LOX-1 Mediates Inflammatory Activation of Microglial Cells Through the p38-MAPK/NF-κB Pathways Under Hypoxic-Ischemic Conditions

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Research

Keywords: chemokine, cytokine, hypoxia, ischemia, microglia, NF-kappa B (NF-κB), p38 MAPK, small interfering RNA (siRNA), LOX-1

Posted Date: November 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-100349/v1

Abstract

Background: Microglial cells play an important role in the immune system in the brain. Activated microglial cells are not only injurious but also neuroprotective. We confirmed marked lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) expression in microglial cells in pathological lesions in the neonatal hypoxic-ischemic encephalopathy model brain. LOX-1 is known to be an activator of cytokines and chemokines through intracellular pathways. Here, we investigated a novel role of LOX-1 in microglial cells under hypoxic and ischemic conditions.

Methods: We isolated primary rat microglial cells from 3-day-old Sprague-Dawley rat brains and confirmed that the isolated cells showed more than 98% Iba-1 positive with immunocytochemistry. We performed oxygen glucose deprivation (OGD) treatment of primary rat microglial cells as an in vitro model of nHIE. Then, we evaluated the expression levels of LOX-1, cytokines and chemokines with or without siRNA and inhibitors, compared with no OGD-treated cells. In addition, we analyzed reactive oxygen species and cell viability.

Results: We found that defects in oxygen and nutrition induced LOX-1 expression and led to the production of inflammatory mediators, such as the cytokines IL-1β, IL-6 and TNF-α, the chemokines CCL2, CCL5 and CCL3 and reactive oxygen/nitrile species. Then, the LOX-1 signal transduction pathway was blocked by inhibitors, LOX-1 siRNA, the p38-MAPK inhibitor SB203580 and the NF-κB inhibitor BAY11-7082 suppressed the production of the inflammatory mediators. Moreover, we demonstrated that LOX-1 in microglial cells was autonomously overexpressed by positive feedback of the intracellular LOX-1 pathway.

Conclusion: The hypoxic/ischemic conditions of microglial cells induced LOX-1 expression and activated immune system. We revealed one of the LOX-1 signaling pathways in microglia and provide a hint of a new treatment strategy for nHIE. It was speculated that Microglial cells are known to contribute to various neurological diseases. Hypoxic and ischemic brain injuries result in lifelong neurological and mental deficiency. LOX-1 and its related molecules or chemicals may be major therapeutic candidates.

Background

Microglial cells are the resident immune cells of the central nervous system (CNS) and have diverse functions that are both beneficial and harmful [1–3]. It has been reported that pathological conditions in CNS disease model animals deteriorate when microglial cells are depleted or do not function properly, or microglial cells help model animals recover from illness [4, 5]. However, the other studies have reported that blocking microglial activities improved disease phenotype [6, 7]. The function of activated microglial cells is not only injurious but also neuroprotective because activated microglial cells can release anti-inflammatory cytokines and trophic factors [1–3]. Activated microglial cells are classified into two major phenotypes, the M1 phenotype (inflammatory phenotype) and the M2 phenotype (anti-inflammatory-

phenotype) [8–10]. Several studies have focused on these microglial phenotypes in a variety of CNS diseases [11–14]. Microglial cells may contribute to form the pathology of the premature brain .

Neonatal hypoxic-ischemic encephalopathy (nHIE) is a major cause of perinatal brain damage that is thought to occur via multiple events such as placental abruption, umbilical cord prolapses, or maternal/fetal infection, ultimately inducing an inadequate supply of oxygen and blood to the brain [15, 16]. The incidence of nHIE is 1 to 8 per 1000 live births in developed countries and is much higher in undeveloped countries [17]. Despite many animal experiments and clinical trials, the pathophysiology is still not clear and an effective therapy has yet to be established [18.19]. We have reported that lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), the gene named *oxidized low-density lipoprotein receptor 1* (*OLR1*), was upregulated in the nHIE rat brain and administration of an anti-LOX-1 neutralizing antibody improved brain pathology [20]. LOX-1 is a scavenger receptor for modified proteins, especially oxidized low-density lipoprotein (oxLDL) [21]. LOX-1 is expressed on endothelial cells, smooth muscle cells and macrophages and is related to the development of atherosclerosis and other cardiovascular diseases. In addition, LOX-1 acts physiologically as an inducer of apoptosis, reactive oxygen species and proinflammatory cytokines [22–24]. We also found that LOX-1 was expressed in microglial cells, the resident brain macrophages, in the nHIE rat model (unpublished data). In addition to other CNS diseases, it is generally recognized that inflammation induced by the peripheral and central immune systems is one of the major pathological factors in nHIE [25–27]. Although microglial cells are thought to be related to the pathology of nHIE, much remains unknown about the role of microglial cells in nHIE and the therapeutic strategy targeting microglial cells.

We hypothesized that LOX-1 mediates the inflammatory activation of microglial cells under hypoxic/ischemic conditions, which induces neuronal death. In the present study, we investigated the alteration in LOX-1 expression in microglia and the influence of LOX-1 on detrimental processes under hypoxic/ischemic conditions using primary rat microglial cell cultures. Furthermore, we explored the signaling pathways related to LOX-1 in microglia to establish a new treatment strategy for nHIE.

Methods

Primary microglial cell isolation from newborn rat brain

We obtained primary microglial cells, according to a previously described technique [28,29]. Briefly, mixed glial cultures were prepared from newborn to 3-day-old Sprague-Dawley rat brains containing the cortex, hippocampus and striatum (CLEA Japan Inc., Tokyo, Japan) and cultured until confluent as described. The cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM high glucose, WAKO, Osaka, Japan) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a 10% CO_2 incubator. After approximately 2 weeks, microglial cells were harvested by the shaking method and replated on 6-well plates at 2×10^6 cells/well, 96-well plates at 1×10^5 cells/well or 8-well chamber slides at 2×10^5 cells/well. The microglial cell cultures consisted of >98% microglial cells (stained with anti-Iba1

antibody (WAKO)) (Supplementary Fig. 1). All animal experiments were performed with the permission of the Animal Experiment Ethics Committee of the National Center of Neurology and Psychiatry.

Experimental groups and oxygen glucose deprivation and LOX-1 knockdown treatments

We performed oxygen glucose deprivation (OGD) treatment as an in vitro model of hypoxia/ischemia [30,31]. The harvested microglial cells were cultured in high glucose DMEM containing 10% FBS for 24 hours. Then, the culture medium was replaced with glucose-free DMEM without FBS. Primary microglial cells were treated with OGD and LOX-1 knockdown.

For OGD treatment, cultured microglial cells were treated with a BIONIX-2 hypoxic cell culture kit (Sugiyamagen, Tokyo, Japan). The concentration of oxygen was maintained at almost 0%. For the controls, the culture medium was replaced with high glucose DMEM without FBS and microglial cells were cultured under normal oxygen concentrations. After OGD treatment for 6 hours, the microglial cells were washed with PBS. Then, total RNA or proteins were extracted for analysis and the culture supernatant was collected for cytokine measurement. For immunocytochemistry, the cells in chamber slides were washed with PBS and then fixed with 4% paraformaldehyde (PFA).

For LOX-1 knockdown, the expression of LOX-1 in primary microglial cells was silenced using the small interfering RNA (siRNA) Silencer Select rat *Olr1* (genetic name of LOX-1) (Thermo Fisher Scientific, Waltham, MA) with Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific), according to the manufacture's instructions. We used Silencer Select negative control No.1 siRNA that did not target any rat genes as a negative control. The diluted siRNA (12.5 pmol/ml) and transfection reagent (3.75 μl/ml) were added to the microglial cell culture. The cells were incubated with the siRNA for 24 hours and then were subjected to the OGD experiments.

Inhibitors of p38-MAPK and NF-kB

SB203580 (199-16551, Wako Pure Chemical Industries, Osaka, Japan), a p38-MAPK inhibitor, was added to cultured microglial cells at a concentration of 20 μmol/l for 60 minutes before OGD treatment [32]. BAY11-7082 (19542-67-7, Wako Pure Chemical Industries), an NF-κB inhibitor, was added to the isolated microglial cells at a concentration of 10 μmol/l for 30 minutes before OGD treatment [33].

RNA extraction and real-time quantitative PCR

Total RNA was extracted from rat brains and cultured microglial cells using the RNeasy Plus Mini kit (Qiagen, Venlo, NLD), according to the manufacturer's protocols. Then, the total RNA concentration was measured using a Nanodrop (Thermo Fisher Scientific). For RT-PCR, cDNA was prepared from total RNA using a high-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). RT-PCR was performed using LightCycler 480 SYBR Green I master mix (Roche, Basel, Switzerland) on a LightCycler 480 System II (Roche, Basel, CHE). The experiments were performed in triplicate. The mRNA levels were normalized relative to the endogenous reference gene *act-b* (β-actin). The results are described as the fold change of the Ct value relative to the control groups.

Transcriptome analysis

Whole transcriptome microarray analysis was performed using a Rat Clariom S assay (Thermo Fisher Scientific) according to the manufacturer's instructions. Analysis and normalization of the raw data was conducted using Transcriptome Analysis Console (TAC) 4.0 (Thermo Fisher Scientific). Differentially expressed genes (DEGs) were determined as genes with an FDR < 0.01 and more than 2-fold change. Gene set analysis of gene ontology (GO) biological processes and KEGG pathways was conducted using GSEA software (http://software.broadinstitute.org/gsea/index.jsp).

Protein extraction and immunoblotting

Total proteins were extracted using RIPA buffer (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Separate cytoplasmic and nuclear protein fractions were extracted using NE-PE nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). Then, the protein concentrations were measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific).

Twenty micrograms of the extracted proteins were loaded in each lane of TruPAG Precast Gels 4-12% (Sigma-Aldrich Corporate, St. Louis, MO) for electrophoresis. After transfer to a polyvinylidine difluoride membrane, each immunoreacted band was detected using Amersham ECL prime western blotting detection reagent (GE Healthcare, Boston, MA) according to the manufacturer's instructions. As a reference, β-actin was detected using a specific antibody. The expression levels of the detected bands were measured and calculated by ImageQuant TL (GE Healthcare).

Immunocytochemistry

The microglial cells on chamber slides or in 96-well plates were washed with PBS and fixed with 4% PFA for 30 minutes. After that, the cells were incubated with the primary antibodies. Then, the cells were incubated with Alex Flour conjugated secondary antibodies (Thermo Fisher Scientific) for 1 hour and were mounted with Hoechst 33342 (Thermo Fisher Scientific). The fluorescent samples were observed with confocal laser scanning microscopy (LSM780; Zeiss, Oberkochen, Germany) or fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

Cytokine measurement

The microglial cell culture medium was collected and centrifuged for 20 minutes at 1,000xg. Then, cytokine/chemokine concentrations in the supernatants were measured using a BioPlex ProTM Rat Cytokine 23-Plex Assay (BioRad, Hercules, CA).

Reactive oxygen species (ROS) detection

After the OGD experiments, microglial cells were subjected to OGD treatment and treated with 4.5% glucose containing original medium as controls. Then, cellular ROS levels were detected using CellRox Green Reagent (C10444: Thermo Fisher Scientific). Briefly, the microglial cells were stained with 5 μM

CellRox Green Reagent by adding the probe to the complete media and incubating at 37°C for 30 minutes. Fifty μM of N-acetyl cysteine (NAC), an antioxidant was added to some of the OGD-treated wells. Then, the cells were washed with PBS and were fixed with 4% PFA. Finally, the microglial nuclei were stained with Hoechst 33342 (Roche). The cells were observed with the microscope (IX71: Olympus, Tokyo, Japan) and the mean fluorescent intensities per cells were analyzed using In Cell Analyzer 2000 (GE Healthcare).

Cell viability

After the OGD experiments, the microglial cells conducted OGD treatment were added with 4.5% glucose as the same glucose concentrations as the CTL. Then, the cells were incubated for 2 hours with CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WA) at 37 °C according to the manufacturer's instructions. This reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. The quantity of formazan product as measured by absorbance at 490 nm is directly proportional to the number of living cells in cultures. After the 2 hours incubation, the absorbance at 490 nm was measured using a 96-well plate reader.

Statistics analysis

All data were described as means ± SD. Data were analyzed with one-way ANOVA followed by Tukey's *post hoc* test or unpaired *t*-test. Statistical analysis was performed using IBM SPSS version 22 (IBM, Armonk, NY). $P < 0.05$ was considered to be statistically significant.

Results

LOX-1, cytokine and chemokine expression and ROS induction in OGD-treated microglial cells

First, we confirmed that the cultured cells in all experience were more than 98% Iba-1 positive (Supplementary Fig. 1). Many oxygen glucose deprivation (OGD)-treated microglial cells had LOX-1 signals in the cytoplasm, although the untreated microglial cells had no LOX-1 signals (Fig. 1A, 1B).

Immunoblotting experiments showed that the expression of LOX-1 was increased in OGD-treated microglial cells compared to control cells (Fig. 1C, 1D). RT-PCR showed that the mRNA levels of LOX-1 were similar to those of the protein levels (data not shown). Thus, the expression of LOX-1 in microglial cells was upregulated under hypoxic and ischemic conditions. OGD treatment also markedly induced the expression levels of the cytokines IL-1β, IL-6 and TNF-α, and the chemokines CCL2, CCL5 and CCL3 (Fig. 2). In addition, the ROS level of OGD-treated microglial cells significantly increased (Supplementary Fig. 2).

LOX-1 knockdown shifted OGD-treated microglial cells to an anti-inflammatory (M2) phenotype

To investigate the relationship between LOX-1 and microglial activation, we analyzed whether microglial cells were activated under hypoxic and ischemic conditions and whether their activation was changed

with LOX-1 siRNA administration. LOX-1 siRNA efficiently knocked down LOX-1 expression in microglial cells, and we confirmed that the expression of LOX-1 (*Olr1*) induced by OGD was suppressed by LOX-1 siRNA to the same level as that of the control (Supplementary Fig. 3). The production of the cytokines IL-1β, IL-6 and TNF-α was increased after OGD and suppressed by LOX-1 siRNA administration (Fig. 2A). Moreover, the production of the chemokines CCL2, CCL5 and CCL3 was increased after OGD and suppressed by LOX-1 siRNA administration (Fig. 2B). The expression level of *IL-4*, an anti-inflammatory cytokine, was not significantly changed (data not shown). However, the *Nos2* (iNOS) expression in OGD-treated microglial cells was increased compared to that of the control and was suppressed by LOX-1 siRNA (data not shown). The ROS level in OGD-treated microglial cells was decreased to that of untreated microglial cells by LOX-1 siRNA treatment (Supplementary Fig. 2). These data clearly indicate that hypoxic/ischemic conditions induce the expression levels of inflammatory cytokines/chemokines, iNOS and ROS in microglial cells through the upregulation of LOX-1, and the downregulation of LOX-1 recovered the expression levels of these inflammatory mediators.

To further investigate the role of LOX-1 in microglial cells, we performed transcriptome analysis to compare the expression patterns in control (CTL)-, OGD- and OGD with LOX-1 siRNA-treated microglial cells. The heatmap indicates that LOX-1 knockdown shifted OGD-treated microglia to the M2-phenotype (Supplementary Fig. 4).

LOX-1 inhibition altered the NF-κB distribution pattern and the phosphorylation of p38-MAPK

NF-κB p65 expression was examined in the cytoplasm in primary microglial cells (Fig. 3). After OGD induction, NF-κB p65 expression was mainly in the nucleus. However, LOX-1 knockdown in OGD-treated microglial cells recovered LOX-1 expression in the cytoplasm. As the intracellular signaling pathway of LOX-1 is known, we investigated p38-MAPK and ERK1/2 activity and phosphorylation. OGD insult induced phosphorylation of both proteins (Fig. 4). LOX-1 siRNA treatment in OGD-treated microglial cells recovered the p38-MAPK phosphorylation level but not the ERK1/2 phosphorylation level. These results indicated that ERK1/2 activation by OGD was independent of the LOX-1 pathway.

Effects of p38-MAPK and NF-κB inhibitors in OGD-treated microglial cells

SB203580 (SB), a p38-MAPK inhibitor, and BAY11-7082 (BAY), an NF-κB inhibitor, did not alter the LOX-1 expression pattern or levels in the cultured cells (Fig. 5). However, both SB and BAY markedly reduced the expression levels of the cytokines IL-1β, IL-6 and TNF-α and the chemokines CCL2, CCL5 and CCL3 (Fig. 6). These data indicate that NF-κB and p38-MAPK are transcription factors associated with cytokines and chemokines. Furthermore, LOX-1 is one of the OGD-induced cytokine/chemokine pathways in microglial cells, and NF-κB and p-38 MAPK are important components of these pathways.

NF-κB expression was analyzed, and both inhibitors suppressed NF-κB p65 nuclear expression (Fig. 7). Furthermore, the ratio of phosphor-p38-MAPK to total p38-MAPK (phospho/total p38-MAPK) was increased in OGD-treated microglia (Supplementary Fig. 5). SB reduced the ratio to the baseline level, but BAY did not have this effect. NF-κB may be downstream of p-38 MAPK.

Furthermore, we found that innate immune signaling pathways were significantly upregulated after OGD induction and that these upregulations were altered by LOX-1 siRNA, as determined by gene set analysis (data not shown). These findings suggested that the genes in the innate immune pathways might be downstream of LOX-1, and the upregulation of LOX-1 leads to microglial activation through these genes.

Microglial viability

Phagocytic activities measured in vitro experiments. Cell viability was not significantly changed after OGD compared to that of the control group (data not shown).

Discussion

In the present study, we found that LOX-1 was induced in microglial cells under oxygen and nutrient deprivation and mediated the inflammatory activation of microglial cells, which led to the production of inflammatory mediators such as cytokines, chemokines, and reactive oxygen/nitrile species. Blocking the LOX-1 signal transduction pathway suppressed the production of inflammatory mediators. The OGD method is widely used to analyze the pathology of neurons, astrocytes, or myocytes as models of hypoxic and ischemic conditions [34–37]. We demonstrated that OGD-induced stress increased microglial cell expression of inflammatory cytokines (IL-1β, IL-6 and TNFα) and chemokines (CCL2, CCL5 and CCL3) (Fig. 2, Supplementary Fig. 3). Transcriptome analysis showed that OGD-induced microglial cells expressed inflammatory gene transcripts. In addition, the expression of LOX-1 was induced in OGD-treated microglial cell, which was similar to the phenomenon of microglial cells in the nHIE rat model (data not shown). On the other hand, LOX-1 knockdown in OGD-treated microglial cells led to the suppression of inflammatory mediators, which was consistent with the change observed in the nHIE rat model brain treated with an anti-LOX-1 neutralizing antibody [20]. Therefore, our OGD microglial cell model is a useful method to develop or screen drugs for HIE and inflammatory conditions in the brain and to analyze the LOX-1 signal transduction pathway as a treatment target. This finding also indicates that microglial cells have an LOX-1-inducible immune system, which acts under hypoxic/ischemic conditions.

It is known that the expression of LOX-1 is induced by external stimuli, such as ox-LDL, apoptotic cells, and cytokines [24, 38]. A study reported that conditioned medium derived from necrotic neuronal cultures induced LOX-1 expression in microglial cells [39]. HSP60 has also been identified as a molecule that induces LOX-1 expression, and HSP60 acted on LOX-1 expressing microglial cells to induce the production of inflammatory mediators [39]. However, we demonstrated that the expression of LOX-1 was induced in primary rat microglial cells after OGD treatment without any external stimulus. In addition, the upregulation of LOX-1 in OGD-treated microglial cells continued despite the suppression of inflammatory cytokines and chemokines with p38-MAPK or NF-κB inhibitors. This finding means that the induction of LOX-1 in microglial cells is not related to inflammatory cytokines or chemokines. Based on these results, we can speculate that the expression of LOX-1 is induced by not only external stimuli but also endogenous factors in microglial cells under hypoxic and ischemic conditions [40, 41].

Based on the results of the human *OLR1* promoter sequence and transcription factor binding site prediction, HIF-1α and NF-κB can directly control LOX-1 expression. Under the conditions of oxygen and nutrient deficiency, HIF-1α may first act on the immune system via microglial cells. In addition, we can speculate that NF-κB induced by the cellular LOX-1 pathway leads to cytokine production. Our results revealed that NF-κB expression was induced by OGD insult and was localized in the nucleus (Fig. 3). It may demonstrate that LOX-1 is overexpressed in microglial cells by positive feedback of the LOX-1 pathway.

Our previous study demonstrated that nHIE model rats expressed LOX-1 and were cured by an anti-LOX-1 neutralizing antibody [20]. In this study, we showed that LOX-1 siRNA downregulated 60–80% of LOX-1 mRNA expression and protein expression to the baseline level (Supplementary Fig. 2). Namely, defects in the brain circulation system lead to LOX-1 expression, and suppression of LOX-1 can markedly alleviate this pathology. Thus, LOX-1 is considered a major candidate for nHIE therapy. OGD insult induced P38-MAPK activation but did not influence ERK1/2 activation; however, both molecules are components of the cellular LOX-1 pathway (Fig. 4). Moreover, inhibitors of P38-MAPK and NF-κB reduced cytokine and chemokine expression to the similar levels as those of LOX-1 siRNA (Fig. 6).

As we previously described, activated microglial cells produced inflammatory cytokines and chemokines and reactive oxygen/nitrogen species under hypoxic and ischemic conditions. Inflammatory cytokines and chemokines activate immune cells, including microglial cells, and induce migration of these immune cells and destruction of blood-brain barrier [42]. Therefore, these cytokines and chemokines may indirectly cause neuronal injuries. In contrast, neurons express TNFα receptors and TNFα can directly induce apoptosis via caspase-8 activation or necrosis RIP1 and RIP3 activation in neurons [42, 43]. Neurons also express IL-1β receptors, and hypoxic microglia-derived IL-1β induces neuronal apoptosis via these receptors [44]. Therefore, in addition to indirect neuronal injury, some cytokines can directly cause neuronal injury. In this study, the production of these cytokines was related to the expression of LOX-1 in hypoxic and ischemic-injured microglial cells, which means that the upregulation of LOX-1 in activated microglial cells is indirectly or directly related to neuronal injuries.

Recently, it has been shown that microglial cells contribute to the pathophysiology of various neurodegenerative diseases, epilepsy, neurodevelopmental diseases, autoimmune encephalitis, neurologic pain and brain trauma [45]. Hypoxic and ischemic brain injuries are caused by many circulatory dysfunctions, extrinsic accidents and various pathological conditions. nHIE is a major perinatal disease and results in lifelong neurological and mental deficiency. We must seek a completely curative therapy. LOX-1 and its related molecules or chemicals are major candidates for nHIE therapy.

Conclusion

We investigated the alteration in LOX-1 expression in microglia and the influence of LOX-1 on detrimental processes under hypoxic/ischemic conditions using primary rat microglial cell cultures. We performed oxygen glucose deprivation (OGD) treatment as an in vitro model of hypoxia/ischemia and LOX-1

knockdown using siRNA and treated with the inhibitors of p38-MAPK or NF-κB. As results, OGD-treated microglial cells induced LOX-1, cytokine and chemokine expression and ROS. It indicates that hypoxic/ischemic conditions of microglial cells induced LOX-1 expression and activated immune system. Moreover, LOX-1 reduction with LOX-1 siRNA shifted microglial cells to an anti-inflammatory phenotype and reduced the expression levels of cytokine and chemokine to the similar levels as those of P38-MAPK or NF-κB inhibitors. Here, we revealed one of the LOX-1 signaling pathways in microglia and provide a hint of a new treatment strategy for nHIE.

List Of Abbreviations

LOX-1; lectin-like oxidized low-density lipoprotein receptor-1, IL-1β; interleukin-1β, IL-6; interleukin-6, TNF-α; tumor necrosis factor-α, CCL2; C-C motif chemokine 2, CCL3; C-C motif chemokine 3, CCL5; C-C motif chemokine 5, CNS; central nervous system, nHIE; neonatal hypoxic-ischemic encephalopathy, OLR1; oxidized low-density lipoprotein receptor 1, oxLDL; oxidized low-density lipoprotein, OGD; oxygen glucose deprivation, CTL; control, ROS; reactive oxygen species, NAC; N-acetyl cysteine, PFA; paraformaldehyde, DMEM; Dulbecco's modified Eagle's medium, FBS; fetal bovine serum, NF-κB; nuclear factor-kappa B, p38-MAPK; p-38 mitogen-activated Protein Kinase.

Declarations

Availability of data and materials

All data are contained within the article and supporting information. The datasets and analyzed data during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Funding

This work was supported by intramural research grants (1-4) for Neurological and Psychiatric Disorders from the National Center of Neurology and Psychiatry (M.I.) and a grant from the Japan Agency for Medical Research and Development (AMED) Grant 19gk0110033h0002 (M.I.).

Authors' contributions

YA and MI designed the study and wrote the paper. TA and TO performed and supported cell culture. YA performed molecular biology experiments and analyzed the data. NT, YG and AO provided experimental instruments and supported the study. MI supervised and finished the whole study. All authors approved the final version of the manuscript.

Acknowledgements

We thank T. Sanagi, PhD (Tohoku University, Japan) for technical support and useful advice on primary rat microglial cell culture.

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Figures

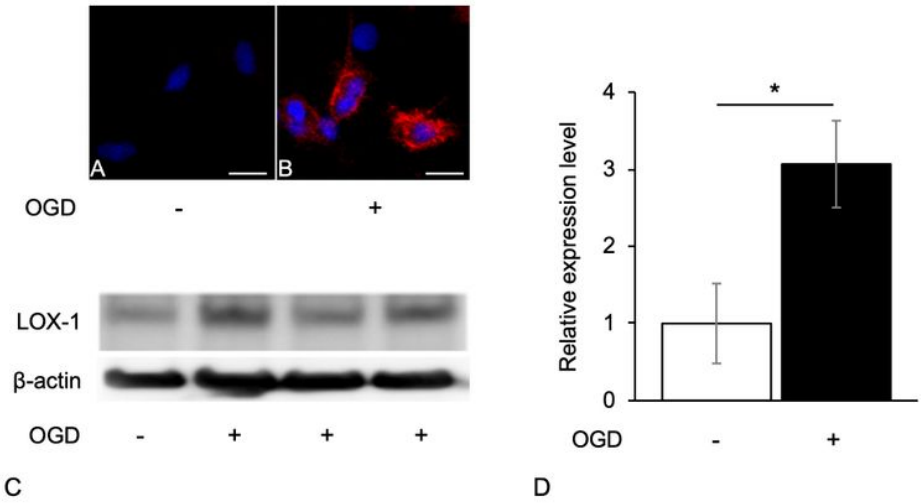

Figure 1

OGD-treated microglial cells express LOX-1. Untreated microglial cells (Control; CTL) does not exhibit LOX-1 expression (A). OGD-treated microglial cells (OGD) exhibit LOX-1 expression in the cytoplasm (B). Immunoblot analysis shows LOX-1 overexpression in OGD-treated microglial cells (C, D). Immunocytochemistry, blue: Hoechst 33342 (A, B), red; LOX-1 (B). *; $P<0.05$. Scale bar = 10 μm.

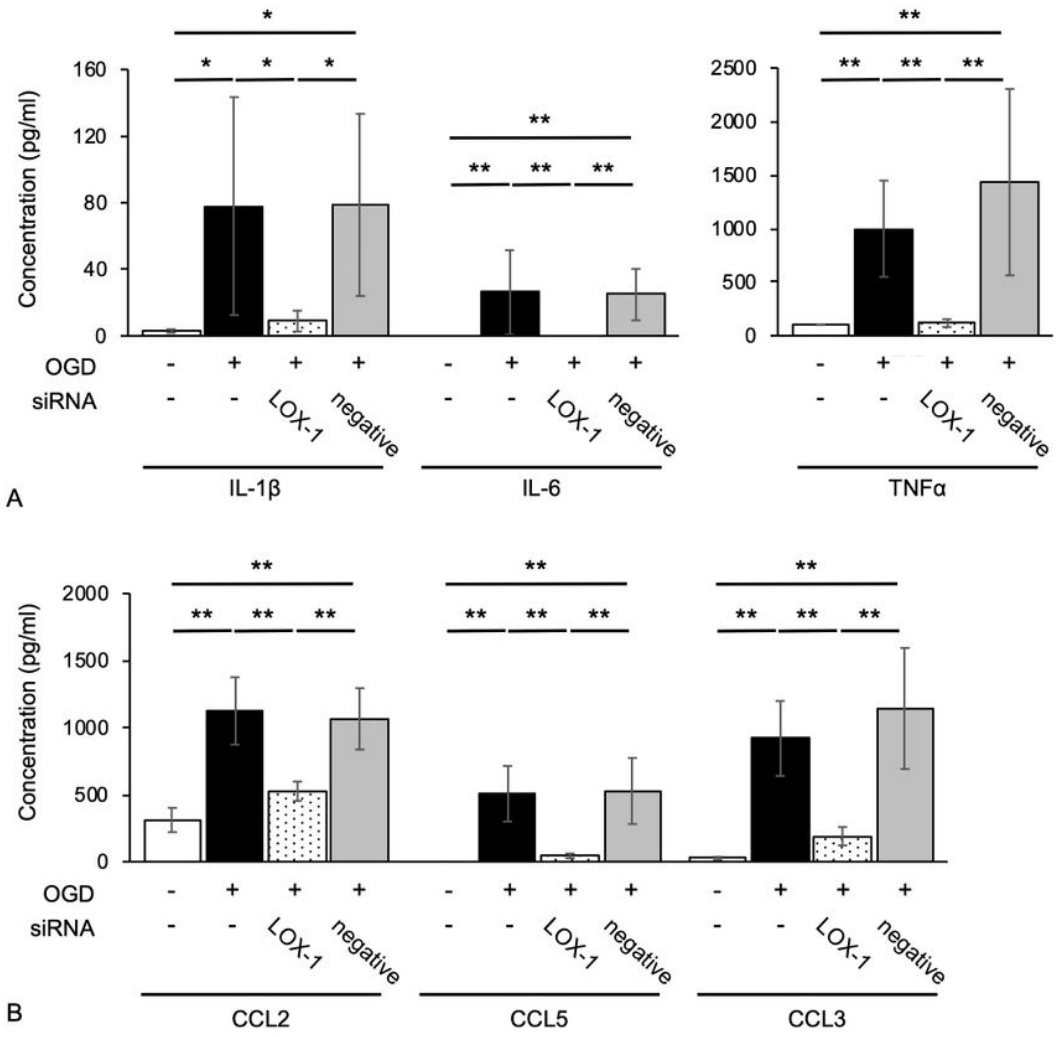

Figure 2

LOX-1 siRNA inhibits OGD-induced cytokine and chemokine production. The cytokines IL-1β, IL-6 and TNF-α (A) and the chemokines CCL2, CCL5 and CCL3 (B) are significantly increased. LOX-1 siRNA downregulates the expression of these molecules. **; P<0.01.

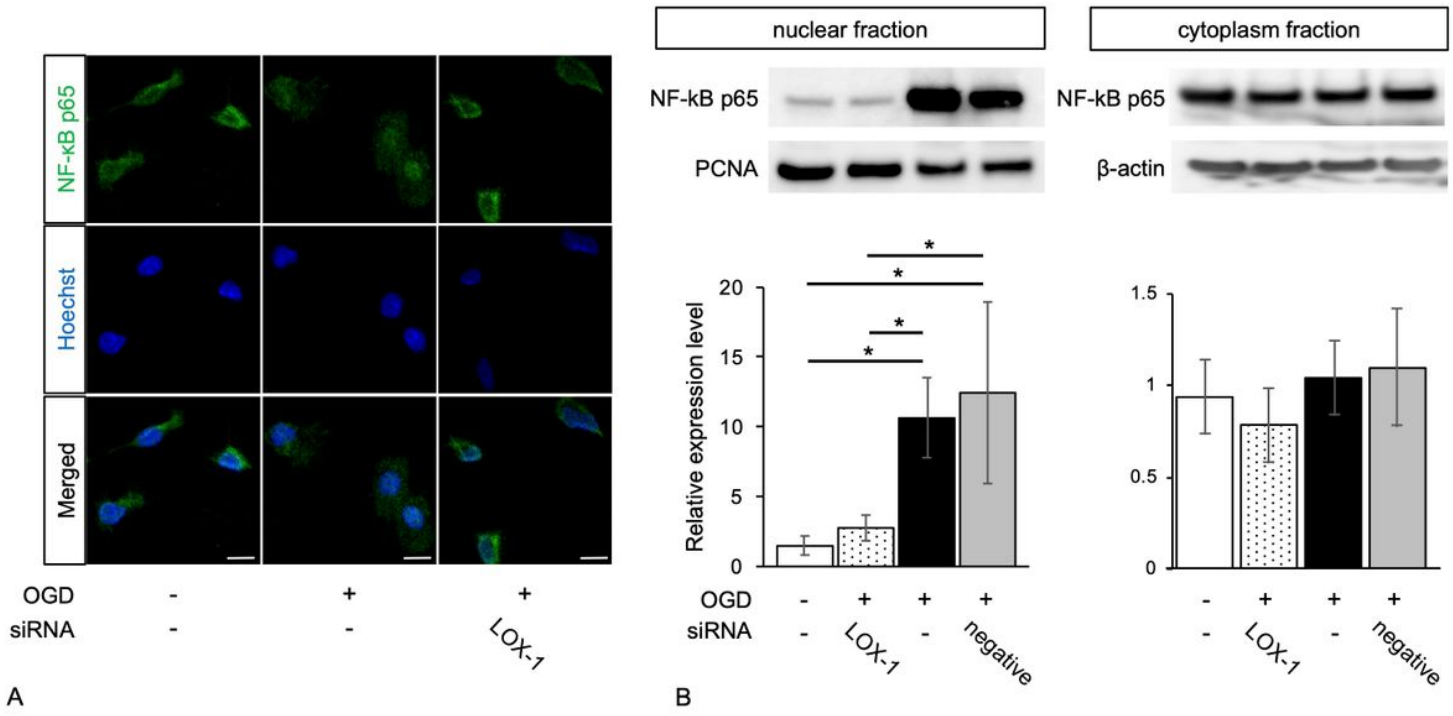

Figure 3

LOX-1 inhibition alters the distribution of NF-κB p65 from the cytoplasm to the nucleus. OGD-treated microglial cells show NF-κB p65 in the nucleus, but LOX-1 siRNA induces cytoplasmic expression of NF-κB p65 (A). Western blots exhibit significantly increased NF-κB p65 expression level in the nuclear fraction of OGD-treated microglial cells but no significant difference in NF-κB p65 expression in the cytoplasm fraction (B). Hoechst; Hoechst 33342, *; P<0.05. Scale bar = 10 μm.

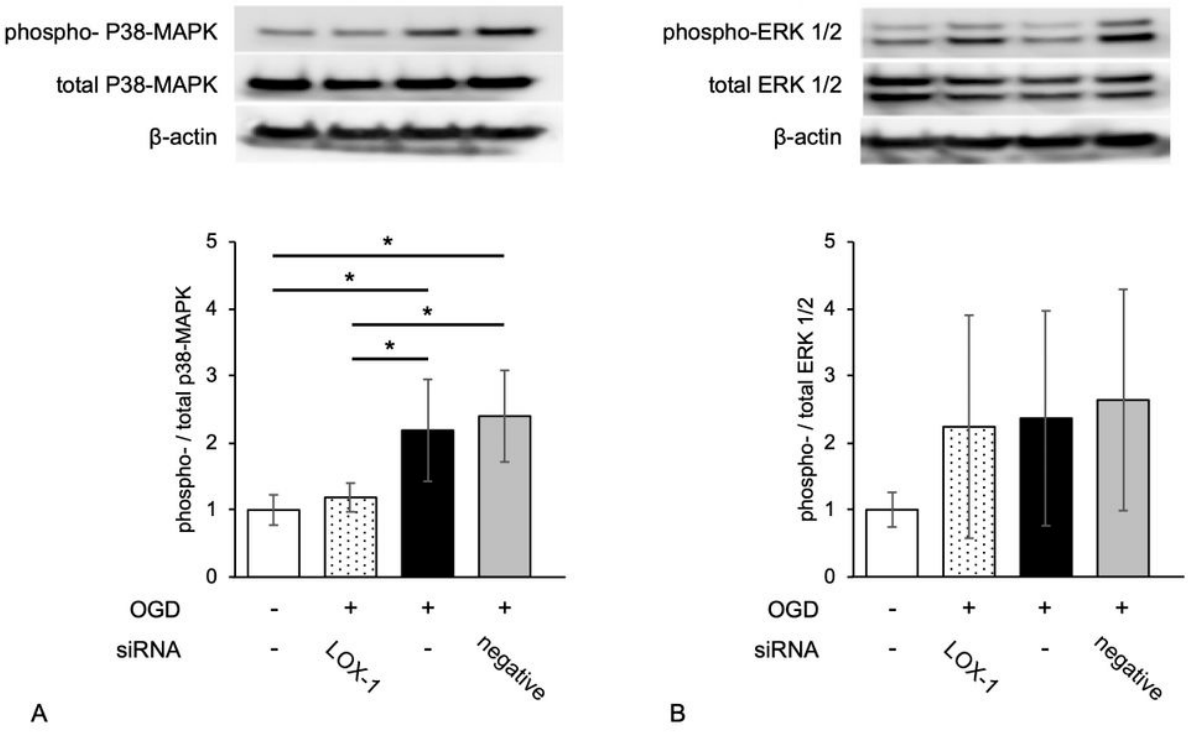

Figure 4

LOX-1 suppression induces different expression patterns of p38-MAPK and ERK phosphorylation in OGD-treated microglial cells. The expression of phospho-p38-MAPK and the ratio of phospho/total p38-MAPK are significantly increased in OGD-treated microglial cells but are reduced to the control level by LOX-1 siRNA (A). However, the expression of phospho-ERK and the ratio of phospho/total ERK are relatively increased in OGD-treated microglial cells but are not significantly reduced to the control level by LOX-1 siRNA (B). *; P<0.05.

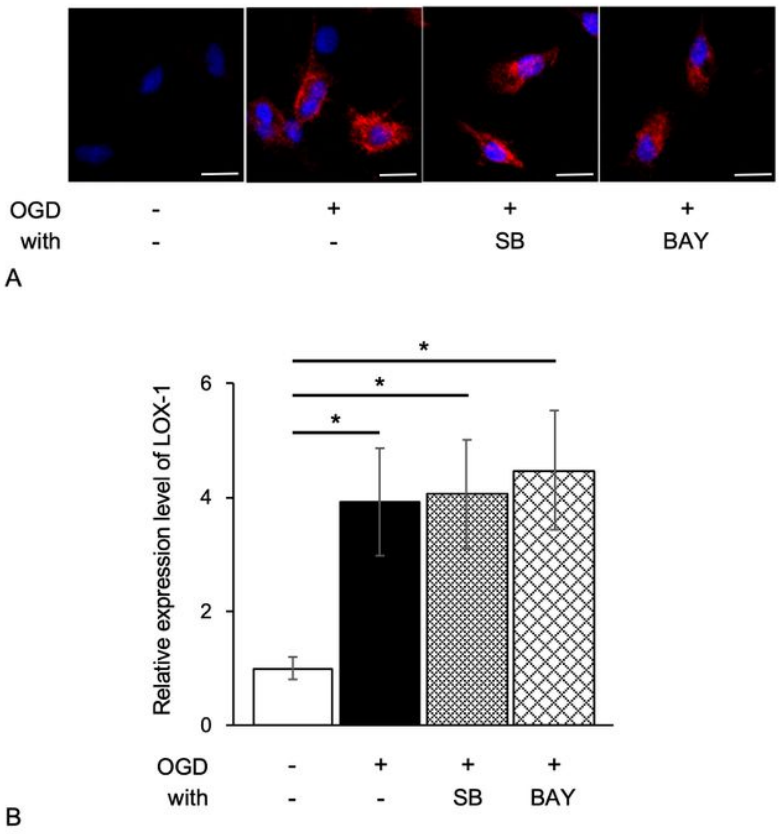

Figure 5

p38-MAPK and NF-κB inhibitors do not change LOX-1 expression levels of OGD-treated microglial cells. OGD-treated microglial cells express LOX-1 protein (red) in cytoplasm (A). No treated microglial cells and OGD-treated microglial cells are the same photographs of Figure 1A and 1B. LOX-1 expression is not influenced by p38-MAPK or NF-κB inhibitors SB or BAY, respectively. The relative LOX-1 expression level is the same as that shown by immunocytochemistry (B). SB; SB203580, BAY; BAY11-7082, Scale bar = 10 μm. *; $P<0.05$.

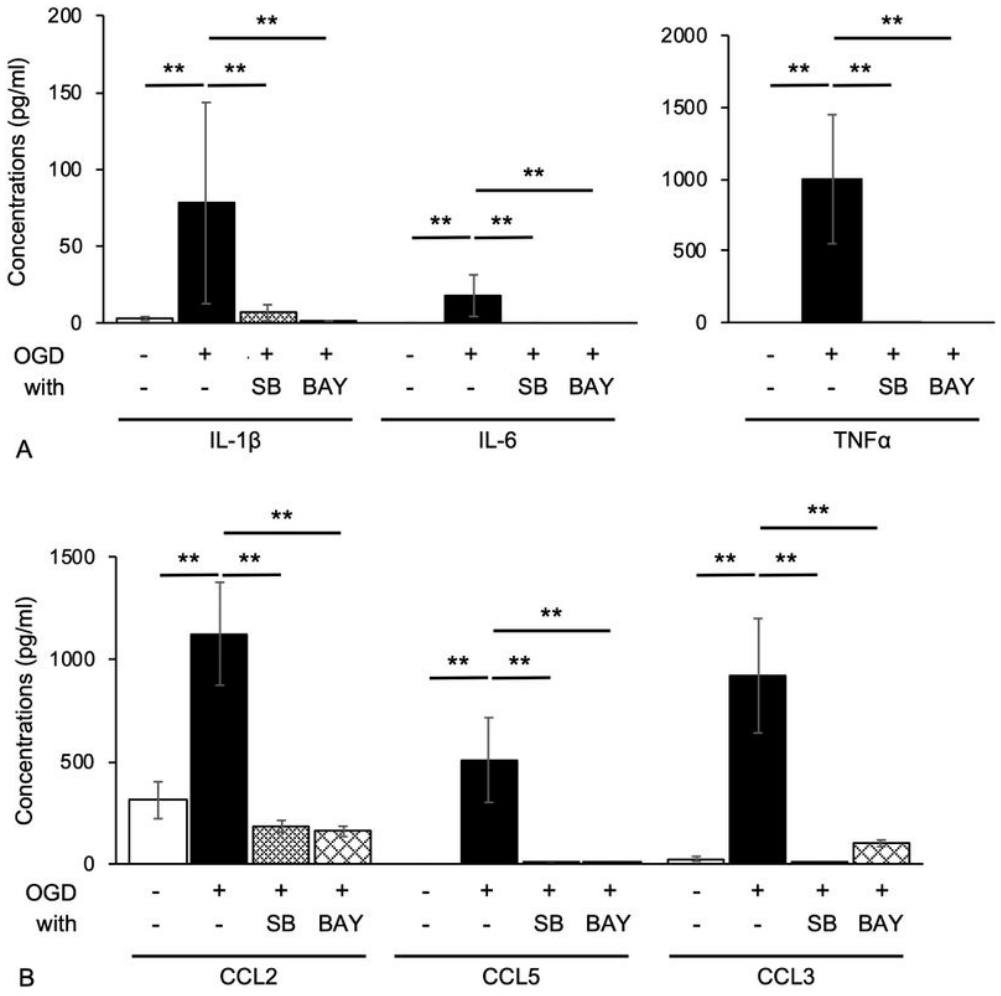

Figure 6

The production of cytokines and chemokines in OGD-treated microglial cells is downregulated by p38-MAPK and NF-κB inhibitors. The production of the cytokines IL-1β, IL-6 and TNF-α (A) and the chemokines CCL2, CCL5 and CCL3 (B), which are increased by OGD treatment, are suppressed by SB and BAY to the same levels as those of the control. SB; SB203580, BAY; BAY11-7082, **; P<0.01.

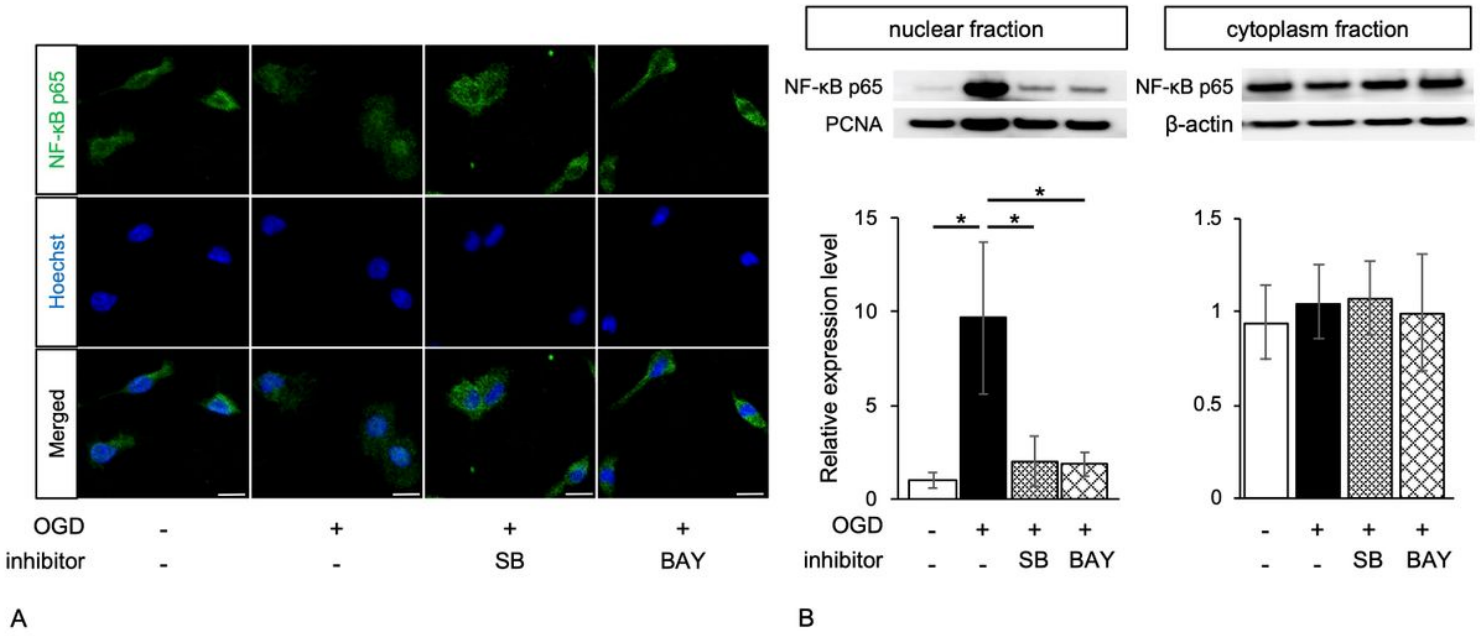

Figure 7

p38-MAPK and NF-κB inhibitors alter the distribution pattern of NF-κB p65 from the cytoplasm to the nucleus. NF-κB p65 is located in the nucleus in OGD-treated microglial cells but in the cytoplasm in other microglial cells (A). No treated microglial cells and OGD-treated microglial cells are the same photographs of Figure 3A. Western blots exhibit significantly increased NF-κB p65 expression levels in the nuclear fraction of OGD-treated microglial cells but no significant differences in NF-κB p65 expression levels in the cytoplasm fraction (B). Hoechst; Hoechst 33342, Scale bar = 10 μm. *; P<0.05.

Supplementary Files

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