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ORIGINAL INVESTIGATION

Duloxetine ameliorates lipopolysaccharide-induced microglial activation by suppressing iNOS expression in BV-2 microglial cells

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Abstract

Rationale It is known that both selective serotonin and serotonin noradrenaline reuptake inhibitors (SSRI, SNRI) are first-line drugs for the treatment of major depressive disorder. It has also been considered that both SSRI and SNRI can improve the symptoms of major depressive disorder by increasing the concentration of monoamine in the synaptic cleft based on the monoamine hypothesis. However, accumulating evidence has indicated that inflammation in the brain may be a key factor in the pathophysiological mechanisms that underlie the development of major depressive disorder.

Objectives It has been advocated that microglial cells may regulate the inflammatory response under pathological conditions such as major depressive disorder. In this study, we focused on whether duloxetine can ameliorate the inflammatory response induced by lipopolysaccharide (LPS) in BV-2 microglial cells.

Results Our results indicated that duloxetine significantly decreased the NO production induced by LPS. The increase in the protein expression level of iNOS induced by LPS was significantly decreased by treatment with duloxetine. Moreover, the increases in the protein expression levels of phosphorylated-IκBα, phosphorylated-Akt and Akt induced by LPS were also significantly decreased. Unexpectedly, the protein expression levels of other pro-inflammatory factors such as COX-2 and the phosphorylation ratios for various molecules including IκBα and Akt were not changed by treatment with duloxetine.

Conclusions These findings suggest that duloxetine may have an anti-inflammatory effect, which could contribute to its therapeutic effectiveness for major depressive disorder.

Keywords BV-2 · Duloxetine · LPS · Nitric oxide · iNOS

Abbreviations

NO	Nitric oxide
DUX	Duloxetine
LPS	Lipopolysaccharide
CORT	Corticosterone
FXT	Fluoxetine
SET	Sertraline
CIT	Citalopram
ESCIT	Escitalopram
MCP	Milnacipran
VEN	Venlafaxine
ATX	Atomoxetine
BUP	Bupropion
iNOS	Inducible nitric oxide synthase
G6PD	Glucose-6 phosphate dehydrogenase
COX-2	Cyclooxygenase-2
MAPKs	Mitogen-activated protein kinases
NFκB	Nuclear factor-kappa B
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
TNF-α	Tumor necrosis factor alpha
IL	Interleukin
JNK	C-Jun N-terminal kinase
JAK/STAT	Janus kinase/signal transduction and activator of transcription

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Introduction

Numerous reports in the literature, including both clinical and pre-clinical studies, have demonstrated that the development of depression may involve inflammatory processes (Dantzer et al. 2008; Dowlati et al. 2010; Han and Ham 2021; Steiner et al. 2011). In particular, it has been considered that microglial cells, which are brain macrophages that play a role in immune defense and inflammatory responses in the brain, regulate the brain microenvironment including neuronal network activation (Hinwood et al. 2012). In addition, it has been suggested that stress-induced microglial activation may contribute to depression by increasing pro-inflammatory cytokines or their mRNA expression, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 (Yirmiya et al. 2015). Considering these reports, it is reasonable that microglial activation followed by irregularity of the brain microenvironment is related to the onset of major depression disorder.

In general, microglial cells are in a "resting" state in the healthy brain. However, once subjected to unusual stimuli, microglial cells change to classical (M1) or alternative (M2) types (Guo et al. 2022). Whereas M2 microglia have neuroprotective effects by producing anti-inflammatory cytokines such as IL4- and IL-13, M1 microglia induce inflammation and neurotoxicity by secreting several pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Colonna and Butovsky 2017). The change in the phenotype of microglial cells from a "resting" state to M1 is typically induced by treatment with interferon-γ (IFN-γ) or lipopolysaccharide (LPS) (Colonna and Butovsky 2017). Activated microglial cells are also linked to the expression of several pro-inflammatory factors including nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and nuclear factor-kappa B (NFκB) (Fu et al. 2017). In addition, several studies have indicated that NFκB, protein kinase B (Akt), and compounds in the mitogen-activated protein kinase (MAPK) signaling pathway such as p38 and extracellular signal-regulated kinase (ERK) are involved in the process of inflammation that accompanies microglial activation (Chu et al. 2021; Qin et al. 2018; You et al. 2018). Since the neurotoxicity produced by microglial activation may be involved in the initiation and progression of irregularity of the brain microenvironment, the inhibition of microglial activation may be an approach to improve depression.

Selective serotonin and serotonin noradrenaline reuptake inhibitors (SSRI, SNRI) are considered to be first-line drugs for the treatment of major depressive disorder. Whereas it is indisputable that the therapeutic effectiveness of both SSRI and SNRI for major depression disorder is due to the selective inhibition of monoamine reuptake in the synaptic cleft, it is also possible that an anti-inflammatory effect via the inhibition of microglial activation produced by both SSRI and SNRI may contribute to their pathophysiological effects as antidepressants. It has been reported that fluoxetine and escitalopram, which are typical SSRIs that are used to treat major depression disorder, inhibited M1 activation and promoted M2 activation of microglial cells in vitro (Su et al. 2015). A comparison of the anti-inflammatory effects of SSRI and SNRI in BV-2 microglial cells treated with LPS demonstrated that fluoxetine, sertraline, and paroxetine significantly decreased the TNF-α secretion and NO production induced by treatment with LPS (Liu et al. 2011; Lu et al. 2019; Tynan et al. 2012). In addition, fluvoxamine and reboxetine inhibited INF-γ-induced microglial production of IL-6 and NO (Hashioka et al. 2007). Considering the studies mentioned above, it is reasonable that the anti-inflammatory effect of SSRI on microglia may be associated with its function as an antidepressant. In contrast, several reports have demonstrated that SNRI also has an anti-inflammatory effect on microglial cells. It has been reported that venlafaxine exhibited an anti-inflammatory effect in an astroglia-microglia co-culture model by reducing IL-6 and IFN-γ secretion (Vollmar et al. 2008). Moreover, it has been demonstrated that both venlafaxine and duloxetine could decrease the serum TNF-α level that has been elevated by a single administration of LPS in mice (Ohgi et al. 2013). However, it is still unknown whether an SNRI, such as duloxetine, venlafaxine, or milnacipran, has anti-inflammatory effects on microglial cells activated by treatment with LPS.

In this study, we examined the effects of duloxetine, venlafaxine, and milnacipran on NO production induced by treatment of BV-2 microglial cells with LPS. We also explored the molecular mechanisms underlying these effects.

Materials and methods

Cell culture

BV-2 microglial cells were cultured as described previously (Nakatani et al. 2012). Briefly, cells were cultured in Dulbecco's modified Eagle's medium with high glucose (Wako, Osaka, Japan) supplemented with 10% Fetal Bovine Serum (Biowest, Nuaillé, France), 100u/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator under a 95%/5% mixture of air and CO_2. Cells were generally given fresh culture media on the second day, and then passaged on the third day.

WST-8 assay

BV-2 microglial cell proliferation was investigated by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Once cells became confluent, they were plated into 96-well microplates at a density of 2×10^5/mL (2×10^4/well) and incubated with various psychotropic drugs, including SSRI or SNRI, which were first dissolved in dimethyl sulfoxide (DMSO). When cells were treated with these compounds, the final concentration of DMSO in the culture medium was set at 0.1%. When cells were treated with lipopolysaccharide (LPS, from *E. coli* O55, Wako), which was first dissolved in phosphate buffer saline (PBS), the final concentration of PBS was also set at 0.1%. In general, this assay was performed after cells were treated with LPS and each compound simultaneously and then cultured for 24 h. After culture, the cells were incubated with WST-8 solution at 37 °C for 2 h. The spectrophotometric absorbance of WST-8-formazan produced by dehydrogenase activity in living cells at 450 nm was measured using a VersaMax (Molecular Devices, Sunnyvale, CA, USA). Absorbance at 650 nm was also measured as a reference.

Nitric oxide measurement

NO production was determined by measuring the amount of nitrite using a method that included Griess reagent as described previously with slight modification (Fu et al. 2017; Ju et al. 2018). Briefly, 100 µL of supernatant from cells cultured for 24 h with LPS and various psychotropic drugs at the concentration indicated was isolated and then mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-1-naphtylethylendiamine dihydrochloride in 2.5% phosphoric acid). After incubation for 20 min at room temperature in the dark, the absorbance at 535 nm was measured using a VersaMax (Molecular Devices). Nitrite concentrations were calculated according to a nitrite standard curve generated using sodium nitrite solution at 0–100 µM.

Western blotting

Protein samples were obtained from BV-2 microglial cells that had been treated with 1 µg/mL LPS, various concentrations of duloxetine or the vehicle used to dissolve LPS or duloxetine for 30 min or 24 h. For the preparation of protein extract from BV-2 microglial cells, the cells were washed with ice-cold homogenizing buffer (pH7.4; 250 mM sucrose, 20 mM Tris–HCl, 10 mM EGTA, and 2 mM EDTA 2Na), and centrifuged to obtain a cell pellet. The cell pellet was treated with lysis buffer (homogenizing buffer with 1% Triton X-100 and a protease inhibitor cocktail) and sonicated on ice. After the sample was centrifuged, the supernatant was collected as a total protein extract. For western blotting, protein extracts were placed on SDS–polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA, USA), and immunoblotted with anti-iNOS (ABclonal Technology, MA, USA) antibody, anti-COX-2 (Cell Signaling Technology, Danvers, MA, USA) antibody, anti-glucose-6-phosphate dehydrogenase (G6PD, Cell Signaling Technology) antibody, anti-phospho Akt (Ser473, Cell Signaling Technology) antibody, anti-Akt (Cell Signaling Technology) antibody, anti-phospho NFκB p65 (Ser536, Cell Signaling Technology) antibody, anti-NFκB p65 (Cell Signaling Technology) antibody, anti-phospho IκBα (Ser32, Cell Signaling Technology) antibody, anti- IκBα (Cell Signaling Technology) antibody, anti-phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling Technology) antibody, anti-ERK1/2 (Cell Signaling Technology) antibody, anti-phospho-p38 MAPK (Thr180/Tyr182, Cell Signaling Technology) antibody, anti-p38 MAPK (Cell Signaling Technology) antibody, and anti-β-tubulin (Wako) antibody. The blots were developed with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA). Western BLoT Quant HRP Substrate (TaKaRa, Ootsu, Japan) was used for detection.

Statistical analysis

The results are presented as the mean ± S.D. One-way analysis of variance was used to detect significant differences between the control and treatment conditions. A statistical analysis was performed using the Dunnett multiple comparisons test. A p value < 0.05 was considered to be statistically significant.

Results

Effect of LPS on NO production and cell viability in BV-2 microglial cells

First, we investigated whether LPS affected NO production by BV-2 microglial cells. As shown in Fig. 1A and 1B, NO production by BV-2 cells was observed under treatment with LPS at a concentration greater than 10^{-1} µg/mL for 24 h and this production increased with the further addition of LPS in a concentration-dependent manner. We also investigated whether LPS affected the viability of BV-2 microglial cells, and found that treatment with 10 µg/mL LPS slightly but significantly decreased cell proliferation (Fig. 1C). According to these results, we decided to use LPS at a concentration of 1 µg/mL in further experiments.

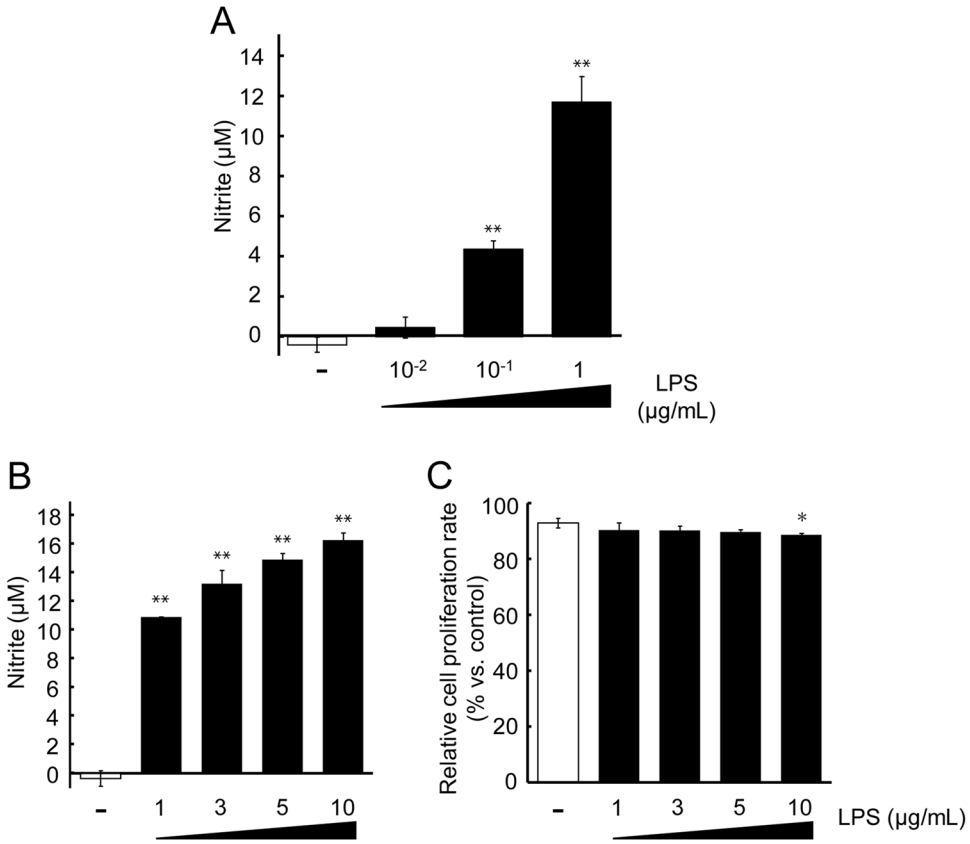

Fig. 1 Effects of LPS on NO production and cell viability in BV-2 microglia cells. Nitrite measurement in LPS-stimulated BV-2 microglial cells. The supernatants were obtained and the concentration of nitrite was measured as NO production in BV-2 microglial cells treated with **A** 10^{-2}, 10^{-1}, and 1 μg/mL LPS and **B** 1, 3, 5, and 10 μg/mL LPS for 24 h. The histogram shows the means ± SD ($n = 3$, *; $p < 0.05$ vs. without LPS treatment). **C** WST-8 assay of BV-2 microglial cells treated with 1, 3, 5, and 10 μg/mL LPS for 24 h. The vertical axis shows the proliferation rate relative to that in the control without LPS. The histogram shows the means ± SD ($n = 3$, *; $p < 0.05$ vs. without LPS treatment)

Effects of psychotropic drugs on NO production and cell viability in LPS-stimulated BV-2 microglial cells

To assess the potential anti-inflammatory effects of psychotropic drugs, we examined which psychotropic drugs could decrease NO production in LPS-stimulated BV-2 microglial cells. In this experiment, four SSRIs (fluoxetine, sertraline, citalopram, and escitalopram), three SNRIs (duloxetine, milnacipran, and venlafaxine), one NRI (atomoxetine), and one NDRI (bupropion) were examined. Since glucocorticoids inhibited NO production induced by the treatment of primary rat microglial cells with LPS, CORT was used as a positive control to decrease NO production induced by the treatment of BV-2 microglial cells with LPS. All compounds were used at a concentration of 10 μM since 10 μM CORT significantly decreased NO production in LPS-stimulated BV-2 microglial cells (data not shown). As shown in Fig. 2A, treatment with CORT significantly decreased NO production to 73.8 ± 7.5% compared to that in LPS-stimulated BV-2 microglial cells. Among the SSRIs, both fluoxetine and sertraline also decreased NO production in LPS-stimulated BV-2 microglial cells to 78.3 ± 1.4 and 63.7 ± 3.8%, respectively, and these results corresponded to previous reports (Tynan et al. 2012). In contrast, duloxetine significantly decreased NO production to 52.8 ± 7.9% compared to an LPS-treated control, which is the greatest decrease among all of the compounds in this study. Of the compounds tested, only venlafaxine affected cell viability in LPS-stimulated BV-2 microglial cells (Fig. 2B).

Effects of duloxetine on NO production and cell viability in LPS-stimulated BV-2 microglial cells

Since only duloxetine had an anti-inflammatory effect, which manifested as an inhibitory effect on NO production, in LPS-stimulated BV-2 microglial cells among various psychotropic drugs, except for some compounds that have already been shown to have anti-inflammatory effects, we focused on the anti-inflammatory effect of duloxetine in detail. As shown in Fig. 3A, duloxetine reduced NO production by LPS-stimulated BV-2 microglial cells in a concentration-dependent manner at concentrations between 10 μM and 20 μM, but not at 1 μM. When we also checked whether the morphological change caused in LPS-stimulated BV-2 restore by 10 μM duloxetine treatment, duloxetine could not restore (supplementary Figure S1). In contrast, treatment of LPS-stimulated BV-2 microglial cells with 20 μM duloxetine decreased cell viability slightly but significantly (Fig. 3B). Since the cell viability of BV-2 microglial cells that were treated with more than 20 μM duloxetine significantly decreased without any effect on NO production (data

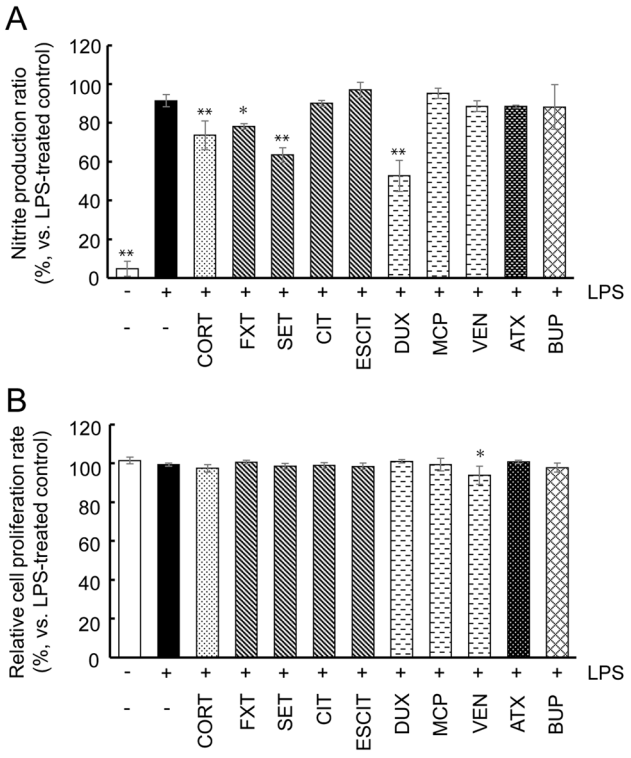

Fig. 2 Effects of corticosterone (CORT) and psychotropic drugs on NO production and cell viability in LPS-stimulated BV-2 microglial cells. **A** Nitrite measurement in LPS-stimulated BV-2 microglial cells treated with CORT and various psychotropic drugs. The supernatants were obtained and the concentration of nitrite was measured as NO production in LPS-stimulated BV-2 microglial cells treated with 10 μM of CORT and psychotropic drugs for 24 h. The vertical axis shows nitrite production relative to that in the control treated with LPS. The histogram shows the means ± SD ($n = 3$–4, *; $p < 0.05$ vs. LPS treatment). **B** WST-8 assay of LPS-stimulated BV-2 microglial cells treated with 10 μM of CORT and various psychotropic drugs for 24 h. The vertical axis shows the proliferation rate relative to that in the control treated with LPS. The histogram shows the means ± SD ($n = 3$–4, *; $p < 0.05$, **; $p < 0.01$ vs. with LPS treatment). DUX, duloxetine; FXT, fluoxetine; SET, sertraline; CIT, citalopram; ESCIT, escitalopram; MCP, milnacipran; VEN, venlafaxine; ATX, atomoxetine; BUP, bupropion

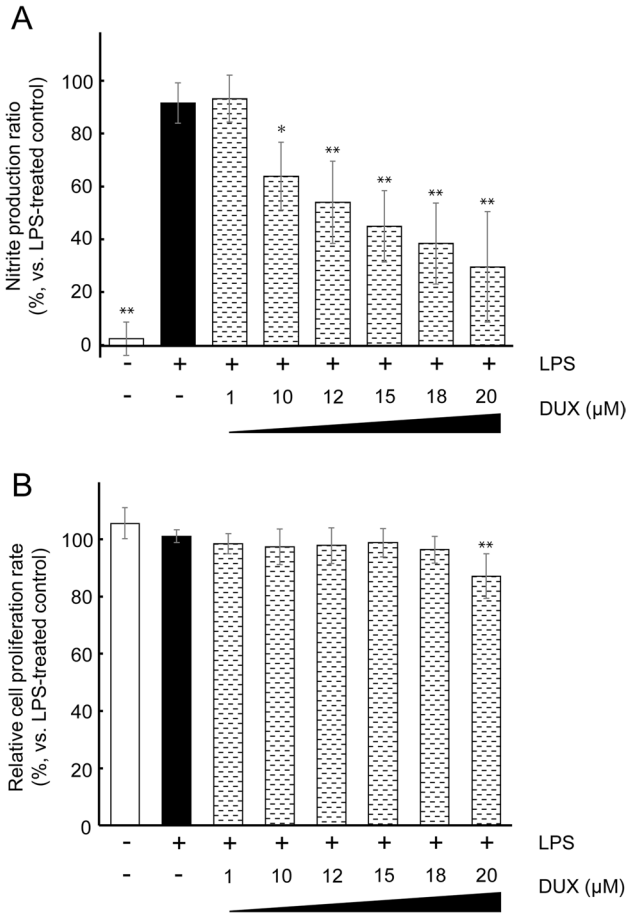

Fig. 3 Effects of duloxetine on NO production and cell viability in LPS-stimulated BV-2 microglial cells. **A** Nitrite measurement in LPS-stimulated BV-2 microglial cells treated with various concentrations of duloxetine. The supernatants were obtained and the concentration of nitrite was measured as NO production in LPS-stimulated BV-2 microglial cells treated with duloxetine at concentrations of 1 to 20 μM for 24 h. The vertical axis shows nitrite production relative to that in the control treated with LPS. The histogram shows the means ± SD ($n = 5$, *; $p < 0.05$, **; $p < 0.01$vs. LPS treatment). **B** WST-8 assay of LPS-stimulated BV-2 microglial cells treated with duloxetine at concentrations of 1 to 20 μM for 24 h. The vertical axis shows the proliferation rate relative to that in the control treated with LPS. The histogram shows the means ± SD ($n = 5$, **; $p < 0.01$ vs. with LPS treatment)

not shown), we decided to examine the effect of duloxetine by using this range of duloxetine concentrations.

Effects of duloxetine on iNOS expression in LPS-stimulated BV-2 microglial cells

To investigate the molecular mechanisms underlying the anti-inflammatory effect, as reflected by the inhibitory effect on NO production, in LPS-stimulated BV-2 microglial cells, we examined the effect of duloxetine on the protein expression level of iNOS in LPS-stimulated BV-2 microglial cells. As shown in Fig. 4, increased protein expression of iNOS induced by LPS treatment was significantly decreased by treatment with more than 10 μM duloxetine. Compared to the results with LPS treatment, 10 μM duloxetine decreased the protein expression level of iNOS to 72.1 ± 16.8% in a concentration-dependent manner. In addition, the observation that 1 μM duloxetine did not induce a decrease in the protein expression level of iNOS in LPS-stimulated BV-2 microglial cells was consistent with the results regarding the inhibitory effect of duloxetine on NO production, as mentioned above.

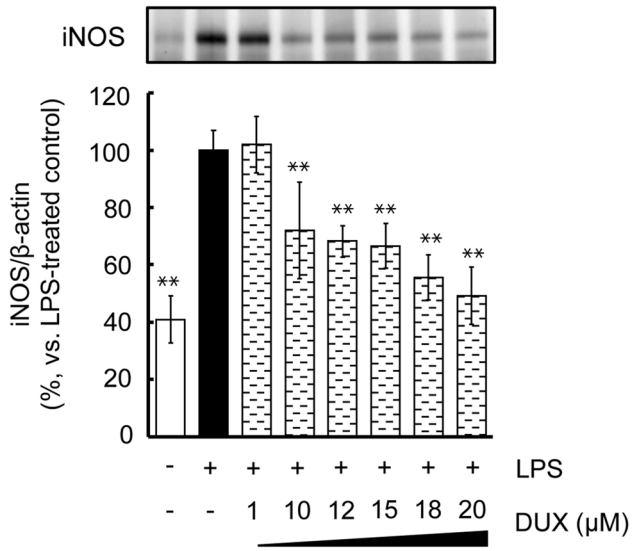

Fig. 4 Protein expression of iNOS in LPS-stimulated BV-2 microglial cells treated with duloxetine at concentrations of 1 to 20 μM for 24 h. The relative expressions of iNOS are shown as the relative ratio based on the respective expression in the control treated with LPS. The histogram normalized by the expression of β-actin shows means ± SD ($n=4$, **; $p<0.01$ vs. with LPS treatment)

Effects of duloxetine on COX-2 and G6PD expression in LPS-stimulated BV-2 microglial cells

It is well known that COX-2 is linked to the activation of microglial cells via the increase in NO production induced by treatment with LPS (Fu et al. 2017). In addition, it has been reported that the induction of G6PD by LPS helps to prevent NO-mediated glutathione depletion in cultured astrocytes (García-Nogales et al. 1999). Therefore, to evaluate the potential of a further anti-inflammatory effect of duloxetine, we examined whether duloxetine affected the protein expression levels of both COX-2 and G6PD in LPS-stimulated BV-2 microglial cells. As shown in Fig. 5A, while duloxetine did not decrease the protein expression level of COX-2 compared to treatment with LPS, the COX-2 expression level was increased in LPS-stimulated BV-2 microglial cells. In contrast, the protein expression level of G6PD did not change in the presence of LPS and duloxetine (Fig. 5B).

Effects of duloxetine on activated-NFκB signaling in LPS-stimulated BV-2 microglial cells

It has been considered that NO production induced by LPS via an increase in the iNOS protein expression level is associated with NFκB signaling. Actually, NFκB regulates several genes involved in the inflammatory response including iNOS and COX-2 (Ju et al. 2018; Kempe et al. 2005). In the inflammatory condition, NFκB is released from nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) via phosphorylation induced by the action of specific kinase proteins such as the IkB kinase complex followed by the degradation of IκBα. In addition, it is well known that the PI3K/Akt signaling pathway regulates NFκB activation as an upstream molecule in the NFκB pathway (Cianciulli et al. 2016; Wang et al. 2015). To investigate the molecular mechanisms underlying the anti-inflammatory effect of duloxetine, NFκB signaling and associated molecules were examined with respect to whether they were affected by duloxetine. As shown in Fig. 6A, the protein expression levels of phosphorylated-NFκB that were elevated by treatment with LPS and NFκB did not change in the

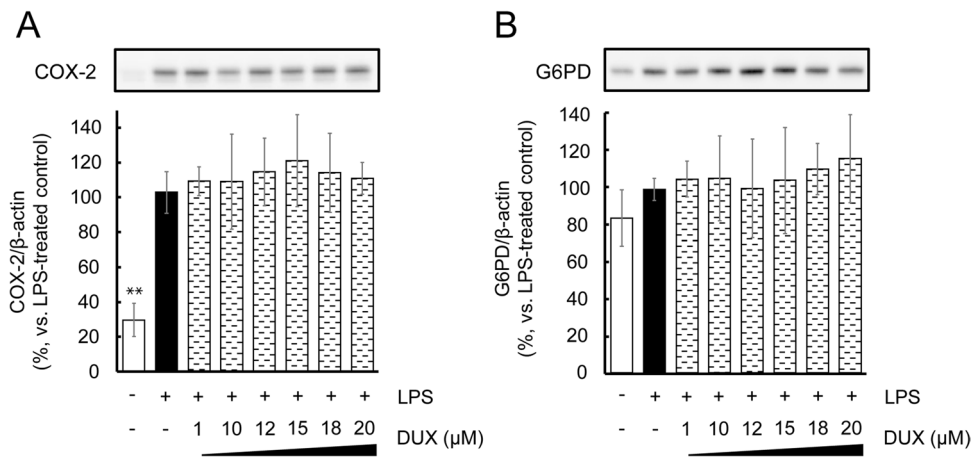

Fig. 5 Protein expressions of COX-2 and G6PD in LPS-stimulated BV-2 microglial cells treated with duloxetine. Protein expressions of **A** COX-2 and **B** G6PD were detected in LPS-stimulated BV-2 microglial cells treated with duloxetine at concentrations of 1 to 20 μM for 24 h. The relative expressions of COX-2 and G6PD are shown as the relative ratio based on the respective expression in the control treated with LPS. The histogram normalized by the expression of β-actin shows means ± SD ($n=4$, **; $p<0.01$ vs. with LPS treatment)

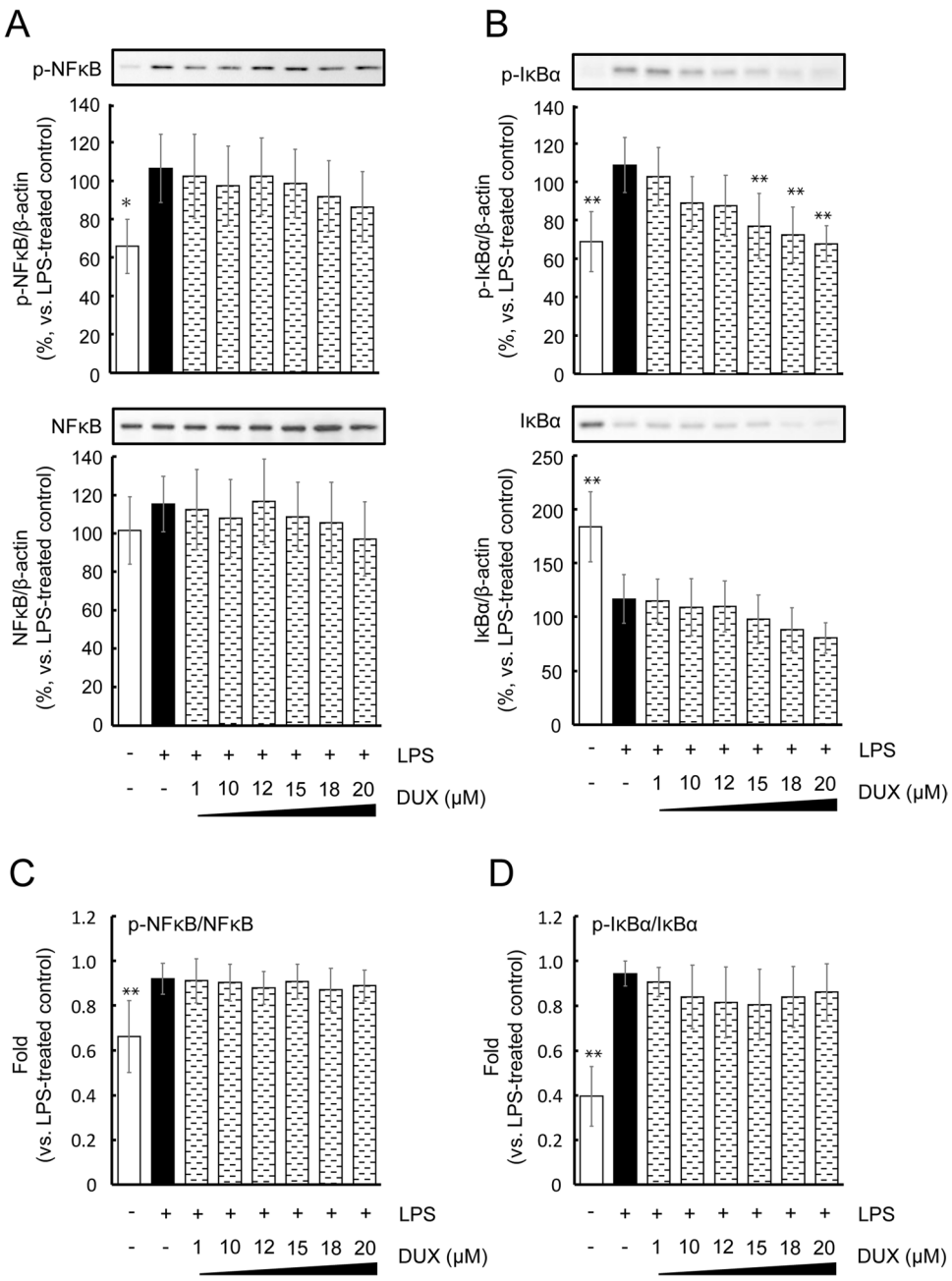

Fig. 6 Expressions of phosphorylated and total proteins of NFκB and IκBα in LPS-stimulated BV-2 microglial cells treated with duloxetine. The expressions of phosphorylated and total proteins of **A** NFκB and **B** IκBα in cells treated with duloxetine at concentrations of 1 to 20 μM for 30 min. The relative expressions of phosphorylated and total proteins of NFκB and IκBα are shown as the relative ratio based on the respective expression in the control treated with LPS. The histogram normalized by the expression of β-actin shows means ± SD ($n=5$, *; $p<0.05$, **; $p<0.01$ vs. with LPS treatment). The phosphorylation ratios of (C) NFκB and (D) IκBα were calculated in LPS-stimulated BV-2 microglial cells treated with duloxetine at concentrations of 1 to 20 μM for 30 min. The relative phosphorylation ratios of NFκB and IκBα are shown as the relative ratio based on the respective expression in the control treated with LPS. The histogram shows means ± SD ($n=5$, **; $p<0.01$ vs. with LPS treatment)

presence of duloxetine. In Fig. 6C, the phosphorylation ratio of NFκB increased in LPS-stimulated BV-2 microglial cells compared to the condition without LPS treatment. However, duloxetine did not alter this increased phosphorylation ratio of NFκB induced by treatment with LPS even though the range of concentration of duloxetine that showed the decrease of protein expression level of iNOS significantly. In contrast, the protein expression levels of phosphorylated-IκBα elevated by treatment with LPS significantly decreased in the presence of more than 15 μM duloxetine to 77.1 ± 17.0% compared to that under treatment with LPS (Fig. 6B). However, duloxetine did not affect the increase in the phosphorylation ratio of IκBα induced by treatment with LPS (Fig. 6D).

Effects of duloxetine on activated-Akt signaling in LPS-stimulated BV-2 microglial cells

It has been considered that Akt is regulated as a pro-inflammatory factor as an upstream molecule of NFκB signaling or via a mechanistic target of rapamycin (mTOR) pathway. As shown in Fig. 7A, the protein expression levels of phosphorylated-Akt elevated by treatment with LPS significantly decreased in the presence of more than 12 μM duloxetine to

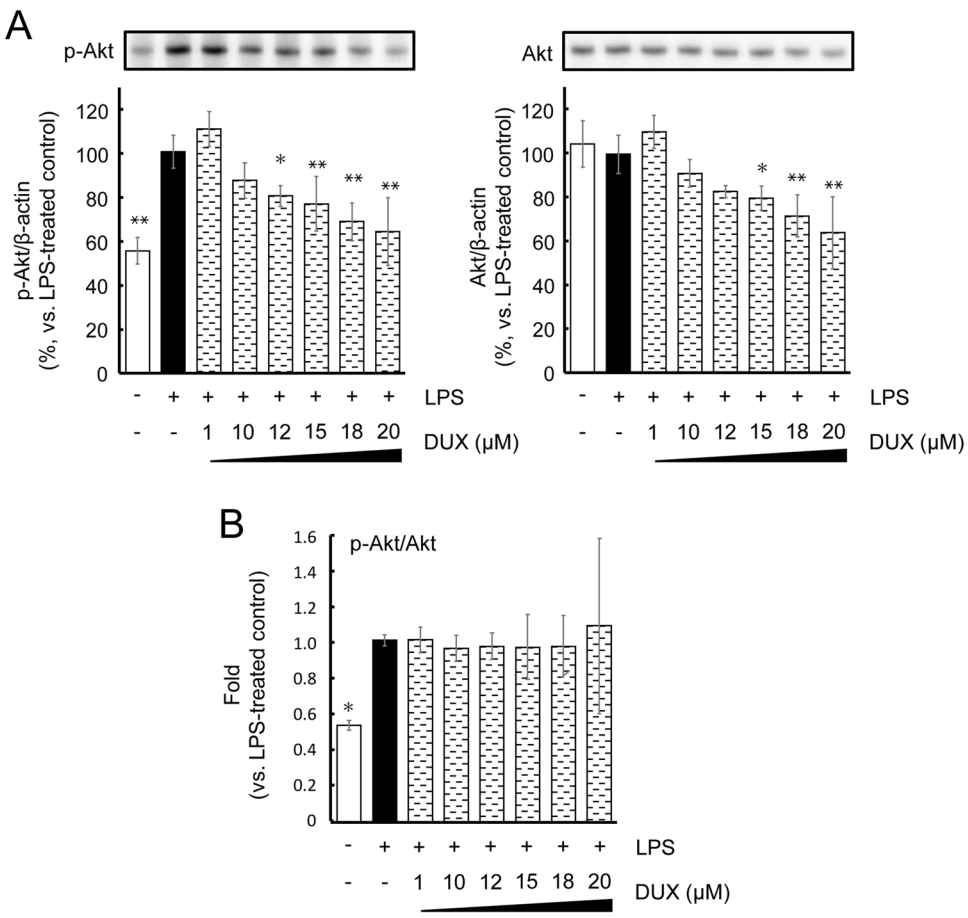

Fig. 7 Expressions of phosphorylated and total proteins of Akt in LPS-stimulated BV-2 microglial cells treated with duloxetine. **A** The expressions of phosphorylated and total proteins of Akt in cells treated with duloxetine at concentrations of 1 to 20 μM for 24 h. The relative expressions of phosphorylated and total proteins of Akt are shown as the relative ratio based on the respective expression in the control treated with LPS. The histogram normalized by the expression of β-actin shows means ± SD ($n=4$, *; $p<0.05$, **; $p<0.01$ vs. with LPS treatment). **B** The phosphorylation ratios of Akt were calculated in LPS-stimulated BV-2 microglial cells treated with duloxetine at concentrations of 1 to 20 μM for 24 h. The relative phosphorylation ratios of Akt are shown as the relative ratio based on the respective expression in the control treated with LPS. The histogram shows means ± SD ($n=4$, *; $p<0.05$ vs. with LPS treatment)

80.1 ± 4.8%. Moreover, the protein expression levels of Akt also significantly decreased in the presence of more than 15 μM duloxetine to 79.7 ± 5.5% (Fig. 7B). When the phosphorylation ratio of Akt was calculated, the results revealed that duloxetine did not alter the increase in the Akt phosphorylation ratio in LPS-stimulated BV-2 microglial cells like NFκB or IκBα (Fig. 7C).

Effects of duloxetine on MAPKs phosphorylation in LPS-stimulated BV-2 microglial cells

It has also been considered that MAPKs plays a role in regulating the expression of inflammatory genes. Indeed, some reports have demonstrated that the inhibition of both p38 MAPK and ERK1/2 activation blocked iNOS expression in LPS-stimulated murine macrophages or microglial cells (Ajizian et al. 1999; Zhang et al. 2012). Therefore, to investigate whether duloxetine decreased the protein expression level of iNOS through MAPK signaling, the effect of duloxetine on the phosphorylation ratios of p38 MAPK and ERK1/2 was examined in LPS-stimulated BV-2 microglial cells. As shown in Fig. 8A and 8B, LPS treatment itself did not change the phosphorylation ratio of either p38 MAPK or ERK1/2 in LPS-stimulated BV-2 microglial cells. The phosphorylation ratios of both p38 MAPK and ERK1/2 also did not change in the presence of LPS and duloxetine in BV-2 microglial cells.

Discussion

In this study, we first demonstrated that duloxetine has an anti-inflammatory effect via NO production in LPS-stimulated BV-2 microglial cells. In addition, since duloxetine decreased the protein expression level of iNOS in LPS-stimulated BV-2 microglial cells, it is considered that the reduction of iNOS expression induced by duloxetine contributed to the inhibitory effect of duloxetine on NO production.

To date, several studies have indicated that psychotropic drugs, such as SSRI or SNRI, may be able to prevent the activation of inflammatory factors in LPS-stimulated microglial cells (Hashioka et al. 2007; Liu et al. 2011, 2014; Lu et al. 2019; Mariani et al. 2022; Ohgi et al. 2013; Su et al. 2015; Tynan et al. 2012). Among various psychotropic drugs, it has been reported that fluoxetine, an SSRI, has potential to show an anti-inflammatory effect in vivo (Ohgi et al. 2013) and in vitro (Liu et al. 2011). Importantly, it has been demonstrated that the anti-inflammatory effect

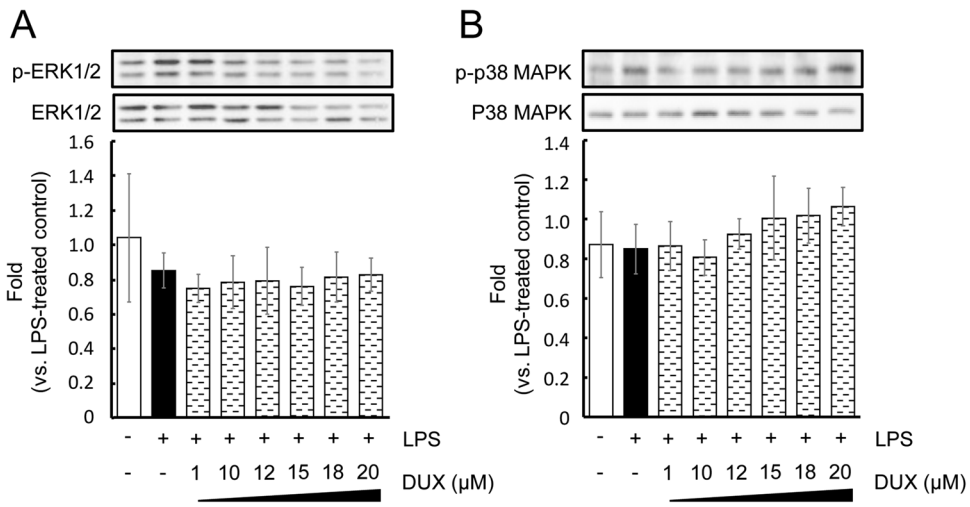

Fig. 8 Phosphorylation ratios of ERK1/2 and p38 MAPK in LPS-stimulated BV-2 microglial cells treated with duloxetine. The phosphorylation ratios of **A** ERK1/2 and **B** p38 MAPK were detected in LPS-stimulated BV-2 microglial cells treated with duloxetine at concentrations of 1 to 20 μM for 30 min. The relative phosphorylation ratios of ERK and p38 MAPK are shown as the relative ratio based on the respective expression in the control treated with LPS. The histogram shows means ± SD ($n=5$)

produced by fluoxetine in LPS-stimulated microglia cells is regulated by the inhibition of NFκB, MAPKs, and ERK signaling (Liu et al. 2011; Yang et al. 2014). Furthermore, fluoxetine prevented LPS-induced NO production in primary microglia cells or BV-2 microglial cells by suppressing the protein expression level of iNOS (Lieb et al. 2003; Liu et al. 2011). Paroxetine and sertraline have also been shown to have these anti-inflammatory effects (Liu et al. 2014; Lu et al. 2019; Ohgi et al. 2013). These results suggest that psychotropic drugs, such as antidepressants, particularly SSRI and SNRI, may have an anti-inflammatory effect by suppressing NO production via a decrease in iNOS expression. While the anti-inflammatory effects of SSRIs have been well documented, whether or not SNRIs, such as duloxetine, venlafaxine, and milnacipran, have anti-inflammatory effects has not yet been adequately investigated. One study indicated that both venlafaxine and duloxetine attenuated LPS-induced increases in TNFα, but increased serum levels of IL-10, in mice treated with LPS in vivo (Ohgi et al. 2013). Moreover, it has been reported that venlafaxine could reduce the increase in the production of TNF-α under treatment with LPS at only certain concentrations but did not affect the increased NO production induced by LPS treatment in an in vitro study (Tynan et al. 2012).

Our results demonstrated that duloxetine, but not venlafaxine or milnacipran, decreased NO production in LPS-stimulated BV-2 microglial cells. Since the result that venlafaxine did not reduce LPS-induced NO production was consistent with the results of a previous study, it is considered that duloxetine has an anti-inflammatory effect, at least with regard to NO production. Considering that epinephrine is potently anti-inflammatory including the inhibitory effect of NO production (de Barros et al. 2012; Dello Russo et al. 2004), the difference in the effect of NO production among three SSRI might be induced by the difference in the potency of inhibitory effect of norepinephrine reuptake. Duloxetine is used for the treatment of neuropathic pain as an inhibitor of $P2X_4$ receptor (Yamashita et al. 2016). Since some reports have indicated that NO release is mediated by $P2X_4$ receptor (Smith et al. 2013; Tu et al. 2017), this might explain the difference between duloxetine and other SNRIs with regard to their inhibitory effect on NO production in LPS-stimulated BV-2 microglial cells.

In an attempt to clarify the mechanisms underlying the decrease in LPS-induced NO production, we found that duloxetine could reduce the increased protein expression level of iNOS induced by treatment with LPS. Unexpectedly, duloxetine did not decrease the increase in the protein expression level of COX-2 induced by treatment with LPS like a SSRI such as fluoxetine. Moreover, duloxetine also did not affect the phosphorylation ratios of NFκB, IκBα, Akt, p38 MAPK, or ERK1/2, although these molecules have been considered to be part of the main signaling pathways to mediate the expression of inflammatory factor such as iNOS, COX-2, or NO and to be target molecules for expression of the anti-inflammatory effects of SSRIs such as fluoxetine (Lieb et al. 2003; Liu et al. 2011; Lu et al. 2019; Yang et al. 2014; Zhang et al. 2012).

Nonetheless, it is interesting to note that the protein expression level of phosphorylated-IκBα was significantly decreased by treatment with more than 15 μM duloxetine. In general, phosphorylated-IκBα proteins are subsequently ubiquitinated and degraded, which results in the translocation of NFκB from the cytoplasm to the nucleus (Viatour et al. 2005; Zhang et al. 2020). Duloxetine might prevent the increase in the protein expression of iNOS in LPS-stimulated BV-2 microglial cells by reducing IκBα phosphorylation although there was no change in its phosphorylation ratio. However, if we consider the phosphorylation level of NFκB under the influence of duloxetine in LPS-stimulated BV-2 microglial cells, other mechanisms may also involve to contribute to suppress iNOS expression by duloxetine in

LPS-stimulated BV-2 microglial cells. In addition, the protein expression levels of both phosphorylated-Akt and Akt were also significantly decreased by treatment with duloxetine. It has been reported that activation of Akt via phosphorylation activates IκB kinase followed by IκBα phosphorylation, or induces the phosphorylation of mTOR followed by the regulation of transcription factors including those related to iNOS expression (Fang et al. 2017). Although the phosphorylation ratio of Akt in LPS-stimulated BV-2 microglial cells was unaffected by treatment with duloxetine, the decrease in phosphorylated-Akt and total Akt might reduce the pro-inflammatory signal itself induced by LPS, followed by the protein expression of iNOS.

As candidate molecules to modify iNOS expression except for molecules analyzed in present study, it is possible to be involved c-Jun N-terminal kinase (JNK) or Janus kinase/signal transduction and activator of transcription (JAK/STAT). Some studies have indicated that inflammatory effects induced by LPS were mediated by the activation of JNK (Lim et al. 2018; Liu et al. 2014). In addition, it has been reported that the inhibitor of JAK/STAT pathway prevented both NO production and protein expression of iNOS in LPS-stimulated BV-2 microglial cells (Liu et al. 2014). Thus, it is possible that duloxetine affects both NO production and iNOS expression via JNK or JAK/STAT signaling modulation. Differences in the signaling molecules affected by each psychotropic drug may explain the differences between their effects.

In summary, duloxetine significantly decreased the increase in the NO production induced by LPS in BV-2 microglial cells. In addition, while the protein expression levels of iNOS, phosphorylated-IκBα, phosphorylated-Akt and Akt increased by LPS were also significantly decreased, the phosphorylation ratio was unaffected in all of these molecules. This anti-inflammatory effect due to the inhibition of NO production by duloxetine may contribute to its antidepressant effect. Further studies will be needed to clarify the mechanisms underlying the regulatory effects of duloxetine on NO production induced by LPS.

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Declarations

Conflict of interest The authors declare no competing interests.

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