Rosiglitazone Protects Dopaminergic Neurons Against Lipopolysaccharide-Induced Neurotoxicity Through Inhibition of Microglia Activation

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ABSTRACT

Recent evidence has suggested that microglia activation plays an important role in the pathogenesis of Parkinson’s disease (PD). Activated microglia secrete various proinflammatory cytokines and neurotoxic mediators, which may contribute to the development of PD. Thus, the inhibition of microglia activation may have a therapeutic benefit in the treatment of PD. In the present study, using mesencephalic neuron–microglia mixed culture and microglia-enriched culture, we investigated whether rosiglitazone (RGZ), a member of peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) agonists, could inhibit microglia activation. Our results showed that RGZ significantly inhibited lipopolysaccharide (LPS)-induced microglia activation and the production of tumor necrosis factor-alpha (TNF-\(\alpha\)), nitric oxide (NO), and superoxide. We further investigated the intracellular signaling pathways regulating the production of TNF-\(\alpha\) and NO in LPS-activated microglia. The results showed that RGZ inhibited the phosphorylation and nuclear translocation of the p65 subunit of NF-\(\kappa\)B, and the phosphorylation of p38 mitogen-activated protein kinase (p38MAPK). Taken together, our results suggested that the therapeutic effects of RGZ were partially mediated by modulating microglia activation.

KEYWORDS: dopaminergic neurons, microglia, Parkinson’s disease, rosiglitazone

INTRODUCTION

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN) and a significant loss of striatal dopamine. Although the etiology of PD remains unclear, recent evidence suggests chronic brain inflammation is involved in the degeneration of the nigral dopaminergic neurons [1].

Microglia, resident immune cells in the central nervous system (CNS), are considered to be the major cell type underlying the inflammatory process. In response to the pathological stimuli, microglia readily become activated and release various neurotoxic factors, such as TNF-\(\alpha\), interleukin-1\(\beta\) (IL-1\(\beta\)), prostaglandin E\(_2\) (PGE\(_2\)), NO, and reactive oxygen species (ROS), which work in concert to trigger neurodegeneration [2–5]. Since McGeer et al. described a large number of activated microglia in the substantia nigra of PD patient brains in 1988 [6], increasing studies demonstrated that activated microglia might be associated with the development of PD and inhibition of microglia activation could protect dopaminergic neurons [7–12]. On this basis, researchers extensively searched for new agents to inhibit microglia activation for therapeutic intervention against neurodegeneration in PD.

Peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily, which regulates transcription of distinct genes through heterodimerization with the retinoid X receptors (RXR) [13, 14]. The receptor is heavily expressed in adipose tissue where it plays a critical role in glucose and lipid metabolism. In addition to adipocytes, cells of the monocyte/macrophage lineage also express PPAR\(\gamma\), suggesting a role for this receptor in the function of these cells [15]. Recently, the PPAR\(\gamma\) ligands have been reported to inhibit the expression of proinflammatory molecules in neurodegenerative diseases [16–18]. Thiazolidinones (TZDs), such as rosiglitazone, pioglitazone, and troglitazone are synthetic PPAR\(\gamma\) agonists.
ligands. Previously, many studies reported that TZDs had potent anti-inflammatory effects, such as the inhibition of macrophage lineage activation, along with the suppression of inflammatory mediators [19, 20]. In the CNS, TZDs also exhibited anti-inflammatory effects which led to neuroprotection[21–24]. Since TZDs had proven successful in various models of neuroinflammation, it was hypothesized that the TZDs could be used as a novel treatment approach to reduce the neuroinflammation in PD.

Nuclear factor kappa-B (NF-κB) is a key regulator of inflammation. In an inactive state, NF-κB heterodimers are sequestered in the cytoplasm by the specific inhibitors IκB. Upon stimulation, degradation of IκB releases NF-κB dimers, causing translocation of NF-κB from cytoplasm to the nucleus and facilitates the transcription of its target genes. Previous studies showed that NF-κB levels were increased in the postmortem PD brain [25] and NF-κB dimers were translocated from cytoplasm to nucleus in LPS-activated microglia in vitro [26]. Numerous reports demonstrated that mitogen-activated protein kinases (MAPKs), especially p38MAPK, played a critical role in microglia-mediated neuronal death in chronic neurodegenerative disease [27–29] and inhibition of MAPKs could protect dopaminergic neurons from microglia-involved insult [30, 31].

Although recent evidence demonstrated that RGZ had neuroprotective effects through the inhibition of microglia activation, the underlying mechanism was far from complete understanding [32, 33]. Therefore, the present study was designed to investigate the effects of RGZ on LPS-induced microglia activation and to elucidate the related signaling pathways.

MATERIALS AND METHODS

Animals

Timed-pregnant Wistar rats were obtained from the laboratory center of the Medical School in Shandong University and were housed in a climate-controlled facility with free access to food and water. All experimental procedures in this study were conducted according to current institutional guidelines for laboratory animal care.

Reagents

Lipopolysaccharide (LPS) (Salmonella minnesota) was purchased from Sigma-Aldrich (St. Louis, MO, USA). RGZ was from Cayman Chemicals (St. Louis, MO, USA). Antibodies used were: monoclonal antibodies against the CR3 complement receptor (OX-42) from Chemicon (Temecula, CA, USA), polyclonal anti-tyrosine hydroxylase (TH) antibody from Freez Biologicals (Rogers, AR, USA), rabbit monoclonal anti-NF-κBp65 antibody from Cell Signaling Technology (Danvers, MA, USA), antibody against phospho-p38MAPK from Cell Signaling Technology (Burlingame, CA, USA). Quantikine Rat TNF-α Immunoassay kits were from R&D Systems (Minneapolis, MN, USA), SB203580 (p38MAPK inhibitor) was from Calbiochem (San Diego, CA, USA), and cell culture ingredients were from Invitrogen (Carlsbad, CA, USA).

Microglia-Enriched Cultures

Microglia were prepared from whole brains of 1–2 day-old Wistar rat pups, as described previously [26]. The enriched microglia were >98% pure, as determined by immunostaining for microglia-specific markers (OX-42). After 24 h, the cells were treated with vehicle, LPS, and RGZ with the indicated concentrations.

Mesencephalic Neuron-Enriched Cultures

Primary rat neuron-glia cultures were prepared by following a previously published protocol [26]. Seven-day-old cultures were used for treatment.

Mesencephalic Neuron–Microglia Mixed Cultures

At DIV7, 2 × 10⁵ microglia were added into primary mesencephalic neuron-enriched cultures containing 2 × 10⁵ cells/well in 24-well plates for immunocytochemistry. After 24 h, the cultures were treated with various protocols and the control groups were only treated with the same amount of DMSO, which was used to dissolve RGZ and SB203580.

Immunocytochemistry

Dopaminergic neurons were detected with the anti-TH antibody and microglia were determined by staining for the CR3 complement receptor with monoclonal antibody OX-42, as described previously [26]. For the detection of NF-κB translocation, microglia were stained with NF-κBp65 antibody. Briefly, the cells were plated on glass coverslips overnight. After drug treatment, they were fixed in 4% paraformaldehyde for 10 min. After permeabilization with 0.3% Triton-X100 in PBS, the cells were blocked with 10% FBS in PBS. Then, the cells were incubated in the primary antibody overnight at 4°C. Following primary antibody incubation, cells were washed again and incubated in the appropriate fluorescent-conjugated secondary antibody for 1 h. The
cells were counterstained with DAPI. Images were captured with an Olympus microscope.

**Cell Quantification**

To quantify TH and OX-42 positive cells, nine representative areas per well of the 24-well plate were counted under the microscope at 100× magnification. Microglia exhibiting expanded transparent extensions from the soma were considered as activated. Cell countings were performed by an investigator blinded to the control and experimental groups.

**TNF-α and Nitric Oxide Assay**

The levels of TNF-α in culture supernatants were measured with commercial ELISA kits according to the manufacturer's instructions. The production of NO in culture supernatants was assessed by the accumulation of nitrite and nitrate, the end products of NO reactions, using a colorimetric assay with the Griess reagent. Culture supernatants were collected at 24 h after LPS treatment and the absorbance at 540 nm was measured with a microplate reader.

**Superoxide Assay**

Extracellular superoxide production from microglia was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C, as previously described [7]. To determine the effect of RGZ on superoxide release, microglia-enriched cultures were preincubated with RGZ at 37 °C for 1 h prior to the addition of LPS. Cell free experiments with and without RGZ were conducted to determine that RGZ did not alter absorbance by itself. The amount of SOD-inhibitable superoxide was calculated and expressed as percent of LPS-treated cultures.

**Western Blotting**

To further discern the mechanism of microglia activation, the signaling pathways of NF-κB and p38MAPK were investigated. After treatment of LPS for 1 h or 30 min, microglia were collected and lysed for western blotting. The band optical density was quantified using the scion image software (Frederick, MD, USA).

**Statistical Analysis**

The data were expressed as the mean±SEM of at least three separate experiments and statistical significance was assessed by an analysis of variances (ANOVA) followed by a Tukey comparisons test. A value of \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**RGZ Protects Dopaminergic Neurons Against LPS Insult**

In neuron-enriched cultures treated with LPS (1 µg/ml), no dopaminergic neuronal loss was found (data not shown), which was in line with that of previous study [26]. In contrast, in neuron–microglia mixed culture, LPS induced significant loss of dopaminergic neurons after a 72 h treatment. Pretreatment with RGZ (50 µM), 1 h prior to LPS treatment, significantly increased the dopaminergic neuron viability rate to 88.9% compared to 56.6% upon LPS alone (Figure 1A, B). At the concentration of 50 µM, RGZ alone did not alter dopaminergic neuron viability.

**RGZ Suppresses LPS-Induced Microglia Activation**

Since LPS-induced dopaminergic neuronal loss was microglia-dependent and RGZ protected dopaminergic neurons against LPS neurotoxicity, we next tested whether the protection was related to the effects of RGZ on microglia activation. Microglia-enriched cultures were treated with LPS (1 µg/ml) in the presence or absence of RGZ (1, 10, and 50 µM) for 24 h. Then, microglia activation was determined by the obvious morphological changes, including an irregular shape with an expanded transparent extension from the soma and intensified OX-42 staining. After 24 h of LPS exposure, 75% of the microglia were activated. In contrast, pretreatment with RGZ (10 µM and 50 µM) 1 h before LPS treatment significantly suppressed microglia activation in a dose-dependent manner (Figure 2A, B).

**RGZ Inhibits Generation of TNF-α, NO, and Superoxide**

Tumor necrosis factor-alpha (TNF-α), NO, and superoxide are three main neurotoxic factors released from activated microglia, which have detrimental effects on neurons, especially on dopaminergic neurons. Since it had been proved that the generation of TNF-α, NO, and superoxide were significantly increased in microglia-enriched cultures after LPS treatment, this study investigated whether the protective effects of RGZ were associated with the reduced level of TNF-α, NO, and superoxide in the culture media. For all subsequent comparison, original data were converted into percentage values. As shown in Figure 3, RGZ suppressed LPS-induced generation of TNF-α, NO, and superoxide in a dose-dependent manner. In addition, RGZ alone did not influence the generation
of TNF-α, NO, and superoxide in the culture media. The suppressive effects of RGZ were stronger at the concentration of 50 μM, therefore, we used RGZ at this concentration in the following experiments.

**RGZ Inhibits LPS-Induced NF-κB Activation**

Nuclear factor kappa-B (NF-κB) has been shown to be one of the most important upstream modulators for proinflammatory cytokines and iNOS expression in microglia. Thus, we next clarified whether the suppressive effects of RGZ on the expression of neurotoxic factors were associated with the blockade of NF-κB activity in microglia.

First, we examined the influence of RGZ on the NF-κB activity by Western blotting. Microglia were pretreated with 50 μM RGZ for 1 h in the presence or absence of LPS for 1 h. As shown in Figure 4A,
FIGURE 3. RGZ inhibits generation of TNF-α, NO, and superoxide in microglia-enriched cultures. Microglia-enriched cultures (2 × 10⁵ cells/well) were treated with RGZ (1, 10, and 50 µM) 1 h before LPS (1 µg/ml) treatment. At different time point, the levels of TNF-α (a), NO (b), and superoxide (c) in the culture supernatants were determined. Supernatants were taken for the measurement of TNF-α at 6 h, for NO at 24 h, and for superoxide at 30 min after the cultures were treated with LPS. LPS significantly induced three neurotoxins generation and pretreatment with RGZ (50 µM) inhibited these neurotoxins generation. In the control cultures, TNF-α levels were under the detection limit of ELISA (5 pg/ml). Data are expressed as a percentage of LPS-treated cultures and are mean±SEM of five experiments. (⁎p < 0.05, ⁎⁎p < 0.01 versus LPS).

RGZ significantly suppressed LPS-induced phosphorylation levels of NF-κBp65. The above results were further confirmed by immunofluorescence analysis. NF-κBp65 was mainly localized in the cytoplasm, treatment with LPS for 1 h induced NF-κBp65 translocation from cytoplasm to the nucleus (Figure 4B). However, the pretreatment of RGZ suppressed the translocation of NF-κBp65 into the nuclei of microglia.

RGZ Inhibits the Generation of NO by Suppression of p38MAPK Activity

To investigate whether the inhibition of NO production was associated with the suppression of p38MAPK activity, microglia were treated with LPS alone or with RGZ. As shown in Figure 5A, pretreatment with RGZ (50 µM) 1 h before LPS exposure decreased phosphorylation of p38MAPK, which suggested that the inhibition of NO production might be due in part to the suppression of p38MAPK. RGZ (50 µM) alone did not influence the expression of p38MAPK in cultures treated with vehicle.

To further demonstrate the involvement of p38MAPK pathway in the increased NO production, SB203580, which prevents the phosphorylation of p38MAPK, was administered to microglia-enriched cultures 1 h before LPS exposure. LPS significantly increased NO generation, and inhibition of p38MAPK activity by the pretreatment with SB203580 (5 µM) decreased the NO production (Figure 5B).

DISCUSSION

In the present study, we demonstrated that PPARγ agonist RGZ significantly suppressed LPS-induced microglia activation. Furthermore, its suppression provided significant protection of dopaminergic neurons by attenuating the generation of TNF-α, NO, and superoxide. In addition, RGZ inhibited the activation of NF-κB and p38MAPK which play critical roles in microglia activation.

Microglia activation had been previously demonstrated in the SN of PD patient brains [6] and considerable evidences suggested that activated microglia might be toxic to dopaminergic neurons by the release of various neurotoxic factors, such as proinflammatory factors (TNF-α, IL-1β, and IL-6), NO, and superoxide [4, 34–39]. Our result that LPS failed to show dopaminergic neurotoxicity in the absence of microglia supported the opinion that the toxic effects of LPS on mesencephalic dopaminergic neurons were dependent on microglia activation and the subsequent release of neurotoxic factors. Whether microglia activation in vivo plays a role in the initiative stage of neuronal damages or occurs merely as a response to neuronal death remains controversial, but increasing evidence and our results demonstrated that microglia activation at least aggravated the injury of dopaminergic neurons.

Increasing studies indicated that certain agents exhibited neuroprotective effects by inhibiting microglia activation in several in vitro and in vivo models of neurodegeneration [7, 40–42]. In the present study, RGZ significantly reduced the LPS-induced dopaminergic...
FIGURE 4. Rosiglitazone inhibits LPS-induced NF-κB activation and translocation. (A) Microglia were treated with RGZ (50 µM) in the presence or absence of LPS (1 µg/ml) for 30 min. Total cell extracts were subjected to immunoblot analysis using antibodies against phosphorylation and total NF-κBp65. Three independent experiments were performed, and the data indicate the mean ± SEM. (∗∗p < 0.01 vs. Control, #p < 0.05 vs. LPS alone). (B) Microglia were treated with RGZ (50 µM) in the presence or absence of LPS for 1 h. NF-κBp65 protein localization was determined using an anti-NF-κBp65 antibody and an FITC-labeled anti-rabbit antibody. Microglia were visualized using a fluorescence microscope. Scale bar = 25µm. Images are representative of triplicate sets.

neuronal loss and microglia activation, suggesting its neuroprotective effects might be associated with the inhibition of microglia activation. The conclusion was in line with several previous studies which demonstrated significant protection with TZDs in the MPTP-induced PD model [23, 24] and the LPS-induced PD model [12, 43]. In addition, previous studies reported RGZ had direct protective effects on primary neurons and neuroblastoma cell lines [12, 21, 44]. These observations indicated that the protective role of RGZ might involve multiple mechanisms. More recently, Mingchang et al. supported the idea that RGZ rescued the...

FIGURE 5. RGZ significantly inhibits LPS-induced p38MAPK phosphorylation. (A) Pretreatment with RGZ (50 µM) inhibited LPS-induced p38MAPK phosphorylation in neuron-microglia mixed cultures. RGZ was added 1 h before LPS (1 µg/ml) treatment. After LPS treatment for 30 min, p-p38MAPK was immunoblotted. Parallel blots were analyzed as controls using the antibodies recognizing β-actin. As shown in Fig.5A, LPS greatly increased phosphorylation of p38MAPK and pretreatment with RGZ inhibited this phosphorylation. Data are expressed as a ratio to control cultures and are mean±SEM of three experiments. (∗∗p < 0.01 versus control, #p < 0.05 versus LPS). (B) A selective p38MAPK inhibitor (SB203580) was added to microglia-enriched cultures 1 h before LPS (1 µg/ml) treatment. After 24 h, NO levels were measured. At the concentration of 5 µM, SB203580 significantly inhibited NO generation. Data are expressed as a ratio to control cultures and are mean±SEM of three experiments. (∗∗p < 0.01 versus control, #p < 0.05 versus LPS).
mitochondrial dysfunction through the upregulation of PPARγ downstream target genes such as PGC-1α [45]. Therefore, the mechanisms underlying the neuroprotective role of RGZ in our study, besides the inhibition of microglia activation, could not exclude the possibility that RGZ directly protected dopaminergic neurons from inflammatory insults through its antioxidant properties and the regulation of mitochondrial function.

Among the neurotoxic factors produced by activated microglia, the consequence of overproduction of TNF-α and NO had been well known. More specifically, McGuire et al. found that TNF-α was toxic to cultured dopaminergic neurons [46]. Excessive accumulation of NO could lead to neurotoxicity due to its diverse detrimental reactivities [31]. Oxidative stress occurs when the intracellular antioxidant defense system is overwhelmed by the generation of ROS, which have the potential to interact with critical proteins, DNA, or RNA to alter their functions or induce lipid peroxidation, leading to cell death [2]. Among the various populations of neurons in the brain, dopaminergic neurons in the SN are uniquely vulnerable to oxidative stress due to their reduced antioxidant capacity, increased accumulation of iron, and high concentration of dopamine [4]. On this premise, among all the neurotoxic factors released by activated microglia, ROS play an especially vital role in the degeneration of dopaminergic neurons. Indeed, recent studies demonstrated that the inhibition of microglia-derived superoxide, but not the other factors, was most effective in protecting neurons in cultures [7, 47]. In our study, RGZ significantly decreased the generation of superoxide in microglia-enriched cultures upon LPS insult, which might provide a partial explanation for the protective role of RGZ.

Numerous studies have demonstrated a crucial role for p38MAPK pathways in the activation of cultured primary microglia [31, 37]. As expected, RGZ abolished LPS-induced phosphorylation of p38MAPK, which was often used as a hallmark of LPS-induced signal transduction in microglia. To further clarify whether p38MAPK signal pathway is involved in the LPS-induced NO generation, a selective inhibitor for p38MAPK (SB203580) was administrated before LPS stimulation. After 24 h treatment, we found that LPS-induced NO production was inhibited by administering SB203580. These data suggested that RGZ-mediated attenuation of NO production seemed to be associated with the inhibition of the p38MAPK signaling pathway. Whether p38MAPK signaling pathway is the main or initiative modulatory pathway in microglia remains unknown, but this study suggested that p38MAPK pathway might be at least partly responsible for NO production in microglia [48, 49].

In addition to direct injury to important biomolecules, ROS can also serve as second mes-sengers to activate diverse downstream signaling molecules, including protein kinase C, MAPKs, and NF-κB [50–54]. In microglia, the similar intracellular signaling pathways responding to ROS had also been found [55, 56]. Since LPS was widely known to induce ROS production in microglia and immortalized cell lines [57, 58], NF-κB and p38MAPK might be downstream targets of ROS signaling pathway [59]. In the present study, RGZ suppressed LPS-induced superoxide generation in microglia, which might explain the inhibition of NF-κB and p38MAPK activity with the administration of RGZ. However, we cannot exclude other mechanisms of the inhibition of NF-κB and p38MAPK activity. In addition, Qin et al. reported that ROS generated from NADPH oxidase was associated with the activation of microglia and the subsequent release of proinflammatory factors [60]. These results and our data suggested that inhibition of ROS generation with the treatment of RGZ might be the main reason for the reduction of microglia-mediated neurotoxicity.

CONCLUSIONS

Our present study shows that the PPARγ agonist RGZ significantly inhibits LPS-induced microglia-derived production of neurotoxic factors, thus, protecting dopaminergic neurons against LPS toxicity. This may be mediated by inhibition of NF-κB and p38MAPK signaling pathway. These results suggest possible therapeutic uses of RGZ in PD, where microglia-mediated neuroinflammation play a significant role.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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