory skills (84).

Schizophrenia and Neuropsychiatric Diseases

Schizophrenia is a psychiatric disease, with complex genetic and neurological contributions of unclear origins, manifesting as a combination of symptoms which includes hallucinations, delusions, motivational and cognitive deficits (85). Although treatments for schizophrenia target psychotic symptoms, most existing drugs do not relieve social and cognitive deficits. The neurochemical changes in schizophrenia typically occur well before formal diagnosis, and, thus, preventive therapies could be beneficial. Schizophrenic patients have lower levels of BDNF so that schizophrenic patients might benefit from use of cysteamine due to its BDNF-enhancing properties and effects on the dopaminergic system (86, 87). In an amphetamine-induced psychosis model of schizophrenia, cysteamine prevents increased locomotor activity by decreasing dopamine release (88). Cysteamine counteracts the BDNF-lowering effects of haloperidol (89). The anti-depressant effect of cysteamine may also benefit other mental conditions (90). These studies are consistent with an earlier study which demonstrated that cysteamine blocked amphetamine-induced deficits in sensorimotor gating in male Sprague-Dawley rats (91). Similarly, cysteamine treatment increases BDNF levels in the frontal cortex and hippocampus and improved spatial memory in heterozygous reeler mice, which exhibit behavioral and neurochemical abnormalities similar to those in schizophrenia (92).

Similarly, cystamine and cysteamine may be beneficial in other conditions involving low neurotrophin levels, such as autism spectrum disorders (ASD). Analysis of postmortem human brain samples revealed increases in TG2 mRNA and protein levels in the middle frontal gyrus of subjects with autism spectrum disorder. Thus, cysteamine may alleviate symptoms of ASD by inhibiting TG2 and increasing BDNF levels (93). The same study demonstrated that ER stress induced TG2 expression and deficits in social behavior. Systemic administration of cysteamine attenuated these behavioral abnormalities. In mice lacking methyl-CpG binding protein 2 (MeCP2), a model of Rett syndrome, associated with decreased BDNF levels and obsessive compulsive phenotypes, cysteamine treatment improved lifespan, and improved motor function (94, 95). In a similar vein, cysteamine counteracted anxiety, and depression-like behaviors in a mouse model of anxiety/depression induced by chronic glucocorticoid exposure (96).

POTENTIAL SIDE-EFFECTS OF CYSTEAMINE AND CYSTAMINE

Although cysteamine and cystamine have several desirable effects in cells and tissues, some studies have reported side-effects. For instance, in the treatment of HD patients using cysteamine (Cystagon) in the CYTE-I-HD clinical trials, rashes, nausea, and motor impairment along with bad breath were observed in a few patients (63). In phase II trials, asthenia or fatigue was more commonly observed (97). Despite these side-effects, cysteamine appeared to be well tolerated by almost all of the patients.

CONCLUDING REMARKS

Some therapies using antioxidants have not yielded satisfactory outcomes in clinical trials (98–101). Several reasons have been attributed to the failure of such trials. Certain antioxidants inhibit fundamental cellular processes such as autophagy, which is crucial to eliminate misfolded proteins and damaged organelles (102). Most antioxidants utilized only target specific free radicals and thus may counteract only selected types of free radicals. Most clinical trials were initiated relatively late in disease progression, when most of the oxidative damage has already accrued. Doses of antioxidants utilized have also not been adequately tested. Durations of several of these trials have also been short, and longer term uses of redox active molecules have not been studied in detail. Thus, development of antioxidant molecules that have multiple targets, while not inhibiting basic cellular processes such as autophagy, is crucial. Cysteamine normalizes the proteostasis machinery by restoring BECN1/Beclin 1-dependent autophagy in cystic fibrosis in mouse models of the disease and also in patients (103). Cysteamine dendrimers have been found to ameliorate autophagy deficits in cystic fibrosis (104). It is evident that signaling pathways modulated by cystamine and cysteamine are diverse (**Figure 1B**), and knowledge of these cascades will yield information that can be harnessed to tailor treatments for diverse diseases. The tissue-specific effects and optimal concentrations of the thiol redox couple that would be beneficial for specific diseases has still not been elucidated. Although these aminothiols possess beneficial disease-modifying effects in several conditions, it is still unclear whether these molecules or their metabolites mediate the cytoprotection observed in neurodegenerative diseases. However, increase in cysteine levels can promote neuroprotection, and some of the beneficial effects can be attributed to increases in cysteine to mitigate oxidative stress as has been observed in HD (56). Similarly, systematic studies measuring the concentration and metabolism of cysteamine and cystamine in pathological conditions have not been conducted and are areas of future investigation. Epigenetic effects of cystamine and cysteamine and cysteaminylation, the posttranslational modification mediated by cystamine and cysteamine await detailed investigation. The use of cystamine and cysteamine is another example of a repurposed drug, which has cytoprotective effects in the brain. Combination therapy of these aminothiols with other approved drugs offer attractive options to arrive at safe and effective drugs for these complex diseases.

AUTHOR CONTRIBUTIONS

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Luteolin Exerts Neuroprotection *via* Modulation of the p62/Keap1/Nrf2 Pathway in Intracerebral Hemorrhage

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Upregulation of neuronal oxidative stress is involved in the progression of secondary brain injury (SBI) following intracerebral hemorrhage (ICH). In this study, we investigated the potential effects and underlying mechanisms of luteolin on ICH-induced SBI. Autologous blood and oxyhemoglobin (OxyHb) were used to establish *in vivo* and *in vitro* models of ICH, respectively. Luteolin treatment effectively alleviated brain edema and ameliorated neurobehavioral dysfunction and memory loss *in vivo*. Also, *in vivo*, we found that luteolin promoted the activation of the sequestosome 1 (p62)/kelch-like enoyl-coenzyme A hydratase (ECH)-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway by enhancing autophagy and increasing the translocation of Nrf2 to the nucleus. Meanwhile, luteolin inhibited the ubiquitination of Nrf2 and increased the expression levels of downstream antioxidant proteins, such as heme oxygenase-1 (HO-1) and reduced nicotinamide adenine dinucleotide phosphate (NADPH): quinine oxidoreductase 1 (NQO1). This effect of luteolin was also confirmed *in vitro*, which was reversed by the autophagy inhibitor, chloroquine (CQ). Additionally, we found that luteolin inhibited the production of neuronal mitochondrial superoxides (MitoSOX) and alleviated neuronal mitochondrial injury *in vitro*, as indicated *via* tetrachloro-tetraethylbenzimidazol carbocyanine-iodide (JC-1) staining and MitoSOX staining. Taken together, our findings demonstrate that luteolin enhances autophagy and anti-oxidative processes in both *in vivo* and *in vitro* models of ICH, and that activation of the p62-Keap1-Nrf2 pathway, is involved in such luteolin-induced neuroprotection. Hence, luteolin may represent a promising candidate for the treatment of ICH-induced SBI.

Keywords: intracerebral haemorrhage, luteolin, p62-Keap1-Nrf2 pathway, autophagy, antioxidant, oxidative stress

Abbreviations: ICH, intracerebral hemorrhage; SBI, secondary brain injury; SD, Sprague-Dawley; OxyHb, oxyhemoglobin; Co-IP, co-immunoprecipitation; PBS, phosphate buffer saline; LC3, microtubule-associated protein 1A/1B-light chain 3; p62, sequestosome 1; Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NADPH, quinine oxidoreductase 1; CQ, chloroquine; ARE, antioxidant response element; ROS, reactive oxygen species; JC-1, tetrachloro-tetraethylbenzimidazol carbocyanine iodide; MitoSOX, mitochondrial superoxide; HRP, horseradish peroxidase; CB, the cerebellum; Ipsi-CX, ipsilateral cortex; Ipsi-BG, the ipsilateral basal ganglia; Cont-BG, the contralateral basal ganglia; Cont-CX, the contralateral basal cortex; PVDF, polyvinylidene difluoride; DCF-DA, 2,7-dichlorofluorescein diacetate.

INTRODUCTION

Intracerebral hemorrhage (ICH) is an important public health problem that has aroused worldwide concern due to its high mortality and morbidity rates (Qureshi et al., 2009). In addition to primary brain injury that disrupts the physical structure of brain tissue, ICH-induced secondary brain injury (SBI) often leads to severe neurological deficits or even death (Xi et al., 2006). Since there has only been minimal progress in the clinical management of ICH, treatment of patients with acute ICH has remained as a challenge for doctors (Law et al., 2017). Therefore, further research is needed for the discovery and development of novel efficacious treatments. There are many pathophysiological changes that have been demonstrated to participate in the process of SBI, including hemoglobin-induced iron overload, oxidative stress, inflammation, cell apoptosis, autophagy, mitochondrial dysfunction, and blood–brain–barrier disruption (Zhou et al., 2014; Duan et al., 2016).

Oxidative stress plays a significant role in ICH-induced SBI. Oxidative stress is involved in pathophysiological processes at multiple stages after ICH (Aronowski and Zhao, 2011). Nuclear factor erythroid-related factor 2 (Nrf2) has been demonstrated to be an important transcription factor that participates in the regulation of oxidative stress and in ameliorating brain damage (Wang et al., 2007; Xu et al., 2017; Zeng and Chen, 2017). Under unstressed states, Nrf2 interacts with its inhibitor, kelch-like enoyl-coenzyme A hydratase (ECH)-associated protein 1 (Keap1), to remain in the cytoplasm. Under conditions of oxidative stress, Nrf2 disassociates from Keap1 and translocates to the nucleus to activate the antioxidant response element (ARE), which leads to an increase in the expression of downstream protective proteins such as heme oxygenase-1 (HO-1) and reduced nicotinamide adenine dinucleotide phosphate (NADPH):quinine oxidoreductase-1 (NQO1) (Wang et al., 2018a).

As a lysosomal degradative pathway, autophagy is essential for survival and maintaining cellular homeostasis. In addition, autophagy is involved in diverse diseases and injuries (Jiang et al., 2015), including the pathological processes during ICH (Duan et al., 2017; Li et al., 2018c). Moreover, recent studies have demonstrated that oxidative stress contributes to autophagy (Duan et al., 2016). Additionally, by engulfing or degrading oxidative-stress products, autophagy may have positive effects on reducing oxidative damage (Filomeni et al., 2015), such as *via* reactive oxygen species (ROS)/Nrf2/p62 autophagy (Jiang et al., 2015). As a form of microtubule-associated protein 1A/1B-light chain 3 (LC3), the amount of LC3II is greatly correlated with the formation of autophagosomes and is considered to be an indicator of the extent of autophagy (Kabeya et al., 2000).

As a member of the flavonoid family, luteolin has been shown to exhibit multiple pharmacological effects, such as antioxidative, anti-inflammatory, autophagic-regulatory, apoptotic, and antitumor effects in many disease models (Xiong et al., 2017; Luo et al., 2019; Ma et al., 2019; Yu et al., 2019). Several studies have shown that luteolin exerts neuroprotective effects both *in vitro* and *in vivo* (Xu et al., 2014; Caltagirone et al., 2016; Kwon, 2017; Luo et al., 2019). However, the effects of luteolin on ICH remain poorly understood.

In this study, we investigated the neuroprotective effects of luteolin in ICH-induced SBI, including potential underlying mechanisms related to regulation of antioxidative processes and autophagy. Moreover, we aimed to assess whether luteolin may represent a potential therapeutic candidate for treating ICH.

MATERIALS AND METHODS

Animals

For all pharmacological experiments *in vivo*, adult male Sprague–Dawley rats (250 to 300 g) were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). The rats had access to water and food *ad libitum* and were group-housed under a 12-h light/dark cycle in animal rooms that had controlled temperature (22 ± 3°C) and humidity (60 ± 5%). All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. All protocols were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Animals.

Reagents

Anti-HO-1 (ab13243), anti-Nrf2 (ab89443), anti-NQO1 (A18; ab28947), anti-histone H3 (ab1791), anti-ubiquitin (ab7780), anti-SQSTM1/p62 (ab56416), and anti-Keap1 antibodies (ab139729) were purchased from Abcam (Cambridge, MA, USA). Anti-β-actin antibody (sc-376421) and normal mouse immunoglobulin G (IgG) (sc-2025) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-tubulin (2128L) and anti-LC3B (2775) antibodies were purchased from Cell Signaling Technology (Beverly MA). Protein A + G agarose (P2012), mitochondrial membrane potential assay kits with tetrachloro-tetraethylbenzimidazol carbocyanine iodide (JC-1) (C2006), and ROS assay kits (S0033) were obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). Mitochondrial superoxide (MitoSOX) Red MitoSOX indicator for live-cell imaging (M36008) was purchased from Thermo Fisher Scientific (USA). Luteolin (T1027) was purchased from TargetMol (USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-rabbit IgG, HRP-linked antibody (7074S), anti-mouse IgG, and HRP-linked antibodies (7076S) were from Cell Signaling Technology (Beverly, MA).

Induction of Intracerebral Hemorrhage

As previously described (Meng et al., 2018), a rat model of ICH was established by injecting 100 μl of autologous blood into the brain of each rat. First, Sprague-Dawley rats were intraperitoneally anesthetized with 4% chloral hydrate and were then mounted onto a stereotactic apparatus (Shanghai Ruanlong Science and Technology Development Co., Ltd., China). After exposing the scalp, we drilled a small hole above the right basal ganglia (1.5 mm posterior to bregma, 3.5 mm

lateral to the midline). Then, autologous whole blood, which was collected by cardiac puncturing, was injected slowly (5.5 mm ventral to the cortical surface, at 20 μl/min) with a microliter syringe (Hamilton Company, NV, USA) into the stereotaxically positioned hole above the right basal ganglia. The needle was required to stay in place for 5 min to prevent reflux. Finally, scalp was sutured. Representative brain slices from each group are shown in **Figure 1A**.

Experimental Design

Part I: Potential Effects of Luteolin on Intracerebral Hemorrhage-Induced Secondary Brain Injury in Rats

In this set of experiments, 96 rats (109 rats were used, but only 96 rats ultimately survived) were randomly and equally divided into the following six groups (n = 16 per group): sham group, ICH group, ICH + vehicle group, and three ICH + luteolin treatment groups (i.e., 5, 10, and 20 mg/kg). Rats in the sham group were intracerebrally injected with physiological saline solution (100 μl) into the right basal ganglia at 20 μl/min, after which the microliter syringe stayed in the place for 5 min to prevent reflux. Then, bone wax was used to seal the burr hole and the skin incision was disinfected and sutured, similar to the procedure for rats in the ICH group. ICH-operated rats were injected with 100 μl of autologous whole blood into the right basal ganglia as mentioned above. Luteolin and vehicle [dimethylsulfoxide (DMSO)] were injected intraperitoneally at different intervals (10 min, 24 h, and 48 h after ICH) according to the prescribed dose. First, stock solution of luteolin was prepared. We dissolved 50 mg of luteolin into 1 ml of DMSO to make the stock solution. Next, we diluted the stock solution into the corresponding doses with phosphate buffer saline (PBS). Subsequently, we administered intraperitoneal injections at 10 min, 24 h, and 72 h after surgery. At 24 h after ICH, 10 rats per group were tested for behavioral impairments. At 72 h after ICH, six rats per group were euthanized and their brain tissues were used for detection of brain edema. Finally, another six rats were tested in the Morris water maze on the third, fourth, fifth, and sixth day after surgery to assess changes in cognition (**Figure 1B**).

Part II: Potential Mechanisms of Luteolin on Intracerebral Hemorrhage-Induced Secondary Brain Injury *In Vivo*

In this set of experiments, we used a total of 86 rats, among which 72 rats ultimately survived. The surviving 72 rats were randomly divided into six groups with six rats in each group (consistent with the groupings of Part I above). Brain tissues were collected at 24 h after surgery for Western blotting, and coimmunoprecipitation (Co-IP) analysis (**Figure 1C**).

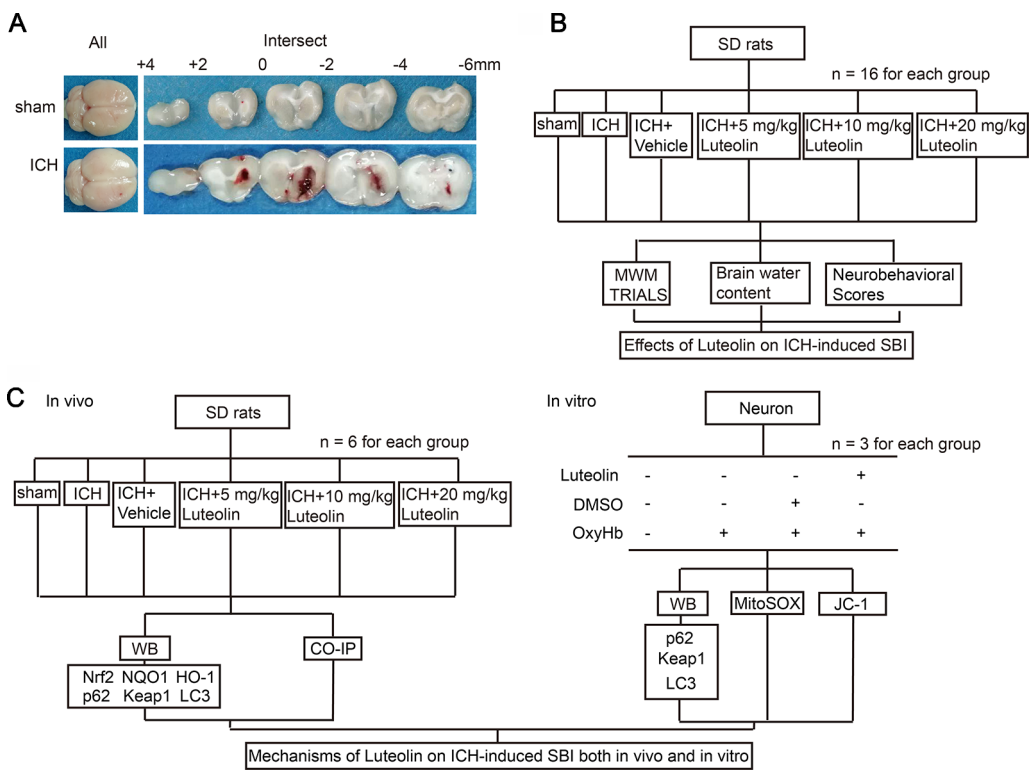

FIGURE 1 | Models of intracerebral hemorrhage (ICH) and experimental designs. **(A)** Coronal brainsections of rats in the sham and ICH groups. **(B)** Effects of luteolin on ICH-inducedsecondary brain injury (SBI) *in vivo*. **(C)** Potential mechanisms of luteolin on ICH-induced SBI both *in vivo* and *in vitro*.

Part III: Potential Mechanisms of Luteolin on Oxyhemoglobin-Induced Secondary Brain Injury *In Vitro*

In this set of experiments, primary neurons were cultured and oxyhemoglobin (OxyHb), as a common irritant, was applied to emulate ICH pathophysiology *in vitro*. Primary neurons were treated with different concentrations of luteolin (5, 10, and 20 μM) and OxyHb (10 μM) for 24 h. Finally, Western blotting, JC-1 staining, and MitoSOX staining were performed to assess potential mechanisms of luteolin on OxyHb-induced SBI.

Neurobehavioral Tests

The effects of luteolin on ICH-induced behavioral impairments were examined by monitoring appetite, locomotor activity, and neurological defects in Sprague-Dawley rats with a scoring system that has been previously published (Li et al., 2018b); **Table 1**). At 24-h post-ICH, 10 rats per group were tested and the data were collected by two investigators blind to the experimental design.

Brain Water Content

As described in a previous study, at 72 h after ICH brain water content was detected by the dry and wet method (Wang et al., 2018b). In brief, at 72 h after ICH induction, the brain of each rat was harvested immediately. Then the harvested brain tissue was subdivided into the following five parts: cerebellum (CB), ipsilateral cortex (Ipsi-CX), ipsilateral basal ganglia (Ipsi-BG), contralateral basal ganglia (Cont-BG), and contralateral cortex (Cont-CX). The wet weight was recorded immediately after the tissues were weighed with an electronic analytical balance. Subsequently, the dry weight was measured after the samples were dried in a thermostatic drier at 100°C for 72 h. Brain water content was calculated with the following formula: (wet weight – dry weight)/wet weight × 100%.

Morris Water Maze

As described previously (Shen et al., 2015), the Morris water maze was performed to assess cognitive function in rats. In short, the rats were trained for 3 days (four trials per day) before the ICH surgery was performed. At 3 to 6 days postsurgery (four trials per day), Sprague-Dawley rats were tested in the Morris water maze.

TABLE 1 | Neurobehavioral tests.

Category	Behavior	Score
Appetite	Finished meal	0
	Left meal unfinished	1
	Scarcely ate	2
Activity	Walked and reached at least three corners of the cage	0
	Walked with some stimulation	1
	Almost always lying down	2
Deficits	No deficits	0
	Unstable walking	1
	Unable to walk	2

The depth of the water tank was half a meter and the diameter was 180 cm. First, the tank was filled with water (20–22°C) to a height of 30 cm, after which ink was added to the water. Black-corded fabric was used to wrap the target platform. The platform, which was 10 cm in diameter, was positioned at 2 cm beneath the surface of the water. The starting location of the rat was altered with each new trial. Moreover, the visual points of reference were kept unchanged around the pool. Each trial was terminated when the rat found the platform or when the trial had lasted for 59 s. Rats were allowed to rest for 20 s on the platform after each trial. During the training phase, the rats were given 1 min to find the platform in the pool. If the rats failed to find the platform, we then guided them to the platform with a rod. The rats were allowed to stay at the platform for 20 s to strengthen their memory before they were removed. The swimming path length, latency, and speed to find the platform for each trial were automatically recorded on a computer. The parameters were used to evaluate learning/memory abilities and cognitive function.

Western Blotting

After induction of ICH for 24 h, brain samples from the right basal ganglia of each rat were collected and homogenized. Both the brain samples collected and extracted cells (for *in vitro* experiments) were lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). After centrifugation at 12,000 rpm at 4°C for 15 min, the supernatant from each sample was collected. Subsequently, we measured protein concentrations *via* a bicinchoninic (BCA) protein assay kit (Beyotime Institute of Biotechnology). After mixing each sample with sodium dodecyl sulfate (SDS) sample buffer, the protein samples were boiled for 5 min at 100°C. After being separated in a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel, the protein samples (30 μg per lane) were electrophoretically transferred to a polyvinylidene-difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA), which was then blocked with non-fat milk in PBS-Tween 20 (PBST) for 1 h at room temperature. The membrane was then incubated with primary antibodies overnight at 4°C. The titers of antibodies were as follows: anti-HO-1 antibody (ab13243, 1:1,000 dilution), anti-Nrf2 antibody (ab89443, 1:1,000 dilution), anti-NQO1 antibody (A180; ab28947, 1:1,000 dilution), anti-SQSTM1/p62 antibody (ab56416, 1:1,000 dilution), anti-Keap1 antibody (ab139729, 1:1,000 dilution), LC3B antibody (Cell Signaling Technology, 2775s, 1:1,000 dilution), and anti-ubiquitin antibody (ab7780, 1:1,000 dilution). Furthermore, anti-β-tubulin antibody (Cell Signaling Technology, 2128L, 1:1,000 dilution), anti-histone H3 antibody (ab1791, 1:1,000 dilution), and anti-β-actin antibody (sc-376421, 1:500 dilution) served as loading controls. On the next day, after being washed with PBST (PBS + 0.1% Tween 20), each membrane was incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, after which each membrane was subsequently washed three times with PBST. Protein bands were then revealed *via* an enhanced chemiluminescence (ECL) kit (Beyotime), and protein bands were analyzed *via* ImageJ software (NIH, Bethesda, MD, USA).

Nuclear and Cytoplasmic Protein Extractions

Nuclear and cytoplasmic proteins were extracted with a nuclear and cytoplasmic protein extraction kit (P0027, Beyotime) according to the manufacturer's instructions.

Ubiquitin Analysis

First, the collected brain samples were lysed in ice-cold RIPA lysis buffer. Then the total protein samples were incubated with 1 μg of anti-Nrf2 antibody or IgG (negative control) overnight at 4°C with agitation. Subsequently, the immune complex was incubated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 4 h and was then precipitated under rotary agitation. Finally, the immunoprecipitated proteins were analyzed by SDS–PAGE and immunoblotting with specific antibodies, including anti-Nrf2 and anti-ubiquitin antibodies.

Cell Culture

As described previously (Sun et al., 2018), primary rat cortical neurons were isolated from 17-day-old rat embryos. In short, we separated the meninges and blood vessels of the brains, which were subsequently rinsed with PBS. Subsequently, the harvested brain tissue was digested with 0.25% trypsin at 37°C for 5 min. The digested brain tissues were then washed with PBS and the resultant brain suspension was centrifuged at 1,500 rpm for 5 min. The resuspended cells were inoculated into 6-well and 12-well plates that were precoated with poly-D-lysine (Sigma, USA). Regarding the inoculation density, we inoculated $2*10^6$ neurons per well into the 6-well plates, and inoculated $1*10^5$ neurons per well into 12-well plates. Subsequently, the dissociated cortical neurons were cultured in Neurobasal medium (Gibco, Carlsbad, CA, USA) that was supplemented with 0.5 mM of GlutaMAX, 2% B-27, 50 U/ml of streptomycin, and 50 U/ml of penicillin (Invitrogen, Grand Island, NY, USA). Finally, the neurons were placed in an incubator at a constant temperature of 37°C and with humidified air containing 5% CO_2. We changed half of the culture medium every 2 days for 1 week, after which the neurons were harvested for subsequent assays.

Intracerebral Hemorrhage Models *In Vitro*

We established an *in vitro* of ICH model by using OxyHb which stimulates neurons and induces pathophysiological changes in neurons that are similar to those from ICH. OxyHb (10 μM) was added in Neurobasal medium to stimulate neurons for 24 h at 37°C in 5% CO_2.

Tetrachloro-Tetraethylbenzimidazol Carbocyanine Iodide Staining

The mitochondrial membrane potential assay kit was used to detect changes in the mitochondrial membrane potential of neurons, while JC-1 staining was used as an indicator of mitochondrial damage (Beyotime, China), both of which were used according to the manufacturer's protocol. After being washed with PBS, pretreated neurons were incubated with 1 ml of JC-1 working solution per sample at 37°C for 20 min. Then neurons were washed twice with JC-1 staining buffer. After adding 4′,6-diamidino-2-phenylindole (DAPI) (DAPI Fluoromount-G, SouthernBiotech, USA), we observed the neurons under a fluorescent microscope (Wang et al., 2018b).

Measurement of Mitochondrial Superoxide

After being treated with OxyHb (10 μM) to mimic ICH *in vitro*, luteolin (10 μM) or vehicle was added into the medium of primary neurons. After 24 h, we firstly prepared the stock solution of 5-mM MitoSOX reagent. Then, 13 μl of DMSO was added to a vial of MitoSOX Red MitoSOX indicator (Thermo Fisher Scientific, USA) containing 50 μg of content. Then, 5-μM MitoSOX reagent working solution was made by diluting the stock solution of 5-mM MitoSOX reagent (mentioned above) with PBS. The neurons of all groups were covered with 5-μM MitoSOX reagent working solution and were incubated at 37°C for 10 min in the dark. Then neurons were mounted in PBS for analysis and imaging after being washed three times with warm PBS.

Statistical Analysis

We used GraphPad Prism 6 to perform statistical analyses of all experimental data. In addition to neurobehavioral scorings, which are expressed as the median with the interquartile range, all other data are expressed as the mean ± standard deviation (SD). The Mann-Whitney U test was used to analyze neurobehavioral scorings. For all other data, one-or two-way analyses of variance (ANOVAs) were applied to determine significant differences among more than two groups, and we used Tukey's *post-hoc* tests to determine pairwise differences among the groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Luteolin Attenuates Intracerebral Hemorrhage-Induced Secondary Brain Injury *In Vivo*

To evaluate the effect of luteolin on brain injury following ICH, autologous blood was injected into the basal ganglia of rats. Coronal brain sections are shown in **Figure 1A**. Behavioral testing was performed at 24 h after ICH. Damage of neurobehavioral abilities of the ICH group was significantly more severe than that of the sham group, and this impairment was partly alleviated after intraperitoneal injection of 10 mg/kg or 20 mg/kg of luteolin for 24 h (**Figure 2A**). We found that sham group *vs.* ICH group, $Z = -4.077$, $P < 0.0001$; ICH + vehicle group *vs.* ICH + 5 mg/kg luteolin group, $Z = -0.390$, $P = 0.8471$; ICH + vehicle group *vs.* ICH + 10 mg/kg luteolin group,

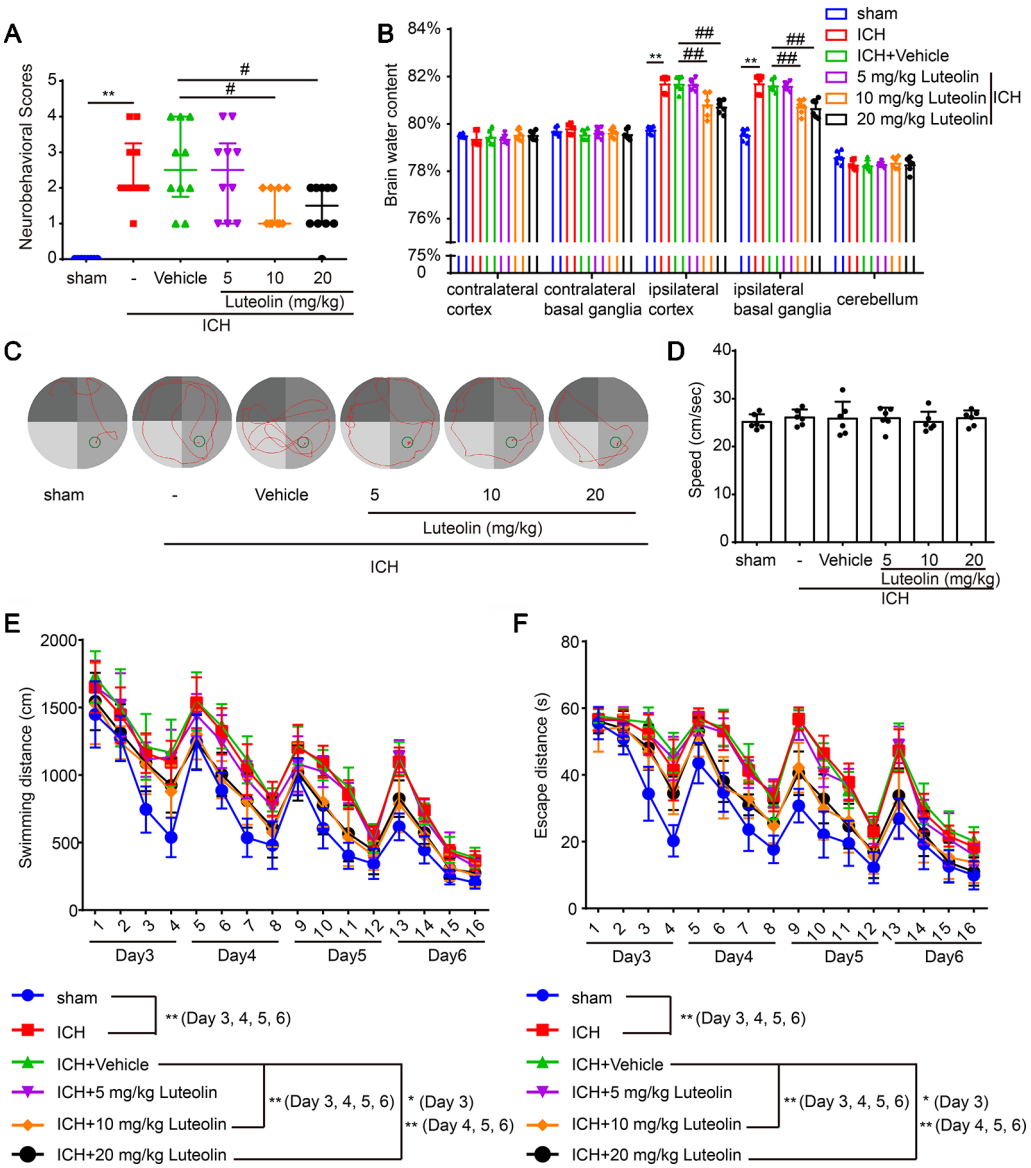

FIGURE 2 | Luteolin ameliorates intracerebral hemorrhage (ICH)-induced neuronal injury. After injection of autologous blood, Sprague-Dawley rats were treated with luteolin (5, 10, 20 mg/kg) or vehicle. **(A)** The neurological scores of rats in the six groups were evaluated and resultant scores are reported in **Table 1** (**p < 0.01 *vs.* sham group; #p < 0.05 *vs.* ICH + vehicle group; n = 10). **(B)** The effects of luteolin on brain water content were examined. All data are shown as the mean ± SD (**p < 0.01 *vs.* sham group; ##p < 0.01 *vs.* ICH + vehicle group; n = 10). **(C–F)** Effects of luteolin treatment on cognitive behavioral impairments induced by autologous blood were tested *via* the Morris water maze. **(C)** Representative swimming-path traces of the rats in each group are displayed. **(D)** Swimming speed at the beginning of the test (third-day postsurgery), **(E)** distances and **(F)** escape latencies from four trials per day for a total of 4 days are shown. The values are shown as the mean ± SD (*P < 0.05, **P < 0.01; n = 6).

Z = −2.403, P = 0.0234; ICH + vehicle group *vs.* ICH + 20 mg/kg luteolin group, Z = −2.262, P = 0.024.

Then, we measured brain water content to assess the effect of luteolin on brain edema after ICH. We found that the brain water content was significantly higher in the ICH group compared with that in the sham group in the Ipsi-BG and Ipsi-CX. However, the rise of brain water content in these brain regions was inhibited *via* luteolin (10 or 20 mg/kg). In contrast, there were no significant differences in the brain water content within the Cont-BG, Cont-CX, or CB among the six experimental groups (**Figure 2B**).

In addition, to examine the role of luteolin in cognitive changes induced by ICH, rats were tested in the Morris water maze test (**Figures 2C–F**). Longer escape latencies and swimming distances were observed in rats from the ICH group compared with these parameters in the sham group. In

contrast, there were no significant differences in these parameters among the ICH group, and ICH + vehicle group, or ICH + 5 mg/kg luteolin group. However, data from rats in the ICH + 10 mg/kg luteolin group and ICH + 20 mg/kg luteolin group demonstrated that the ICH-induced increases in escape latencies and swimming distances were partially ameliorated *via* luteolin treatments (**Figures 2E, F**). For latencies, the following results were found: third-day postsurgery, $F(3, 120) = 88.48$, $P < 0.0001$; on the fourth-day postsurgery, $F(3, 120) = 157.4$, $P < 0.0001$; on the fifth-day postsurgery, $F(3, 120) = 139.6$, $P < 0.0001$; and on the sixth-day postsurgery, $F(3, 120) = 112.4$, $P < 0.0001$. For swimming distance, the following results were found: on the third-day postsurgery, $F(3, 120) = 72.53$, $P < 0.0001$; on the fourth-day postsurgery, $F(3, 120) = 119.2$, $P < 0.0001$; on the fifth-day postsurgery, $F(3, 120) = 115.7$, $P < 0.0001$; and on the sixth-day postsurgery, $F(3, 120) = 279.9$, $P < 0.0001$. Additionally, we found there were no significant differences in the swimming speed of all the groups at the beginning of the test (third-day postsurgery) (**Figure 2D**). Overall, luteolin exerted a partial rescuing effect on the brain injury induced by ICH, and this effect was evident at doses of 10 and 20 mg/kg.

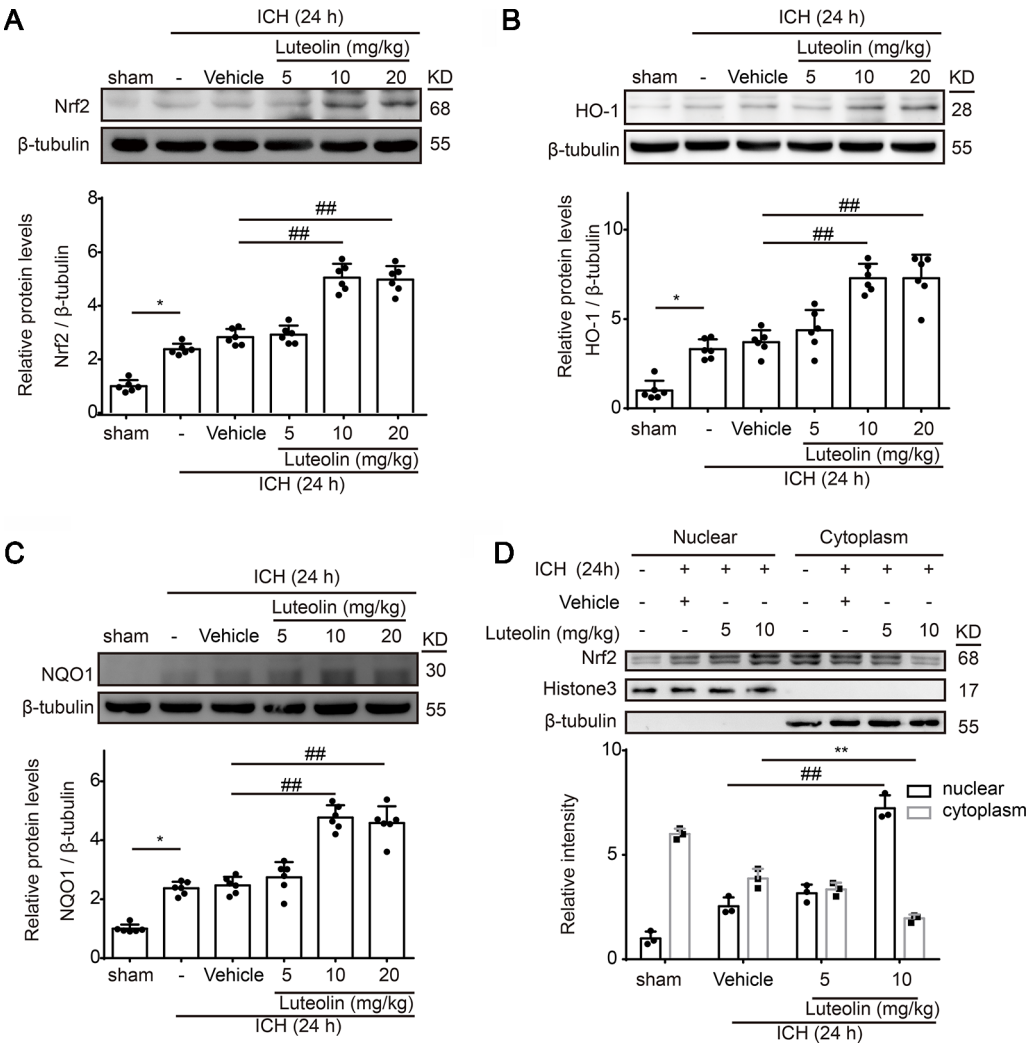

FIGURE 3 | Luteolin treatment promotes intracerebral hemorrhage (ICH)-induced activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway and enhances Nrf2 nuclear translocation. After injecting of autologous blood and luteolin (5, 10, 20 mg/kg), we extracted brain tissue proteins in each group at 1 day after ICH. **(A–C)** Protein levels of Nrf2, heme oxygenase-1 (HO-1), and reduced nicotinamide adenine dinucleotide phosphate (NADPH):quinine oxidoreductase 1 (NQO1) were examined by Western blotting. In the quantitative analysis of protein levels, the mean values of proteins in the corresponding sham groups were normalized to 1.0. Data are presented as the mean ± SD (*$p < 0.05$ *vs.* sham group and ##$p < 0.01$ *vs.* ICH + vehicle group; n = 6). **(D)** Western-blot analysis of Nrf2 in the nucleus and cytoplasm at 24 h after ICH. Relative protein levels are shown. H3 and β-tubulin served as loading controls. All data are shown as the mean ± SD [##$p < 0.01$ *vs.* ICH + vehicle group (nuclear); **$p < 0.01$ *vs.* ICH + vehicle group (cytoplasmic); n = 3]. Full images for Western blots in figures were shown in **Supplementary Material**.

Luteolin Promotes Activation of the Nrf2 Pathway and Enhances Nrf2 Nuclear Translocation Following Intracerebral Hemorrhage *In Vivo*

To explore the effects of luteolin on the Nrf2 signaling pathway after ICH, at 24 h after ICH, we detected the protein levels of both Nrf2 and downstream antioxidative proteins of Nrf2 (HO-1 and NQO1) *via* Western blotting. At 24 h after ICH Nrf2 levels were not significantly elevated compared to those of the sham group; however, treatment with luteolin significantly elevated Nrf2 levels at 24 h after ICH (**Figure 3A**). We obtained similar results when we detected the protein levels of HO-1 and NQO1 (**Figures 3B, C**). The promotion effect was only apparent when the dose of luteolin reached 10 and 20 mg/kg. Moreover, to further explore the mechanisms of luteolin on regulating the Nrf2 signaling pathway, we evaluated Nrf2 nuclear translocation by extracting and assaying nuclear and cytoplasmic proteins. As shown in **Figure 3D**, after ICH, nuclear Nrf2 protein levels were increased and corresponded to concomitantly decreased levels of cytoplasmic Nrf2 protein compared to those in the sham group,

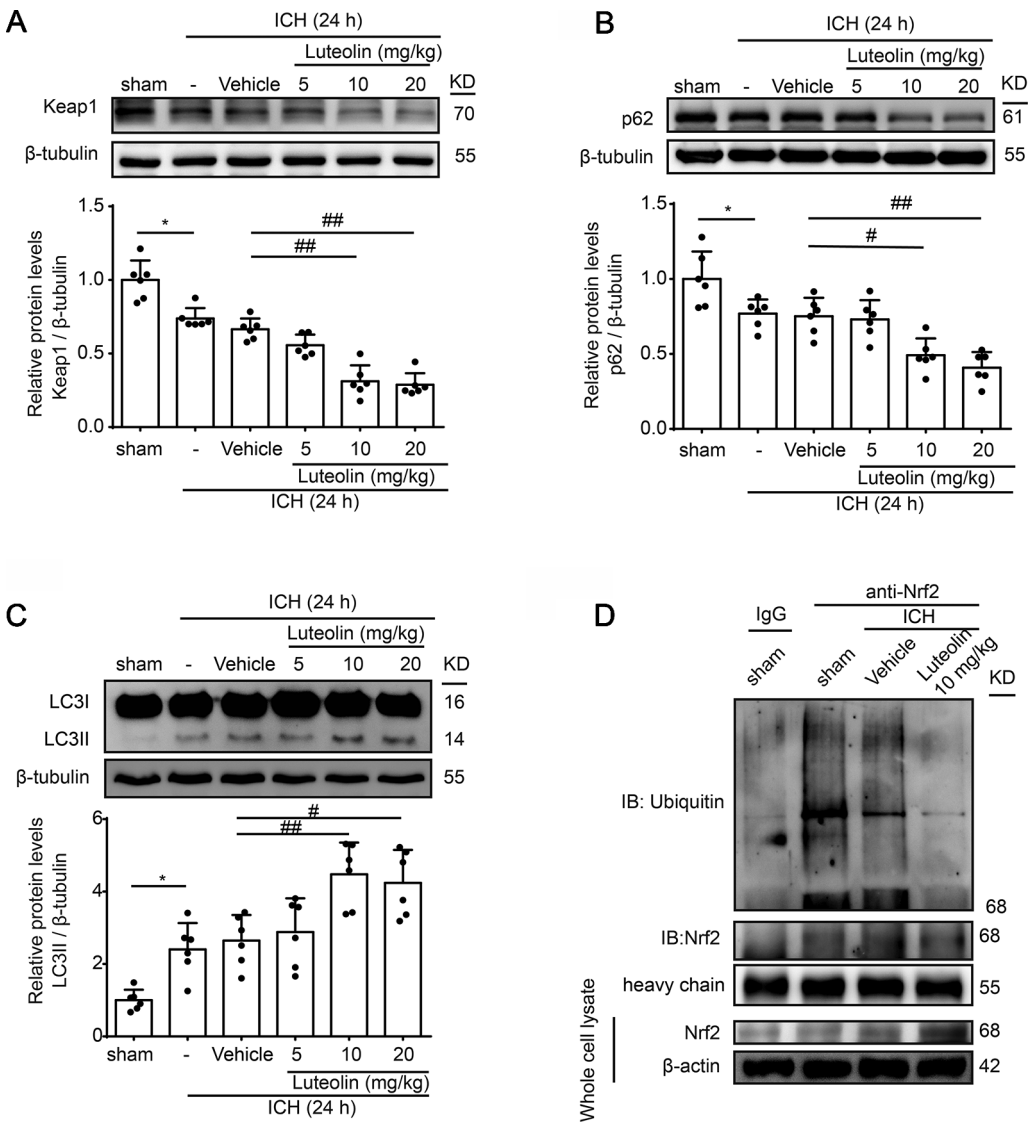

FIGURE 4 | Luteolin promotes autophagy to activate the sequestosome 1 (p62)/kelch-like enoyl-coenzyme A hydratase (ECH)-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and inhibits the ubiquitination of Nrf2 *in vivo*. Rats were subjected to intracerebral hemorrhage (ICH), and were then injected with luteolin (5, 10, 20 mg/kg) or vehicle. At 24 h after ICH, brain tissues were collected and Western-blot analysis was performed. The protein levels of Keap1 **(A)**, p62 **(B)**, and LC3 **(C)** were evaluated in the six groups. Data are presented as the mean ± SD (*P < 0.05 *vs.* sham group; #P < 0.05, ##P < 0.01 *vs.* ICH + vehicle group; n = 6). **(D)** The interaction between Nrf2 and ubiquitin *in vivo* was analyzed *via* coimmunoprecipitation. Ubiquitin was immunoprecipitated with the anti-Nrf2 antibody and immunoglobulin G (IgG) was used as a negative control. Luteolin inhibited the ubiquitination of Nrf2. Full images for Western blots in figures were shown in **Supplementary Material**.

and this effect was significantly amplified after administration of luteolin (10 mg/kg). These findings suggest that luteolin increased Nrf2 nuclear translocation to activate subsequent pathways at 24 h after ICH, possibly to induce anti-oxidative processes.

Luteolin Activates the P62-Keap1-Nrf2 Pathway and Enhances Autophagy After Intracerebral Hemorrhage by *In Vivo*

We next further explored the effects and possible mechanisms of luteolin on the Nrf2 signaling pathway after ICH. Here, we focused on Keap1, which is an important Nrf2 repressor that binds to Nrf2 in the absence of stimulation and is related to ubiquitination of Nrf2 to mediate proteasomal degradation. As shown in **Figure 4A**, the level of Keap1 in brain tissue was decreased at 24 h after ICH compared to that in the sham group but it was not obvious. However, the administration of medium and high doses of luteolin (10, 20 mg/kg) significantly decreased Keap1 levels compared to that in the ICH + vehicle group. p62, which is another type of autophagy-adaptor protein, has been documented to associate with Nrf2 signaling and autophagy *via* binding with Keap1. Finally, p62 sequesters Keap1 into autophagosomes for degradation during autophagy (Jiang et al., 2015). Hence, we next examined the expression of p62 as an indicator of autophagy. As is shown in **Figure 4B**, a lower expression of p62 was found in the ICH group compared with that in the sham group. In contrast, luteolin (10, 20 mg/kg) reduced the expression of p62 compared to that in the ICH + vehicle group. Next, we evaluated the level of LC3II, which is another autophagy-related marker. The expression of LC3II was increased at 24 h after ICH and the treatment of luteolin (10, 20 mg/kg) further increased LC3II expression. This finding suggests that luteolin enhanced autophagy and led to the activation of the downstream Nrf2 signaling pathway (**Figure 4C**).

Luteolin Protects Intracerebral Hemorrhage-Induced Injury *Via* Inhibition of Nrf2 Ubiquitination *In Vivo*

Reduced Nrf2 ubiquitylation has been recognized to enhance the stability of Nrf2 and to promote the activation of the Nrf2 signaling pathway (Jiang et al., 2015). Therefore, we examined the level of Nrf2 ubiquitination *via* Co-IP assays to further explore the mechanism of luteolin in influencing the Nrf2 signaling pathway. As shown in **Figure 4D**, the interaction between Nrf2 and ubiquitin was obvious in the sham group. Compared with that in the ICH + vehicle group, treatment with luteolin (10 mg/kg) inhibited the interaction between Nrf2 and ubiquitin.

Luteolin Ameliorates Oxyhemoglobin-Induced Mitochondrial Injury *In Vitro*

The Nrf2 signaling pathway has been recognized as a significant pathway for exerting antioxidative processes and downregulating the accumulation of ROS (Zeng and Chen, 2017). As an important indicator for assessing the level of oxidative-stress damage, we used the MitoSOX Red MitoSOX indicator to measure mitochondrial ROS. OxyHb was used to simulate ICH pathophysiology *in vitro* in cultured primary neurons. After being treated with OxyHb (10 μM), using a fluorescent microplate reader, we found increased ROS in the OxyHb group and OxyHb + vehicle group compared to that in the sham group, but this OxyHb-induced increase was inhibited *via* luteolin (10 μM) treatment (**Figures 5A, B**). JC-1 staining is an ideal fluorescent probe for examining changes in the mitochondrial membrane potential ($\triangle\Psi t$). In the absence of stimulation, JC-1 binds to the mitochondrial matrix in the form of J-aggregates, producing red fluorescence. As shown in **Figure 5C**, after treatment with OxyHb, a decrease in red fluorescent intensity and an increase in green fluorescence intensity were observed in the OxyHb group and the OxyHb + vehicle group, which indicated a loss of the mitochondrial membrane potential and openings of mitochondrial permeability transition pores (MPTPs). However, the administration of luteolin (10 μM) reversed such effects. In conclusion, luteolin reduced the production of mitochondrial ROS and played a significant role in mitochondrial protection following OxyHb.

Luteolin Protects Neurons From Oxyhemoglobin-Induced Injury *via* Activation of the P62/Keap1/Nrf2 Pathway *In Vitro*

To further investigate the role of luteolin in the p62/Keap1/Nrf2 pathway after ICH, we examined the protein levels of p62, Keap1, and LC3II *via* Western blotting of primary neurons *in vitro*. The expression levels of both p62 and Keap1 were decreased after treatment with OxyHb (10 μM), as compared with these levels in the control group. However, the protein levels of both p62 or Keap1 were significantly decreased following co-treatment with OxyHb (10 μM) and luteolin (10 μM), as compared with these levels following OxyHb (10 μM) + vehicle. In order to explore the potential mechanisms of luteolin in the correlation between the p62/Keap1/Nrf2 pathway and autophagy, chloroquine (CQ) —which is an autophagy inhibitor—was used. We found that the luteolin-induced decreases in the protein levels of p62 or Keap1 were reversed after the co-treatment with OxyHb (10 μM), CQ (30 μM), and luteolin (10 μM). Additionally, analysis of LC3II protein levels recapitulated this phenomenon. Pre-treatment with OxyHb (10 μM) and luteolin (10 μM) up-regulated the expression of LC3II, which suggested that there were elevated levels of autophagy, compared with those in the OxyHb (10 μM) + vehicle group. Moreover, this change was reversed *via* CQ (**Figures 6A–D**). In summary, we obtained similar results to those in our *in vivo* experiments, which confirmed the role of luteolin in promoting the activation of the p62/Keap1/Nrf2 pathway.

DISCUSSION

Over the past several decades, oxidative stress has been found to be involved in the pathogenesis and development of many

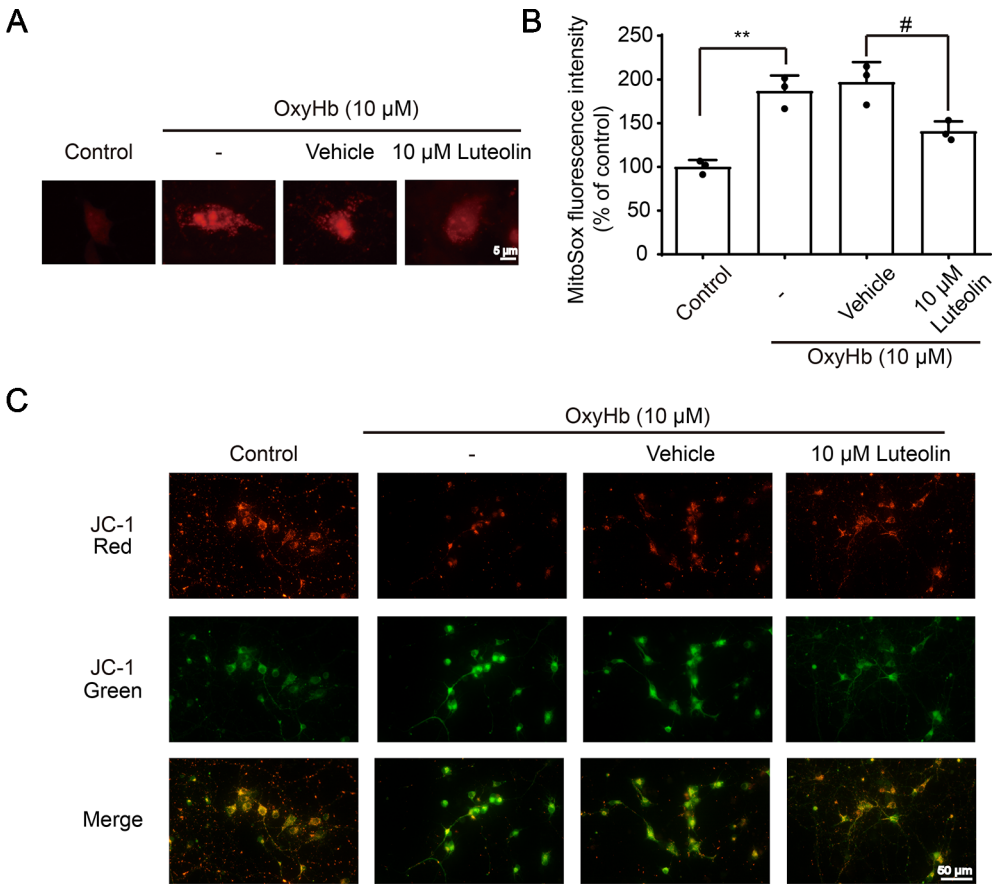

FIGURE 5 | Luteolin attenuates oxyhemoglobin (OxyHb)-induced increases in mitochondrial reactive oxygen species (ROS) and mitochondrial injury *in vitro*. Primary neurons were cultured and were incubated with or without OxyHb (10 μM) and/or co-incubation with luteolin (10 μM) for 24 h. Representative images of mitochondrial superoxide (MitoSOX) staining **(A)**, relative MitoSOX fluorescence intensities analysis **(B)**, and tetrachloro-tetraethylbenzimidazol carbocyanine iodide (JC-1) staining images **(C)** are shown, which indicated the levels of mitochondrial ROS and the degrees of mitochondrial injury. The scale bar = 5 μm **(A)**. Data are presented as the mean ± SD (**P < 0.01 vs. control group; #P < 0.05 vs. OxyHb [10 μM] + vehicle group; n = 3) **(B)**, whereas the scale bar = 50 μm **(C)**.

diseases, including ophthalmic diseases (Nishimura et al., 2017; Pinazo-Duran et al., 2018), diabetes (Rochette et al., 2018), cardiovascular diseases (Luscher, 2015; Munzel et al., 2015; Schiattarella and Hill, 2017), atherosclerosis (Forstermann et al., 2017), arthritis (Bala et al., 2017; Kardes and Karagulle, 2018), dermatological diseases (Rojo de la Vega et al., 2018), respiratory diseases (Hecker, 2018), hepatic diseases (Lee et al., 2019), urinary system diseases (Andersson, 2018), cancer (Poprac et al., 2017), neurodegenerative disorders (Jiang et al., 2016), and other nervous system diseases (D'Amico et al., 2013; Patel, 2016; Pei and Fan, 2017). Moreover, oxidative stress also participates in pathological processes after ICH (Aronowski and Zhao, 2011). Inhibition of oxidative stress has been demonstrated to improve the prognosis of ICH, ameliorate neurobehavioral impairments, and reduce brain edema (Wei et al., 2017; Zeng and Chen, 2017; Sosa et al., 2018; Wang et al., 2018b). Autophagy is involved in the pathophysiological processes of various diseases, as well as in ICH (He et al., 2008). In recent years, the crosstalk between autophagy and anti-oxidative processes has received considerable attention, and related studies have suggested that autophagy may enhance antioxidative processes in a variety of disease models (Giordano et al., 2014; He et al., 2017; Li et al., 2018a).

ICH exhibits high disability and mortality rates. Moreover, ICH has become a heavy burden for global health care systems and societies (Selim et al., 2019). Supportive medical care has represented the main treatment for ICH but has yielded an insufficient efficacy (Hanley et al., 2019). Numerous studies have been carried out in order to further investigate the mechanisms of ICH-induced SBI. Many kinds of recombinant proteins, compounds, drugs, and other agents—including recombinant complement component 1q (C1q)/tumor necrosis factor (TNF)-related protein 9 (rCTRR9), recombinant osteopontin (rOPN), isoliquiritigenin, andrographolide, and melatonin—have been reported to exert neuroprotective effects after ICH by alleviating brain injury, inhibiting neuronal apoptosis, suppressing oxidative stress, down-regulating inflammatory damage, and protecting the blood–brain barrier (Zeng and

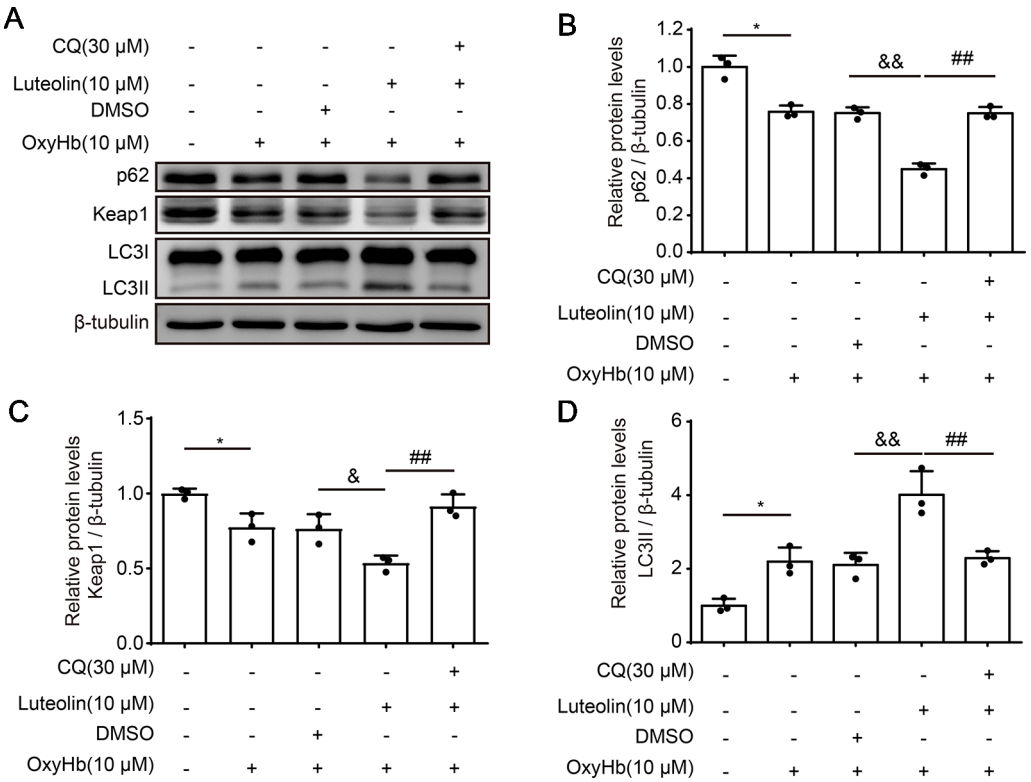

FIGURE 6 | Luteolin promotes the activation of the sequestosome 1 (p62)/kelch-like enoyl-coenzyme A hydratase (ECH)-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway *in vitro*. **(A)** Western blotting showing that compared to that in the control group, after oxyhemoglobin (OxyHb) stimulation, the protein level of p62 or Keap1 was decreased and the expression of LC3II was increased. Additionally, these changes were magnified with co-treatment of OxyHb (10 μM) and luteolin (10 μM). Moreover, the above changes were reversed *via* chloroquine (CQ). **(B)** Quantification of the protein levels of p62 in the various groups. Data are presented as the mean ± SD [$^{*}P < 0.05$ *vs.* control group; $^{\&\&}P < 0.01$ *vs.* OxyHb (10 μM) + vehicle group; $^{\#\#}P < 0.01$ *vs.* OxyHb (10 μM) + luteolin (10 μM) group; n = 3]. **(C)** Quantification of the expression of Keap1 in the various groups. Data are presented as the mean ± SD [$^{*}P < 0.05$ *vs.* control group; $^{\&}P < 0.05$ *vs.* OxyHb (10 μM) + vehicle group; $^{\#\#}P < 0.01$ *vs.* OxyHb (10 μM) + luteolin (10 μM) group; n = 3]. **(D)** Quantification of the relative levels of LC3II in the various groups. Data are presented as the mean ± SD [$^{*}P < 0.05$ *vs.* control group; &&P < 0.01 *vs.* OxyHb (10 μM) + vehicle group; $^{\#\#}P < 0.01$ *vs.* OxyHb (10 μM) + luteolin (10 μM) group; n = 3]. Full images for Western blots in figures were shown in **Supplementary Material**.

Chen, 2017; Gong et al., 2018; Li et al., 2018d; Wang et al., 2018b; Zhao et al., 2018). However, the protective effects of these neuroprotective agents are still lacking in clinical applications, and many such agents include problematic side effects. Therefore, there is a continued need to further identify and develop novel drugs that are both safe and efficacious in the treatment of ICH.

Luteolin is a member of the naturally occurring flavonoid family and has various beneficial bioactivities. Numerous studies have revealed anti-inflammatory, antioxidative, anti-apoptotic, autophagic-regulatory, anti-viral, anticancer, and metabolic effects of luteolin, which have been confirmed in many different disease models (Hu et al., 2016; Zhang et al., 2016; Peng et al., 2017; Du et al., 2018; Liu et al., 2018; Tan et al., 2018b; Yang et al., 2018b; Kang et al., 2019). In addition, in studies on ischemic stroke (Qiao et al., 2012; Tan et al., 2018a; Luo et al., 2019), traumatic brain injury (Xu et al., 2014), neurodegenerative diseases (Kwon, 2017; Zhang et al., 2017), and other neurological diseases, luteolin has been shown to exert therapeutic effects. Compared with other properties of agents, luteolin has a wide range of sources and is cost-effective. Moreover, because of its lipophilicity, luteolin is able to freely penetrate the blood-brain barrier even if it is administered peripherally (Sawmiller et al., 2014). However, to the best of our knowledge, the impact of luteolin on ICH-induced SBI has remained unclear. Hence, our study focused on this direction and attempted to elucidate any underlying mechanisms.

In this study, we demonstrated that luteolin enhanced the activation of the Nrf2 pathway and enhanced Nrf2 nuclear translocation after ICH. Nrf2 is known to regulate various antioxidant enzymes to protect cells against oxidative stress and is essential for the clearance of hematomas (Zhao et al., 2015; Kang et al., 2019). Numerous studies have demonstrated that activation of the Nrf2 signaling pathway is beneficial in alleviating ICH-induced SBI. Additionally, activation of the Nrf2 signaling pathway has been suggested to be an underlying mechanism related to the efficacies of other agents in ICH treatment (Lan et al., 2017; Wei et al., 2017; Zeng and Chen, 2017).

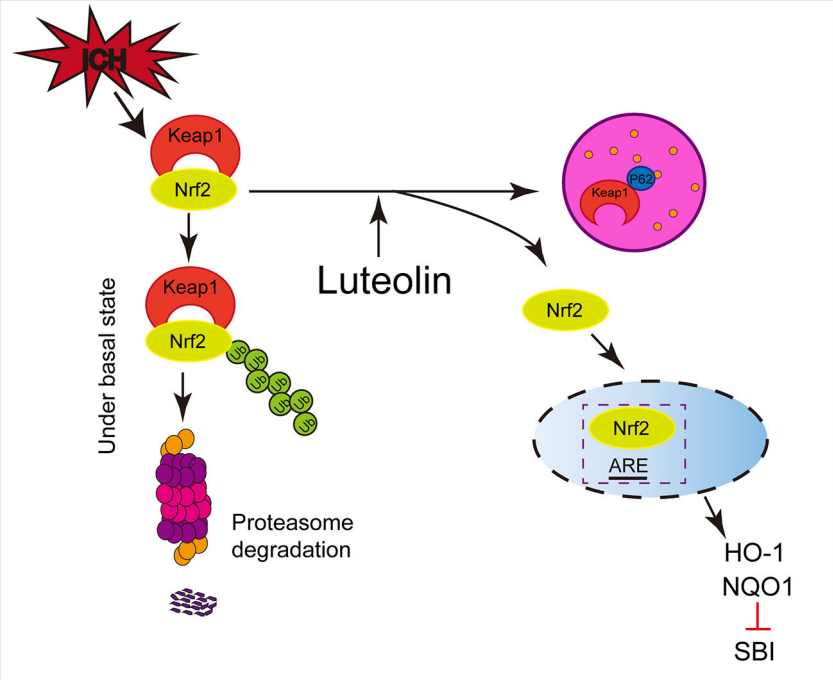

FIGURE 7 | Potential mechanisms of luteolin in ameliorating intracerebral hemorrhage (ICH)-induced secondary brain injury (SBI). Luteolin enhances autophagy, activates the sequestosome 1 (p62)/kelch-like enoyl-coenzyme A hydratase(ECH)-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, and plays an important role in neuroprotection and anti-oxidative processes, which suggests that luteolin may represent a promising drug for ameliorating ICH-induced SBI.

Moreover, previous studies have revealed that luteolin upregulates Nrf2 expression and triggers Nrf2 translocation in various disease models, including brain diseases (Xu et al., 2014; Liu et al., 2018; Tan et al., 2018b; Ma et al., 2019). Our present findings were consistent with those of the previous studies.

Previous studies have revealed that there are differential effects of luteolin on the Nrf2 signaling pathway in different cell lines. For example, luteolin was recognized as an Nrf2 inhibitor and suppressed the activity of the Nrf2/ARE pathway in human lung carcinoma A549 cells (Tang et al., 2011). Son et al. found that luteolin has a bidirectional regulation of the Nrf2 pathway at different stages of disease development (Son et al., 2017). These findings indicate that the role of luteolin in the regulation of the Nrf2/ARE pathway may be different in different cell types. At the same, the biological timing context may be a significant factor.

As a type of LC3-binding protein, p62 functions as a critical autophagy-adaptor protein and promotes the selective degradation of proteins *via* autophagy (Komatsu et al., 2007). The results of related studies have indicated that by physically isolating Keap1 and impairing the ubiquitylation of Nrf2, p62 mediates the activation of Nrf2 and its downstream pathways and plays an important role in antioxidative processes (Jiang et al., 2015). In the process of exploring new drugs, up-regulation of autophagy has been found in numerous disease-model studies (Yang et al., 2018a; Rusmini and Cortese, 2019) and activation of the p62/Keap1/Nrf2 pathway has been shown to play a role in alleviating systemic diseases (Sun et al., 2016; Su et al., 2018) and in ameliorating brain injury, such as ischemic stroke (Wu et al., 2019). Our present findings were similar to those described above. In addition, luteolin has been found to enhance autophagy in studies of other diseases (Xu et al., 2014; Hu et al., 2016; Cao et al., 2017). Additionally, the administration of autophagic inhibitors such as CQ, has been shown to be associated with exacerbating disease progression (Yang et al., 2018a; Wu et al., 2019). These findings are consistent with our present results, such that we found that luteolin enhanced autophagy and activated the p62/Keap1/Nrf2 pathway, and this effect was reversed by the autophagic inhibitor, CQ, in our ICH model (**Figure 7**).

Above all, our findings suggest that luteolin may represent a novel treatment for ICH-induced SBI. However, our study had some limitations. Our study used Sprague–Dawley male rats as animal models. However, in clinical epidemiological studies, there are also female patients with ICH, and the incidence of ICH in elderly patients is higher than in younger patients. Furthermore, the specific details of luteolin promoting autophagy and affecting the p62/Keap1/Nrf2 pathway remain unclear.

CONCLUSION

Taken together, our results demonstrate that autophagy increases slightly after ICH, which activates the p62/Keap1/Nrf2 pathway and upregulates the expression levels of its downstream antioxidant proteins, HO-1 and NQO1, but that effect was not obvious. In contrast, the administration of luteolin significantly amplified the above effects and may have the potential to attenuate ICH-induced SBI in ICH patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. All protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Animals.

AUTHOR CONTRIBUTIONS

WD and XL were responsible for the conception and design of the experiments. XT, YY, and JX performed the experiments. PZ, RD, YM, JH, YC, YZ and JD participated in data analysis. XT wrote the manuscript. HL, HS, GC, and YY was responsible for its revision. All the authors read and approved the final version of the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found onlineat : https://www.frontiersin.org/articles/10.3389/fphar.2019.01551/full#supplementary-material

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Sphingosine-1-Phosphate Receptors Modulators Decrease Signs of Neuroinflammation and Prevent Parkinson's Disease Symptoms in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model

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Sphingosine-1-phosphate (S1P) is a potent bioactive lipid mediator that acts as a natural ligand upon binding to five different receptors that are located in astrocytes, oligodendrocytes, microglial and neuronal cells. Recently, global activation of these receptors by FTY720 (fingolimod) has been suggested to provide neuroprotection in animal model of Parkinson's disease (PD). Among S1P receptors, the subtype 1 (S1P1R) has been linked to features of neuroprotection and, using the selective agonist SEW2871, the present investigation assessed potential benefits (and mechanisms) of this receptor subtype in an established animal model of PD. We demonstrated that oral treatments with SEW2871 are able to provide protection to the same levels as FTY720 against loss of dopaminergic neurons and motor deficits in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (30 mg/kg, i.p., 5 days) mouse model of PD. At the molecular level, we observed that the beneficial effects of both S1PR agonists were not associated with alterations in ERK and Akt levels, two markers of molecular adaptations in the striatum neurons. However, these compounds have the capacity to prevent signs of neuroinflammation such as the activation of astrocytes and glial cells, as well as MPTP-induced reduction of BDNF levels in key regions of the brain implicated in motor functions. These findings suggest that selective S1P1R modulation has the ability to provide neuroprotection in response to MPTP neurotoxicity. Targeting S1P1R in PD therapy may represent a prominent candidate for treatment of this neurodegenerative conditions.

Keywords: S1P receptors, FTY720, SEW2871, MPTP, neuroinflammation, neuroprotection

INTRODUCTION

Parkinson's disease (PD) is a common age-related neurodegenerative condition, characterized by progressive loss of the nigrostriatal dopaminergic pathway and erosion of several neurological functions. Biochemical studies performed on *postmorterm* brains suggest that pathogenic factors most likely contributing to PD include a progressive neuroinflammatory reaction involving microglial activation and subsequent formation of pro-inflammatory cytokines such as the tumor necrosis factor (TNF-α) (Nagatsu and Sawada, 2007). Reduction in neurotrophins synthesis such as brain-derived nerve growth factor (BDNF) is another important feature associated with PD pathology (Shen et al., 2018). In support to this theory, several *in vitro* and *in vivo* studies propose that BDNF depletion occurring in various neuropathological conditions is mediated by the release of pro-inflammatory cytokines (Calabrese et al., 2014).

Although symptomatic improvement can be achieved by pharmacologically restoring dopaminergic transmission, the development of neuroprotective treatments that prevent or halt PD pathogenic processes is still awaiting. Emerging evidence have established that Fingolimod (FTY720), a non-selective sphingosine-1-phosphate receptors (S1PRs) modulator approved for the treatment of multiple sclerosis, can provide significant protection in mouse models of neurodegenerative conditions including two recent studies on PD (Aytan et al., 2016; Zhao et al., 2017). These later studies suggest that neuroprotective properties of FTY720 in a murine model of PD require direct effects of the drug on neuronal cells, which are presumably dependent on ERK activity. However, which specific S1PRs is responsible for these beneficial effects and whether non-neuronal mechanisms such as neuroinflammation are associated with brain damages is still unknown.

The present study will examine the efficacy of an oral treatment with the non-selective agonist FTY720 to subtype 1, 3, and 5 of S1PRs, or the selective agonist SEW2871 to subtype 1 of S1PRs (S1P1R), to prevent the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced nigrostriatal loss and motor deficits in mice. In addition, potential mechanisms of action that include neuronal signaling, BDNF synthesis, and neuroinflammatory pathways will be investigated.

MATERIALS AND METHODS

Animals

Twelve week-old male C57BL/6j mice (Charles River Laboratories, QC, CAN) were individually housed in a controlled room under a 14 h light/10 h dark cycle. Food and water were available *ad libitum*. All experiments were approved and carried out with the recommendations of the UQTR Institutional Animal Care and Use Committee (protocol #2016-MiC.24) in accordance with the Canadian Council on Animal Care.

Pharmacological Treatments

The experimental design is detailed in **Figure 1**. FTY720 and SEW2871 [5-(4-phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole] were purchased from Cayman Chemical (Ann Arbor, MI, USA) and MPTP hydrochloride from Toronto Research Chemicals (North York, ON, CAN). All mice ($n = 24$) were orally treated with either vehicle [10% dimethyl sulfoxide (DMSO) and 25% Tween 20 v/v dissolved in saline 0.9% sodium chloride], 1 mg/kg FTY720 (dissolved in vehicle) (Yazdi et al., 2015), or 20 mg/kg SEW2871 (dissolved in vehicle) (Dong et al., 2014) daily for 14 days. Mice were also

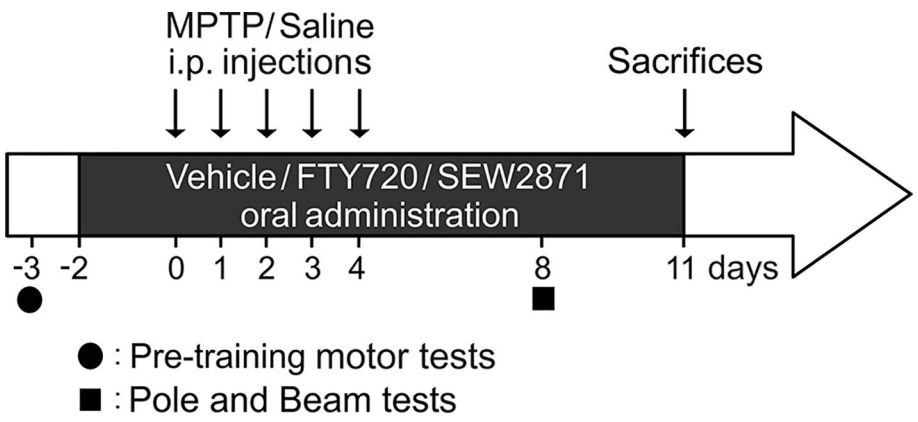

FIGURE 1 | Experimental design. Three days before 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injections, mice were pre-trained at the pole and beam tests by completing three trials within a day. Two days before MPTP injections, mice received an oral treatment with either vehicle [10% dimethyl sulfoxide (DMSO) and 25% Tween 20 v/v dissolved in saline 0.9% sodium chloride], FTY720 (1 mg/kg), or SEW2871 (20 mg/kg) once a day for a total of 14 days. At day 0, saline or MPTP (30 mg/kg/day) treatments were administered i.p. during 5 days. Motor abilities at the pole and beam tests were evaluated 8 days after the first MPTP injections. On day 11, all mice were sacrificed. The experimental setting involved six groups: 1) vehicle with saline treatments, $n = 3$; 2) FTY720 with saline treatments, $n = 3$; 3) SEW2871 with saline treatments, $n = 3$; 4) vehicle with MPTP treatments, $n = 5$; 5) FTY720 with MPTP treatments, $n = 5$; (6) SEW2871 with MPTP treatments, $n = 5$.

intraperitoneally (i.p.) injected with saline or 30 mg/kg MPTP (dissolved in saline) (Xiao-Feng et al., 2016) once a day for five consecutive days. Our experimental groups were defined as follow: 1) vehicle + saline, n = 3 mice; 2) FTY720 + saline, n = 3; 3) SEW2871 + saline, n = 3; 4) Vehicle + MPTP, n = 5; 5) FTY720 + MPTP, n = 5; and 6) SEW2871 + MPTP, n = 5.

Behavioral Assessments

Motor behavior was assessed using the pole and beam tests, as previously described (Chagniel et al., 2016; Bergeron et al., 2017). Briefly, at the pole test, mice were placed at the upper end of a vertical pole (diameter: 1.5 cm; length: 50 cm). The time required for each mouse to turn down and reach the base of the pole was recorded for three trials. The balance beam assessed two mouse abilities: the first one is the time taken to go across a narrow beam (width: 6 mm; length: 100 cm) to reach a dark goal box whereas the second one measured the stepping errors, i.e., foot-faults, occurring during the same trial. Mouse performances were recorded for three trials. A foot-fault was considered each time a paw fell under the beam midline. In all tests, mice were pre-trained three consecutive periods to remove the learning variables and a maximum time of 60 s was allowed to execute the tasks.

Protein Levels Quantification

Mice were sacrificed by decapitation and brains were divided in half. The posterior part of the brain was immediately processed for immunofluorescence analyses. In the anterior part of the brain, we rapidly dissected out the striatum structure in both hemispheres and immediately freeze them on powdered dry ice. The striatum were pooled together and stored at −80°C until we performed protein extractions for Western blot analysis. Samples were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN, USA). Protein concentrations were quantified by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (30–40 µg) were separated on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) gels and transferred onto nitrocellulose membranes. Immunoblots were performed overnight using the following primary antibodies: mouse monoclonal antibody against tyrosine hydroxylase (TH; 1:2,000; Millipore, Billerica, MA, USA), rabbit polyclonal antibody against dopamine transporter (DAT; 1:1,000; Millipore), rabbit polyclonal antibody against sphingosine 1-phosphate receptor 1 (EDG-1; 1:10,000; Abcam, Cambridge, MA, USA), rabbit polyclonal antibody against phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204; 1:1,000; Cell Signaling Technology, Whitby, ON, CAN), mouse monoclonal antibody against p44/42 MAPK (ERK1/2; 1:2,000; Cell Signaling Technology), rabbit monoclonal antibody against protein kinase B (p-Akt-Thr308; 1:1,000; Cell Signaling Technology), rabbit monoclonal antibody against total protein kinase B (Akt; 1:1,000; Cell Signaling Technology), rabbit polyclonal antibody against BDNF (1:400; Abcam), mouse monoclonal antibody against tumor necrosis factor alpha (TNF-α; 1:2,000; Abcam), mouse monoclonal antibody against glial fibrillary acidic protein (GFAP; 1:1,000; Cell Signaling Technology), and mouse monoclonal antibody against GAPDH (1:10,000; Abcam). Membranes were washed in tris-buffered saline (TBS)-Tween 0.1% and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:5,000; Thermo Scientific, Ottawa, ON, CAN). To visualize protein bands, chemiluminescence reactions were performed (SuperSignal West Femto Chemiluminescence Kit, Pierce Chemical Co, IL, USA). Densitometry analysis were achieved using the VisionWorks LS software (UVP Bioimaging Upland, Upland, CA, USA) and expressed as relative optical density.

Immunofluorescence Analysis

Posterior part of the brain was post-fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.5, and a few hours in 10% sucrose/4% paraformaldehyde (wt/vol). They were frozen in isopentane and stored at −80°C. Coronal brain sections (60 µm) containing the subtantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) (−2.92 to −3.64 mm from the Bregma; Paxinos and Franklin, 2001) were sliced using a Leica CM3050S Cryostat (Leica, Richmond Hill, ON, Canada) and kept at 4°C in PBS. For free-floating immunofluorescence, sections were incubated in permeabilizing solution containing 1.2% Triton X-100 in PBS followed by blocking solution containing 10% normal goat serum in PBS to avoid non-specific binding. They were then incubated with primary antibodies: rabbit polyclonal antibody or mouse monoclonal antibody against TH (1:500; Millipore), mouse monoclonal antibody against neuronal nuclear antigen (NeuN; 1:200; Millipore), mouse monoclonal antibody against GFAP (1:300; Cell Signaling Technology), and rabbit monoclonal antibody against ionized calcium binding adaptor molecule 1 (Iba-1; 1:100; Abcam). The sections were rinsed in PBS and incubated in appropriate secondary antibody: goat anti-mouse conjugated with fluorescein isothiocyanate (FITC) or DyLight 594-conjugated goat anti-rabbit (1:500; Cell Signaling Technology), diluted in PBS containing 0.3% Triton X-100 and 2% next-generation sequencing (NGS) for 1 h at room temperature. After several washes in PBS, they were incubated with Hoescht 33342 (1:10,000/PBS, Invitrogen, Burlington, ON, CAN) for 15 min. Finally, the sections were rinsed several times in PBS and mounted in VECTASHIELD medium on Superfrost slides for visualization under a confocal spinning disk microscope (MBF Bioscience, Williston, VT, USA).

TH and NeuN positive cells were determined by unbiased stereological quantification using the optical fractionator of Stereo Investigator software (MBF Bioscience, Vermont, USA). Three coronal sections containing the SNc and VTA of both hemispheres were considered per animal: −3.16, −3.28, and −3.40 mm from the bregma. Borders of the SNc and VTA were defined using TH-immunostaining from a random starting point with 2X objective. Inside these borders, positive cells were counted with a 60X PlanApo oil-immersion objective and 1.4 numerical aperture attached to an Olympus BX51 microscope. A systematic sampling of the outlined area was made from a random starting point. Counts were recorded at predetermined intervals (x = 250, y = 150) and a counting frame

(50x50 µm) was superimposed on the live image of each tissue section. Section thickness was measured by focusing on the top of the section, zeroing the z-axis, and focusing on bottom of the section (average section thickness was 60 µm with a range of 58.9–61.1 µm). The dissector height was set at 50 µm. Immunolabeled neurons were counted only if the first recognizable profile came into focus within the counting frame. This method certified a uniform, random, and systematic cell count. Focusing through the z-axis revealed that NeuN and TH antibodies penetrated the full depth of tissue sections. Positive cell counts were expressed as total number/mm^3.

Semiquantitative optical densitometry measurements in the SNc and VTA structures were also conducted to evaluate Iba-1 and GFAP immunofluorescences. Three sections of both hemispheres were considered per animal: −3.16, −3.28, and −3.40 mm from Bregma. To delineate SNc and VTA, coronal midbrain sections labeled with Iba-1 or GFAP were co-immunostained with TH. Images of Iba-1 and GFAP immunolabeling were captured bilaterally with 2X objective using Olympus BX51 microscope and optical densitometry measurements were obtained using ImageJ software (NIH, USA).

Data Analysis

The data were analyzed using the GraphPad Prism software (version 5.0, Graph Pad Software, San Diego, CA, USA) to perform one-way ANOVA followed by the Newman-Keuls *post hoc* tests. Data were reported as mean ± SEM. and statistical significance was set at $P < 0.05$.

RESULTS

S1PRs Modulators Reduced MPTP-Induced Inflammation and BDNF Depletion Without Interfering With S1P1R in Striatum Homogenates

We first examined the manifestation of neuroinflammation in the striatum by the determination of GFAP and TNF-α levels using Western blot analyses. While the SEW2871 and FTY720 treatments alone have no effect, they were able to prevent the surge of striatal GFAP [**Figure 2A**; F(5,23) = 5.31, P < 0.01] and TNF-α [**Figure 2B**; F(5,23) = 8.20, P < 0.001] levels induced by MPTP. In parallel, expression levels of BDNF proteins have also been investigated and we observed a significant decrease of its protein levels after MPTP administrations, an effect that was totally prevented by SEW2871 and FTY720 treatments [**Figure 2C**; F(5,23) = 10.77, P < 0.01].

The levels of S1P1R were estimated by Western blot analysis in the striatum of MPTP mice treated with the SEW2871 or FTY720. Statistical analyses did not revealed any difference between all groups [**Figure 3A**; F(5,23) = 0.20, P > 0.05]. Levels of phosphorylated ERK1/2 and Akt proteins were also investigated by Western blot in order to assess adaptive response to neuronal activation. We observed that in addition to total ERK1/2 and total Akt, levels of phospho-Thr202-ERK1 [**Figure 3B**; F(5,23) = 1.62, P > 0.05] and phospho-Thr308-Akt [**Figure 3C**; F(5,23) = 1.69, P > 0.05] were not altered by the different treatments.

S1PRs Modulators Reduced MPTP-Induced Astrogliosis and Microgliosis in the SNc

Because MPTP injections were associated with signs of inflammation in the striatum, we next examined astrocytic and microglial activation in the two major midbrain DAergic centers, the SNc and VTA, using immunofluorescence technique. Astrocytic and microglial activation were revealed by GFAP and Iba-1 immunostaining, respectively. We observed the occurrence of faint immunostaining of Iba-1 positive microglia and GFAP positive astrocytes in the SNc of vehicle-treated mice (**Figure 4A**). However, in the SNc of MPTP-treated mice, an augmentation in the staining of Iba-1 as well as GFAP were observed, phenotypes known to be reminiscent of reactive microglia and astrocytes, respectively (**Figure 4A**). FTY720 and SEW2871 were able to prevent the occurrence of microgliosis and astrocytosis as the staining of Iba-1 and GFAP were similar to vehicle-treated mice (**Figure 4A**). In order to quantify these observations, we performed semi-quantitative optical densitometry measurements in the SNc and VTA of mice treated with MPTP and S1PR modulators. These analyses confirmed our qualitative observations and

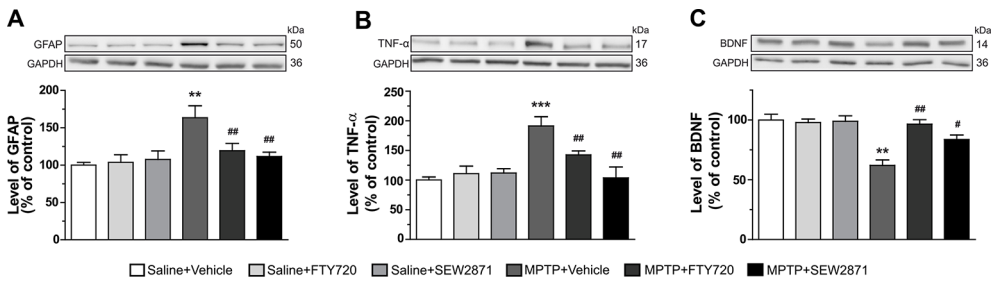

FIGURE 2 | Levels of inflammatory markers are affected in the striatum. Levels of GFAP **(A)**, TNF-α **(B)**, and BDNF **(C)** were assessed by Western blot in the striatum of mice. The data, expressed relative to GAPDH, represent the mean of relative optical density in triplicate experiments of GFAP, TNF-α, and BDNF (expressed as a percentage of control values) ± S.E.M., n = 3–5 mice/group. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. vehicle + saline; #$p < 0.05$, ##$p < 0.01$ *vs*. vehicle + MPTP.

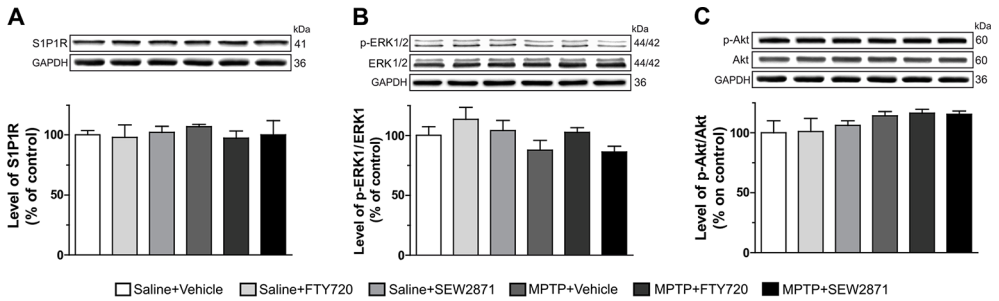

FIGURE 3 | Levels of neuronal activity markers were unaltered in the striatum. Protein levels were evaluated by Western blotting with proteins extracted (30–40 μg of protein) from the mouse striatum. Levels of S1P1R **(A)**, phospho-Thr202-ERK1 **(B)**, and phospho-Thr308-Akt **(C)** were determined. GAPDH was used as loading control. Data represent the mean of relative optical density in triplicate experiments of S1P1R, phospho-Thr202-ERK1 and phospho-Thr308-Akt (expressed as a percentage of control values) ± S.E.M. Values are respectively expressed relative to GAPDH, total ERK, and total Akt, n = 3–5 mice/group.

revealed that FTY720 and SEW2871 treatments prevented the robust increase of Iba-1 [**Figure 4B**; $F_{(5,23)} = 48.66$, $P < 0.001$] and GFAP [**Figure 4D**; $F_{(5,23)} = 11.43$, $P < 0.001$] staining in the SNc of mice after MPTP treatments. No effect of FTY720 and SEW2871 treatments alone were noticed in the SNc and no signs of reactive microglia [**Figure 4C**; $F_{(5,23)} = 0.24$, $P > 0.05$] or astrocytes [**Figure 4E**; $F_{(5,23)} = 0.19$, $P > 0.05$] were observed in the VTA following the different treatments.

S1PRs Modulators Protects Against MPTP-Induced Nigrostriatal Cellular Loss

The extent of MPTP-induced midbrain dopaminergic denervation was estimated by immunofluorescence techniques using two independent antibodies (**Figure 5A**), one raised against the neuronal marker NeuN and the second raised against TH. This rate-limiting enzyme for dopamine synthesis is widely used as a marker of dopaminergic depletion (Chagniel et al., 2012). In the SNc of MPTP-treated mice, our stereological method revealed a reduction in TH immunopositive cells that was totally prevented by the SEW2871 and FTY720 treatments [**Figure 5B**; $F_{(5,23)} = 5.31$, $P < 0.01$]. In contrast, no effect of either MPTP, SEW2871, or FTY720 administration was observed on TH immunopositive cells of the VTA [**Figure 5C**; $F_{(5,23)} = 0.13$, $P > 0.05$]. These data were replicated using the NeuN marker in the SNc [**Figure 5D**; $F_{(5,23)} = 3.57$, $P < 0.05$] and the VTA [**Figure 5E**; $F_{(5,23)} = 0.15$, $P > 0.05$].

In addition to the VTA and SNc, we also investigated the detrimental effects of MPTP on striatal dopamine terminals by using Western blot analysis. Two well-known markers of dopaminergic neuron terminals were employed and robust decreases in TH [**Figure 6A**; $F_{(5,23)} = 5.92$, $P < 0.001$] and DAT [**Figure 6B**; $F_{(5,23)} = 3.98$, $P < 0.01$] levels were observed after MPTP injections. SEW2871 and FTY720 administrations were both able to prevent this effect of MPTP (**Figures 6A, B**).

Motor Abilities Were Preserved With FTY720 and SEW2871 Administrations

To assess the effect of treatments on motor abilities, we performed two behavioral tests, namely the pole and beam tests. In particular, the pole test estimated bradykinesia and motor coordination whereas the beam test analyzed skilled walking and overall coordination. It is noteworthy that mice were pre-trained in order to remove the learning variables associated to these tests. We observed that while SEW2871 or FTY720 treatments alone were without effects on motor behaviors, they prevented the MPTP-induced motor deficits in all tasks. We observed impaired performances of MPTP treated mice at the pole test [**Figure 7A**; $F_{(5,23)} = 3{,}47$, $P < 0.05$] as well as an increase in the time taken to go across the beam [**Figure 7B**; $F_{(5,23)} = 2{,}81$, $P < 0.05$] and in the number of stepping errors at the beam test [**Figure 7C**; $F_{(5,23)} = 13{,}48$, $P < 0.001$]. The time required to perform the pole test and the beam test, as well as the average foot-faults were returning to control values when MPTP mice received either SEW2871 or FTY720 administrations. Note that statistical significance was not reached for the time required to perform the beam test in both groups.

DISCUSSION

The present study demonstrates that oral treatments with FTY720 and SEW2871 provide protection against loss of dopaminergic neurons and motor deficits in a mouse model of PD. In addition to PD, FTY720 was known to exert several beneficial pharmacological effects on central nervous system (CNS) cells contributing to the treatment of various neuropathological conditions, such as stroke (Brunkhorst et al., 2014), Huntington's (Di Pardo et al., 2014), and Alzheimer's diseases (Martin and Sospedra, 2014). However, this study is the first to demonstrate such CNS positive outcomes for the S1P1R-selective agonist SEW2871. In addition, we provide evidence that FTY720 and SEW2871 treatments have the capacity to prevent activation of astrocytes and glial cells, as well as prevent the decrease of BDNF levels, in regions of the brain involved in the control of motor functions. Our finding propose that drugs acting *via* the S1P1R have the capacity to provide neuroprotection to the detrimental effects of MPTP treatments.

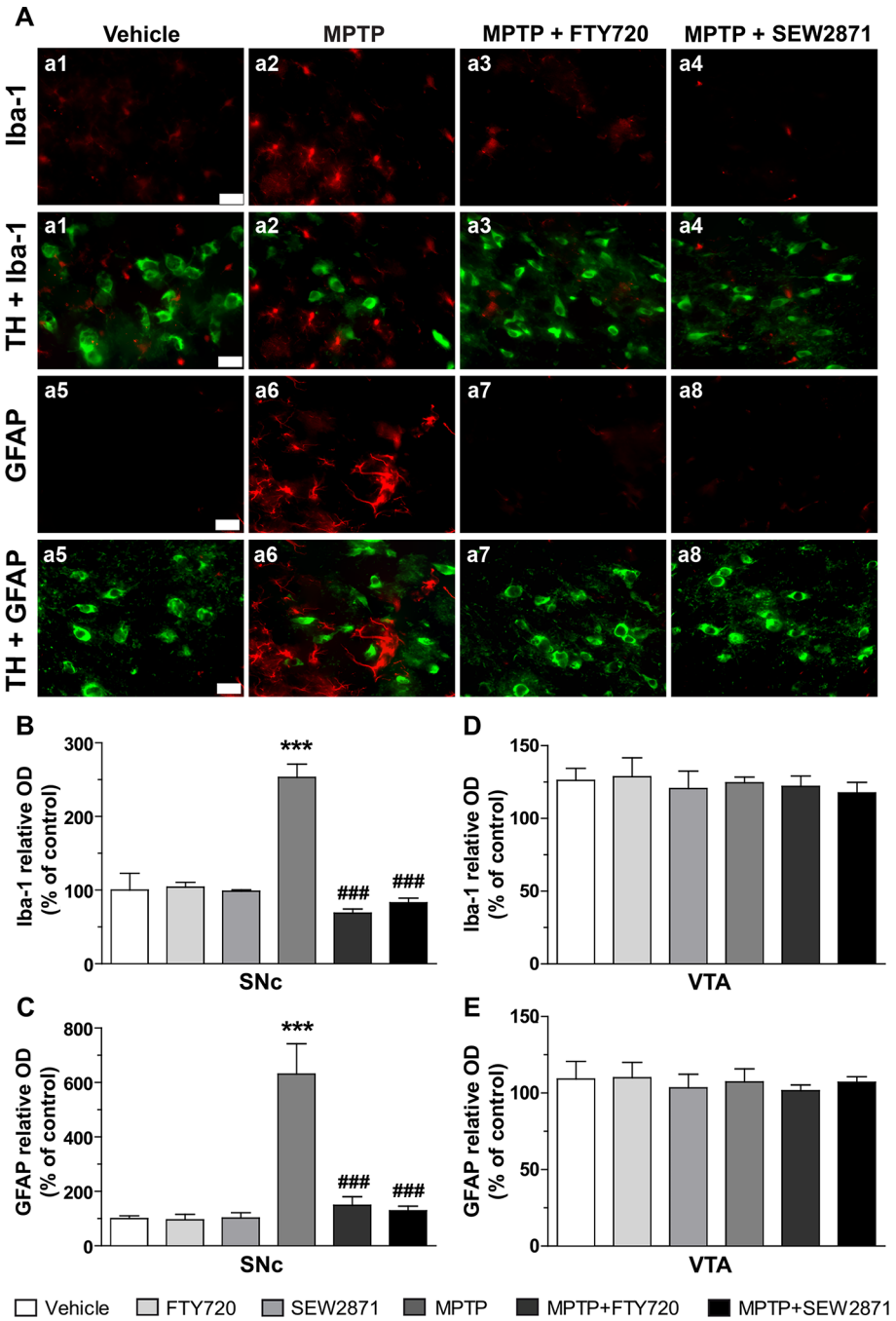

FIGURE 4 | Signs of astrogliosis and microgliosis induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are prevented by S1P1R modulators. **(A)** Representative examples of Iba-1 immunoreactive microglia (panel a1 to a4; red color), GFAP-positive astrocytes (panel a5 to a8; red color) and TH-positive neurons (panel a1 to a8; green color) using epifluorescence microscope (40X objective) in the mouse subtantia nigra pars reticulate (SNr; –3.16 mm from the Bregma). Bar equals 50 µm. Fluorescence intensity of Iba-1 and GFAP were measured in the SNc **(B, D)** as well as in the ventral tegmental area (VTA) **(C, E)**. The data represent the mean of Iba-1 and GFAP relative optical density (expressed as a percentage of control values) ± S.E.M., n = 3–5 mice/group. $^{***}p < 0.001$ *vs*. vehicle + saline; $^{###}p < 0.001$ *vs*. vehicle + MPTP.

At this point it is important to emphasize that the MPTP treatment (5 days, 30 mg/kg, once daily) used in this mouse study is a well validated model and that the results obtained in our experiments are in line with previous observations (Jackson-Lewis et al., 2012; Wang et al., 2015). The loss of nigrostriatal dopaminergic neurons is parallel by a robust increase of glial and astrocyte reactivity in the SNc and striatum, in addition to impaired motor behavior. It is interesting that no alteration at

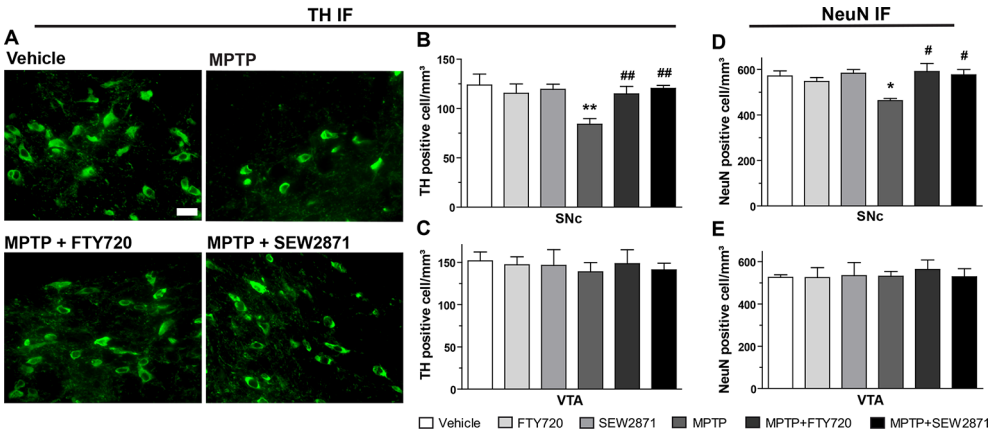

FIGURE 5 | S1PR modulators protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced subtantia nigra pars compacta (SNc) cellular loss. Representative examples of tyrosine hydroxylase (TH)-immunostaining (green) using the 40X objective of the epifluorescence microscope in coronal sections (–3.16 mm from the Bregma) of mouse subtantia nigra pars reticulate (SNr). Bar equals 50 µm **(A)**. Stereological counts of TH- and neuronal nuclear antigen (NeuN)-positive neurons in the SNc **(B, D)** and ventral tegmental area (VTA) **(C, E)**. The data represent the mean of TH- and NeuN-positive cell numbers/mm^3 (expressed as a percentage of control values) ± S.E.M., n = 3-5 mice/group. $^*p < 0.05$, $^{**}p < 0.01$ *vs*. vehicle + saline; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. vehicle + MPTP.

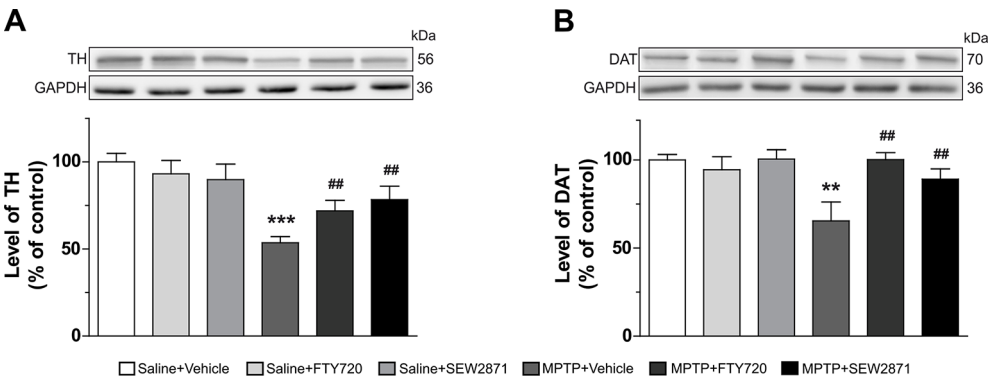

FIGURE 6 | Dopamine terminals were spared in the striatum following S1P1Rs modulators treatments. Levels of tyrosine hydroxylase (TH) **(A)** and dopamine transporter (DAT) **(B)** were determined in the mouse striatum by Western blot experiments. The data, expressed relative to GAPDH, represent the mean of relative optical density in triplicate experiments of TH and DAT (expressed as a percentage of control values) ± S.E.M., n = 3–5 mice/group. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. vehicle + saline; $^{\#\#}p < 0.01$ *vs*. vehicle + MPTP.

the striatal levels of phosphorylated ERK and Akt, two markers of molecular adaptations of striatal spiny projection neurons, are observed after MPTP treatments. This finding was somewhat expected as variation in the activity of these striatal kinases is associated with high dopaminergic depletion; observed for instance in unilateral 6-hydroxydopamine (OHDA) treated mice (Zhao et al., 2017) or more severe MPTP mouse model (Motyl et al., 2018). In our study, the degree of dopaminergic depletion is of 50%, as evaluated by TH levels in the striatum of MPTP mice, which is reminiscent of a mild dopaminergic-depletion. Limitations of MPTP administrations as a model of PD has been well documented previously (Bezard et al., 2013). Part of the problem with the MPTP toxin models is their acute nature, which is completely different from the insidious progression of PD observed in patients. Compensatory changes may arise in patients over the course of the disease that would not have an opportunity to occur in the acute animal models. In addition, PD occurs most frequently in elderly patients, usually around the age of 60 or older. Unfortunately, because of the inconvenience and cost of housing the animals for an extended period our study did not use older animals. In addition, a closer look at the differences in behavior, physiology, and gene expression between rodents and humans partially reveals why the animal studies do not translate well to clinical studies. With that said, while it may not be realistic to obtain a single animal model that completely reproduces every feature of a human

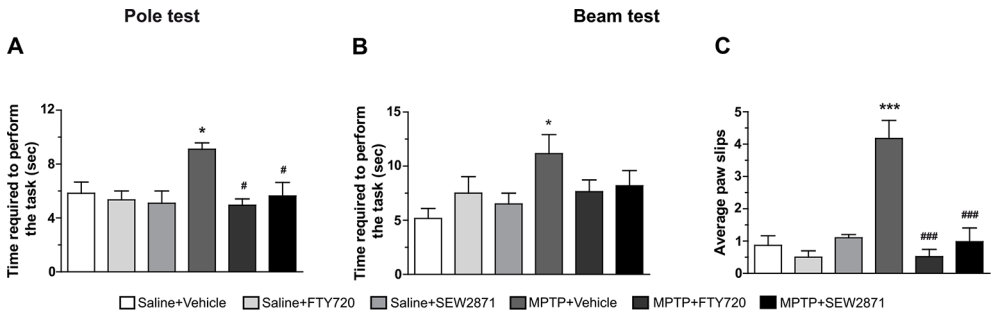

FIGURE 7 | Motor behavioral outcomes. Motor abilities on the pole and beam tests were evaluated. Data represent the mean time require to perform the pole **(A)** and beam **(B)** tests and the mean number of foot-faults occurring during the beam test **(C)** ± S.E.M., n = 3–5 mice/group. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs*. vehicle + saline; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *vs*. vehicle + MPTP.

disease as complex as PD, it would still be interesting to verify whether FTY720 and SEW2871 provide also protection during the formation of α-synuclein aggregates that resemble Lewy bodies or Lewy neurites. For instance, chronic administration of neurotoxins induces progressive PD rodent models that include α-synuclein aggregates in addition to motor deficits and rapid nigrostriatal dopaminergic cell death (Creed and Golberg, 2018). Furthermore, genetic-based approaches including transgenic models, viral vector-mediated models based on genes linked to monogenic PD as well as introduction of α-synuclein preformed fibrils all induce toxic protein aggregates in rodents. It would have been interesting to investigate the effects of FTY720 and SEW2871 in these particular models or in a combination of models to study the interplay between genetics and environment in an attempt to untangle the heterogeneity and mechanisms underlying PD.

One striking original finding in our study is the demonstration that SEW2871 and FTY720 prevent the occurrence of neuroinflammatory signs associated with MPTP treatments in mice. In particular, we establish that the SEW2871 and FTY720 treatments have the capacity to prevent the robust increase in GFAP and TNF-α expression levels in the striatum as well as the increased expression of GFAP and Iba-1 of the SNc observed in the MPTP treated mice. While the mechanism of action is still unclear, some multiple sclerosis studies performed *in vitro* and in animal models have demonstrated that FTY720 reduced microglia mediated inflammation and also diminished astrocyte activation in association to the neuronal protection (Choi et al., 2011; Miguez et al., 2015; Rothhammer et al., 2017; O'Sullivan et al., 2018). Whether the ongoing astrocytosis and microgliosis in the brain of our MPTP treated mice is responsible for the dopamine neuronal loss is unknown, this question is still under extensive debate in PD field (Kaur et al., 2017). However, in the VTA, a region where no effect of the MPTP treatment on dopaminergic neurons is noticed, no signs of astrocytosis or microgliosis are observed. This is at least one evidence showing that neuroinflammatory signs are selectively detected in brain regions where neuronal death is observed (Mosharov et al., 2009). Our data support a new role for S1P1R agonists in mediating anti-inflammatory effects in the MPTP mouse model of PD.

Treatments with FTY720 and SEW2871 did not alter the levels of phosphorylated ERK or Akt in the striatum of MPTP treated mice. In model cell lines transfected with the S1P1R subtype, the natural ligand S1P, FTY720, and SEW2871 appear to activate ERK and Akt pathways (Jo et al., 2005). In mouse models of PD, two recent studies have respectively reported that FTY720 increases levels of p-ERK or p-Akt in the striatum of 6-OHDA or MPTP treated mice (Zhao et al., 2017; Motyl et al., 2018). Again, the mild dopaminergic depletion in our MPTP mice may be responsible for the divergence with previous findings. Another interesting possibility is the fact that FTY720 and SEW2871 may have different effects on S1P1 expression on neuronal *versus* glial cells. However, we believe that this particular question would necessitate further research as only few studies have addressed this topic and the effect of SEW2871 is not assessed *in vivo* or in the context of PD. It is however clear from our data that activation of ERK and Akt pathways would play a minor role in the beneficial effects of FTY720 and SEW2871 on dopamine neuronal survival and neuroinflammation. On the other hand, both treatments have the capacity to prevent the reduction of BDNF levels observed in the striatum of MPTP mice. The link between FTY720, increased BDNF levels and neuroprotection has been well described in cell cultured and animal models of Rett syndrome (Deogracias et al., 2012), HD (Miguez et al., 2015), AD (Doi et al., 2013; Fukumoto et al., 2014) and PD (Giasson et al., 2002). For instance, in cultured neurons, FTY720 increases BDNF levels and counteracts N-methyl-d-aspartate (NMDA)-induced neuronal death in a BDNF-dependent manner (Di Menna et al., 2013; Cipriani et al., 2015). In PD animal models, BDNF treatment has been shown to reduce the loss of dopaminergic neurons (Tsukahara et al., 1995; Levivier et al., 1995). Our findings are suggesting that the decrease of BDNF levels may be one contributing factor responsible for the observed dopaminergic neuronal loss, and that rescuing this deficit may underlies the protective effect of FTY720 and SEW2871 treatments.

Despite that our study raised the interesting possibility that prevention of neuroinflammation and striatal BDNF levels

recovery, rather than striatal Akt and ERK signaling modulation, are responsible for the neuroprotective effects of FTY720 and SEW2871 in the MPTP mouse model, the exact target mechanisms remain to be determined. For instance, we cannot exclude that brain inflammation could rely on peripheral mechanisms. Yang et al. (2018) demonstrate that lymphocyte infiltration contributes to brain neurodegeneration in the MPTP mouse model. On that line and, given the ability of FTY720 and SEW2871 to produce lymphopenia (Sanna et al., 2004; Morris et al., 2005), we cannot exclude that the neuroprotection we observed from these drugs in the MPTP-treated mice is dependent on reduced neuroinflammatory processes subsequent to peripheral mechanisms. Administration of S1P modulators after MPTP exposure would have help separating out an effect of drugs on peripheral immune cell recruitment and direct glial modulation. Of course, the precise contributions of central and peripheral mechanisms to the benefit of FTY720 and SEW2871 in PD pathology warrants further investigations. On the other hand, our data suggest that the anti-inflammatory effects of FTY720 and SEW2871 may be responsible for rescuing BDNF levels in the striatum of MPTP-treated animals. This scenario is consistent with the recent literature indicating that production of TNFα and subsequent activation of TNFα receptor 1 can be responsible for down-regulating BDNF expression in the hippocampus. Precisely, Liu et al. (2017) demonstrated that hippocampal BDNF expression is markedly reduced in mice after peripheral nerve injury, an effect that was totally abolished following genetic deletion of TNFα receptor 1. In addition, it has been documented that BDNF is synthesized not only in neurons, but also in astrocytes (Saha et al., 2006). It is therefore tempting to propose the interesting possibility that astrocytes are also mediating these effects given that S1PR expression are enriched in these cells in addition to their capacity to produce BDNF. It would be of interest to find out to what extent the beneficial effects on BDNF expression exerted by FTY720 and SEW2871 are dependent on S1PR and TNFα receptor activation in the striatum of MPTP-treated mice.

Our results show that similar beneficial effects observed with either SEW2871 or FTY720 treatments in the MPTP mouse model of PD suggest a crucial role for the subtype 1 of S1PRs. The specificity of SEW2871 to bind to S1P1R is undeniably well recognized (Bolick et al., 2005; Blaho and Hla, 2014). On the other hand, FTY720 is less selective to that particular S1PR. FTY720 need to be phosphorylated *in vivo* by sphingosine kinase-2 to form the active moiety FTY720-phosphate known to bind subtypes −1, −3, −4, and −5 (Brinkmann et al., 2002; Soliven et al., 2011). At the exception of S1PR4, all S1PRs subtypes are expressed in the central nervous system and S1P1R and three levels are substantially high in the brain (MacLennan et al., 1994; Zhang et al., 1999). Interestingly, S1P1R is located on astrocytes, oligodendrocytes, microglial and neuronal cells throughout the brain, including region of the brain associated with the control of motor function (Chae et al., 2004; Aktas et al., 2010). Notably, S1P1R responses in astrocytes has been well studied in human and rodent studies (Healy et al., 2013; Wu et al., 2013). For instance, our finding confirmed S1P1R protein expression in the striatum structure. Whether S1P1R is exclusively responsible for the neuroprotection we report using SEW2871 or FTY720 treatments is undetermined, but some evidence are supporting this contention in the literature. For instance, the role of S1P1R in the beneficial action of FTY720 is reported in animal models of stroke and PD (Dev et al., 2008; Hasegawa et al., 2010; Imeri et al., 2014; Brunkhorst et al., 2014; Sun et al., 2016; Zhao et al., 2017; Motyl et al., 2018). These studies all demonstrate that the effect of FTY720 is largely mediated by the S1P1R.

Consistent with other investigations, we observed that FTY720 could exert beneficial effects in PD mouse model (Zhao et al., 2017; Motyl et al., 2018). This drug has been shown to exert several pharmacological effects on CNS cells which may contribute to the treatment of various neuropathological conditions, such as stroke (Brunkhorst et al., 2014) Huntington's (Di Pardo et al., 2014) and AD (Martin and Sospedra, 2014). In that line, it was recently found to reduce the density of pathological plaques and decreased the number of pro-inflammatory cells in animal models of AD (Fukumoto et al., 2014; Aytan et al., 2016). Unfortunately, both *in vitro* and *in vivo* experiments have demonstrated that FTY720, accumulating above a certain threshold in the brain, becomes less effective (Aytan et al., 2016) and even neurotoxic (van Echten-Deckert et al., 2014). In fact, studies have documented the possibility that FTY720 can alter normal brain physiology *via* a mechanism involving hyperphosphorylation of Tau proteins (Attiori Essis et al., 2015).

In conclusion, we observed that pharmacological targeting of S1P1R with the specific agonist SEW2871 conferred strong resistance to MPTP-induced dopaminergic depletion, inflammation processes and motor dysfunctions. From a clinical perspective, it seems thus plausible that selective activation of S1P1R by SEW2871 (or other chemical analogs) might be more effective and probably safer, knowing that this drug is not prone to induce Tau hyperphosphorylation (St-Cyr Giguere et al., 2017). In addition, many adverse events, including hypertension, macular edema, pulmonary toxicity, and hepatotoxicity, have been associated with FTY720, because of its off-target interactions with other S1PRs subtypes, particularly with S1PR3. The activation of S1PR3 by FTY720 was at least partially responsible for its side effects on the cardiovascular system and organ fibrosis, which may cause prominent safety issues (Cohen and Chun, 2011). Several drugs acting more selectively on S1PRs subtypes have been developed in recent years (Guerrero et al., 2016) and future experimentation are required to test for there efficiencies and safeties in the context of PD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the UQTR Institutional Animal Care and Use Committee and performed in accordance with the Canadian Council on Animal Care.

AUTHOR CONTRIBUTIONS

ÉP and MC designed the study. ÉP conducted this research. TJ and GL contributed for the behavioral experiments. ÉP, MC, and GM wrote the manuscript. MC and GM contributed to the conceptual frame of the study and edited the manuscript.

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MINI REVIEW
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From *Cannabis sativa* to Cannabidiol: Promising Therapeutic Candidate for the Treatment of Neurodegenerative Diseases

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Cannabis sativa, commonly known as marijuana, contains a pool of secondary plant metabolites with therapeutic effects. Besides Δ9-tetrahydrocannabinol that is the principal psychoactive constituent of *Cannabis*, cannabidiol (CBD) is the most abundant nonpsychoactive phytocannabinoid and may represent a prototype for anti-inflammatory drug development for human pathologies where both the inflammation and oxidative stress (OS) play an important role to their etiology and progression. To this regard, Alzheimer's disease (AD), Parkinson's disease (PD), the most common neurodegenerative disorders, are characterized by extensive oxidative damage to different biological substrates that can cause cell death by different pathways. Most cases of neurodegenerative diseases have a complex etiology with a variety of factors contributing to the progression of the neurodegenerative processes; therefore, promising treatment strategies should simultaneously target multiple substrates in order to stop and/or slow down the neurodegeneration. In this context, CBD, which interacts with the eCB system, but has also cannabinoid receptor-independent mechanism, might be a good candidate as a prototype for anti-oxidant drug development for the major neurodegenerative disorders, such as PD and AD. This review summarizes the multiple molecular pathways that underlie the positive effects of CBD, which may have a considerable impact on the progression of the major neurodegenerative disorders.

Keywords: ***Cannabis sativa*, oxidative stress, phytocannabinoids, cannabidiol, Alzheimer's disease, Parkinson's disease**

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INTRODUCTION

Oxidative stress (OS) plays a crucial role in aging and occurs manly when the activity of the anti-oxidants enzymes is not sufficient to counterbalance the generation of reactive oxygen species (ROS). In the latter condition, high production of ROS can alter the structure of proteins, lipids, nucleic acids, and matrix components leading to programmed cell death (Cassano et al., 2016). Different tissues present different susceptibility to OS. The central nervous system (CNS) is

extremely sensitive to this type of damage for several reasons. To this regard, the CNS has a low level of antioxidant enzymes, a high content of oxidizable substrates, and a large amount of ROS produced during neurochemical reactions (Trabace et al., 2004; Uttara et al., 2009). In addition to several other environmental or genetic factors, OS contributes to neurodegeneration since free radicals attack neural cells. Therefore, neurons suffer a functional or sensory loss during the neurodegenerative process. Even if oxygen is indispensable for life, an unbalanced metabolism and an excess production of ROS ends up in a series of pathological conditions, such as Alzheimer's disease (AD), Parkinson's disease (PD), and many other neural disorders. Free radicals cause lesions to protein and DNA, activate inflammatory process and subsequent cell apoptosis (Cassano et al., 2012).

In the last years, there is an urgent need to discover new drug targets that can effectively combat cell alteration caused by the stress of cell membranes. In this perspective, the endocannabinoid (eCB) system has attracted considerable interest due to the current interplay between eCB and different redox-dependent signaling pathways. The two well-characterized eCBs are N-arachidonoyl-ethanolamine or anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG), which are synthesized on demand in response to elevations of intracellular calcium (Howlett et al., 2002; Di Marzo et al., 2005) and respectively metabolized by fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MAGL) (Piomelli, 2003; Di Marzo, 2008; Kunos et al., 2009). Cannabinoid (CB) receptors exist in two different subtypes: type 1 (CB1) and type 2 (CB2) (Matsuda et al., 1990; Munro et al., 1993; Howlett et al., 2002). The CB1 receptors, first cloned in 1990, are widely distributed in the body and in the CNS are distributed at the level of basal ganglia, cerebellum, hippocampus, caudate nucleus, putamen, hypothalamus, amygdala, and spinal cord (Matsuda et al., 1990). The CB2 receptors, cloned in 1993, are mainly located in cells of the immune system with high density in the spleen, T lymphocytes, and macrophages (Munro et al., 1993). Their anatomical distribution correlates them to the actions for which they are responsible: the activation of the CB1 receptors has euphoric effects and an antioxidant, antiemetic, analgesic, antispasmodic, and appetite stimulating actions. As for CB2 receptors, their stimulation is attributable to the anti-inflammatory and immunomodulatory actions of CB (Cassano et al., 2017).

Converging evidence strongly suggests that eCBs act as retrograde synaptic messengers (Kano et al., 2002; Freund et al., 2003). This phenomenon is initiated postsynaptically by an elevation of cytoplasmic calcium concentration that induces the production and release into the synaptic space of eCBs. Thereafter, eCBs activate CB1 receptors at presynaptic levels and block the release from the terminals of neurons of different transmitters, such as gamma-aminobutyric acid (GABA), glutamate, dopamine (DA), noradrenaline, serotonin, and acetylcholine (Howlett et al., 2002; Pertwee and Ross, 2002; Szabo and Schlicker, 2005). These mechanisms mediated by the activation of presynaptic CB1 receptors are termed depolarization-induced suppression of inhibition (DSI) and excitation (DSE), respectively when are involved the inhibitory (GABA) or excitatory (glutamate) synaptic transmissions (Kano et al., 2002; Freund et al., 2003). Likewise, CB2 receptors can modulate the production and function of certain inflammatory cytokines at multiple levels by activating the immune cells and modulating their migration both within and outside the CNS (Freund et al., 2003; Walter and Stella, 2004). Antioxidant enzymes can be modulated by eCBs, not only acting on the CB1 and CB2 receptors, but also through the transient receptor potential vanilloid-1 (TRPV1), the peroxisome proliferator-activated receptor alpha (PPAR-alpha), and the orphan receptors N-arachidonyl glycine receptor or G-protein-coupled receptors 18 (GPR18) GPR19 and GPR55 (Piomelli, 2003; McHugh et al., 2010; Howlett et al., 2011; McHugh, 2012). Therefore, the direct and/or indirect modulation of pathways through which the eCBs damper the OS may represent a promising strategy for reducing the damage caused by a redox imbalance (Gallelli et al., 2018). Moreover, antioxidants are now seen as a convincing therapy against severe neurodegeneration, as they have the ability to fight it by blocking the OS. Diet and medicinal herbs are an important source of antioxidants. The recognition of antioxidant therapy upstream and downstream of OS has proven to be an effective tool to improve any neuronal damage as well as to eliminate free radicals. Antioxidants have a wide field of application and can prevent OS interacting with the metal ions, which play an important role in the build-up of neuronal plaque (Uttara et al., 2009).

In the last decade there are increasing evidences that secondary plant metabolites, extracted from medicinal herbs, may represent lead compounds for the production of medications against inflammation and OS, protecting from neuronal cell loss (Giudetti et al., 2018). Among these medicinal herbs, *Cannabis sativa*, commonly known as marijuana, contains a pool of secondary plant metabolites with therapeutic effects (Gugliandolo et al., 2018). In this context, cannabidiol (CBD) the nonpsychotropic CB extract from *Cannabis sativa* may represent a prototype for anti-inflammatory drug development for those human pathologies where both the inflammation and OS play a key role to their etiology and progression (Izzo et al., 2009). To this regard, therapies that effectively combat disease progression are still lacking in the field of neurodegenerative disorders, and mostly with AD. CBD, which modulates the eCB system, but has also CB receptor-independent mechanism, seems to be a prototype for anti-inflammatory drug development.

Therefore, the present review summarizes the main molecular mechanisms through which CBD exerts its beneficial effects that may have a considerable impact on the progression of the major neurodegenerative disorders.

Cannabis sativa

The medical and psychotropic effects of *Cannabis sativa* have been well known since long time. A multitude of secondary metabolites was extracted from this plant and most of them were used for therapeutic purpose by many cultures. So far more than 400 chemical compounds have been isolated from *Cannabis sativa* and among them more than 100 terpeno-phenol compounds named phytocannabinoids have been detected (Mechoulam and Hanus, 2000; Mechoulam et al., 2007). As such *Cannabis sativa* can be regarded as a natural library of unique compounds. The most abundant phytocannabinoid is the

Δ9-tetrahydrocannabinol (delta-9-THC), responsible for the psychotropic effect associated with *Cannabis* consumption, and then the nonpsychoactive constituent CBD and cannabigerol (CBG) (Mechoulam and Hanus, 2000; Mechoulam et al., 2007; Gugliandolo et al., 2018). **Table 1** shows the list of the most abundant nonpsychoactive phytocannabinoids isolated from *Cannabis sativa.* Phytocannabinoids mimic the effects of eCBs that regulate the transmission of nerve impulses in some synapses of the nerve pathways, causing in particular a reduction in the release of signals between the cells (Piomelli, 2003).

Due to its high lipophilicity and its affinity for lipid membranes, delta-9-THC was supposed to bind non-specifically variety of cell membranes modifying their fluidity rather than to activate a specific receptor (Hillard et al., 1985). Later this first hypothesis was completely discarded and was demonstrated that delta-9-THC exerts its effects by combining with a selective receptor (Devane et al., 1988; Howlett et al., 1990; Matsuda et al., 1990). In fact, many authors have demonstrated that delta-9-THC exerts its psychoactive effects acting on CB1 receptors, whereas CDB and CBG, two nonpsychoactive CBs, have low affinity for both CB1 and CB2 receptors and inhibit FAAH, resulting in increased levels of eCBs, which in turn further activate the CB1 receptor (Devane et al., 1988; Howlett et al., 1990; Matsuda et al., 1990; Appendino et al., 2011). Among the nonpsychoactive phytocannabinoids, most of the evidences have focused on CBD, which possesses a high antioxidant and anti-inflammatory activity, together with neuroprotective, anxiolytic and anticonvulsant properties (Pellati et al., 2018).

Mechanisms of CBD Action

After delta-9-THC, CBD is the second most abundant phytocannabinoids and is one of the major nonpsychoactive CB constituents in the plant of *Cannabis sativa* representing up to 40% of *Cannabis* extract. Adams and colleagues first isolated the CBD, while Mechoulam and colleagues analyzed its structure and stereochemistry (reviewed in Pertwee, 2006). Therapeutically CBD is already available alone and in formulation with delta-9-THC (Booz, 2011). In particular, a drug containing only CBD (Epidiolex) is used for children affected by epilepsy resistant to other treatments, as well as in combination (1:1 ratio) with delta-9-THC (CBD/delta-9-THC, Sativex/Nabiximols) is currently used to treat the spasticity observed in patients affected by multiple sclerosis (Pertwee, 2008; Devinsky et al., 2016). Compared to delta-9-THC, CBD possess a better safety profile and it is well tolerated when administered at animals and patients even at high doses (up to 1,500 mg/day) (Bergamaschi et al., 2011). In fact, authors demonstrated that CBD did not alter cardiovascular parameters, body temperature, psychomotor, and psychological functions, as well as did not induce catalepsy like delta-9-THC (Bergamaschi et al., 2011). Unlike delta-9-THC, CBD does not target directly the CB receptors and this characteristic may justify its better safety profile compared to delta-9-THC (Pertwee, 2006; Thomas et al., 2007).

Although the pharmacodynamics of CBD is not fully clarified, different evidences have been accumulated showing that CBD seems to act throughout different pathways. To this regard, although CBD shows much lower affinity than delta-9-THC for CB1 and CB2 receptors, it is able to antagonize CB1/CB2 receptor agonists *in vitro* at reasonably low concentrations (nanomolar range) (Thomas et al., 2007). In particular, it has been shown by *in vitro* studies that CBD is able to act as CB1/CB2 receptors inverse agonist an action that underlies its antagonism of CP55940 and R-(+)-WIN55212 at the CB1/CB2 receptor (Thomas et al., 2007). It has been hypothesized that the anti-inflammatory actions of CBD might be due to its ability to act as a CB2 receptor inverse agonist (Pertwee, 2006). Besides CB receptors, CBD has been profiled also towards other pharmacological substrates. To this regard, CBD showed also affinity to the peroxisome proliferation-activated receptors (PPARs), which are a family of ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily. In humans, there are three PPAR isoforms PPARα, PPARβ/δ, and PPARγ that are encoded by separate genes and are differently expressed in organs and tissues (Michalik et al., 2006). CBD seems to activate the transcriptional activity of PPARγ, which play a primary role in the regulation of adipocyte formation, insulin sensitivity and activation of inflammatory response (O'Sullivan, 2007; O'Sullivan and Kendall, 2010; Hind et al., 2016; O'Sullivan, 2016). To this regard, CBD activates PPARγ receptors leading to a lower expression of proinflammatory genes, which were inhibited by PPARγ antagonists (Esposito et al., 2007; Esposito et al., 2011; O'Sullivan, 2016).

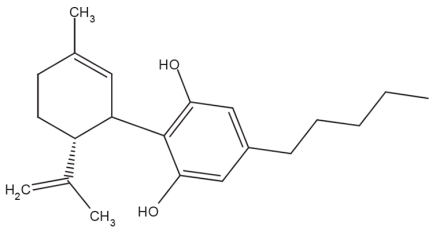
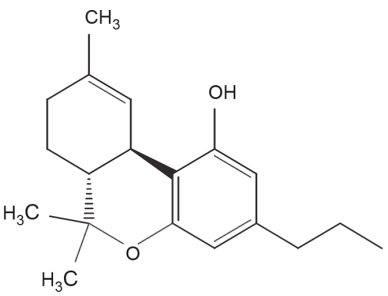
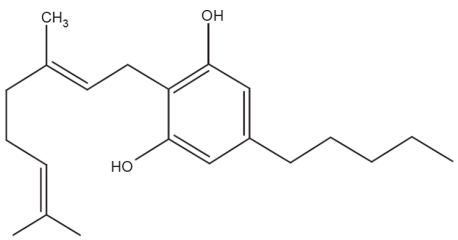
Moreover, CBD exerts a more potent antioxidant effects than other antioxidants, such as ascorbate or α-tocopherol, in *in vitro* study where cortical neurons were treated with toxic concentrations of glutamate (Hampson et al., 1998; Campos et al., 2016).

The neuroprotective effect was present regardless of whether the insult was due to the activation of N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, or kainate receptors and, more interestingly, it was not mediated by CB receptors since the CB antagonist was unaffected (Hampson et al., 1998). The latter result suggests that CBD may be a potent antioxidant without psychotropic side effects, which are mediated by the direct action on CB receptors.

The anti-inflammatory effect of CBD is also mediated by the adenosine A_{2A} (A_{2A}) receptor whose activation dampers the immune system, leading to a reduction of the antigen presentation, immune cell trafficking, immune cell proliferation, production of the proinflammatory cytokine, and cytotoxicity (Magen et al., 2009). In fact, it has been shown that CBD enhances A_{2A} receptor signaling by the inhibition of cellular update of an adenosine transporter leading to anti-inflammatory and antioxidant effects (Carrier et al., 2006). Likewise, CGS-21680, which is an agonist of the A_{2A} receptor, mimics the actions of CBD that were suppressed by an A_{2A} antagonist (i.e. ZM241,385) (Martín-Moreno et al., 2011).

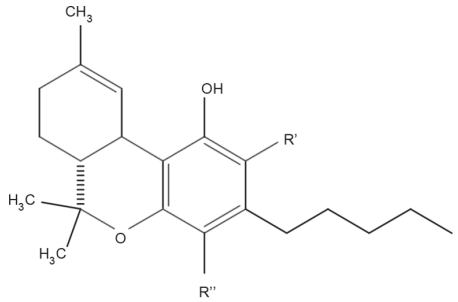
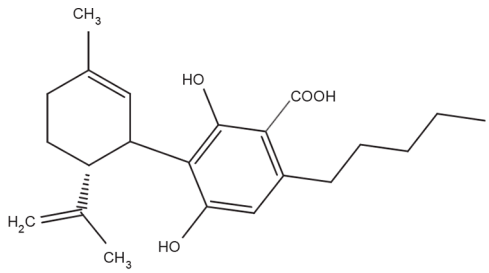
The CBD neuroprotective property seems to be due also to the activation of 5-hydroxytryptamine subtypes 1A (5-HT1A) receptors, which are located in pre- and post-synaptic membranes in several brain regions (Hoyer et al., 1986). Russo and colleagues first demonstrated that CBD is able to activate the 5-HT1A receptors (Russo et al., 2005). Further support to this first observation was given by a recent study where the authors

TABLE 1 | Most abundant nonpsychoactive phytocannabinoids isolated from *Cannabis sativa*: chemical structures and pharmacological actions.

Phytocannabinoids	Mechanisms	Effects	References
 **Cannabidiol (CBD)**	CB_2 inverse agonist CB_1, CB_2 antagonist	Anti-inflammatory effects Antispasmodic effect	Thomas et al., 2005
	FAAH inhibition	Reduces FAAH expression in the inflamed intestine	Ligresti et al., 2006
	TRPA1 agonist TRPM8 antagonist TRPV1 agonist	Analgesic effects Analgesic effects. Potential role in prostate carcinoma Antipsychotic and analgesic effects	De Petrocellis et al., 2008
	Adenosine uptake competitive inhibitor	Anti-inflammatory effects	Carrier et al., 2006
	PPARγ agonist	Vasorelaxation and stimulation of fibroblasts into adipocytes	O'Sullivan et al., 2009
	$5HT_{1A}$ agonist	Anti-ischemic and anxiolytic properties	Campos and Guimarães, 2008 Resstel et al., 2009
	Ca^{2+} channel	Neuroprotective and antiepileptic properties	Drysdale et al., 2006 Ryan et al., 2009
	Suppressor of tryptophan degradation	Potential role in pain, inflammation and depression	Jenny et al., 2009
 **Δ^9-tetrahydrocannabivarin (Δ^9-THCV)**	CB_1 antagonist	Increases central inhibitory neurotransmission	Thomas et al., 2005 Dennis et al., 2008 Ma et al., 2008
	CB_2 partial agonist	Stimulates mesenchymal stem cells	Scutt and Williamson, 2007
 **Cannabigerol (CBG)**	TRPV1 agonist TRPA1 agonist TRPM8 antagonist	Potential role in analgesia	Ligresti et al., 2006 De Petrocellis et al., 2008

(Continued)

TABLE 1 | Continued

Phytocannabinoids	Mechanisms	Effects	References
 **Δ9-tetrahydrocannabinolic acid (Δ9-THCA)** **R' = COOH; R'' = H D9-THCA A** **R' = H; R''= COOH D9-THCA B**	TRPA1 partial agonist TRPM8 antagonist	Potential role in analgesia	De Petrocellis et al., 2008
 **Cannabidiolic acid (CBDA)**	TRPA1 partial agonist TRPM8 antagonist TRPV1 agonist	Potential role in analgesia	De Petrocellis et al., 2008 Ligresti, A., et al., 2006
	COX-2 inhibitor	Potential role in inflammation	Takeda et al., 2008

found that the effect of CBD was blocked by WAY-100135, a selective 5-TH1A receptor antagonist (Galaj et al., 2019).

Finally, it has been demonstrated that CBD has a direct effect on mitochondria (da Silva et al., 2018). To this regard, it has been widely accepted that mitochondrial dysfunction can contribute to neurodegeneration due to the overproduction of ROS and iron accumulation (Mills et al., 2010; Serviddio et al., 2011; Cassano et al., 2012; Cassano et al., 2016; Romano et al., 2017). In particular, iron overload induces several mitochondrial alterations, such as increased mitochondrial DNA (mtDNA) deletions and reduction of epigenetic mtDNA modulation, mitochondrial ferritin levels, and succinate dehydrogenase activity, which may altogether alter cellular viability leading to neurodegenerative process (da Silva et al., 2018). Interestingly, all these iron-induced mitochondrial alterations were completely reversed by CBD, which promotes neural cell survival (da Silva et al., 2018). Moreover, doxorubicin, a broad-spectrum chemotherapeutic drug, induces a dose-dependent cardiotoxicity through the dysregulation of various metabolic signaling pathways, including mitochondrial dysfunction (Hao et al., 2015). In particular, doxorubicin reduces the activity of myocardial mitochondrial complexes (I and II) and glutathione peroxidase leading to an increase of ROS generation (Hao et al., 2015). Interestingly, CBD significantly attenuated doxorubicin-induced cardiotoxicity and cardiac dysfunction by improving mitochondrial complex I activity and enhancing mitochondrial biogenesis (Hao et al., 2015).

Since CBD targets multiple substrates, it may be a good candidate as a multimodal drug for the major neurodegenerative disorders, such as PD and AD. **Figure 1** shows the effects of CBD in PD and AD.

CBD and PD

PD is a progressive neurodegenerative disorder characterized manly by motor alterations, such as akinesia, bradykinesia, tremors, postural instability, and rigidity. Although the etiology of PD is still largely elusive, its pathophysiology is characterized by loss of midbrain substantia nigra DA neurons and overwhelming evidence indicates that OS is a central factor in PD pathophysiology (Hirsch et al., 1988; Branchi et al., 2010; Aureli et al., 2014). It has been demonstrated in animal model of PD that CBD exerts a neuroprotective effect as antioxidant compound acting through a mechanism that is CB receptor-independent (Fernández-Ruiz et al., 2013). In fact, in 6-hydroxydopamine-lesioned mice CBD was able to significantly reduce the DA depletion and to attenuate the OS increasing the expression of Cu,Zn-superoxide dismutase (SOD), which is an important endogenous mechanism that defences cell against OS (Fernández-Ruiz et al., 2013; Martinez et al., 2015). The latter evidence indicates that CBs having antioxidant CB receptor-independent properties attenuate the neurodegeneration of nigrostriatal dopaminergic fibers occurring in PD (García-Arencibia et al., 2007). This thesis is reinforced by the observation that CBD reduces the neuronal cell death in the striatum occurring after the administration of 3-nitropropionic acid (3NP), an inhibitor of mitochondrial complex II. In

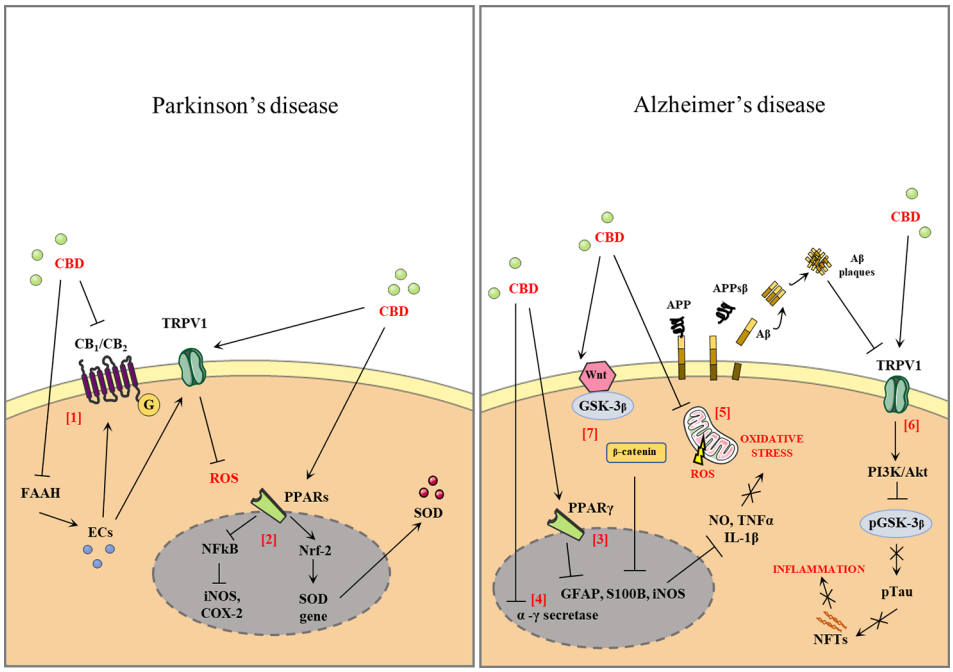


FIGURE 1 | Effect of cannabidiol (CBD) in Parkinson's disease and Alzheimer' disease (AD). CBD antagonizes the action of cannabinoid receptors (CB1, CB2) acting as a reverse agonist and negative allosteric modulator of both receptors. CBD also inhibits fatty acid amide hydrolase (FAAH), resulting in increased levels of endocannabinoids (ECs). ECs activate the anti-oxidant and anti-inflammatory effects that are partially mediated by the actions of the CBD of transient receptor potential cation channel subfamily V member 1 (TRPV1) [1]. CBD binds the peroxisome proliferator-activated receptors (PPARs), antagonizes the action of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), and reduces the expression of proinflammatory enzymes such as inducible nitric oxide synthases (iNOS), cyclooxygenase-2 (COX-2), and proinflammatory cytokines [2]. Activation of PPARγ by modulating the expression of proinflammatory mediators such as nitric oxide (NO), tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), iNOS, and COX-2 [3]. The CBD downregulates the β- and γ-secretase genes leading to a reduction in amyloid-β (Aβ) production [4]. CBD is able to reduce the oxidative stress (OS) through the attenuation of mitochondrial dysregulation and reactive oxygen species (ROS) generation or by the decrease of the expression of several ROS generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoforms [5]. The stimulation of transient receptor potential vanilloid-1 (TRPV1) by CBD can activate phosphoinositide 3-kinases/protein kinase B (PI3K/Akt) signaling, which in turn inhibits glycogen synthase kinase 3 β (GSK-3β) by phosphorylation of Ser9, thus reducing tau phosphorylation [6]. CBD reduces the activity of p-GSK-3β, the active phosphorylated form of GSK3-β, and causes an increase in the Wnt/β-catenin pathway. The activation of this pathway can protect against OS and Aβ neurotoxicity in AD [7].

particular, the authors demonstrated that 3NP administration causes a reduction of both GABA levels and striatal atrophy of the GABAergic neurons as indicated by a depletion of mRNA levels of proenkephalin (PENK), substance P (SP), and neuronal-specific enolase (NSE). Moreover, the inhibition of mitochondrial complex II induced by 3NP reduces the mRNA expression of superoxide dismutase-1 (SOD-1) and -2 (SOD-2), which are endogenous defences against the OS. Interestingly, after 3NP administration CBD can completely abolish the atrophy of the GABAergic neurons and significantly increase the mRNA levels of SOD-2, as well as attenuate the reduction of mRNA levels of SOD-1 and PENK. Differently, after 3NP administration the administration of arachidonyl-2-chloroethylamide (ACEA) or HU-308, respectively agonist of CB1 and CB2 receptor, did not revert the striatal atrophy of the GABAergic neurons, as well as did not restore the endogenous defences against the OS induced by 3NP (Sagredo et al., 2007). Taken together, these results suggest that CBD exerts a neuroprotective role on the GABAergic neurons that project from the striatum to the substantia nigra and further confirm that its mechanism is CB receptor-independent (Sagredo et al., 2007). Furthermore, in another study authors explored whether CBD was able to attenuate the pathological symptoms of PD modulating the GPR55. In particular, mice were treated for 5 weeks with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/probenecid (MPTPp), which induced motor function impairment and loss of tyrosine hydroxylase-positive neurons and DA levels in the brain. This chronic mouse model of PD was treated with abnormal-CBD (Abn-CBD), a synthetic CBD isomer and GPR55 agonist. Authors found that the key features of PD induced by MPTPp were prevented by the pharmacological treatment, suggesting that the activation of GPR55 may be a good strategy for the treatment of PD (Celorrio et al., 2017).

CBD and AD

AD is the most common form of dementia affecting elderly people and its pathology is characterized by the accumulation of amyloid-β (Aβ) plaques and tau neurofibrillary tangles (NFTs) in the brain (Querfurth and LaFerla, 2010).

Although the etiology of AD appears to be linked to a multitude of mechanisms, inflammation seems to play a crucial

role in its pathogenesis (Bronzuoli et al., 2018; Scuderi et al., 2018). Expected benefits of current therapies are limited (Sabbagh, 2009; Neugroschl and Sano, 2010), so that there is pressing demand for discovering new treatments able to slow disease progression or prevent its onset.

In this contest, the anti-inflammatory properties of CBD were evaluated by both *in vitro* and *in vivo* studies in an animal model of Aβ-induced neuroinflammation (Iuvone et al., 2004; Esposito et al., 2006; Esposito et al., 2007; Esposito et al., 2011). In particular, authors demonstrated that CBD reduces the tau protein hyperphosphorylation through the inhibition of Wingless-type MMTV integration site family member (Wnt) pathways and significantly attenuates all the markers of the Aβ-induced neuroinflammation, including the glial fibrillary acidic protein (GFAP) and inducible nitric oxide synthase (iNOS) protein expression, nitrite production, and interleukin 1 β (IL-1β) (Iuvone et al., 2004; Esposito et al., 2006; Esposito et al., 2007; Iuvone et al., 2009; Esposito et al., 2011). CBD pre-treatment induces a reduction of ROS production, lipid peroxidation, caspase-3 levels, and DNA fragmentation in PC12 cells stimulated by Aβ, an *in vitro* model of AD (Iuvone et al., 2004; Bedse et al., 2014; Gallelli et al., 2018).

The beneficial effects of CBD were further confirmed by another study where mice were chronically treated (for 3 weeks) with CBD after been injected intracerebroventricularly with fibrillar Aβ (Martín-Moreno et al., 2011). CBD counteracts the Aβ-induced microglial activation, the production of proinflammatory cytokine tumor necrosis factor α (TNF-α) and ameliorates the memory alterations observed in a spatial memory task (Martín-Moreno et al., 2011).

Moreover, Aβ can gradually accumulate in mitochondria, where it can cause reduction of both activity of the respiratory chain complexes and the rate of oxygen consumption leading to a free radical generation and oxidative damage (Caspersen et al., 2005; Lin and Beal, 2006; Manczak et al., 2006; Cassano et al., 2012). To this regard, CBD is able to counteract mitochondrial alterations by the reduction of ROS production induced by both the Aβ and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase (NOX) (Hao et al., 2015; Vallée et al., 2017).

It is well know that tau hyperphosphorylation, mostly at serine (Ser) or threonine (Thr) residues, plays a crucial role in the pathogenesis of AD, thereby molecules that reduce phospho-tau aggregates may represent a good candidate for the AD treatment. To this regard, it has been demonstrated that CBD reduces the expression of genes, which encode kinases (GSK-3β, CMK, and MAPK) responsible for aberrant tau phosphorylation, leading to a reduction of tau hyperphosphorylation and subsequent NFT formation (Libro et al., 2016). Likewise, CBD activates the PI3K/Akt signaling through the TRPV1, which is able to inhibit the kinase GSK-3β, thereby decreasing tau phosphorylation (Libro et al., 2016). Finally, CBD downregulates β- and γ-secretase genes leading to a reduction of Aβ production (Libro et al., 2016).

CONCLUSION

The present review provided evidence that the nonpsychoactive phytocannabinoids CBD could be a potential pharmacological tool for the treatment of neurodegenerative disorders; its excellent safety and tolerability profile in clinical studies renders it a promising therapeutic agent.

The molecular mechanisms associated with CBD's improvement in PD and AD are likely multifaceted, and although CBD may act on different molecular targets all the beneficial effects are in some extent linked to its antioxidant and anti-inflammatory profile, as observed in *in vitro* and *in vivo* studies. Therefore, this review describes evidence to prove the therapeutical efficacy of CBD in patients affected by neurodegenerative disorders and promotes further research in order to better elucidate the molecular pathways involved in the therapeutic potential of CBD.

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α-Cyperone Attenuates H_2O_2-Induced Oxidative Stress and Apoptosis in SH-SY5Y Cells *via* Activation of Nrf2

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α-Cyperone, extracted from *Cyperus rotundus*, has been reported to inhibit microglia-mediated neuroinflammation. Oxidative stress and apoptosis play crucial roles in the course of Parkinson's disease (PD). PD is a common neurodegenerative disease characterized by selective death of dopaminergic neurons. This study was designed to investigate the neuroprotective effects of α-cyperone against hydrogen peroxide (H_2O_2)-induced oxidative stress and apoptosis in dopaminergic neuronal SH-SY5Y cells. Neurotoxicity was assessed by MTT assay and the measurement of lactic dehydrogenase (LDH) release. The level of reactive oxygen species (ROS) was measured by dichlorodihydrofluorescin diacetate (DCFH-DA) staining. The apoptosis of SH-SY5Y cells was evaluated by annexin-V-FITC staining. The translocation of NF-E2-related factor 2 (Nrf2) was determined by western blot and immunofluorescence staining. Western blot analysis was conducted to determine the expression level of cleaved-caspase-3, the pro-apoptotic factor Bax, and the anti-apoptotic factor, Bcl-2. The results showed that α-cyperone substantially decreased H_2O_2-induced death, release of LDH, and the production of ROS in SH-SY5Y cells. In addition, we found that α-cyperone attenuated H_2O_2-induced cellular apoptosis. Moreover, α-cyperone remarkably reduced the expression of cleaved-caspase-3 and Bax, and upregulated Bcl-2. Furthermore, α-cyperone enhanced the nuclear translocation of Nrf2. Pretreatment with brusatol (BT, an Nrf2 inhibitor) attenuated α-cyperone-mediated suppression of ROS, cleaved-caspase-3, and Bax, as well as α-cyperone-induced Bcl-2 upregulation in H_2O_2-treated SH-SY5Y cells. α-cyperone neuroprotection required Nrf2 activation. In conclusion, α-cyperone attenuated H_2O_2-induced oxidative stress and apoptosis in SH-SY5Y cells *via* the activation of Nrf2, suggesting the potential of this compound in the prevention and treatment of PD.

Keywords: α-cyperone, oxidative stress, apoptosis, ROS, Nrf2

INTRODUCTION

Parkinson's disease (PD) is the second most common age-associated neurodegenerative disorder, affecting about 2% of subjects older than 60 years and more than 5 million people worldwide (Olanow et al., 2009). PD is mainly characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta region of the midbrain (Obeso et al., 2010). Its most common features include resting tremor, rigidity, and bradykinesia (Coelho and Ferreira, 2012). Previous studies have reported that age, genetic, and environmental factors are major determinants in PD pathogenesis. Moreover, the role of oxidative stress and mitochondrial dysfunction is increasingly recognized. For example, it has been demonstrated that oxidative stress and mitochondrial dysfunction can cause neuronal damage and degeneration in PD pathogenesis (Henchcliffe and Beal, 2008; Hauser and Hastings, 2013; Subramaniam and Chesselet, 2013). Therefore, inhibition of these events is a potential strategy to protect dopaminergic neurons.

Oxidative stress is a damaging response resulting from an imbalance between the generation of oxygen-derived radicals and the organism's antioxidant potential, and implies excessive production of reactive oxygen species (ROS) (Schieber and Chandel, 2014). Excessive ROS production and accumulation damage the structure of cell membranes and the biological functions of lipids, proteins and DNA, and ultimately cause the initiation of cell apoptosis (Ma, 2012). Additionally, hydrogen peroxide (H_2O_2)-induced ROS production and release can cause a series of oxidative stress responses, which in turn lead to mitochondrial dysfunction, cell damage, and death (Cheng et al., 2010). Apoptosis induced by mitochondrial dysfunction is regulated by the anti-apoptotic protein, Bcl-2, and the pro-apoptotic protein Bax (D'orsi et al., 2017). Caspase-3, a key component of the apoptotic machinery, is activated to generate cleaved-caspase-3 leading to cell death (Porter and Janicke, 1999). Therefore, suppression of cleaved-caspase-3 and Bax, or Bcl-2 upregulation, may prevent neuronal apoptosis due to oxidative stress.

α-Cyperone, extracted from *Cyperus rotundus*, has been demonstrated to weaken the inflammatory response by microtubule destabilization in the brain (Azimi et al., 2016). Sutalangka et al. has reported *Cyperus rotundus* has neuroprotective and cognitive-enhancing effects in AF64A-treated rats (Sutalangka and Wattanathorn, 2017). We previously showed that α-cyperone exerts neuroprotective effects by inhibiting microglia-mediated neuroinflammation (Huang et al., 2018). The SH-SY5Y neuroblastoma cell line has been previously used as a cellular model of PD (Xie et al., 2010). H_2O_2 is commonly used to reproduce oxidative stress *in vitro*. It has been reported that oxidative stress plays a crucial role in PD (Jenner, 2003; Puspita et al., 2017). Some natural products were reported to prevent oxidative stress in H_2O_2-treated SH-SY5Y cells (Alvarino et al., 2017; De Oliveira et al., 2017; De Oliveira et al., 2018). However, the effects of α-cyperone in H_2O_2-treated SH-SY5Y cells have not been explored. Here, we investigated whether α-cyperone prevented oxidative stress-induced apoptosis of SH-SY5Y cells, and addressed the underlying mechanisms.

MATERIALS AND METHODS

Materials

3-(3,4-Dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), brusatol (BT, an Nrf2 inhibitor), and dimethylsulfoxide (DMSO) were obtained from Sigma Aldrich (St Louis, MO, USA). The Bicinchoninic acid protein H_2O_2 assay (BCA) kit, RIPA lysis buffer, ROS detection kit, and LDH detection kit were obtained from Beyotime Biotechnology (Shanghai, China). α-Cyperone (> 98% purity; Yuan ye Biotech, Shanghai, China) was dissolved in DMSO and freshly diluted to the final concentration of 0.05% DMSO. The Annexin V-FITC/PI Apoptosis Detection Kit was obtained from Solarbio (Beijing Solarbio Science & Technology, Beijing, China). Phosphate buffered saline (PBS), Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY, United States). Primary antibodies against Nrf2, Bax, Bcl-2, cleaved-caspase-3, PCNA, and β-actin were obtained from Proteintech Group (Wuhan China). Secondary goat anti-rabbit or goat anti-mouse antibodies were obtained from Santa Cruz, CA, USA.

Cell culture

The SH-SY5Y cells were obtained from the Cell Culture Center at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Peking, China). The cell lines were seeded on 25 cm^2 cell culture flasks and maintained in DMEM containing 10% FBS, at 37°C in a 5% CO_2 incubator. When cells were approximately at 80% confluence, they were subcultured by trypsinization (0.05%, w/v) and seeded into 6-well or 96-well plates. SH-SY5Y cells were cultured for five days with 10 μM retinoic acid (RA) in DMEM plus 1% FBS, 2 mM glutamine and the necessary antibiotics, followed by another five days of culture in serum-free DMEM with BDNF (brain-derived neurotrophic factor, 50 ng/mL), glutamine (2 mM), and antibiotics (P/S). After culture in serum-free DMEM for 4 h, the cells were pretreated with α-cyperone (15 μM or 30 μM) for 2 h and then co-treated with H_2O_2 (200 μM) for 24 h. To investigate whether the neuroprotective effect of α-cyperone was associated with the activation of Nrf2, SH-SY5Y cells were pretreated with brusatol (BT, an Nrf2 inhibitor dissolved in 0.05% DMSO) for 4 h, co-treated with α-cyperone (30 μM) for 2 h, and then post-treated with H_2O_2 for 24 h. Control cells were cultured in medium containing 0.05% DMSO.

MTT Assay

SH-SY5Y cells (1×10^4 cells/well) were seeded and cultured into a 96-well plate for 12 h. Cells were supplied with fresh serum-free DMEM for 4 h, pretreated with α-cyperone for 2 h, and then co-treated with H_2O_2 for 24 h. The cell viability was determined by MTT assay according to the manufacturer's instructions. First, MTT (0.5 mg/mL) was transferred to the cell cultures, and the cells were incubated for an additional 4 h. The supernatant was removed, DMSO (150 μL/well) was added, and the plates were shaken for 15 min. The absorbance was measured using an absorbance reader (iMark, BioRad, USA) with a testing wavelength of 570 nm and a reference wavelength of 630 nm.

LDH Assay

The release of LDH in the medium was determined by an LDH assay kit in accordance with the supplier's instructions. In brief, α-cyperone was transferred into the medium for 2 h before co-treatment with H_2O_2. After stimulation with H_2O_2 for 24 h, the culture medium (120 μL/well) was collected and transferred to a new 96-well plate. The reaction solution (60 μL/well) was added, and the plate was shaken for 30 min. The release of LDH was evaluated by measuring the absorbance at 490 nm and 600 nm using an absorbance reader (iMark, BioRad, USA).

Measurement of Intracellular ROS

Intracellular ROS was measured with a ROS detection kit according to the manufacturer's instructions. Briefly, SH-SY5Y cells were seeded into 96-well plates (2×10^4 cells/well) for 12 h, and then incubated with serum-free DMEM for 4 h. The cells were pretreated with α-cyperone for 2 h, and then incubated with 50 μM of DCFH-DA for 30 min prior to stimulation with H_2O_2 for 10 min to induce ROS generation. The cells were washed twice with PBS (100 μL/well), incubated with PBS (100 μL/well), and then analyzed by a multi-detection reader at excitation and emission wavelengths of 485 and 535 nm, respectively.

Apoptosis Assay

SH-SY5Y cells apoptosis were assessed by Annexin V-FITC/PI Apoptosis Detection Kit. Briefly, cells (1×10^6 cells/well) were seeded in 6-well plates for 12 h and then pretreated with α-cyperone for 2 h followed by co-treatment with H_2O_2 for an additional 24 h. Cells were mildly washed twice with ice-cold PBS, collected, and centrifuged at 1500 rpm for 5 min at 4 °C. Next, cells were harvested, and the percentage of apoptotic cells was assessed by flow cytometry. Gated cells were separated into four quadrants: early apoptotic cells (Annexin positive/PI negative), necrotic cells (Annexin negative/PI positive), late apoptotic cells (Annexin positive/PI positive), and viable cells (Annexin negative/PI negative). The regions LR, UL, UR, and LL represent early apoptotic, necrotic, late apoptotic, and live cells, respectively.

Western Blot

After treatment with α-cyperone or H_2O_2, the cells were harvested and lysed in RIPA lysis buffer containing phenylmethylsulfonyl fluoride (PMSF). The total protein concentration was evaluated by using a BCA kit with bovine serum albumin as a standard. Equal amounts of proteins (30 μg/well) were separated by a 12% SDS-polyacrylamide gel, and then transferred to polyvinylidene difluoride membranes (PVDF: Millipore, Bedford, MA). The membrane was blocked with 5% (w/v) non-fat milk for 2 h at 25°C. The membrane was incubated with the primary and then with the secondary antibody. The membranes were visualized by the ECL western blot detection system according to the manufacturer's instruction. The band intensities were quantified using Image J gel analysis software. All experiments were performed in triplicate.

Immunofluorescence Assay

SH-SY5Y cells were seeded on poly-L-lysine-coated coverslips in 24-well plates for 12 h, and then treated with α-cyperone (30 μM) for 2 h. To analyze the intracellular distribution of Nrf2, the cells were harvested and fixed with Immunol Staining Fix Solution for 10 min. After three washes with PBS, the cells were permeabilized for 10 min, incubated with 5% normal goat serum for 2 h, incubated with the anti-Nrf2 antibody (1:200) overnight, and then washed with PBS. After incubation with the secondary antibody for 1 h, the cells were stained with DAPI. The coverslips were washed with PBS and then mounted onto slides with fluorescent mounting medium. Representative images were chose from 9 fields of view per treatment group.

Nrf2 Silencing by siRNA Transfection

An Nrf2-specific siRNA was used to knock down Nrf2 expression. SH-SY5Y cells (1 × 105 cells/well) were grown in 6-well plates. After treatment with RA and BDNF, the cells were allowed to reach 50%-60% confluence. For each transfection, 80 pmol of Nrf2 siRNA duplex were diluted into 100 μL of siRNA transfection medium (serum-free Opti-MEM). In a separate tube, 4 μL of the transfection reagent, LipofectamineTM 3000, were diluted into 100 μL of siRNA transfection medium (serum-free Opti-MEM). The dilutions were mixed gently and incubated for 20 min at 25°C. Next, 200 μL of the siRNA/Lipofectamine complex were added to cell culture medium serum-free DMEM (2 mL/well). After siRNA transfection for 12 h, the transfected cells were exposed to α-cyperone. The sequences targeting Nrf2 were as follows: sense, 5′ GAA UUA CAG UGU CUU AAU A 3′; antisense, 5′ UAU UAA GAC ACU GUA AUU C 3′.

Statistical Analysis

The data were presented as the means ± S.E.M and analyzed with SPSS 19.0 (IBM). The comparisons between the different experimental groups were evaluated by one-way ANOVA, whereas multiple comparisons were performed using the LSD method. P values < 0.05 were considered statistically significant.

RESULTS

Effect of α-Cyperone on H_2O_2-Induced Neurotoxicity in SH-SY5Y Cells

To verify possible protective effects of α-cyperone against H_2O_2-induced neurotoxicity in SH-SY5Y cells, MTT and LDH assays were performed. In brief, SH-SY5Y cells were pretreated with α-cyperone (15 or 30 μM) for 2 h and then incubated with H_2O_2 (200 μM) for 24 h. We found that 30 μM α-cyperone had no toxic effect on SH-SY5Y cells (**Figure 2A**). However, the cell viability was remarkably reduced in H_2O_2-treated cells compared to controls (**Figure 2A**). The pretreatment with α-cyperone increased the viability of H_2O_2-treated SH-SY5Y cells (**Figure 2A**). The release of LDH, a cytosolic enzyme, is a commonly used indicator of decreased cell integrity. Whereas cell treatment with H_2O_2 increased LDH release (**Figure 1B**), pretreatment with α-cyperone markedly attenuated this effect (**Figure 2B**). Taken

together, these results revealed that α-cyperone exerted a neuroprotective effect against H_2O_2-induced neurotoxicity in SH-SY5Y cells.

Effect of α-Cyperone on H_2O_2-Induced ROS Production in SH-SY5Y Cells

Mitochondria are the major source of ROS in mammalian cells, and excessive ROS production causes oxidative stress and mitochondrial dysfunction, resulting in cell apoptosis. The production of ROS in SH-SY5Y cells was measured using a DCFH-DA fluorescence assay. We found that 30 μM α-cyperone alone did not affect ROS production (**Figure 3**). Treatment with H_2O_2 markedly increased the intracellular level of ROS in SH-SY5Y cells (**Figure 3**). However, pretreatment with α-cyperone dramatically prevented ROS production (**Figure 3**), suggesting that the compound protected dopaminergic neurons against oxidative stress damage.

Effect of α-Cyperone on H_2O_2-Induced Cell Apoptosis

To assess the effect of α-cyperone on H_2O_2-induced apoptosis in SH-SY5Y cells, the apoptotic rate was measured by flow cytometry. We found that 30 μM α-cyperone alone did not affect the apoptotic rate of SH-SY5Y cells (**Figure 4B**). After treatment with H_2O_2 for 24 h, the apoptotic rate of SH-SY5Y cells increased to approximately

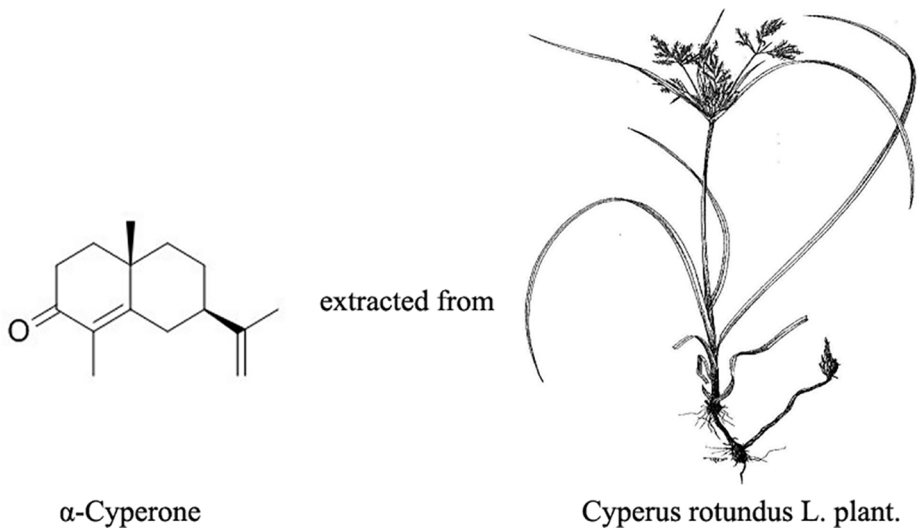

FIGURE 1 | Structure of α-cyperone and *Cyperus rotundus* L. plant. *Cyperus rotundus* L. Traditional uses have been summarized and reported (Pirzada et al., 2015; Kamala et al., 2018).

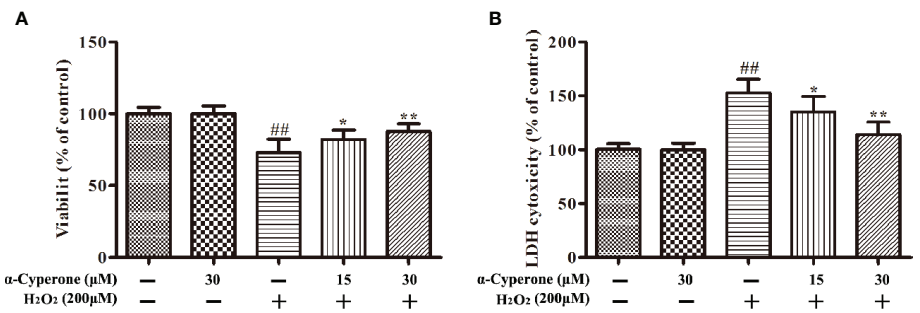

FIGURE 2 | Effect of α-cyperone on H_2O_2-induced neurotoxicity in SH-SY5Y cells. After being pretreated with α-cyperone (15 or 30 μM) for 2 h, SH-SY5Y cells were stimulated with H_2O_2 (200 μM) for 24 h. **(A)** Cell viability was measured by MTT assay. **(B)** The release of LDH was assessed by LDH assay. All experiments were repeated at least three times and similar results were obtained. Data are presented as the mean ± SE, (n = 5 samples per group). $^{\#\#}p < 0.01$ vs. the control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the H_2O_2-treated group.

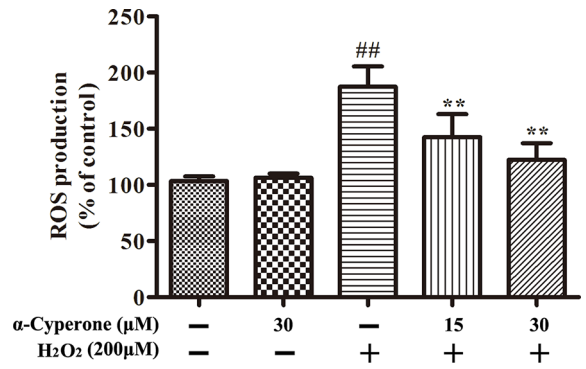

FIGURE 3 | Effect of α-cyperone on H_2O_2-induced ROS in SH-SY5Y cells. After treatment with α-cyperone (15 or 30 μM) for 2 h, SH-SY5Y cells were incubated with 50 μM of DCFH-DA for 30 min prior to stimulation with H_2O_2 (200 μM) for 10 min. The production of ROS was determined by an ROS detection kit. All experiments were repeated at least three times and similar results were obtained. Data are presented as the mean ± SE, (n= 5 samples per group). $^{\#\#}p < 0.01$ vs. the control group, $^{**}p < 0.01$ vs. the H_2O_2-treated group.

50% (**Figure 4C**). However, pretreatment with α-cyperone clearly reduced the H_2O_2-induced effect (**Figures 4D–F**), indicating that α-cyperone inhibited H_2O_2-induced cell apoptosis. Moreover, whereas the fraction of annexin-negative/PI-positive necrotic cells was about 6.39% after 24 h of incubation with H_2O_2, pretreatment with 30 μM α-cyperone brought this proportion to about 4.47%.

Effect of α-Cyperone on H_2O_2-Induced Changes in Bax, Bcl-2, and Cleaved-Caspase-3 Expression

To further explore the effects of α-cyperone on H_2O_2-induced apoptosis, we analyzed the expression level of Bax, Bcl-2, and cleaved-caspase-3 by western blot. The level of Bax and Bcl-2 expression may be indicative of mitochondrial dysfunction, affecting cell apoptosis. We found that H_2O_2 upregulated the pro-apoptotic protein, Bax, while pretreatment with α-cyperone attenuated this effect in SH-SY5Y cells (**Figures 5A, B**). Moreover, 30 μM α-cyperone alone enhanced the expression level of the anti-apoptotic protein, Bcl-2. Notably, while 200 μM H_2O_2 downregulated Bcl-2 expression, cell pretreatment with α-cyperone reduced the latter effect (**Figure 5C**). The expression level of cleaved-caspase-3 protein is a marker of cell apoptosis. We found that α-cyperone suppressed cleaved-caspase-3 expression in H_2O_2-treated SH-SY5Y cells (**Figure 5D**). Taken together, our results suggested that α-cyperone prevented the apoptosis of dopaminergic neurons due to mitochondrial dysfunction.

Effect of α-Cyperone on the Nuclear Translocation of Nrf2 in SH-SY5Y Cells

Nrf2 is a transcription factor regulating basal and inducible transcription of genes encoding factors protective against

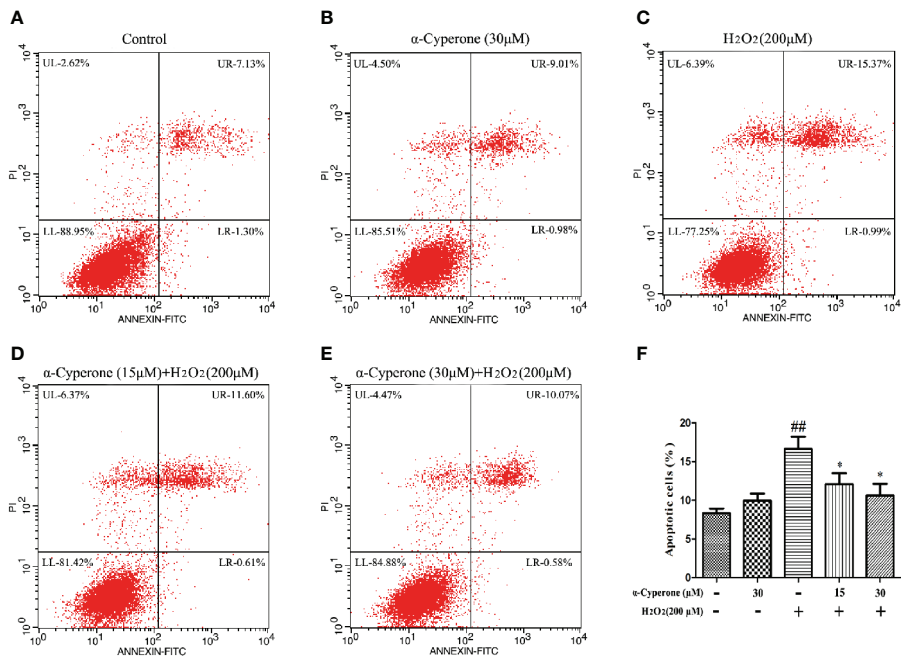

FIGURE 4 | Effect of α-cyperone on H_2O_2-induced cell apoptosis. After being pretreated with α-cyperone (15 or 30 μM) for 2 h, SH-SY5Y cells were stimulated with H_2O_2 (200 μM) for 24 h. **(A–F)** The apoptosis were assessed by an Annexin V-FITC/PI Apoptosis Detection Kit. All experiments were repeated at least three times and similar results were obtained. Data are presented as the mean ± SE, (n= 5 samples per group). $^{\#\#}p < 0.01$ vs. the control group, $^{*}p < 0.05$ vs. the H_2O_2-treated group.

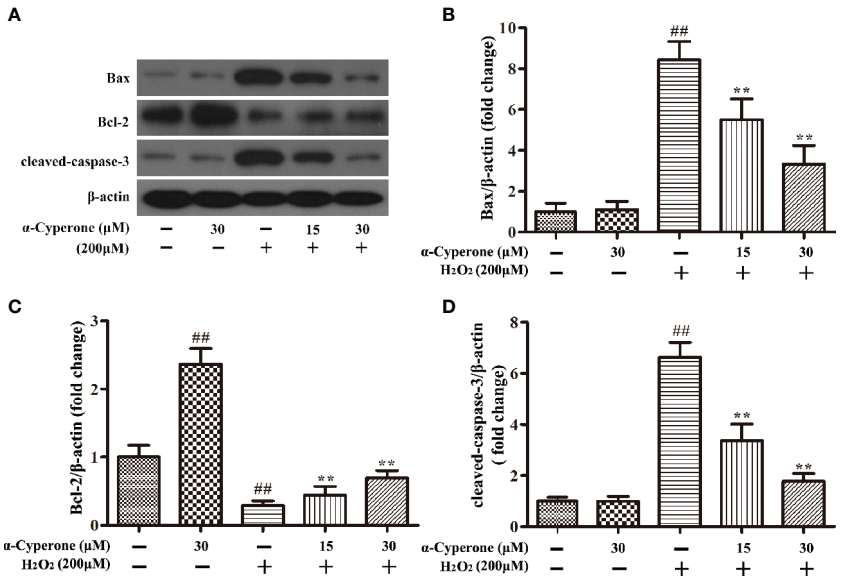

FIGURE 5 | Effect of α-cyperone on Bax, Bcl-2, and cleaved-caspase-3 expression in SH-SY5Y cells pre-challenged with H_2O_2. After being pretreated with α-cyperone (15 or 30 μM) for 2 h, SH-SY5Y cells were stimulated with H_2O_2 (200 μM) for 24 h. **(A–D)** The expression levels of Bax, Bcl-2, and cleaved-caspase-3 were determined by western blot. All experiments were repeated at least three times and similar results were obtained. Data are presented as the mean ± SE, (n= 5 samples per group). $^{\#\#}p < 0.01$ vs. the control group, $^{**}p < 0.01$ vs. the H_2O_2-treated group.

various oxidative stresses. Nrf2 activation causes its nuclear translocation, leading to the activation of antioxidant or detoxifying genes. To assess the effect of α-cyperone or H_2O_2 on the activation of Nrf2, we used western blot and immunofluorescence assay to measure Nrf2 nuclear translocation in SH-SY5Y cells. We observed that 30 μM α-cyperone enhanced the nuclear translocation of Nrf2 over a period of 6 h (**Figure 6A**). However, the effect of α-cyperone on Nrf2 translocation was already detectable after 2 h. And we also found that 30 μM of α-cyperone observably activated the nuclear translocation of Nrf2 for 2 h (**Figures 6B, D**). In addition, 200 μM H_2O_2 had no effect on Nrf2 activation in SH-SY5Y cells, as assessed by both western blot and immunofluorescence (**Figures 6A, C, E**). Overall, these results suggested that α-cyperone promoted the nuclear translocation of Nrf2 in SH-SY5Y cells.

α-Cyperone Effects on ROS Production, as Well as on the Expression of Bax, Cleaved-Caspase-3, and Bcl-2, Depend on Nrf2 Activation

To explore whether α-cyperone effects on oxidative stress, mitochondrial dysfunction, and apoptosis were dependent on Nrf2 activation, we pretreated SH-SY5Y cells with BT (an inhibitor of Nrf2) or knocked down Nrf2 expression by specific siRNAs. Western botting results showed that both conditions dramatically attenuated the inhibitory effect of α-cyperone on ROS production, as well as on Bax and cleaved-caspase-3 expression (**Figures 7A–C**, **E**). Moreover, the inhibition of Nrf2 activation reduced the expression of the anti-apoptotic protein, Bcl-2 (**Figure 7D**). MTT, LDH, and ROS assays revealed the absence of cytotoxicity only in cells cultured in DMEM, control cells, and cells transfected with Nrf2-siRNA (not shown). These results indicated that α-cyperone inhibited oxidative stress, mitochondrial dysfunction, and apoptosis in H_2O_2-treated SH-SY5Y cells *via* the activation of Nrf2.

DISCUSSION

In the present study, we reported the neuroprotective effect of α-cyperone in an *in vitro* model of PD based on the exposure of SH-SY5Y human neuroblastoma cells to H_2O_2. This cells have many characteristics of human dopaminergic neuron and, therefore, are commonly employed to *in vitro* mimic PD alterations. Accumulating evidence has demonstrated that oxidative stress, associated with mitochondrial dysfunction, contributes to the development of PD (Dias et al., 2013; Mullin and Schapira, 2013; Yan et al., 2013; Kong et al., 2014). Our results revealed that α-cyperone reduced the excessive production of ROS, and attenuated mitochondrial dysfunction and cellular apoptosis in H_2O_2-induced SH-SY5Y cells. Pretreatment with BT (an inhibitor of Nrf2) attenuated the neuroprotective effects of α-cyperone, suggesting that α-

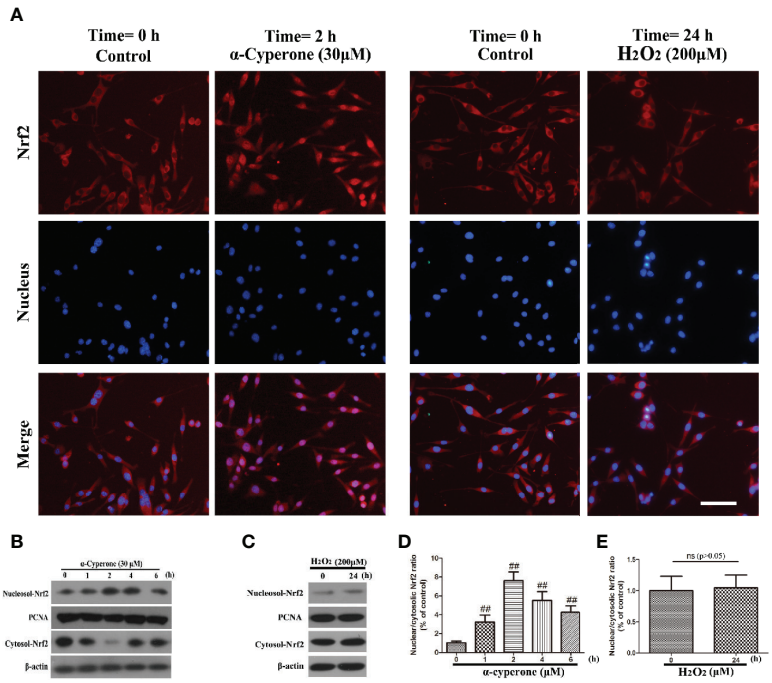

FIGURE 6 | Effects of α-cyperone and H_2O_2 on the nuclear translocation of Nrf2 in SH-SY5Y cells. **(A)** SH-SY5Y cells were treated with α-cyperone (30 μM) for 2 h or H_2O_2 (200 μM) for 24 h, and the nuclear translocation of Nrf2 was determined by immunofluorescence assay. **(B–E)** After incubation with α-cyperone (30 μM) for various time periods (1-6 h) or with H_2O_2 (200 μM) for 24 h, the nuclear and cytosolic Nrf2 levels were measured by western blot. All experiments were repeated at least three times and similar results were obtained. Representative photomicrographs of Nrf2 are shown. The scale bar represents 100 μ. Data are presented as the mean ± SE, (n= 5 samples per group). $^{\#\#}p < 0.01$ vs. the control group.

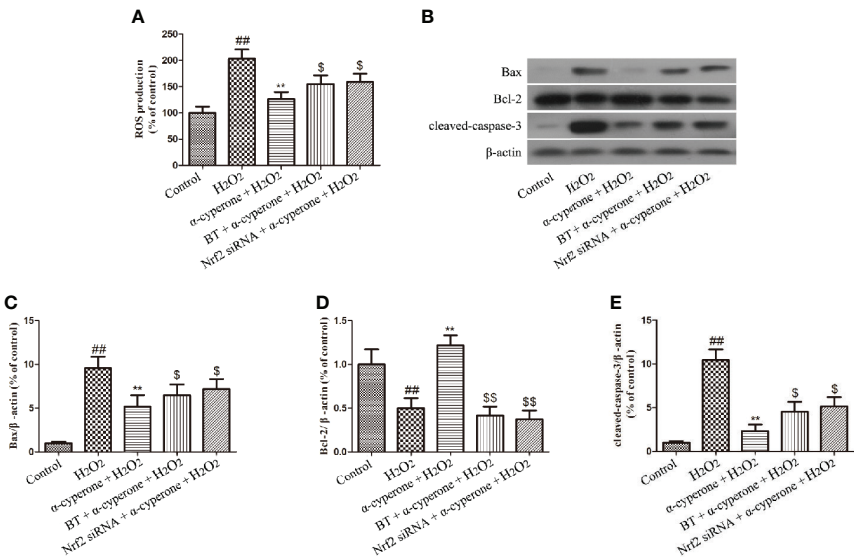

FIGURE 7 | Role of Nrf2 in the protective effects of α-cyperone against H_2O_2-induced oxidative stress, mitochondrial dysfunction, and apoptosis. After cell pretreatment with the Nrf2 inhibitor, BT (250 nM), for 4 h or with Nrf2 siRNA for 12 h, SH-SY5Y cells were treated with α-cyperone (30 μM) for 2 h. **(A)** Then, the cells were incubated with 50 μM of DCFH-DA for 30 min prior to stimulation with H_2O_2 (200 μM) for 10 min. The production of ROS was determined by ROS detection kits. **(B-E)** The cells were also stimulated with H_2O_2 (200 μM) for 24 h, and the expression levels of Bax, Bcl-2, and cleaved-caspase-3 were measured by western blot. All experiments were repeated at least three times and similar results were obtained. Data are presented as the mean ± SE, (n= 5 samples per group). $^{\#\#}p < 0.01$ vs. the control group, $^{**}p < 0.01$ vs. the H_2O_2-treated group, $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ vs. the α-cyperone + H_2O_2 group.

cyperone-mediated protection of dopaminergic neurons from oxidative stress-induced apoptosis, as well as from mitochondrial dysfunction, occurred *via* the activation of Nrf2.

We previously reported that α-cyperone inhibits neuroinflammation in activated-microglia. Moreover, it has been reported that α-cyperone inhibits LPS-induced COX-2 expression and PGE2 production through the negative regulation of NF-κB signaling in RAW 264.7 cells (Jung et al., 2013). To further explore whether α-cyperone protected dopaminergic neurons against oxidative stress-induced damage, we investigated the effect of α-cyperone in H_2O_2-treated SH-SY5Y cells. We confirmed the neuroprotective effect of α-cyperone against H_2O_2-induced neurotoxicity by both MTT and LDH assay. Cell pretreatment with H_2O_2 is known to cause the excessive release of ROS, which initiates DNA and mitochondrial damage, resulting in cell apoptosis (Dai et al., 2014; Ding et al., 2016; Fu et al., 2016). Accumulating evidence has shown that ROS production is associated with oxidative stress and mitochondrial anomalies (Nesi et al., 2017; Sundqvist et al., 2017; Franco-Iborra et al., 2018). We found that α-cyperone reduced the production of ROS in H_2O_2-treated SH-SY5Y cells. It has been demonstrated that cell apoptosis caused by oxidative stress may be related to various diseases (Cervellati et al., 2014; Paradies et al., 2014; Wu et al., 2014; Li and Hu, 2015). In the present study, α-cyperone notably suppressed the apoptosis of SH-SY5Y cells. To explore this phenomenon, we investigated the effect of α-cyperone on mitochondrial dysfunction. We found that α-cyperone upregulated Bcl-2 and downregulated Bax in H_2O_2-treated SH-SY5Y cells, suggesting that α-cyperone suppressed mitochondrial dysfunction. Sanga et al. have proved that the inhibition of cleaved-caspase-3 production protects a SH-SY5Y against toxic injury (Sang et al., 2018). In addition, Wu et al. have reported that carnosic acid protects against 6-hydroxydopamine-induced neurotoxicity in *in vivo* and *in vitro* models of PD, by inducing antioxidative enzymes and inhibiting the production of cleaved-caspase-3 (Wu et al., 2015). We demonstrated that α-cyperone effectively inhibited the expression of cleaved-caspase-3 in H_2O_2-induced SH-SY5Y cells. These results suggested that α-cyperone may protect dopaminergic neurons against mitochondrial dysfunction induced by oxidative stress.

The rhizome of *C. rotundus* has been reported to exert anti-apoptotic and anxiolytic activity in SH-SY5Y cells (Awad et al., 2003; Hemanth Kumar et al., 2013). Moreover, Hu et al. have demonstrated that the essential oil from *C. rotundus rhizomes*, containing α-cyperone (38.46%), possesses an excellent antioxidant activity, protects against DNA damage in human neuroblastoma SH-SY5Y cells (Hu et al., 2017). We found that α-cyperone promoted the nuclear translocation of Nrf2 in SH-SY5Y cells. Nrf2 is a potent transcriptional activator, controlling the transcription of many cytoprotective genes in response to oxidative stress (Maruyama et al., 2011; Qaisiya et al., 2014). Under normal conditions, Nrf2 is present in the cell cytoplasm and maintains the basal levels of cytoprotective enzymes. Upon activation, Nrf2 translocates to the nucleus and upregulates various cytoprotective enzymes (Baird and Dinkova-Kostova, 2011). Several studies have reported Nrf2-dependent neuroprotective effects of natural products in SH-SY5Y cells (Lou et al., 2014; Jin et al., 2015; Kwon et al., 2015; De Oliveira et al., 2016). We found that cell pretreatment with BT (an inhibitor of Nrf2) attenuated α-cyperone-induced neuroprotection.

ROS may be a critical mediator of H_2O_2 neurotoxicity. Mitochondria, endoplasmic reticulum, α-synuclein, and dopamine may contribute to oxidative stress in dopaminergic neurons. Our results showed that BT or Nrf2 silencing attenuated α-cyperone effects on cleaved caspase-3 expression in H_2O_2-treated SH-SY5Y cells (**Figure 7E**). However, α-cyperone neuroprotection may be associated with other signaling pathways and need be further explored. PD models based on Nrf2 knockout mouse will be a suitable tool to in-depth characterize α-cyperone neuroprotection and its therapeutic potential in the context of PD.

In conclusion, our results showed that α-cyperone exerted neuroprotective effects against H_2O_2-induced cell apoptosis in SH-SY5Y cells. In addition, α-cyperone inhibited excessive ROS production and mitochondrial dysfunction. Moreover, α-cyperone downregulated the expression of Bax and cleaved-caspase-3, and upregulated Bcl-2 expression. Finally, Nrf2 inhibition attenuated α-cyperone-induced neuroprotection. The neuroprotective actions of α-cyperone are summarized in **Figure 8**. Taken together, these results suggest that α-cyperone is a potential candidate for the prevention of apoptosis in dopaminergic neurons.

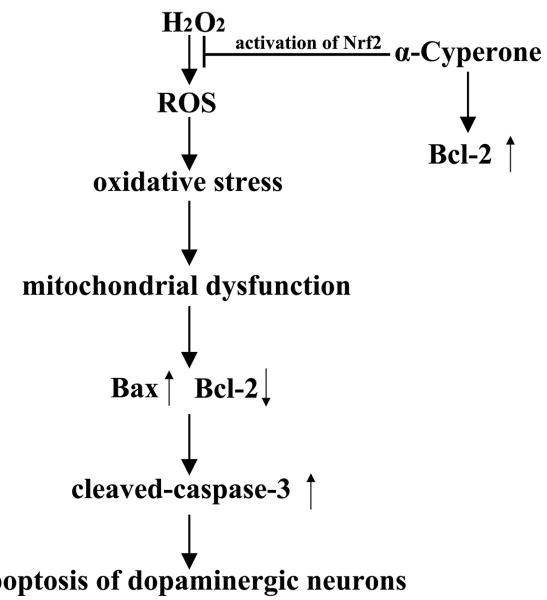

FIGURE 8 | Proposed schematic mechanisms of α-cyperone neuroprotection in an *in vitro* PD model. α-cyperone attenuates H_2O_2-induced oxidative stress and apoptosis in SH-SY5Y cells *via* the activation of Nrf2.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JL and DL conceived and designed the study. BH and SF performed the experiments, analyzed the data, and wrote the paper. YL, DH, XR, XY, TM, JD, XG, and YZ collected the samples and information. All authors reviewed the manuscript. In addition, all authors have read and approved the manuscript.

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