Research Article

Quercetin and Sesamin Protect Dopaminergic Cells from MPP⁺-Induced Neuroinflammation in a Microglial (N9)-Neuronal (PC12) Coculture System

Julie Bournival, 1 Marilyn Plouffe, 1 Justine Renaud, 1 Cindy Provencher, 1 and Maria-Grazia Martinoli 1, 2

1 Department of Biochemistry and the Neuroscience Research Group, Université du Québec, Trois-Rivières, Québec, Canada G9A 5H7
2 Neuroscience Research Unit, Centre de Recherche du CHUL, Université Laval, Ste-Foy, Québec, Canada G1V 4G2

Correspondence should be addressed to Maria-Grazia Martinoli, maria-grazia.martinoli@uqtr.ca

Received 9 March 2012; Revised 11 May 2012; Accepted 20 May 2012

A growing body of evidence indicates that the majority of Parkinson’s disease (PD) cases are associated with microglia activation with resultant elevation of various inflammatory mediators and neuroinflammation. In this study, we investigated the effects of 2 natural molecules, quercetin and sesamin, on neuroinflammation induced by the Parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP⁺) in a glial-neuronal system. We first established that quercetin and sesamin defend microglial cells against MPP⁺-induced increases in the mRNA or protein levels of 3 pro-inflammatory cytokines (interleukin-6, IL-1β and tumor necrosis factor-alpha), as revealed by real time-quantitative polymerase chain reaction and enzyme-linked immunoabsorbent assay, respectively. Quercetin and sesamin also decrease MPP⁺-induced oxidative stress in microglial cells by reducing inducible nitric oxide synthase protein expression as well as mitochondrial superoxide radicals. We then measured neuronal cell death and apoptosis after MPP⁺ activation of microglia, in a microglial (N9)-neuronal (PC12) coculture system. Our results revealed that quercetin and sesamin rescued neuronal PC12 cells from apoptotic death induced by MPP⁺ activation of microglial cells. Altogether, our data demonstrate that the phytoestrogen quercetin and the lignan sesamin diminish MPP⁺-evoked microglial activation and suggest that both these molecules may be regarded as potent, natural, anti-inflammatory compounds.

1. Introduction

Parkinson’s disease (PD) is a progressive, neurodegenerative disorder characterized by the loss of dopaminergic (DAergic) neurons in the substantia nigra (SN) and glial dysfunction. A new flow of information indicates that inflammation-derived oxidative stress and cytokine-dependent toxicity contribute to nigrostriatal pathway degeneration [1–3]. Postmortem studies have shown that microglia are activated regionally in the SN of PD patients as well as in PD animal models [4–6]. Microglia, resident immune cells of the brain, are activated in response to initiation factors (i.e., toxins, bacteria or viruses, pesticides, neuronal injury, etc.). These factors may also trigger a self-perpetuating cycle of chronic neuroinflammation, increasing the release of inflammatory chemical substances and promoting microglia activation. Besides, the SN is the brain region with the highest density of microglial cells [7]; thus, the neurons of this region are particularly susceptible to microglial-mediated toxicity in vitro and in vivo [8].

Proinflammatory cytokines and prostaglandins, identified in the SN, striatum and cerebrospinal fluid of PD patients postmortem, include tumor necrosis factor-alpha (TNFα), interleukin-1beta, -2, and -6 (IL-1β, IL-2, IL-6) [9, 10]. TNFα, IL-1α, IL-1β, and IL-6 have also been identified in PD animal models [11–13].

MPP⁺ (1-methyl-4-phenylpyridinium), the active neurotoxic metabolite of the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), inhibits complex I of the mitochondrial respiratory chain, inducing energy depletion and producing reactive oxygen species (ROS), such as superoxide anion (\(\cdot\)O₂⁻) [14]. The latter can react with nitric oxide (NO) to generate the potent oxidant...
2. Materials and Methods

2.1. Drugs and Chemicals. All reagents and chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

2.2. Cell Culture and Treatments. PC12 cells, obtained from the American Type Culture Collection (Rockville, MD), were maintained in a humidified environment at 37°C and 5% CO2 atmosphere. They were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated horse serum (HS), 5% (v/v) heat-inactivated fetal bovine serum (FBS), and gentamicin (50 μg/mL). Neuronal PC12 cell differentiation was evoked by nerve growth factor-7S (50 ng/mL) in DMEM supplemented with 1% FBS for 5 days, as already described [33]. The microglial cell line N9 (a generous gift from Dr. L. Vallières, Centre de recherche, CHUL, Quebec, QC, Canada) was grown in 10% HS in DMEM nutrient mixture F12-ham (DMEM-F12). To assess the influence of quercetin and sesamin on MPP+-induced N9 inflammation, the cells were pretreated with quercetin (0.1 μM) or sesamin (1 pM) for 3 h and then exposed to MPP+ (500 μM) for 12 or 24 h. Quercetin and sesamin concentrations in these experiments were determined by previous dose-response curves and kinetic studies [25, 32, 33]. All experiments were performed in medium with charcoal-stripped serum to remove steroids from the medium.

Neuronal PC12 cells and N9 microglia were cocultured to study the impact of MPP+-activated microglia on the survival of neuronal PC12 cells. N9 microglial cells were grown in culture inserts (pore size 0.4 μm, BD Falcon, Oakville, ON, Canada); then, MPP+ was added, and inserts containing N9 cells were transferred on neuronal PC12 cells grown previously on coverslips. In this coculture system, microglial cells communicate with neuronal PC12 cells through the semipermeable membrane, avoiding direct contact between the 2 cell populations [26, 34]. The PC12 supernatant was collected 24 h later for cell death measurement, according to the lactate dehydrogenase (LDH) cytotoxicity test and DNA fragmentation assay described below. To control for possible MPP+ crossing the insert membrane and causing neuronal PC12 death, we performed control experiments on inserts without microglia and after treatment of the medium with MPP+.

2.3. Cytotoxicity Measurements. Cytotoxicity was evaluated in control and MPP+ conditions by colorimetric assay, which is based on the measurement of LDH activity released from damaged cells into the supernatant, as already described [33]. LDH, a stable cytoplasmic enzyme present in all cells, is rapidly released into the cell culture supernatant upon plasma membrane damage. Enzyme activity in the culture supernatant correlates with the proportion of lysed cells [35]. Briefly, 50 μL of cell-free supernatant served to quantify LDH activity by measuring absorbance at a wavelength of 490 nm in a microplate reader (Thermo Lab Systems, Franklin, MA). Total cellular LDH was determined by lysing the cells with 1% Triton X-100 (high control); the assay medium with 1% Triton X-100 served to quantify LDH activity in control and MPP+ conditions by colorimetric assay, which is based on the measurement of LDH activity released from damaged cells into the supernatant, as already described [33].

\[
\text{Cytotoxicity} (\%) = \frac{(\text{Experimental value} - \text{Low control})}{(\text{High control} - \text{Low control})} \times 100. \tag{1}
\]

2.4. DNA Fragmentation Analysis. DNA fragmentation was assessed with the single-stranded DNA (ssDNA) apoptosis

peroxynitrite, which has been implicated in the development of several neurological diseases [15, 16]. Accumulation of activated microglia around DAergic neurons has been found in postmortem human brains with MPTP-induced parkinsonism [17]. In addition, MPTP primate models confirm that serum TNFα levels are elevated without changes in IL-1β levels after toxin administration [18]. Furthermore, the proinflammatory cytokines TNFα and IL-1β are involved in DAergic neuronal death in MPTP-treated mice [19]. Together, these data indicate a close association between MPP+-induced microglial activation and the degeneration of DAergic neurons.

Recent investigations have disclosed the powerful properties of various natural polyphenols against oxidative stress in several cellular and in vivo paradigms of neurodegenerative diseases [20–23]. In particular, quercetin, a flavonoid possessing free radical scavenging properties, may protect against oxidative injury by its ability to modulate intracellular signals and promote cell survival [24]. Several studies suggest its potential as a cardioprotective, antioxidant, and antiapoptotic molecule (see references in [25]). Quercetin also exerts a protective effect against microglia activation and NO production and defends DAergic cells against inflammatory damage induced by the potent inflammatory molecule lipopolysaccharide (LPS) [26, 27].

Sesamin as well as sesamol and sesaminol, the other 2 primary compounds in sesame seeds, is likely responsible for the increased stability of sesame oil against autoxidation and the development of rancidity caused by free radicals [28]. Sesamin is also recognized for its positive physiological actions, regulation of lipid and alcohol metabolism in the liver [29–31], and protection against oxidative stress and inflammation in PC12 cells [25, 32]. Currently, no data on the effects of natural antioxidant molecules against MPP+-induced neuroinflammation have been reported.

The objective of this study was to investigate the influence of quercetin and sesamin on MPP+-induced inflammation in a microglial-neuronal coculture system. Our results demonstrate that quercetin and sesamin reduce the gene expression and protein concentrations of 3 proinflammatory cytokines (IL-6, IL-1β, and TNFα) in N9 microglial cells. Also, quercetin and sesamin decrease inducible nitric oxide synthase (iNOS) protein expression and *O₂⁻ production and rescue neuronal PC12 cells from glial-evoked apoptotic death.

\[ \text{Cytotoxicity} (\%) = \frac{\text{Experimental value} - \text{Low control}}{\text{High control} - \text{Low control}} \times 100. \tag{1} \]
ELISA kit (Chemicon International, Temecula, CA). This procedure is based on selective DNA denaturation by formamide in apoptotic cells but not in necrotic cells or in cells with DNA damage in the absence of apoptosis. The detection of denatured DNA was performed with a monoclonal antibody to ssDNA. The staining of ssDNA in early apoptosis was undertaken with a mixture of antibody and peroxidase-labelled secondary antibody. The reaction was then stopped, and ssDNA fragmentation was quantified by measuring absorbance at a wavelength of 405 nm in a microplate reader (Thermo Lab Systems). ssDNA was calculated with reference to control conditions. Absorbance of positive and negative controls served as quality control of ELISA.

2.5. Detection of Mitochondrial \( \text{O}_2^- \). Microglial cells were grown and treated on collagen-coated circular glass coverslips, and MitoSOX Red (Invitrogen, Burlington, ON, Canada) was deployed to estimate intracellular \( \text{O}_2^- \) production. This fluorogenic dye is a highly selective indicator of \( \text{O}_2^- \) in the mitochondria of live cells. After treating microglia for 9 h with MPP+ with or without quercetin or sesamin, the medium was removed and the cells were incubated with MitoSOX Red (5 mM) for 10 min at 37°C (Invitrogen). MitoSOX Red rapidly and selectively targets the mitochondria. Once in the mitochondria, it is oxidized by \( \text{O}_2^- \) and exhibits red fluorescence. The cells were washed with Hanks’ buffered salt solution and 4',6'-diamidino-2-phenylindole (DAPI) counterstained all nuclei. Then, the cells were fixed in 4% paraformaldehyde for 6 min at 37°C. Coverslips were mounted with Molecular Probes ProLong Antifade kit (Invitrogen). Images were acquired by Leitz inverted microscope with a high-pressure mercury burner and necessary filters, and analyzed with NIS-Element 2.2 software (Nikon, Mississauga, ON, Canada). To demonstrate MitoSOX Red selectivity, sodium diethyldithiocarbamate (DDC), an inhibitor of superoxide dismutase, was used as positive control.

2.6. Electrophoresis and Immunoblot Analysis. N9 cells were grown and treated in 6-well plates. Total proteins were extracted with nuclear extraction kit (Active Motif, Brockville, ON, Canada). Proteins were assessed by bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL), and equal amounts were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel. After electrophoretic separation (180 volts, 45 min), the gels were transferred onto polyvinylidene difluoride membranes (0.22 μm pore size, BioRad, Hercules, CA). The blots were blocked for 1 h at room temperature (RT) in 5% nonfat powder milk. Immunoblotting was performed overnight at RT with anti-iNOS antibody (1:50) (StressGen, Biotech, Ann Arbor, MI). The following day, the blots were washed and then incubated with peroxidase-conjugated secondary antibody (1:10,000), for 1 h at RT, for development with enhanced chemiluminescence substrate solution.

2.7. Real-Time-Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted with Sigma’s GenElute Mammalian Total RNA extraction kit. RNA was spectrophotometrically measured for each condition, and 1 μg of total RNA was reverse-transcribed with 25 U of M-MLV reverse transcriptase, 1.5 μM of dNTP, and 10 μM of random hexamers. RT-qPCR was then performed in a MiniOpticon RT-PCR system (BioRad) in 20 μL-sized reactions containing 4 μL of cDNA mixture, 0.3 μM of each forward and reverse primer and 10 μL of iQ SYBR Green Supermix (BioRad). Incubation at 95°C for 3 min was followed by 40 cycles of 15 s at 95°C and 30 s at 61°C. Primers for TNFa (5′-TTCTGTCATTCTGAACTTCGGGGTGATCGGTCC-3′ and 5′-GTA-TGAGATAGCAAATCGGCTGACGTTGTTGGG-3′), IL-1β (5′-GCCCATCCTCTGTTAGCTACAT-3′ and 5′-AGGCCAAGGTATTTTGTCG-3′), IL-6 (5′-TTCCATCCAGTT-GCCCTCTT-3′ and 5′-ATTCCACGATTTCCCAGAG-3′), ubiquitin C (5′-AGCCACGTDTACCAACAG-3′ and 5′-TCACACCAAGAAACACGAC-3′), β-microglobulin (5′-ATGGGAACCGGAACTACTG-3′ and 5′-CAGTCTCAG-TGGGTTGAAT-3′) were designed by BLAST sequences with PRIMER3 web-based software and synthesized at Sigma Genosys (Oakville, ON, Canada). Reactions were performed in duplicate, and 3 independent preparations of cDNA were studied. A 10-fold dilution series was obtained from a random pool of cDNA ranging from ×10 to ×100,000 dilution. Mean cycle threshold values (Ct) for each dilution were plotted against log10 of cDNA input to generate efficiency plots. The reaction efficiency of each gene assay was calculated according to the equation E = 10(−1/slope), where E was reaction efficiency and “slope” was the slope of the line generated in efficiency plots. All PCR efficiencies were above 90%. In all PCR experiments, post-PCR DNA-melting curve analysis was undertaken to assess amplification specificity. DNA melting was carried out at a temperature ramping rate of 1°C per step with 1 s rest at each step. Relative gene transcription was calculated by the comparative Ct method, using the real-time efficiency values of each gene. cDNA levels among the samples were normalized by the expression of 2 internal control genes: ubiquitin and β-microglobulin. These housekeeping genes were chosen with the geNorm algorithm [36]. A normalization factor was calculated with the geometric mean of the 2 reference genes. The normalized expression of each gene of interest was calculated by dividing the raw quantities of each sample by the appropriate normalization factor [36].

2.8. ELISAs for IL-6, IL-1β, and TNFa. IL-6, IL-1β, and TNFa were measured by specific ELISAs (BioLegend, San Diego, CA). After incubation with MPP+, with or without quercetin or sesamin, for 24 h, the supernatants were collected for each respective ELISA. Mouse-specific monoclonal antibody (IL-6, IL-1β, and TNFa) was first coated on 96-well plates. Standards and samples were then added to the wells for 2 h, where IL-6, IL-1β, or TNFa were bound to the immobilized capture antibody. A biotinylated anti-mouse detection antibody was added for 1 h, producing an antibody-antigen-antibody “sandwich” to which an avidin-horseradish peroxidase solution was added for 30 min. Finally, a tetramethylbenzidine solution was included for 15 min in the dark. Reaction with horseradish peroxidase
resulted in conversion of the substrate to a blue-colored product. Addition of 2 N sulfuric acid stop solution yielded a yellow color. Microwell absorbance was read at 450 nm with a microplate reader (Thermo Lab Systems).

2.9. Statistical Analysis. Significant differences between groups were ascertained by 1-way analysis of variance (ANOVA), followed by Tukey’s post-hoc analysis with the GraphPad InStat program, version 3.06 for Windows (San Diego, CA; http://www.graphpad.com/). All data, analyzed at the 95% confidence interval, were expressed as means ± SEM from 3 independent experiments. Asterisks indicate statistical differences between the treatment and control condition (**P < 0.001, *P < 0.01, and ^P < 0.05), diamonds denote statistical differences between the treatment and MPP+ condition (○○○P < 0.001, ○○P < 0.01, and ▽P < 0.05), and □ empty circle indicates difference between MPP+-treated cells and their respective control conditions (^P < 0.05).

3. Results

3.1. Quercetin and Sesamin Decrease MPP+ -Induced IL-6, IL-1β, and TNFα mRNA and Protein Concentrations. We measured the expression of the potent proinflammatory cytokines IL-6, IL-1β, and TNFα by RT-qPCR. Figures 1(a), 1(b), and 1(c) show that MPP+ induced N9 microglial cell activation by dramatically increasing these cytokine mRNA levels. No significant difference from the control condition was detected when quercetin and sesamin were administered alone. On the other hand, quercetin or sesamin pretreatment of microglial N9 cells 3 h before MPP+ administration elicited a reduced pattern of IL-6, IL-1β, and TNFα...
gene expression, suggesting that quercetin and sesamin are notably involved in the expression of these cytokines. We also evaluated the protein expression of IL-6, IL-1β, and TNFα by specific ELISAs, as described in Section 2. Our results illustrate that the administration of quercetin or sesamin alone does not modulate IL-6, IL-1β, or TNFα protein expression (Figures 2(a), 2(b), and 2(c)). On the other hand, treatment with MPP+ alone considerably increases the presence of proinflammatory cytokines, indicating that MPP+ can induce an inflammatory process in microglial cells. The MPP+-evoked elevation of IL-6, IL1-β, or TNFα protein expression was strongly attenuated to control levels in microglial cells pretreated with quercetin or sesamin (Figures 2(a), 2(b), and 2(c)), suggesting that these natural substances play a role as anti-inflammatory molecules by impacting both the gene and protein expression of proinflammatory cytokines.

3.2. Quercetin and Sesamin Modulate MPP+-Induced iNOS Protein Expression. Early studies using iNOS inhibitors provided evidence of their potential as neuroprotective agents in the treatment of PD [37]. Here, we observed a very significant rise of iNOS expression by western blotting in microglia cells after only 1 h of MPP+ administration (Figure 3(a)). Then, iNOS expression decreased during a 24 h period (Figure 3(a)). We thus analyzed whether quercetin or sesamin might modulate iNOS protein expression after 1 h of MPP+ administration (Figure 3(b)). Our results illustrate that the polyphenol quercetin and the lignan sesamin consistently decreased MPP+-induced iNOS expression at 1 h.

3.3. Quercetin and Sesamin Counteract MPP+-Induced •O₂⁻ Production in N9 Microglial Cells. To investigate the mechanism underlying the protective properties of quercetin and sesamin against MPP+ treatment in N9 microglial cells,
**Figure 3:** (a) Histogram of kinetic studies showing iNOS protein expression for 24 h in neuronal PC12 cells as revealed by western blotting. Ctrl represents 100% of the controls for each time period. (b) iNOS protein expression after MPP⁺ administration with or without sesamin or quercetin. Quercetin or sesamin alone did not alter iNOS protein expression, whereas MPP⁺ increased iNOS protein levels by 79% in our cellular paradigm. When sesamin or quercetin was administered prior to MPP⁺, a significant decline of iNOS was detected. ***P<0.001 and *P<0.05 versus Ctrl; ○○○ P<0.001 versus MPP⁺, as determined by 1-way ANOVA, followed by Tukey’s multiple comparison test.

**Figure 4:** Effects of quercetin and sesamin on MPP⁺-induced superoxide anion (·O₂⁻) in N9 microglia cells. (a) Fluorescence photomicrographs. Ctrl: cells were treated with control medium. quercetin, sesamin: cells were treated with quercetin or sesamin in control medium. MPP⁺: cells were treated with 500 μM MPP⁺. MPP⁺ quercetin or MPP⁺ sesamin: cells were treated with quercetin or sesamin plus MPP⁺. A marked red signal was evident only in neuronal PC12 cells treated with MPP⁺. Red fluorescence was less intense in cells treated with control medium (Ctrl) or when quercetin or sesamin was added to MPP⁺ medium (MPP⁺ quercetin or MPP⁺ sesamin). Magnification 400x. n = 3. DDC: sodium diethyldithiocarbamate. (b) Semiquantitative image analysis. ***P<0.001 and **P<0.01 compared to the control (CTRL), ○○○ P<0.001 compared to MPP⁺, as determined by 1-way ANOVA, followed by Tukey’s multiple comparison test.

we estimated ·O₂⁻ production with MitoSOX Red, a derivative of ethidium bromide, as already described [37], after MPP⁺ administration or not, with or without quercetin for 9 h. This time period was considered since ROS, and eventually oxidative stress, are early events in the causative process of cellular death [37]. MitoSOX Red, a fluorogenic dye, was highly selective in detecting ·O₂⁻ in the mitochondria of live cells. Low fluorescence levels were apparent in control microglial cells as well as in cells treated with quercetin or sesamin alone (Figure 4(a): Ctrl, quercetin, sesamin), whereas a marked signal was detected in MPP⁺-treated microglial cells (Figure 4(b), MPP⁺). Figure 4(b) reports
MPP+ was added to the medium of the insert without microglial cells compared to the control (Ctrl), and no neuronal death was detected. MPP+:MPP+-activated microglial nonactivated microglial cells were placed on neuronal PC12 cells; DNA fragmentation in neuronal cells was detected with a monoclonal antibody to single-stranded DNA (ssDNA). Ctrl: non-activated microglial cells were placed on neuronal PC12 cells; a significant increase of neuronal cell death was apparent, indicating that cytokines produced by microglial cells induce neuronal death. Ctrl MPP+: MPP+ was added to the medium of the insert without microglial cells. Pretreatment of N9 cells with quercetin or sesamin prior to MPP+ clearly reduced neuronal PC12 cell death. Pretreatment of non-MPP+-activated N9 cells with quercetin or sesamin did not produce cellular death (quercetin, sesamin) n = 3. **P < 0.01 compared to the control (Ctrl), *P < 0.05 compared to their respective controls (quercetin, sesamin, or Ctrl), as determined by 1-way ANOVA, followed by Tukey’s multiple comparison test.

3.4. Quercetin and Sesamin Reduce MPP+-Induced Cytotoxicity and Apoptotic Cell Death in Microglial (N9)-Neuronal (PC12) Coculture. To investigate microglial-activated neuronal cell death, we tested a microglial-neuronal coculture system described elsewhere [26]. N9 microglial cells were cocultured in inserts on neuronal, differentiated PC12 cells to evaluate the effect of MPP+-induced cytokine secretion from microglial cells on neuronal PC12 cell death. The inserts have a pore size of 0.4 μM; thus, they allow cytokines to pass through but prevent cell contact [38]. Neuronal cells placed beneath non-MPP+-activated N9 cells did not present any significant cell death (Figure 5: Ctrl), whereas neuronal cells cocultured with MPP+-activated microglial cells displayed a high level of cell death (Figure 5: MPP+), demonstrating that MPP+-activated microglia secrete cytokines transported through the membrane insert inducing neuronal death. Figure 5 also reveals that neuronal cell death is diminished to 1.9% when microglial cells are treated with quercetin, and to 1.1% when they were treated with sesamin prior to MPP+ administration. In addition, our results show no significant neuronal death of PC12 cells exposed to inserts containing MPP+ without microglial (Ctrl MPP+), demonstrating that in our co-culture system, cytokines secreted by microglial cells cross the membrane and elicit neuroinflammation with consequent neuronal PC12 cell death.

To determine whether quercetin and sesamin can protect neuronal DAergic cells from inflammation-induced apoptosis, we also measured ssDNA fragmentation (Figure 6), a marker of late apoptosis. Microglial cells treated with MPP+ for 24 h manifested a 167% increase in DNA fragmentation—in comparison to control cells (Figure 6)—that was strongly and significantly prevented by quercetin and sesamin (Figure 6). These results disclose that quercetin or sesamin administration to microglial cells can efficiently reduce the apoptotic death of neuronal PC12 cells induced by microglial activation, thus supporting an anti-inflammatory role of these 2 natural molecules.

4. Discussion

Currently, several studies have described microglia activation in the SN pars compacta of PD patients and PD animal models [4–6]. As such, neuroinflammation is considered a feature of PD progression and pathogenesis.
Our present data highlight the neuroprotective properties of quercetin and sesamin, 2 natural molecules that reduce the expression of IL-6, IL-1β, and TNFα, 3 cytokines associated with neuroinflammation. We also show that quercetin and sesamin prevent the production of two cellular markers of inflammation, iNOS and *O₂−*, as well as the apoptosis of DA-producing neurons provoked by microglial stimulation. Our previous studies have already revealed that quercetin and sesamin have neuroprotective, antiapoptotic and antioxidative properties, reducing MPP+ and LPS-induced neuronal death [25, 26, 32]. Here, we further demonstrate that quercetin and sesamin can also act as potent anti-inflammatory compounds, restraining microglia activation and oxidative stress.

Activated microglial cells contribute to DAergic cell death by releasing cytotoxic inflammatory compounds, such as the proinflammatory cytokines IL-6, IL-1β, TNFα, and interferon-gamma. Among them, IL-6, IL-1β and TNFα have attracted much attention with regard to neuroinflammatory processes in PD [4]. DAergic degeneration induced by MPTP or MPP+ is linked with an inflammatory response in vitro [39] as well as in mouse and primate models of PD [18, 40]. On the other hand, inhibition of microglia activation is neuroprotective [41, 42] and coupled with the attenuation of TNFα expression [43]. The mechanisms by which microglia are activated are not fully understood. However, very recent data on a mouse MPTP model of PD as well as mesencephalic culture support a role of brain angiotensin II as one of the most potent inducers of inflammation and ROS [44, 45].

In this study, we clearly demonstrated that the flavonoid quercetin and the lignan sesamin strongly reduce the expression of proinflammatory cytokines in N9 microglia cells, indicating an interesting anti-inflammatory role of these natural molecules. We also analyzed the effects of quercetin and sesamin on parameters of oxidative cell distress. Activated microglia and excessive NO production by the high-output NO-synthesizing enzyme iNOS are observed in various neurological diseases, including PD [46]. NO is an apoptosis inducer, and iNOS is a key enzyme that produces large quantities of NO. Quercetin exerts a broad inhibitory effect on iNOS gene expression [27]. Sesamin and sesamin metabolites are known to induce endothelial NOS (eNOS) and thus have a significant antihypertensive function [30]. In this study, we demonstrated that quercetin and sesamin markedly reduced MPP+-evoked upregulation of iNOS expression in microglial cells, corroborating previous data from our group and others [32, 47].

It should be noted that, in our microglial cellular system, MPP+ increased iNOS production, after only 1 h of administration, and iNOS protein expression then declined constantly. This might be explained by early iNOS production followed by other intracellular apoptotic mechanisms. Certainly, in-depth studies should be performed to analyze the kinetics of iNOS production in this particular cellular system. Several investigations have determined that quercetin and sesamin can decrease ROS production to near-normal levels in various cellular systems [21, 48]. With MitoSOX Red, a selective indicator of mitochondrial *O₂−*-production, we illustrated an increase of fluorescence, when MPP+ was administered alone, and a substantial reduction with quercetin or sesamin treatment, supporting a potent scavenging role of quercetin and sesamin.

Microglia activation leads to increased production of cytokines that could mediate neuronal apoptosis and precede DAergic nigrostriatal neuron degeneration in a PD mouse model [49]. In this study, we show that quercetin and sesamin reduce the cellular death induced by MPP+ administration. We also document that quercetin and sesamin are potent modulators of apoptosis, opposing MPP+-induced DNA fragmentation. Our results demonstrate that when neuronal PC12 cells are cocultured with MPP+-treated N9 cells in vitro, their levels of cellular death increase to 267%. Quercetin or sesamin administration to MPP+-activated microglia reduces apoptotic DAergic neuronal PC12 cell death to 125% and 112%, respectively.

It should be noted that, although natural polyphenols are being studied intensively in vitro and in vivo for their neuroprotective properties, our knowledge about their bioavailability and possible target organs is far from being complete ([50] for review). In mammals, flavonoids, such as quercetin, as well as the lignan sesamin, are absorbed in the gut and that their bioavailability is much greater than previously believed [51–53]. Flavonoid concentrations in human plasma vary from 3 to 30 microM and certainly more, so if red blood cell-associated flavonoids are taken into consideration [54]. In addition, polyphenols can cross the blood-brain barrier to varying degrees depending on their chemical structure [55–57]. Recent studies have reported that their bioavailability in the nervous system may be improved by designing specific synergies between orally consumed polyphenols ([58] for review). Recent critical and comprehensive reviews report that quercetin and its metabolite isorhamnetin are found in the brain of rats and pigs in measurable levels ([50, 59] and references within), while nowadays less is known regarding the degree of brain bioavailability of sesame lignans. Sesamin can be converted to the mammalian lignans (enterodiol, enterolactone, and sesamol) by the intestinal microflora and is found in circulating blood. Recent results report that in rats orally administered sesamin can improve oxidative stress induced by kainic acid-induced status epilepticus and middle cerebral artery occlusion [60, 61]. In this study, we have used quercetin at 10−7 M which is a relative high concentration compared to that reported in vivo in the pmol and nmol/g/tissue range [50]. However, for sesamin, we used 10−12 M concentration that should be physiologically attainable in vivo.

Currently, more and more in vivo studies point out the importance of the biotransformation of natural molecules and the magnitude of bioavailability of the parental molecule and/or its metabolites for the prevention of human diseases [59]. With respect to these in vivo studies, our in vitro experiments document for an anti-inflammatory response with both quercetin and sesamin in a glial-neuron coculture system. Although far from an in vivo trial, it remains one of the best in vitro paradigms to study the possible relationship between microglial production of proinflammatory cytokines and neuronal cellular death.
Nevertheless, the information obtained with this study is also valuable providing new insights into the cellular mechanisms of natural compounds as preventive and/or complementary therapies for human diseases.

Acknowledgments

This work was funded by a Natural Sciences and Engineering Research Council of Canada grant to M.-G. Martinoli. The authors thank F. Longpré and A. Kervadec for their technical assistance and O. Da Silva for editing their paper.

References

[1] P. L. McGeer and E. G. McGeer, “Glial reactions in Parkinson’s disease,” Movement Disorders, vol. 23, no. 4, pp. 474–483, 2008.
[2] K. A. Frankola, N. H. Greig, W. Luo, and D. Tweedie, “Targeting TNF-alpha to elucidate and ameliorate neuroinflammation in neurodegenerative diseases,” CNS and Neurological Disorders, vol. 10, no. 3, pp. 391–403, 2011.
[3] L. Qian, P. M. Flood, and J. S. Hong, “Neuroinflammation is a key player in Parkinson’s disease and a prime target for therapy,” Journal of Neural Transmission, vol. 117, no. 8, pp. 971–979, 2010.
[4] Y. Ouchi, S. Yagi, M. Yokokura, and M. Sakamoto, “Neuroinflammation in the living brain of Parkinson’s disease,” Parkinsonism and Related Disorders, vol. 15, no. 3, pp. S200–S204, 2009.
[5] B. Mirza, H. Hadberg, P. Thomsen, and T. Moos, “The absence of reactive astrocytosis is indicative of a unique inflammatory process in Parkinson’s disease,” Neuroscience, vol. 95, no. 2, pp. 425–432, 1999.
[6] E. C. Hirsch and S. Hunot, “Neuroinflammation in Parkinson’s disease: a target for neuroprotection?” The Lancet Neurology, vol. 8, no. 4, pp. 382–397, 2009.
[7] L. J. Lawson, V. H. Perry, P. Dri, and S. Gordon, “Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain,” Neuroscience, vol. 39, no. 1, pp. 151–170, 1990.
[8] W. G. Kim, R. P. Mohney, B. Wilson, G. H. Jeohn, B. Liu, and J. S. Hong, “Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia,” Journal of Neuroscience, vol. 20, no. 16, pp. 6309–6316, 2000.
[9] S. Hunot, N. Dugas, B. Faucheux et al., “FcεRII/CD23 is expressed in Parkinson’s disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-α in glial cells,” Journal of Neuroscience, vol. 19, no. 9, pp. 3440–3447, 1999.
[10] M. Mogi, M. Harada, H. Narabayashi, H. Inagaki, M. Minami, and T. Nagatsu, “Interleukin (IL)-1β, IL-2, IL-4, IL-6 and transforming growth factor-α levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson’s disease,” Neuroscience Letters, vol. 211, no. 1, pp. 13–16, 1996.
[11] M. Kohutnicka, E. Lewandowska, I. Kurkowska-Jastrzębska, A. Członkowski, and A. Członkowska, “Microglial and astrocytic involvement in a murine model of Parkinson’s disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),” Immunopharmacology, vol. 39, no. 3, pp. 167–180, 1998.
[12] A. Członkowska, M. Kohutnicka, I. Kurkowska-Jastrzębska, and A. Członkowski, “Microglial reaction in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinson’s disease mice model,” Neurodegeneration, vol. 5, no. 2, pp. 137–143, 1996.
[13] K. Sriram, D. B. Miller, and J. P. O’Callaghan, “Minocycline attenuates microglial activation but fails to mitigate striatal dopaminergic neurotoxicity: role of tumor necrosis factor-α,” Journal of Neurochemistry, vol. 96, no. 3, pp. 706–718, 2006.
[14] J. Segura-Aguilar and R. M. Kostrzewa, “Neurotoxins and neurotoxic species implicated in neurodegeneration,” Neurotoxicity Research, vol. 6, no. 7–8, pp. 615–630, 2004.
[15] Y. Zhang, V. L. Dawson, and T. M. Dawson, “Oxidative stress and genetics in the pathogenesis of parkinson’s disease,” Neurobiology of Disease, vol. 7, no. 4, pp. 240–250, 2000.
[16] T. Obata, “Nitric oxide and MPP+-induced hydroxyl radical generation,” Journal of Neural Transmission, vol. 113, no. 9, pp. 1131–1144, 2006.
[17] J. W. Langston, L. S. Forno, J. Tetrad, A. G. Reeves, J. A. Kaplan, and D. Karluk, “Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure,” Annals of Neurology, vol. 46, no. 4, pp. 598–605, 1999.
[18] C. Barcia, V. De Pablos, V. Bautista-Hernández et al., “Increased plasma levels of TNF-α but not of IL1-β in MTPP-treated monkeys one year after the MPTP administration,” Parkinsonism and Related Disorders, vol. 11, no. 7, pp. 435–439, 2005.
[19] C. Zhao, Z. Ling, M. B. Newman, A. Bhatia, and P. M. Carvey, “TNF-α knockout and minocycline treatment attenuates blood-brain barrier leakage in MPTP-treated mice,” Neurobiology of Disease, vol. 26, no. 1, pp. 36–46, 2007.
[20] A. P. Lakshmanan, K. Watanabe, R. A. Thandavarayan et al., “Curcumin attenuates hyperglycaemia-mediated AMPK activation and oxidative stress in cerebral of streptozotocin-induced diabetic rat,” Free Radical Research, vol. 45, no. 7, pp. 788–795, 2011.
[21] M. Zhang, S. G. Swarts, L. Yin et al., “Antioxidant properties of quercetin,” Advances in Experimental Medicine and Biology, vol. 701, pp. 283–289, 2011.
[22] M. Singh, M. Arseneault, T. Sanderson, V. Murthy, and C. Ramassamy, “Challenges for research on polyphenols from foods in Alzheimer’s disease: bioavailability, metabolism, and cellular and molecular mechanisms,” Journal of Agricultural and Food Chemistry, vol. 56, no. 13, pp. 4855–4873, 2008.
[23] K. B. Pandey and S. I. Rizvi, “Plant polyphenols as dietary antioxidants in human health and disease,” Oxidative Medicine and Cellular Longevity, vol. 1999, pp. 270–278, 2009.
[24] L. D. Mercer, B. L. Kelly, M. K. Horne, and P. M. Beart, “Dietary polyphenols protect dopamine neurons from oxidative insults and apoptotic investigations in primary rat mesencephalic cultures,” Biochemical Pharmacology, vol. 69, no. 2, pp. 339–345, 2005.
[25] J. Bourinval, P. Quessy, and M. G. Martinoli, “Protective effects of resveratrol and quercetin against MPP+ -induced oxidative stress act by modulating markers of apoptotic death in dopaminergic neurons,” Cellular and Molecular Neurobiology, vol. 29, no. 8, pp. 1169–1180, 2009.
[26] G. Bureau, F. Longpré, and M. G. Martinoli, “Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation,” Journal of Neuroscience Research, vol. 86, no. 2, pp. 403–410, 2008.
[27] T. K. Kao, Y. C. Ou, S. L. Raung, C. Y. Lai, S. L. Liao, and C. J. Chen, “Inhibition of nitric oxide production by quercetin in endotoxin/cytokine-stimulated microglia,” *Life Sciences*, vol. 86, no. 9–10, pp. 315–321, 2010.

[28] F. Shahidi and P. K. Wanasundara, “Phenolic antioxidants,” *Critical Reviews in Food Science and Nutrition*, vol. 32, no. 1, pp. 67–103, 1992.

[29] F. Hirata, K. Fujita, Y. Ishikura, K. Hosoda, T. Ishikawa, and F. Shahidi, “Phenolic antioxidants,” *In Vitro Cellular and Developmental Biology*, vol. 27, no. 3, pp. 183–184, 1991.

[30] C. C. Lee, P. R. Chen, S. Lin et al., “Sesamin induces nitric oxide and decreases endothelin-1 production in HUVECs: possible implications for its antihypertensive effect,” *Journal of Hypertension*, vol. 22, no. 12, pp. 2329–2338, 2004.

[31] T. Noguchi, K. Ikeda, Y. Sasaki et al., “Effects of vitamin E and sesamin on hypertension and cerebral thrombogenesis in stroke-prone spontaneously hypertensive rats,” *Hypertension Research*, vol. 24, no. 6, pp. 735–742, 2001.

[32] V. Lahee-Collins, J. Bournival, M. Plouffe, J. Carange, and M. G. Martinoli, “Sesamin modulates tyrosine hydroxylase, superoxide dismutase, catalase, inducible NO synthase and interleukin-6 expression in dopaminergic cells under MPP+-induced oxidative stress,” *Oxidative Medicine and Cellular Longevity*, vol. 1, no. 1, pp. 54–62, 2008.

[33] S. Gélinas and M. G. Martinoli, “Neuroprotective effects of estradiol and phytoestrogens on MPP+-induced cytotoxicity in neuronal PC12 cells,” *Journal of Neuroscience Research*, vol. 70, no. 1, pp. 90–96, 2002.

[34] F. Q. Li, T. Wang, Z. Pei, B. Liu, and J. S. Hong, “Inhibition of microglial activation by the herbal flavonoid baikalen attenuates inflammation-mediated degeneration of dopaminergic neurons,” *Journal of Neural Transmission*, vol. 112, no. 3, pp. 331–347, 2005.

[35] A. Martin and M. Clynès, “Acid phosphatase: endpoint for in vitro toxicity tests,” *In Vitro Cellular and Developmental Biology*, vol. 27, no. 3, pp. 183–184, 1991.

[36] J. Vandesompele, K. De Preter, F. Pattyn et al., “Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes,” *Genome Biology*, vol. 3, no. 7, p. RESEARCH0034, 2002.

[37] K. Aquilano, S. Baldelli, G. Rotilio, and M. R. Cirilo, “Role of nitric oxide synthases in Parkinson’s disease: a review on the antioxidant and anti-inflammatory activity of polyphenols,” *Neurochemical Research*, vol. 33, no. 12, pp. 2416–2426, 2008.

[38] Y. Li, L. Liu, S. W. Barger, R. E. Mrak, and W. S. T. Griffin, “Vitamin E suppression of microglial activation is neuroprotective,” *Journal of Neuroscience Research*, vol. 66, no. 2, pp. 163–170, 2001.

[39] G. Gille, W. D. Rausch, S. T. Hung et al., “Protection of dopaminergic neurons in primary culture by lisuride,” *Journal of Neural Transmission*, vol. 109, no. 2, pp. 157–169, 2002.

[40] M. Mogi, A. Togari, M. Ogawa et al., “Effects of repeated systemic administration of 1-methyl-4-phenyl1,2,3,6-tetrahydropyridine (MPTP) to mice on interleukin-1β and nerve growth factor in the striatum,” *Neuroscience Letters*, vol. 250, no. 1, pp. 25–28, 1998.

[41] R. I. Hunter, N. Dragicevic, K. Seifert et al., “Inflammation induces mitochondrial dysfunction and dopaminergic neurodegeneration in the nigrostriatal system,” *Journal of Neurochemistry*, vol. 100, no. 5, pp. 1375–1386, 2007.

[42] Y. Liu, L. Qin, G. Li et al., “Dextromethorphan protects dopaminergic neurons against inflammation-mediated degeneration through inhibition of microglial activation,” *Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 1, pp. 212–218, 2003.

[43] S. L. Liao, W. Y. Chen, and C. J. Chen, “Estrogen attenuates tumor necrosis factor-α expression to provide ischemic neuroprotection in female rats,” *Neuroscience Letters*, vol. 330, no. 2, pp. 159–162, 2002.

[44] B. Joglar, J. Rodriguez-Pallares, A. I. Rodriguez-Perez, P. Rey, M. J. Guerra, and J. L. Labandeira-Garcia, “The inflammatory response in the MPTP model of Parkinson’s disease is mediated by brain angiotensin: relevance to progression of the disease,” *Journal of Neurochemistry*, vol. 109, no. 2, pp. 656–669, 2009.

[45] B. Villar-Cheda, A. Dominguez-Mejíide, B. Joglar, A. I. Rodriguez-Perez, M. J. Guerra, and J. L. Labandeira-Garcia, “Involvement of microglial RhoA/Rho-Kinase pathway activation in the dopaminergic neuron death. Role of angiotensin via angiotensin type 1 receptors,” *Neurobiology of Disease*, vol. 47, no. 2, pp. 268–279, 2012.

[46] F. González-Scarano and G. Baltuch, “Microglia as mediators of inflammatory and degenerative diseases,” *Annual Review of Neuroscience*, vol. 22, pp. 219–240, 1999.

[47] J. Chen, X. Q. Tang, J. L. Zhi et al., “Curcumin protects PC12 cells against 1-methyl-4-phenylpyridinium ion-induced apoptosis by bcl-2-mitochondria-ROS-iNOS pathway,” *Aptoptosis*, vol. 11, no. 6, pp. 943–953, 2006.

[48] R. C. W. Hou, H. M. Huang, J. T. C. Zhen, and K. C. G. Jeng, “Protective effects of sesamin and sesamolin on hypoxic neuronal and PC12 cells,” *Journal of Neuroscience Research*, vol. 74, no. 1, pp. 123–133, 2003.

[49] G. T. Liberatore, V. Jackson-Lewis, S. Vukosavic et al., “Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease,” *Nature Medicine*, vol. 5, no. 12, pp. 1403–1409, 1999.

[50] S. Schaffer and B. Halliwell, “Do polyphenols enter the brain and does it matter? Some theoretical and practical considerations,” *Genes and Nutrition*, vol. 7, no. 2, pp. 99–109, 2012.

[51] T. Clavel, J. Doré, and M. Blaut, “Bioavailability of lignans in human subjects,” *Nutrition Research Reviews*, vol. 19, no. 2, pp. 187–196, 2006.

[52] J. A. Ross and C. M. Kasum, “Dietary flavonoids: bioavailability, metabolic effects, and safety,” *Annual Review of Nutrition*, vol. 22, pp. 19–34, 2002.

[53] E. N. Papadakis, D. Lazarou, R. Groungnet et al., “Effect of the form of the sesame-based diet on the absorption of lignans,” *British Journal of Nutrition*, vol. 100, no. 6, pp. 1213–1219, 2008.

[54] M. Fiorani, A. Accorsi, and O. Cantoni, “Human red blood cells as a natural flavonoid reservoir,” *Free Radical Research*, vol. 37, no. 12, pp. 1331–1338, 2003.

[55] E. M. Janle, M. A. Lila, M. Grannan et al., “Pharmacokinetics and tissue distribution of 14C-Labeled grape polyphenols in the peripheral and the central nervous system following oral administration,” *Journal of Medicinal Food*, vol. 3, no. 4, pp. 926–933, 2010.

[56] K. A. Youdim, M. Z. Quiser, D. J. Begley, C. A. Rice-Evans, and N. J. Abbott, “Flavonoid permeability across an in situ model of the blood-brain barrier,” *Free Radical Biology and Medicine*, vol. 36, no. 5, pp. 592–604, 2004.

[57] A. Faria, D. Pestana, D. Teixeira et al., “Insights into the putative catechin and epicatechin transport across blood-brain barrier,” *Food and Function*, vol. 2, no. 1, pp. 39–44, 2011.
[58] A. Scheepens, K. Tan, and J. W. Paxton, “Improving the oral bioavailability of beneficial polyphenols through designed synergies,” *Genes and Nutrition*, vol. 5, no. 1, pp. 75–87, 2010.

[59] D. Vauzour, A. Rodriguez-Mateos, G. Corona, M. J. Oruna-Concha, and J. P. E. Spencer, “Polyphenols and human health: prevention of disease and mechanisms of action,” *Nutrients*, vol. 2, no. 11, pp. 1106–1131, 2010.

[60] M. M. Khan, T. Ishrat, A. Ahmad et al., “Sesamin attenuates behavioral, biochemical and histological alterations induced by reversible middle cerebral artery occlusion in the rats,” *Chemico-Biological Interactions*, vol. 183, no. 1, pp. 255–263, 2010.

[61] P. F. Hsieh, C. W. Hou, P. W. Yao et al., “Sesamin ameliorates oxidative stress and mortality in kainic acid-induced status epilepticus by inhibition of MAPK and COX-2 activation,” *Journal of Neuroinflammation*, vol. 8, article 57, 2011.