Research Article

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Corticosterone potentiates ochratoxin A-induced microglial activation

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Abstract: Microglial activation in the central nervous system (CNS) has been associated with brain damage and neurodegenerative disorders. Ochratoxin A (OTA) is a mycotoxin that occurs naturally in food and feed and has been associated with neurotoxicity, while corticosteroids are CNS’ physiological function modulators. This study examined how OTA affected microglia activation and how corticosteroids influenced microglial neuroinflammation. Murine microglial cells (BV-2) were stimulated by OTA, and the potentiation effects on OTA-induced inflammation were determined by corticosterone pre-treatment. Expressions of pro-inflammatory mediators including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS) were determined. Phosphorylation of mitogen-activated protein kinases (MAPKs) was analyzed by western blotting. OTA significantly increased the mRNA expression of IL-6, TNF-α, IL-1β, and iNOS and also elevated IL-6 and NO levels. Corticosterone pre-treatment enhanced the neuroinflammatory response to OTA in a mineralocorticoid receptor (MR)-dependent mechanism, which is associated with increases in extracellular signal-regulated kinase (ERK) and p38 MAPK activation. In response to OTA, microglial cells produced pro-inflammatory cytokines and NO, while corticosterone increased OTA-induced ERK and p38 MAPK phosphorylation via MR. Findings indicated the direct role of OTA in microglia activation and neuroinflammatory response and suggested that low corticosterone concentrations in the brain exacerbated neurodegeneration.

Keywords: corticosterone, mitogen-activated protein kinases, microglia, neuroinflammation, ochratoxin A

Abbreviations

- BSA: bovine serum albumin
- COX-2: cyclooxygenase-2
- DMSO: dimethyl sulfoxide
- DMEM: Dulbecco’s modified eagle medium
- ERK: extracellular signal-regulated kinase
- GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- GR: glucocorticoid receptor
- iNOS: inducible nitric oxide synthase
- JNK: c-Jun N-terminal kinase
- LPS: lipopolysaccharide
- MAPKs: mitogen-activated protein kinases
- MR: mineralocorticoid receptors
- NF-κB: nuclear factor-κB
- NO: nitric oxide
- PBS: phosphate-buffered saline
- PCR: polymerase chain reaction
- PMS: N-methyl dibenzopyrazine methyl sulfate
- PVDF: polyvinylidene fluoride
- ROS: reactive oxygen species
- SD: standard deviation
- TBST: tris-buffered saline tween-20
- TMB: tetramethylbenzidine
- TNF-α: tumor necrosis factor-α
- OTA: ochratoxin A

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Introduction

Microglial cells are brain-resident macrophage-like cells that are involved in the neuroinflammatory response. Neurodegenerative diseases such as multiple sclerosis, Parkinson’s disease (PD), and Alzheimer’s disease (AD) are caused by overactive microglial cells which produce pro-inflammatory cytokines, mediators, and reactive oxygen species (ROS). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and nitric oxide (NO) [1–3]. Mycotoxins have been shown to be toxic to humans and animals. Mycotoxins contaminate nearly 25% of world-wide cereal yield, according to the United Nations Food and Agriculture Organization [4]. Ochratoxin A (OTA) is a frequent food and feedstock contaminant produced by *Aspergillus* and *Penicillium*. The International Agency for Research on Cancer (IARC) classified OTA as a human carcinogen of class 2B (possibly carcinogenic to humans) [5]. OTA is concentrated in the cerebellum,pons, and cerebral cortex, resulting in neurotoxicity and neuroinflammation [6,7]. Numerous research groups have recognized the neurotoxic potential of OTA in the development of neurodegenerative disorders [7–14]. It has been hypothesized that OTA-mediated neurotoxicity occurs as a result of mitogen-activated protein kinase (MAPK) activation [15,16]. MAPK signaling has been shown to play a critical role in microglial cell activation by regulating the production of pro-inflammatory mediators [17,18]. However, it remains unclear whether microglia respond to OTA and induce neuroinflammatory cascades.

The hypothalamic–pituitary–adrenal (HPA) axis is a neurohumoral signaling system associated with stress response that releases glucocorticoid stress hormones, primarily cortisol in humans and corticosterone in rodents [19]. Chronic stress causes a significant increase in systemic baseline glucocorticoid release, which might exacerbate neurodegenerative disorders [20–22]. Glucocorticoids enter the brain and activate the glucocorticoid and mineralocorticoid receptors (GR and MR), which are critical for controlling the brain’s response to stress [23]. Both GR and MR are expressed differently in neurons, microglia, astrocytes, and oligodendrocytes, and have opposing affinity and sensitivity to corticosteroid concentrations [24,25]. In various studies, glucocorticoids have been shown to promote neuroinflammation and enhance neurotoxicity. For instance, pre-exposure to corticosterone enhanced the neuroinflammatory response and neurotoxicity to methamphetamine [26], organophosphates [27,28], alcohol [29], and lipopolysaccharides (LPS) [30–33]. Chronic stress and glucocorticoids have been demonstrated to sensitize microglia, causing them to respond exaggeratedly to a second stimulus, such as LPS [22,30,34]. Thus, the present study wondered whether persistent stress may make microglia more vulnerable to OTA-induced neuroinflammation by promoting microglia activation.

As mentioned above, corticosterone may modulate microglial cell vulnerability to OTA-induced neuroinflammation. Here the expression of different pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and the release of IL-6 and NO in murine microglia in response to OTA exposure were measured to investigate the influence of corticosterone pre-treatment on microglial cell activation by OTA and the underlying key signaling molecules. Findings showed that corticosterone exacerbated OTA-induced pro-inflammatory cytokine production in microglia through MR modulation of the extracellular signal-regulated kinase (ERK) and p38 MAPK signaling pathways.

Materials and methods

Reagents and antibodies

Cell culture medium and supplements such as RPMI-1640, fetal bovine serum (FBS), 1-glutamine, penicillin, streptomycin, fungizone, and trypsin were acquired from Gibco (Waltham, MA, USA). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, ochratoxin A (from *Aspergillus ochraceus*), sodium 3′-1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), N-methyl dibenzopyrazine methyl sulfate (PMS), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against actin (cat no. 4970), both phosphorylated and unphosphorylated ERK (cat no. 4376 and 4695), p38 MAPK (cat no. 9215 and 9212), and c-Jun N-terminal kinase (JNK) (cat no. 4671 and 9252) were obtained from Cell Signaling Technology (Danvers, MA, USA). The Bradford solution, non-fat dry milk, and polyvinylidene fluoride (PVDF) membrane were purchased from Bio-Rad (Bencia, CA, USA). The 2x qPCR Bio SyGreen 1-step Lo-ROX was obtained from PCR Biosystems (Wayne, PA, USA). Tri-RNA Reagent was purchased from FavorGen (Kaohsiung, Taiwan). IL-6 quantitative sandwich ELISA kit and radioimmunoprecipitation assay (RIPA) buffer were obtained from Abcam (Cambridge, MA, USA). The Griess reagent and CellTiter-Glo® Luminescence assay kits were purchased from Promega (Madison, WI, USA). Protease inhibitor cocktail was
and purchased from Roche (Shanghai, China). Immobilon Forte Western HRP substrate was obtained from Merck Millipore (Burlington, MA, USA). Unless otherwise noted, all additional reagents were from Sigma-Aldrich. OTA was dissolved in DMSO and serially diluted in a cell culture medium. In the cultured cells experiments, the final concentration of DMSO was 0.5% (v/v) for the highest mycotoxin concentrations.

**Cell culture and treatment**

**BV-2 cell culture**

Immortalized mouse microglial cells (BV-2) were obtained from the Interlab Cell Line Collection (Genoa, Italy), and cultured as previously described [30] in 75 cm² flasks with RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), fungizone (2.5 µg/mL), non-essential amino acids (0.1 mM), and HEPES (10 mM), at pH of 7.4 and at 37°C in a humidified incubator containing 5% CO₂. Cells were trypsinized after they reached approximately 80% confluence.

**Treatment**

Before each experiment, cells were seeded into 96-well plates (1 × 10³ cells/well for the XTT reduction assay and adenosine triphosphate (ATP) determination), 24-well plates (2 × 10⁴ cells/well for ELISA and NO measurement), 12-well plates (5 × 10⁵ cells/well for qPCR), and 6-well plates (1 × 10⁶ cells/well for western blotting), and incubated for 12 h at 37°C in FBS-free DMEM.

To determine the cytotoxicity and inflammatory activity of OTA on microglia, BV-2 cells were treated with various concentrations of OTA (32.25–2,000 nM) for 24 h before analysis.

To investigate the role of MAPKs on the inflammatory effects of OTA, BV-2 cells were pre-incubated for 1 h with PD98059 (ERK inhibitor; 20 µM), SP600125 (p38 MAPK inhibitor; 20 µM), and SB203580 (JNK inhibitor; 10 µM) before treatment for another 24 h with OTA (50, 250, and 500 nM).

To assess the effects of corticosterone and the receptor involved in OTA-induced microglia activation, cells were treated for 12 h with corticosterone (25 nM) in the absence or presence of spironolactone (1 µM) or mifepristone (1 µM), specific receptor antagonists of MR and GR, respectively, followed by treatment with OTA (250 nM).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Following treatment, total RNA was extracted from cells using Tri-RNA Reagent, following the manufacturer’s instructions. Nanodrop ND-1000 spectrophotometry was used to analyze the RNA quality and concentration (NanoDrop Technologies, Wilmington, DE, USA). The qRT-PCR reaction mixture, qPCRBI SyGreen 1-step Lo-ROX, was prepared according to the manufacturer’s recommendations (PCR Biosystems, Wayne, PA, USA). The qTOWER3 Real-Time PCR Systems (Analytik Jena, Langewiesen, Germany) were used to conduct qRT-PCR, and the mRNA expression levels were normalized to GAPDH. Each experiment was replicated at least three times, and the primer sequences are detailed in Table 1.

**XTT reduction assay**

The XTT reduction test was employed to assess cell viability. After treatment, the medium was withdrawn and solution containing XTT at 0.3 mg/mL and PMS at 125 mM was added in each well. Cells were incubated for 4 h at 37°C. The absorbance at 450 nm was measured by a microplate reader (BioTek, Winooski, VT, USA), while cell viability was quantified as a percentage of the control.

**ATP level determination**

Total ATP content was evaluated using the manufacturer’s suggested CellTiter-Glo® Luminescence test kit. The assay buffer and substrate were pre-warmed. The buffer was then mixed gently with the substrate.

| Genes         | Primers | Sequences                        |
|---------------|---------|----------------------------------|
| GAPDH (mouse) | Forward | CTGTCGGAGGCTTGCTGGTG             |
|               | Reverse | GTCATCACTTGGCCAGGT              |
| NOS (mouse)   | Forward | ATGAGGGTACCTCGTGCTCCAC          |
|               | Reverse | CCACCAATGACCATCATTACTCTGG       |
| IL-1β (mouse) | Forward | CGACAAATATACCTGTCGCT            |
|               | Reverse | TCTTTCGGGTATGCTGCGTGG           |
| IL-6 (mouse)  | Forward | GGAGGGCTTAAATTACATGTT           |
|               | Reverse | TGATTCAAGATGAAATTGGAT           |
| TNF-α (mouse) | Forward | TTCGTCCTCAGGACTTCCGG            |
|               | Reverse | GTATGAGATAGCAAAATCGGC           |
Following cell treatment, 100 µL of the assay reagent was added to each well and stirred for 30 min under light protection. Luminescence was measured by a microplate reader (BioTek, Winooski, VT, USA). The luminescence signal was quantified as a percentage of the control.

**Determination of NO production**

The amount of nitrite released in the supernatant was determined using Griess reagent. Briefly, supernatant was mixed with reagent A (1% w/v sulfanilamide in 5% phosphoric acid) for 10 min then followed by incubation with reagent B (0.1% w/v N-1-naphthylethylene diamine dihydrochloride) for 10 min. The absorbance at 540 nm was determined by a microplate reader (BioTek, Winooski, VT, USA).

**IL-6 level measurement by ELISA**

The levels of IL-6 were determined using a sandwich ELISA kit from Abcam (Cambridge, MA, USA). Briefly, 96-well ELISA plates (NUNC, Roskilde, Denmark) were pre-coated with murine monoclonal anti-mouse IL-6 antibody. Following addition of the supernatants, incubation, and washing, the biotinylated murine anti-IL-6 antibody was added. After incubation, HRP-streptavidin enzyme was added, followed by a substrate solution (TMB). Sulfuric acid (1 M) was added to stop the reaction. The absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

**Western blotting**

Whole-cell lysates were prepared using RIPA buffer with protease inhibitors. Protein concentration was quantified by the Bradford assay. Equal amounts of protein (30 µg) were separated on a 10% SDS-polyacrylamide gel and then electro-blotted onto 0.2 µm PVDF membranes. Blots were blocked in 5% non-fat milk in TBST (10 mM Tris (pH 8.0) and 150 mM NaCl solution containing 0.05% Tween-20) for 1 h. After washing, the membrane was incubated overnight at 4°C with the following primary antibodies: anti-actin (1:5,000), anti-p-p38 (1:1,000), anti-p38 (1:1,000), anti-p-JNK (1:1,000), anti-JNK (1:1,000), anti-p-ERK (1:1,000), and anti-ERK (1:1,000). The membranes were washed with TBST and further incubated with anti-rabbit IgG-horseradish peroxidase (1:5,000) for 1 h. Immunolabeling was detected using the Immobilon Forte Western HRP substrate and visualized by Gel doc (Bio-Rad, Benicia, CA, USA). The density of the immunoreactive bands was quantitated using ImageJ software and normalized using actin as a loading control.

**Statistical analysis**

Data are expressed as mean value ± SD of the indicated number of independent experiments. All experiments were carried out in at least three replicates. Results were analyzed by one-way ANOVA followed by Tukey’s multiple comparison tests using GraphPad Prism (GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered statistically significant at p < 0.05.

**Results**

**Cytotoxicity of ochratoxin A on microglial BV-2 cells**

To determine the OTA cytotoxicity in BV-2 microglia, the XTT reduction assay and ATP levels were employed. The maximal OTA concentration in the present study was ten-fold higher than those found in plasma [35]. The results revealed that 24 h treatments with OTA concentrations ranging from 15 to 2,000 nM did not decrease the viability of the microglial BV-2 cells, as determined by XTT reduction (Figure 1a) as well as ATP levels (Figure 1b).

**Ochratoxin A-induced microglial activation**

Neuronal injury is a significant element that contributes to microglial activation, which results in an inflammatory response by releasing numerous inflammatory markers [2]. As a result, we investigated the effects of OTA on microglial activation through the production of inflammatory markers, including IL-6, TNF-α, IL-1β, iNOS, and NO. After exposure to OTA, the responses of these inflammatory markers were determined. Exposure of BV-2 cells with OTA (50–2,000 nM) for 24 h significantly upregulated the expression of IL-6, TNF-α, IL-1β, and iNOS at mRNA levels.
in a dose-dependent fashion, albeit with different levels of expression. iNOS mRNA expression was the highest compared to the other genes. The concentrations of IL-6 and NO released into the culture media were determined using ELISA and Griess reagent, respectively. The results showed that OTA-treated BV-2 significantly increased IL-6 and NO production in a concentration-dependent manner when compared to controls. Consistent with the results in Figure 2a, increased IL-6 and NO were observed when OTA at 500 nM was applied. The morphology of the control BV-2 cells was mostly spherical with small dark nuclei. Cells treated with OTA and LPS became elongated with short thick processes (Figure 2d), confirming BV-2 activation by OTA. Overall, the data indicate that the non-toxic doses of OTA triggered microglial activation within 24 h of treatment in a dose-dependent fashion.

Figure 1: Cytotoxicity of ochratoxin A on microglial BV-2 cells. BV-2 cells were incubated with different concentrations of OTA (31.25, 62.5, 125, 250, 500, 1,000, and 2,000 nM) for 24 h. (a) Cell viability examined by XTT reduction assay. (b) ATP levels measured by CellTiter-Glo® luminescence assay kit. Results are expressed as a percentage of the control (100%). Values are mean values ± SD of at least three independent experiments.

Figure 2: Effects of ochratoxin A on microglial inflammatory responses. BV-2 cells were incubated with different concentrations of OTA (50, 100, 250, 500, 1,000, and 2,000 nM) for 24 h. (a) mRNA levels of IL-6, TNF-α, IL-1β, and iNOS examined by qRT-PCR. (b) The IL-6 levels measured by ELISA. (c) NO levels measured by Griess reagent. (d) Cell morphology at 24 h obtained by taking phase-contrast pictures with an inverted Nikon microscope (10×). Data were expressed as mean value ± SD from at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005, and ****p < 0.0001 compared to the control medium.
ERK and p38 MAPK are essential for induction of microglial activation by ochratoxin A

MAPK signaling activity has been involved in the production of pro-inflammatory mediators during microglial activation [36]. Thus, we conjectured that OTA may activate MAPKs, particularly the ERK, JNK, and p38 pathways, during microglial activation. To study the involvement of MAPKs in microglia activation triggered by OTA, three specific inhibitors as PD98059 (ERK inhibitor; 20 μM), SB202190 (p38 MAPK inhibitor; 10 μM), and SP600125 (JNK inhibitor; 20 μM) were co-treated with BV-2 and OTA. The BV-2 cells were pre-incubated with each specific inhibitor for 1 h before exposure with OTA (50, 250, and 500 nM) for 24 h (Figure 2). The cytokine mRNA expression, IL-6 levels, and NO levels were examined, as shown in Figure 2. Compared to cells treated only with OTA, cells pre-incubated with PD98059 (ERK inhibitor) and SB202190 (p38 MAPK inhibitor) significantly decreased the mRNA expression of IL-6, TNF-α, IL-1β, and iNOS induced by OTA (Figure 3a–d). Consistent with the mRNA data, PD98059 and SB202190 significantly reduced IL-6 protein and NO levels in OTA-treated BV-2 cells (Figure 3e and f). Nevertheless, pre-treatment with a JNK inhibitor did not affect microglial activation induced by OTA. Western blot analysis was used to confirm whether OTA has a concentration-dependent effect on MAPK phosphorylation. As illustrated in Figure 3d, OTA treatment significantly increased ERK and p38 MAPK phosphorylation levels compared to the control group. Cotreatment with MAPK inhibitors decreased OTA-mediated ERK and p38 MAPK activation, while OTA was unable to stimulate JNK phosphorylation (data not shown). In conclusion, OTA activated microglia through activating ERK and p38 MAPK but not JNK.

Corticosterone exacerbates ochratoxin A-induced activation of microglial cells through the MR

Increased glucocorticoid stress hormones (cortisol in humans and corticosterone in rodents) produced by the HPA axis are an important component of organismal stress responses (Scheuer, 2010). Furthermore, glucocorticoids have been reported to enhance neuroinflammation-mediated neurotoxicity [37]. Hence, it is probable that glucocorticoids enhance neuronal susceptibility to OTA via microglial activation. To clear this point, BV-2 cells were treated with or without corticosterone (25 nM) for 12 h and then exposed to OTA (250 nM) for an additional 24 h. Cell viability was not significantly altered by corticosterone at the concentrations used (data not shown). As illustrated in Figure 4a–d, pre-exposure to corticosterone significantly increased OTA-induced mRNA expression of IL-6, TNF-α, IL-1β, and iNOS (p < 0.01). These findings were confirmed by investigating the IL-6 and NO protein levels. Corticosterone significantly increased OTA-induced IL-6 and NO generation, consistent with the mRNA data (Figure 4e and f). To establish the role of GR and MR in corticosterone’s impact on OTA-treated microglia cells, mifepristone (GR antagonist) and spironolactone (MR antagonist) were employed. Following corticosterone pre-exposure in the presence or absence of spironolactone (1 μM) or mifepristone (1 μM), cells were incubated with OTA for 24 h. Spironolactone, but not mifepristone, reduced the mRNA expression of cytokines by OTA (Figure 5a–d). Similarly, spironolactone substantially decreased the generation of IL-6 and NO (Figure 5e and f). The involvement of ERK and p38 MAPK was studied in corticosterone-mediated microglia activation induced by OTA. Corticosterone significantly increased OTA-induced ERK and p38 MAPK phosphorylation, whereas spironolactone significantly attenuated this effect (Figure 4g). Taken together, the results indicated that corticosterone promoted OTA-induced microglia activation via MR regulation of ERK and p38 MAPK signaling.

Discussion

OTA triggered the neurotoxicity and the development of various neurodegenerative disorders including AD, PD, and neuropsychiatric disorders [11–13]. Stress hormones have been demonstrated to boost neuroinflammation and activate microglia [20–22]. Hence, it is probable that glucocorticoids enhance neuronal susceptibility to OTA via microglial activation. The purpose of this research was to determine the impact of corticosterone pre-treatment on microglial cell activation by OTA and the underlying key signaling molecules. We found that OTA activated microglia and enhanced the expression of pro-inflammatory mediators. We also showed that corticosterone amplified microglial activation produced by OTA. Microglial activation serves essential functions in the brain, while the imbalance between the M1 (activated) and M2 (inactivated) phenotypes of microglial contributes to brain damage and pathologies such as ischemia, AD, PD, and multiple sclerosis [1–3,38]. Microglia undergo...
Figure 3: Involvement of MAP kinases in OTA-induced pro-inflammatory cytokine and iNOS expression in glial cells. BV-2 cells were pretreated with or without PD98059 (20 μM), SP600125 (20 μM), or SB203580 (10 μM) for 1 h, and then treated with OTA (500 nM) for 24 h. (a–d) mRNA levels of IL-6, TNF-α, IL-1β, and iNOS examined by real-time PCR. (e) IL-6 levels detected by ELISA. (f) NO levels measured by Griess assay. (g) Phosphorylation levels of ERK and p38 MAPK determined by western blot analysis. Data were expressed as mean value ± SD of at least three independent experiments. *p < 0.05, **p < 0.01, and ****p < 0.0001 compared to medium control and †p < 0.05 compared with OTA.
Figure 4: Potentiation effects on OTA-induced cytokine mRNA expression and IL-6 and NO production in BV-2 cells. BV-2 cells were pretreated with corticosterone (25 nM) in the absence or presence of spironolactone (1 μM) or mifepristone (1 μM) for 12 h and then exposed to OTA (250 nM) for 24 h. (a–d) mRNA levels of IL-6, TNF-β, IL-1β, and iNOS examined by real-time PCR. (e) IL-6 levels detected by ELISA. (f) NO levels measured by Griess reagent. (g) Phosphorylation levels of ERK and p38 MAPK determined by western blot analysis. Data were expressed as mean value ± SD of at least three independent experiments. *p < 0.05 and ****p < 0.0001 compared with OTA; #p < 0.05 and ####p < 0.0001 compared with OTA and corticosterone.
morphological changes during the M1 stage through the secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and other inflammatory mediators, including NO and ROS [3,38–40]. Therefore, the mRNA expression of IL-6, TNF-β, IL-1β, and iNOS and the release of IL-6 and NO were employed as bio-indicators of activated microglia. This study provided evidence that OTA promoted inflammation-mediated microglia activation by increasing pro-inflammatory cytokine mRNA expression and elevating IL-6 and NO levels in a concentration-dependent manner. This finding was consistent with previous studies exhibiting pro-inflammatory mediator induction of OTA in various cell types including microglia, macrophages, and Kupffer cells [14,41,42]. OTA has previously been demonstrated to have pro-inflammatory activities in a variety of acute and chronic inflammatory conditions of the liver, kidney, and gastrointestinal system [43–45]. However, the ability of OTA to produce neuroinflammation remains unclear although several studies have established neurotoxicity [46].

While various signaling molecules are involved in the activation of microglia, MAPKs have been demonstrated to be crucial in the activation of microglia, leading to the etiology of neurodegenerative illnesses [17,18,36]. As a result, we investigated the involvement of MAPKs in OTA-induced microglia activation. Pre-treatment with an ERK inhibitor (PD98059) and p38 MAPK inhibitor (SB202190) but not with a JNK inhibitor (SP600125) dramatically reduced the elevation and production of inflammatory molecules produced by OTA. Moreover, suppressing ERK and p38 MAPK with PD98059 and SB202190 abolished IL-6 and NO generation. Western blot results demonstrated that OTA treatment of BV-2 microglia increased ERK and p38 MAPK phosphorylation but not JNK phosphorylation. This finding concurred with previous studies in epithelial cells, chicken heterophils, and porcine alveolar macrophages that OTA activated ERK and/or p38 MAPK [47–50]. However, in this study, OTA failed to induce JNK phosphorylation in BV-2 cells. OTA activated JNK, stimulated JNK phosphorylation, and potentiated the effect of TNF-α on JNK activation in renal epithelial kidney cells [51].

It has been demonstrated that dysregulation of the HPA axis, as well as elevated cortisol and corticosterone levels, are associated with microglia activities, which contribute to the development of neuroinflammatory processes [20–22]. Animal studies have shown that corticosterone activates microglia [26,52–55]. Consequently, the function of corticosterone on OTA-induced inflammation in microglia was studied. The current study found that pre-treatment with corticosterone (25 nM) enhanced OTA-induced inflammation in BV-2 microglial cells. Consistent with the western blot result, corticosterone with OTA caused higher phosphorylation of ERK and p38 MAPK than OTA alone. Consistent with our study, corticosterone enhanced microglia activity and sensitivity to LPS in the hippocampus of rodents [31,34,52]. Chronic exogenous corticosterone amplified methamphetamine/diisopropyl fluorophosphate-induced activation of microglia in the striatum, resulting in increased production of pro-inflammatory cytokines/chemokines [26,53].

In microglia cells, corticosterone has been demonstrated to activate both MR and GR [25,56–58]. Therefore, the receptor dependence of corticosterone-potentiated microglia activation by OTA was examined using mifepristone and spironolactone as GR and MR antagonists, respectively. Subsequent studies showed that spironolactone diminished the action of corticosterone on the potentiation effect of OTA on microglia activation and inhibited corticosterone-induced activation of ERK and p38 MAPK. Consistently, aldosterone, an MR agonist,
increased cytokine expression and activation of the ERK and p38 MAPKs and was then abolished by spironolactone. This finding is consistent with the binding affinity of the corticosterone receptors, since MR is a high-affinity receptor for corticosterone and GR is a low-affinity receptor for corticosterone. Corticosterone reduced LPS-induced inflammatory production through a GR-dependent mechanism at high physiological concentrations. On the other hand, low corticosterone concentrations increased cytokine expression in an MR-dependent manner [24,25,30,59,60]. MRs are highly expressed in immune cells, particularly microglia, and their involvement in neuroinflammation is well established [38]. For instance, aldosterone induced an inflammatory response by MR activation, characterized by microglial activation [30]. Increased MR expression in the hippocampus of spontaneously hypertensive rats was related to increased ramiﬁed glial cells [61]. Aldosterone raised the number of microglia in the retina and promoted the generation of ROS and pro-inﬂammatory mediators [62].

Our findings have signiﬁcant implication for the role of stress hormone released by the HPA axis in promoting neuroinflammation caused by OTA through microglia activation. Chronic stress primes microglia, resulting in an increased pro-inﬂammatory response through MR-mediated ERK and p38 MAPK pathways. Furthermore, mediators produced during microglial activation might activate the HPA axis, causing glucocorticoid production. [63]. Consequently, glucocorticoid persistence can activate microglia, resulting in a vicious cycle (Figure 5).

Conclusion

The molecular mechanism(s) through which OTA activates microglial cells via the ERK and p38 MAPK pathways were deﬁned. Corticosterone enhanced OTA by activating the MAPK signaling pathway through MR. Results suggested that stress hormones contributed to OTA-induced neurodegeneration via microglia activation.

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Data availability statement: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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