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Aspirin-triggered lipoxin A₄ attenuates LPS-induced pro-inflammatory responses by inhibiting activation of NF- κ B and MAPKs in BV-2 microglial cells

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Abstract

Background: Microglial activation plays an important role in neurodegenerative diseases through production of nitric oxide (NO) and several pro-inflammatory cytokines. Lipoxins (LXs) and aspirin-triggered LXs (ATLs) are considered to act as 'braking signals' in inflammation. In the present study, we investigated the effect of aspirin-triggered LXA₄ (ATL) on inflammatory responses induced by lipopolysaccharide (LPS) in murine microglial BV-2 cells.

Methods: BV-2 cells were treated with ATL prior to LPS exposure, and the effects of such treatment production of nitric oxide (NO), inducible nitric oxide synthase (iNOS), interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α) were analysed by Griess reaction, ELISA, western blotting and quantitative RT-PCR. Moreover, we investigated the effects of ATL on LPS-induced nuclear factor- κ B (NF- κ B) activation, phosphorylation of mitogen-activated protein kinases (MAPKs) and activator protein-1 (AP-1) activation.

Results: ATL inhibited LPS-induced production of NO, IL-1 β and TNF- α in a concentration-dependent manner. mRNA expressions for iNOS, IL-1 β and TNF- α in response to LPS were also decreased by ATL. These effects were inhibited by Boc-2 (a LXA₄ receptor antagonist). ATL significantly reduced nuclear translocation of NF- κ B p65, degradation of the inhibitor I κ B- α , and phosphorylation of extracellular signal-regulated kinase (ERK) and p38 MAPK in BV-2 cells activated with LPS. Furthermore, the DNA binding activity of NF- κ B and AP-1 was blocked by ATL.

Conclusions: This study indicates that ATL inhibits NO and pro-inflammatory cytokine production at least in part via NF- κ B, ERK, p38 MAPK and AP-1 signaling pathways in LPS-activated microglia. Therefore, ATL may have therapeutic potential for various neurodegenerative diseases.

Background

There is increasing awareness that inflammation may play a role in various neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, HIV-associated dementia, trauma, multiple sclerosis and stroke [1,2]. Microglial cells are generally considered to

be the immune cells of the central nervous system (CNS). They respond to neuronal injury or immunologic challenges with a reaction termed microglial activation. Activated microglial cells can serve diverse beneficial functions essential to neuron survival, which include cellular maintenance and innate immunity [3,4]. However, overactivated microglia can induce significant and highly detrimental neurotoxic effects through excess production of a large array of cytotoxic factors such as superoxide, nitric oxide (NO), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) [1]. Overactivation of

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microglia followed by overproduction of neurotoxic factors results in deleterious and progressive neurotoxic consequences [5,6]. In several studies it has been shown that reduction of pro-inflammatory mediators produced by microglia may attenuate the severity of neuronal damage [7]. Therefore, inhibiting inflammatory cytokine production by activated microglia may be useful for preventing neurodegeneration [8-10].

Lipoxins (LXs) are endogenous lipid mediators with potent anti-inflammatory and pro-resolving actions [11]. Of special interest, aspirin can also trigger transcellular biosynthesis of 15-epimers of LX, termed aspirin-triggered LX (ATL) [12], that share the potent anti-inflammatory actions of LX but are more resistant to metabolic inactivation [13]. LXs and ATL elicit multicellular responses via a specific G protein-coupled receptor termed the LXA₄ receptor (ALX) that has been identified in human [14], mouse [15] and rat [16] tissues. In our previous papers, we evaluated the anti-inflammatory activity of an LXA₄ analogue, 5(S), 6(R)-LXA₄ methyl ester, in a rat model of permanent focal cerebral ischemia and focal cerebral ischemia reperfusion [17,18]. Our results showed that this LXA₄ analogue could attenuate focal ischemia-induced inflammatory responses and inhibit activation of microglia *in vivo*. Expression of functional ALXs was identified in neural stem cells, neurons, astrocytes and microglia [19-23]. Microglial cells are key sensors and versatile effectors in normal and pathologic brain [24]. These findings suggest that microglia may be a target for LXs in brain. However, the effects of LXs on expression of inflammation-related genes and molecular mechanisms in microglia have not been demonstrated.

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, initiates a number of major cellular responses that play critical roles in the pathogenesis of inflammatory responses and has been commonly used to model proinflammatory and neurotoxic activation of microglia [25,26]. We used LPS as a stimulant of the microglial reactivity in the current study.

In the present study, we investigated the impact of ATL on the inflammatory response induced by LPS in murine microglial BV-2 cells, as well as the signaling pathways involved in these processes. Our data suggest that ATL inhibits NO and pro-inflammatory cytokine production in LPS-activated microglia at least in part via NF- κ B, ERK, p38 MAPK and AP-1 signaling pathways.

Methods

Cell culture

The immortalized murine microglia cell line BV-2 was purchased from Cell Resource Centre of Peking Union Medical College (Beijing, China) and maintained in

Dulbecco's modified Eagle's medium with F12 supplement (DMEM/F12, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 95% air, 5% CO₂. Confluent cultures were passaged by trypsinization. BV-2 cells were seeded onto 96-well plates (10⁴ cells/well for cell viability assay), 24-well-culture plates (10⁵ cells/well for ELISA and NO measurement, 10⁴ cells/well for immunofluorescence), 6-well plates (2.5 \times 10⁵ cells/well for PCR) or 100 mm culture dishes (1.2 \times 10⁶ cells/dish for western blotting and EMSA). Before each experiment, cells were serum-starved for 12 h. BV-2 cells were incubated in the initial experiments with different concentrations (1 nM, 10 nM or 100 nM) of ATL (Cayman Chemical, Ann Arbor, MI), leading to a concentration of 100 nM ATL used in further experiments or vehicle (0.035% ethanol) for 30 min before addition of 100 ng/ml LPS (*Escherichia coli* O26:B6, Sigma-Aldrich, St. Louis, MO) under serum-free conditions. To investigate the involvement of ALXs in the anti-inflammatory effects of ATL, the cells were treated with 100 μ M Boc-2 (Phoenix Pharmaceuticals), a specific receptor antagonist, prior to the treatment with ATL for 30 min.

RNA isolation, reverse-transcriptase (RT) PCR and real-time PCR

Total RNA was extracted from BV-2 cells with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. 1.0 μ g of total RNA was subjected to oligo-dT-primed RT with ReverTra Ace Kit (Toyobo, Osaka, Japan).

Semi-quantitative PCR was carried out with DNA polymerase (Toyobo) by using specific primers (Invitrogen): 5'-GGCAACTCTGTTGAGGAAAG-3' and 5'-GGCTCTCGGTAGACGAGA-3', which amplify the 423 bp product for ALX1/FPR-rs1; and 5'-GTCAAGATCAACAGAAGAAACC-3' and 5'-GGGCTCTCTCAAGACTATAAGG-3', which amplify 298 bp product for ALX2/FPR2; and 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAAACGCAGCTCAGTAA-CAGTCCG-3', which amplify 349 bp product for β -actin. The amplified PCR products were resolved by 2% agarose gel electrophoresis.

Real-time PCR was performed for a quantitative analysis of iNOS, IL-1 β and TNF- α mRNA expression using SYBR Green real-time PCR Master Mix (Toyobo) on an MX3000P real-time PCR system (Stratagene). The following primers were used (Invitrogen): 5'-CAGCTGGGCTGTACAAACCTT-3' and 5'-CATTGGAAGTGAAGCGTTTCG-3', which amplify the 95 bp product for iNOS; 5'-CAACCAACAAGTGATATTCTCATG-3' and 5'-GATCCACACTCTCCAGCTGCA-3', which amplify the 152 bp product for IL-1 β ; 5'-

CATCTTCTCAAAATTCGAGTGACAA-3' and 5'-TGGGAGTAGACAAGGTACAACCC-3', which amplify the 175 bp product for TNF- α ; and 5'-TGTCCACCTTCCAGCAGATGT-3' and 5'-AGCT-CAGTAACAGTCCGCCTAGA-3', which amplify the 101 bp product for β -actin. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method [27].

Cell viability assay

Cell viability was measured by quantitative colorimetric assay with MTT (Sigma-Aldrich), showing the mitochondrial activity of living cells. BV-2 cells in 96-well plates were pretreated with various concentrations of ATL for 30 min and incubated with or without LPS for 24 h in the continued presence of ATL. Upon termination of the experiments, the culture media were aspirated and MTT (0.5 mg/ml) was added to cells and then incubated at 37°C for 4 h. The supernatant was aspirated and dimethyl sulfoxide (Sigma-Aldrich) was added to the wells. Insoluble crystals were dissolved by mixing and the plates were read on an automated Tecan Sunrise absorbance reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Nitrite measurements

Production of NO was determined by measuring the level of accumulated nitrite, a metabolite of NO in the culture supernatant using Griess reagent (Sigma-Aldrich). After 24 h of treatment with LPS with or without ATL, the culture supernatants were collected and mixed with an equal volume of Griess reagent in 96-well culture plates and incubated at room temperature for 10 min. The absorbance was measured at 540 nm and nitrite concentrations were calculated by reference to a standard curve generated by known concentrations of sodium nitrite.

ELISA for IL-1 β and TNF- α

BV-2 cells in 24-well plates were stimulated for 24 h, and then culture supernatants were harvested. Levels of IL-1 β and TNF- α in 100 μ l medium were measured by commercial ELISA kits (Boster Biological Technology, Wuhan, China) according to the manufacturer's instructions.

Immunofluorescence confocal microscopy

For the detection of intracellular location of NF- κ B p65, BV-2 cells were cultured on sterile glass cover slips in 24 well plates and treated with ATL and LPS as described above. At various times after the LPS treatment, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. After rinsing, cells were blocked with 3% BSA in PBS for 1 h and incubated with rabbit anti-NF- κ B p65 antibodies

(1:200, Santa Cruz Biotechnology, Santa Cruz) overnight at 4°C. After washing, cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:400, Pierce, Rockford, IL) for 1 h and counterstained with 4, 6-diamidino-2-phenylindole (DAPI, Roche, Shanghai, China) for the identification of nuclei. After washing with PBS, the cover slips were mounted with antifade mounting medium (Beyotime, China) on slides, and the cells were observed with a confocal microscope Olympus Fluoview FV500.

Protein extraction

For making whole cell lysates, the cells were lysed in radioimmune precipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (Roche). Nuclear and cytoplasmic fractionations were performed with Proteo JET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas Life Science) according to manufacturer's protocol.

Western blot analysis

Equal amounts of cytoplasmic, nuclear, or whole cell extracts were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, and then transferred onto a polyvinylidene difluoride membrane (Millipore). The transformed membrane was blocked for 1 h and incubated with indicated primary antibodies (Santa Cruz Biotechnology) at 4°C overnight. The primary antibodies used were as follows: rabbit anti-iNOS (1:500), β -actin (1:1000), p65 (1:1000), Lamin B (1:1000), I κ B- α (1:500), ERK1/2 (1:1000), p38 (1:1000), JNK (1:1000) and mouse anti-phosphorylated ERK1/2, p38, JNK antibody (1:1000). The membrane was washed three times with Tris-buffered saline containing 0.05% Tween 20 (TBST) for 10 min and incubated with anti-rabbit or anti-mouse IgG-horseradish peroxidase (1:5000, Pierce) at room temperature for 1 h. The Supersignal West Pico chemiluminescent substrate system (Pierce) was used to detect immunoreactive bands. The intensity of protein bands after western blotting were quantitated by using Quantity One Version 4.6.3 Image software (Bio-Rad) and normalized against proper loading controls.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described above. Oligonucleotides corresponding to the NF- κ B (5'-AGTT-GAGGGGACTTTCAGGC-3') and AP-1 (5'-CGCTTGATGAGTCAGCCGAA-3') binding site consensus sequences were synthesized and end-labeled with biotin by Invitrogen. EMSAs were performed using the LightShift chemiluminescent EMSA kit (Pierce). Briefly, 20 fmol of biotin-labeled, double strand probe was incubated for 20 min at room temperature in 20 μ l of EMSA binding buffer containing 2.5% glycerol, 5 mM

MgCl₂, 50 ng/μl poly (dI-dC), 0.05% Nonidet P-40, and 6 μg of nuclear proteins. For competition EMSA, 200-fold (4 pmol) excess unlabeled, double strand probe was added to the binding reaction. The DNA-nuclear protein complexes were resolved by electrophoresis in 6% nondenaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA (TBE) buffer at 100 V. Gels were then electroblotted onto Hybond nylon membranes (GE Healthcare) at 380 mA for 50 min. The membranes were then cross-linked for 15 min with the membrane face down on a transilluminator at 312 nm, and the biotinylated protein-DNA bands were detected with HRP-conjugated streptavidin using the chemiluminescent nucleic acid detection system (Pierce).

Statistical analysis

Data are expressed as means ± SEM of the indicated number of independent experiments. Changes in IκB protein levels were analyzed by two-way ANOVA (treatment and time). All other data were analyzed by one-way ANOVA. Least significant difference (LSD) post hoc test was used for multiple comparisons. Statistical analysis was performed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

Results

ALXs are expressed in BV-2 microglial cells

Using RT-PCR, we showed that both ALX1/FPR-rs1 and ALX2/FPR2 were expressed in BV-2 microglial cells. The mRNA expression levels of these two receptors were significantly enhanced when the cells were exposed to LPS (100 ng/ml) for 6 h (Figure 1).

ATL inhibits LPS-induced NO, IL-1β and TNF-α production in BV-2 cells

Initially, we evaluated the effects of ATL on NO, IL-1β and TNF-α production in LPS-stimulated BV-2 microglia. BV-2 cells were incubated with vehicle or different concentrations of ATL (1, 10 and 100 nM) for 30 min and stimulated with 100 ng/ml LPS for 24 h. To determine NO production, we measured nitrite released into the culture medium using the Griess reagent. Stimulation of BV-2 cells with LPS markedly increased (about 7.5-fold) NO production, compared with that generated under control conditions. Pretreatment with ATL significantly inhibited this increase in a concentration-dependent manner (Figure 2A).

We then tested whether ATL reduces the production of LPS-induced pro-inflammatory cytokines IL-1β and TNF-α using ELISA. As shown in Figure 2B and 2C, stimulation of BV-2 cells with LPS led to a significant increase in the levels of IL-1β and TNF-α in the cell-conditioned media after 24 h. Pretreatment of BV-2

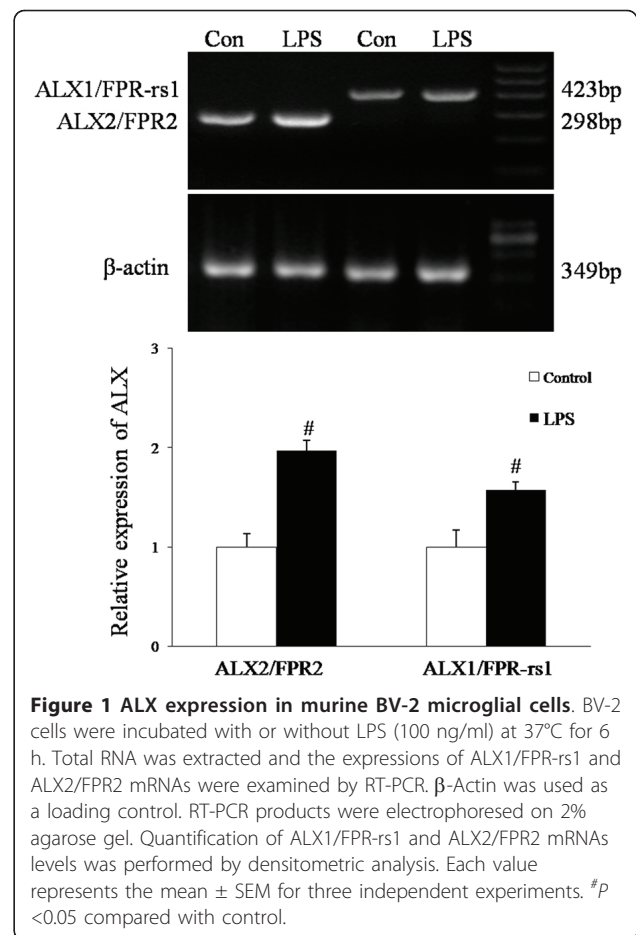
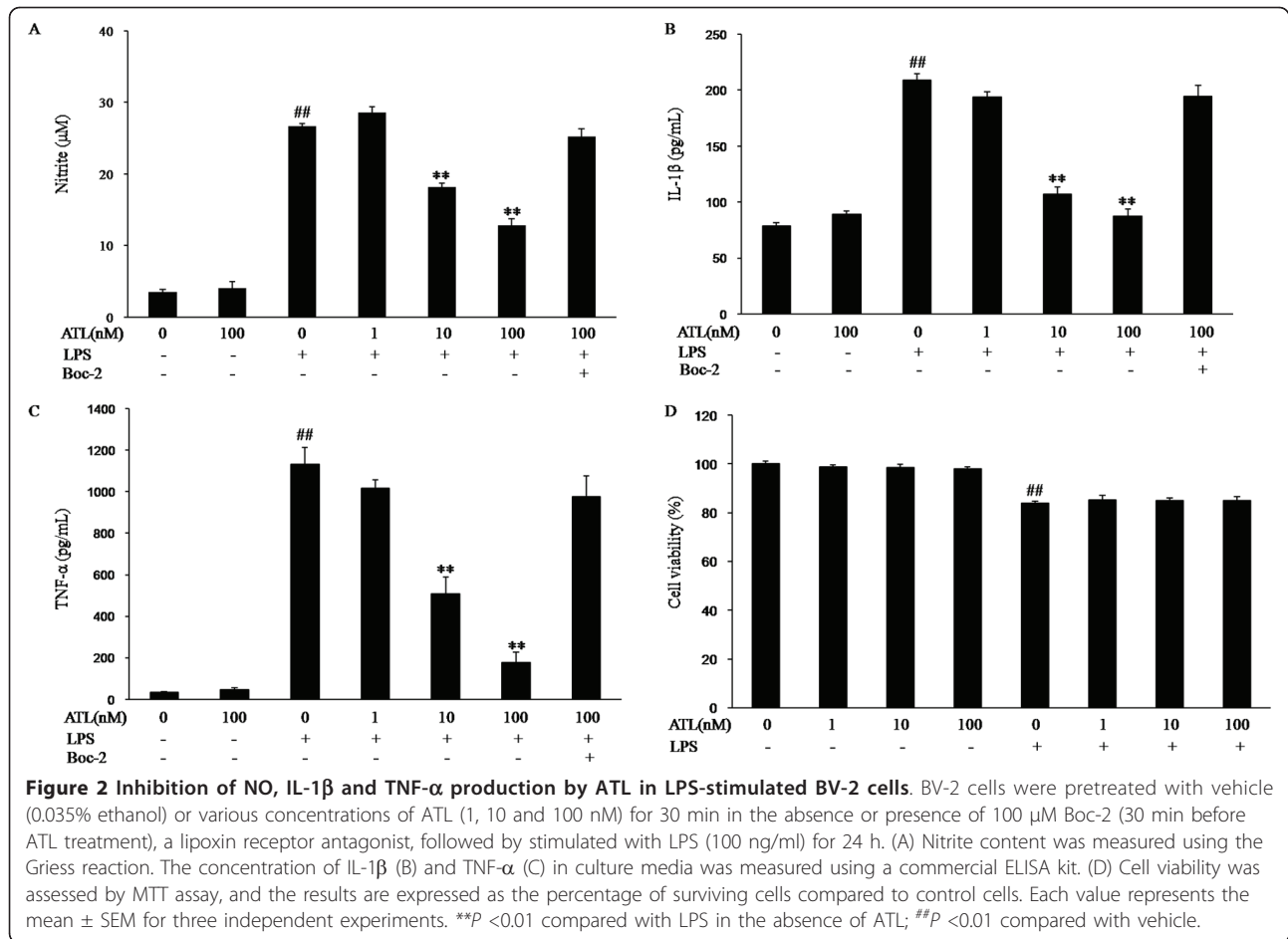


Figure 1 ALX expression in murine BV-2 microglial cells. BV-2 cells were incubated with or without LPS (100 ng/ml) at 37°C for 6 h. Total RNA was extracted and the expressions of ALX1/FPR-rs1 and ALX2/FPR2 mRNAs were examined by RT-PCR. β-Actin was used as a loading control. RT-PCR products were electrophoresed on 2% agarose gel. Quantification of ALX1/FPR-rs1 and ALX2/FPR2 mRNA levels was performed by densitometric analysis. Each value represents the mean ± SEM for three independent experiments. [#]*P* < 0.05 compared with control.

cells with ATL significantly inhibited the LPS-induced IL-1β and TNF-α production, concentration dependently.

To evaluate the role of the ALXs in the anti-inflammatory effects of ATL, BV-2 cells were treated with an ALX antagonist, Boc-2 (100 μM, 30 min) prior to treatment with ATL. Pretreatment with Boc-2 inhibited these effects in response to ATL (Figure 2).

To exclude the possibility that the decrease in the NO and cytokines levels was simply due to the cytotoxicity of the drug, cell viability was evaluated. The cytotoxic effects of ATL in BV-2 cells were evaluated in the absence or presence of LPS using MTT assays. ATL (1, 10 and 100 nM) and vehicle did not affect cell viability (Figure 2D). When cells were treated with 100 ng/ml LPS only, a decrease in viability was detected compared with the control cells. However, cells pretreated with ATL for 30 min showed no significant increase compared with cells that were treated with LPS only (Figure 2D). Therefore, the inhibitory effect of ATL on LPS-induced, inflammation-related responses in activated BV-2 cells was not the result of ATL effects on cell survival.



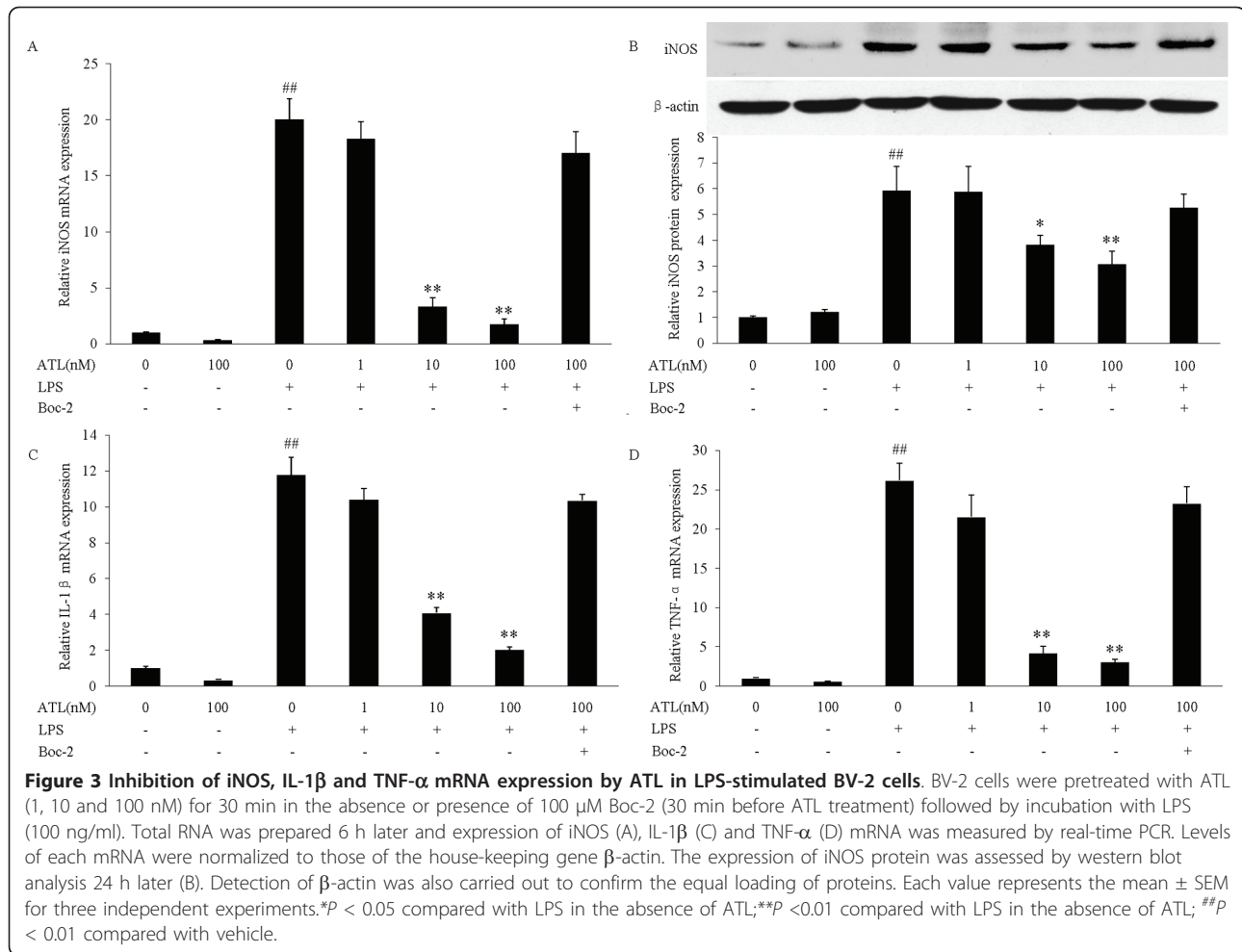
ATL inhibits mRNA expressions of iNOS, IL-1β, and TNF-α

To find out whether ATL suppresses iNOS, IL-1β and TNF-α expression at the transcriptional level, BV-2 cells were incubated for 30 min with the indicated concentrations of ATL and then incubated with 100 ng/ml LPS for 6 h. The relative amounts of iNOS, IL-1β and TNF-α mRNA were determined by real-time RT-PCR. As anticipated, LPS induced a marked increase in iNOS, IL-1β and TNF-α mRNA in BV-2 cells, about 20, 11, 26-fold increase, respectively (Figure 3). Pretreatment with ATL reduced LPS-induced up-regulation of iNOS, IL-1β and TNF-α mRNA levels in a dose-dependent manner (Figure 3). The inhibitory effects of ATL on LPS-induced iNOS mRNA up-regulation were accompanied by attenuation of iNOS protein induction (Figure 3B). ATL inhibition of LPS-induced expression of iNOS, IL-1β and TNF-α was reversed after pre-exposure of BV-2 cells to the ALX antagonist Boc-2 (100 μM) for 30 min (Figure 3). Taken together, our current data prove that ATL inhibits the inflammatory activation of BV-2 microglia cells with respect to NO production and pro-inflammatory cytokine expression.

ATL inhibits nuclear translocation of NF-κB and degradation of IκB-α

Because ATL reduced the transcriptional activation of iNOS, IL-1β and TNF-α genes, it is likely that it blocks signaling events involved in transcriptional activation of these genes. Expression of iNOS and cytokines genes requires NF-κB activation and nuclear translocation to interact with DNA. Therefore, the involvement of NF-κB nuclear translocation in ATL-induced suppression of NO and cytokines was examined by fluorescence microscopy. LPS stimulation caused obvious translocation of NF-κB p65 from the cytoplasm into the nucleus 60 min after activation (Figure 4A), whereas the presence of 100 nM ATL reduced this (Figure 4B). To further verify the p65 nuclear translocation data, we analyzed the cells by western blotting and found that pretreatment of cells with 100 nM ATL prevented p65 nuclear localization induced by LPS (Figure 4C and 4D).

To address the possibility that the impaired nuclear translocation of p65 was due to inhibition of degradation of IκB-α, we examined the effect of ATL on IκB-α degradation induced by LPS. Western blot analysis



showed that LPS-induced degradation of I κ B- α was significantly reversed by 100 nM ATL in BV-2 cells (Figure 4E).

ATL inhibits LPS-induced ERK and p38 MAPK activation

Along with NF- κ B, MAPKs are known to play an important role in the signaling pathways that induce proinflammatory cytokines and iNOS in glial cells [28]. To investigate whether the inhibition of inflammation by ATL is regulated by the MAPK pathway, we examined the effects of ATL on LPS-induced phosphorylation of ERK, p38 MAPK and JNK in BV-2 microglia by western blot analysis. Cells were pretreated with 100 nM ATL for 30 min and then incubated with 100 ng/ml LPS for 30 min. The 30-min treatment of LPS was determined to be optimal in a preliminary study that examined MAPK phosphorylation at 0, 10, 20, 30, and 60 min after LPS treatment (data not shown). ATL (100 nM) markedly inhibited ERK and p38 MAPK activation, while phosphorylation of JNK was not affected

(Figure 5A-C). Strikingly, ATL could induce JNK phosphorylation without effect on ERK and p38 MAPK activity.

ATL inhibits LPS-induced NF- κ B and AP-1 DNA binding activity

To determine the effects of ATL on transcription factor signaling pathways that might mediate LPS-induced proinflammatory cytokines production, EMSA was performed. BV-2 cells were pretreated with vehicle and 100 nM ATL for 30 min before stimulation with LPS (100 ng/ml) for 1 h. NF- κ B and AP-1 binding activities were induced by LPS treatment (Figure 6A and 6B, lane 3). Binding specificity was verified by incubating nuclear extracts from LPS-stimulated BV-2 cells with excess unlabeled specific competitor oligonucleotide probe (Figure 6A and 6B, lane 5). Pretreatment with ATL markedly reduced the LPS-induced DNA-binding activity of NF- κ B and AP-1 (Figure 6A and 6B, lane 4).

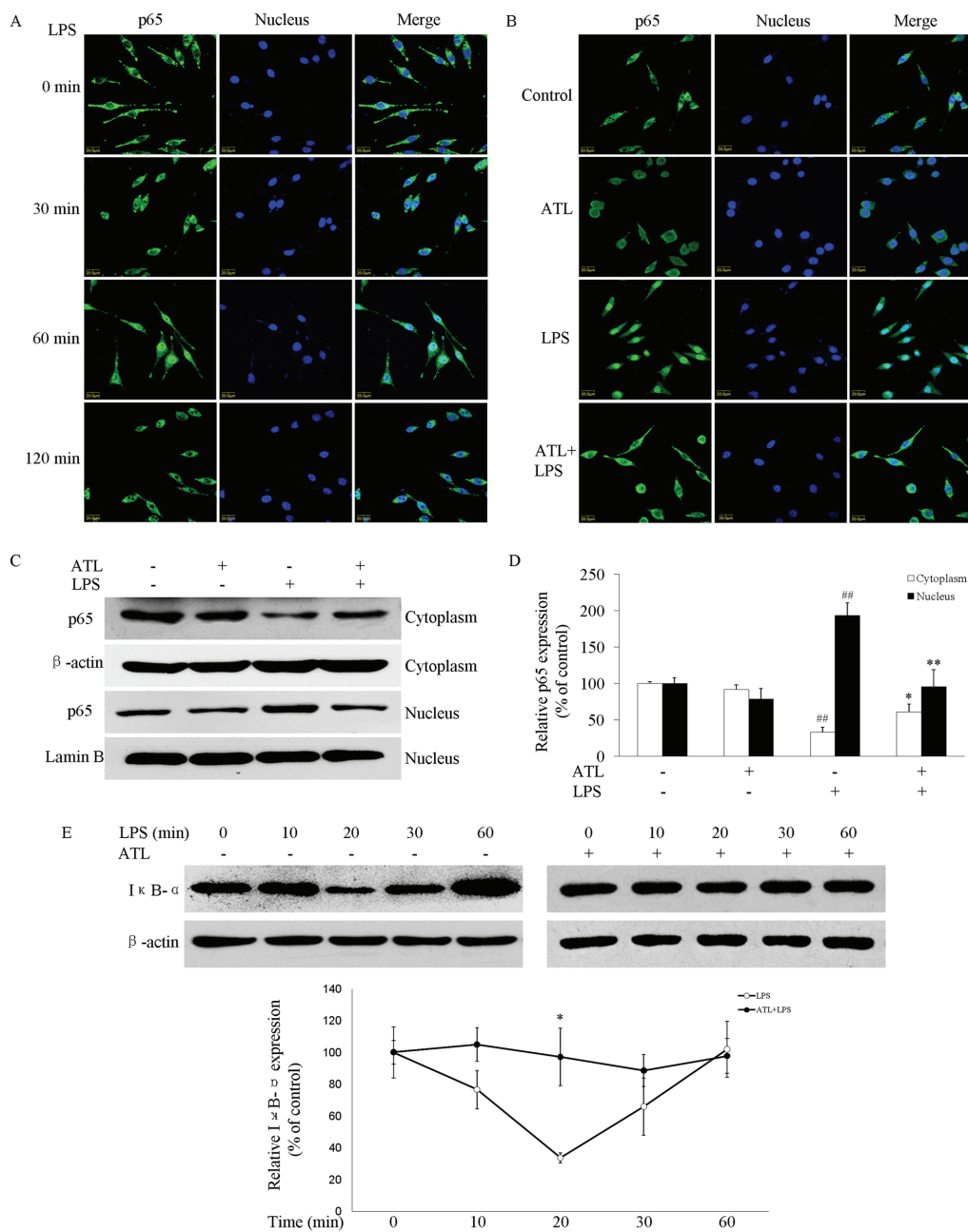
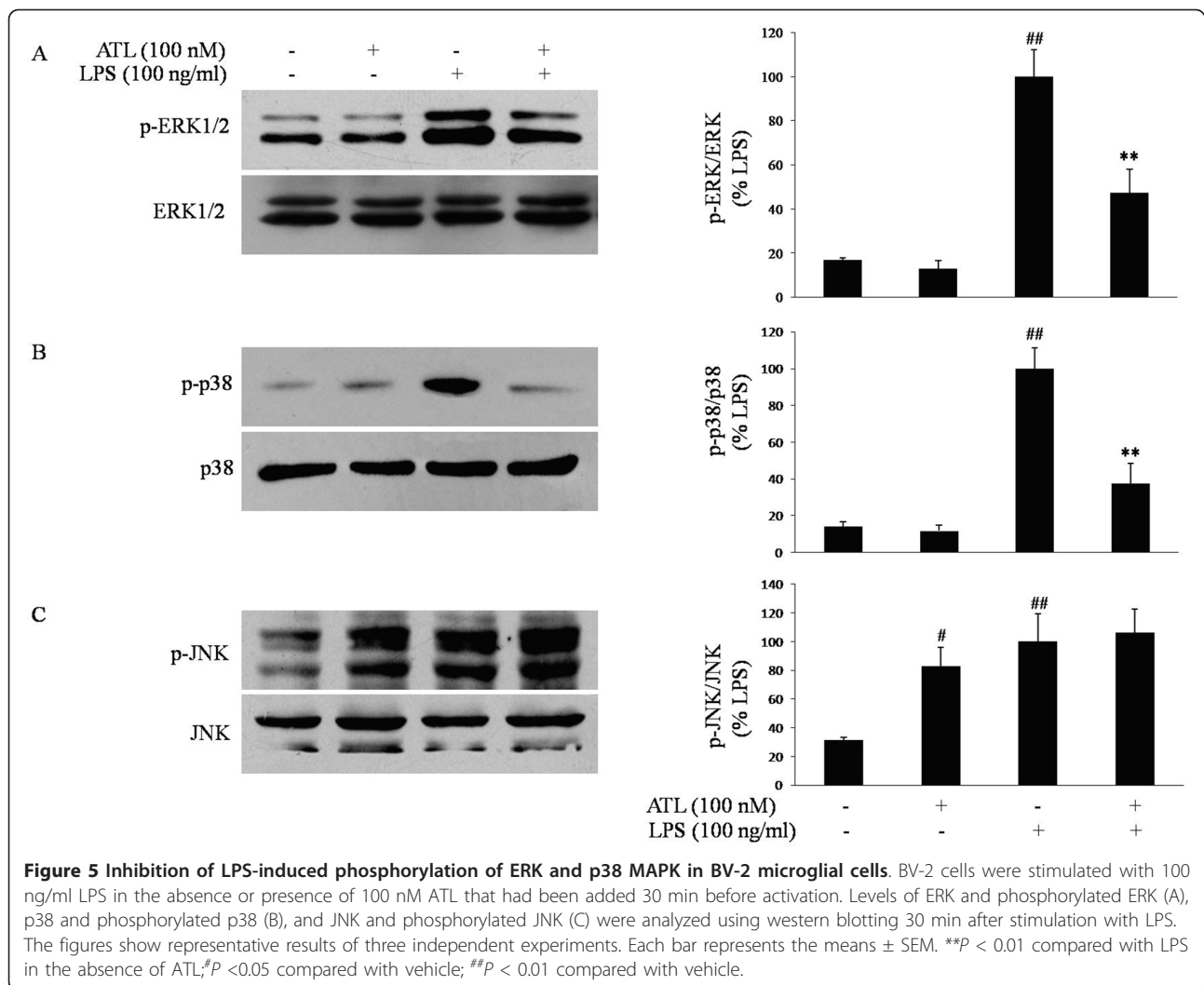


Figure 4 Inhibition of the nuclear accumulation of the NF- κ B p65 subunit and degradation of I κ B- α by ATL in LPS-stimulated BV-2 microglial cells. (A) BV-2 cells were stimulated with 100 ng/ml LPS for the indicated times. Subcellular localization of p65 subunit was evaluated using an anti-p65 antibody and a FITC-labelled anti-rabbit IgG antibody. DNA was stained using DAPI to visualize nuclei, and cells were visualized using laser confocal scanning microscopy. Note that nuclear translocation of the p65 subunit is not complete, but that part of the cytoplasmic p65 is translocated to the nucleus so that the distinction between the nucleus and the cytoplasm blurs. This is obvious 60 min after activation. (B) BV-2 cells were stimulated with 100 ng/ml LPS in the absence or presence of 100 nM ATL that had been added 30 min before activation. Subcellular location of the p65 subunit was tested using immunofluorescence assay 60 min after activation. (C) BV-2 cells were stimulated as in B. Cytoplasmic and nuclear extracts were separated by SDS-PAGE and immunoblotted with anti-p65 antibody. The same extracts were re-electrophoresed and immunoblotted for β -actin or lamin B to monitor loading. A representative result from three independent experiments is shown. (D) Quantification of cytoplasmic and nuclear p65 bands from the experiments in C was normalized by β -actin or lamin B. (E) BV-2 cells were pretreated with vehicle or 100 nM ATL for 30 min and stimulated with LPS (100 ng/ml). Levels of I κ B- α in cellular lysates were analyzed using western blotting at indicated times. Quantification of I κ B- α protein levels was performed by densitometric analysis. Data are presented as mean \pm SEM for three independent experiments. * P < 0.05 compared with LPS in the absence of ATL; ** P < 0.01 compared with LPS in the absence of ATL; ## P < 0.01 compared with vehicle.



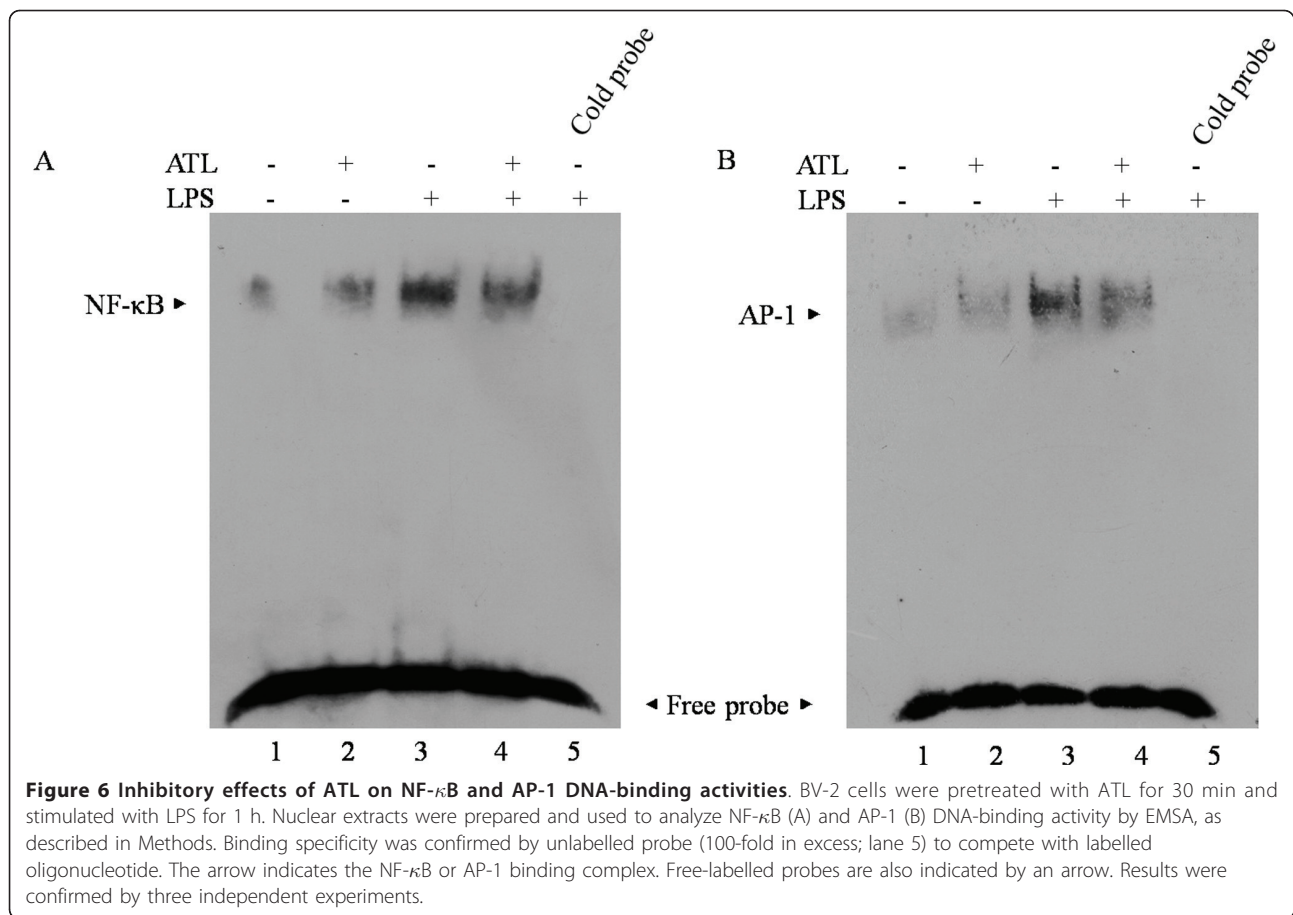
Discussion

Our present data provide the first evidence that ATL inhibits the inflammatory activation of microglia. To date, two separate LXA₄ receptors (ALX1/FPR-rs1 and ALX2/FPR2) have been identified in mice [15,29]. Mouse ALX2/FPR2 is expressed by neutrophils, monocytes, macrophages, dendritic cells, and microglial cells, and its transcripts are detected at high levels in spleen and lung [30]. ALX1/FPR-rs1 and ALX2/FPR2 are both expressed in the mouse pituitary gland, hypothalamic tissue and vomeronasal organ [31,32]. As demonstrated by RT-PCR analysis, ALX1/FPR-rs1 and ALX2/FPR2 are both expressed in BV-2 microglial cells. ATL reduced LPS-induced production of NO, IL-1 β and TNF- α in BV-2 microglial cells. This is a receptor-mediated effect as it disappeared when microglial cells were pretreated with Boc-2 before ATL treatment. Quantitative PCR analysis showed that ATL markedly suppresses iNOS, IL-1 β and TNF- α gene expression in BV-2 microglia

cells. Similarly, this effect was abrogated by the use of Boc-2. NF- κ B, ERK and p38 MAPK pathways are at least partly involved in the anti-inflammatory mechanisms of ATL in BV-2 cells. Thus, ATL is a promising agent for preventing and treating neuroinflammation and may be useful for mitigating a dysregulated linkage between the immune system and brain.

Although microglial activation has important reparative functions in the CNS, microglial cell activation in infection, inflammation, or injury may go beyond control and eventually produce detrimental effects that override the beneficial effects. Activation of microglia leads to release of various toxic molecules such as superoxide, NO, IL-1 β and TNF- α , contributing to neuronal damage in various neurodegenerative disorders [1].

LX possesses dual anti-inflammatory and pro-resolution activities that have been demonstrated in a multitude of acute and chronic inflammatory conditions [11]. Previously, LXA₄, ATL and their stable analogues have



been shown to play a major role in important functional properties of the central nervous system, such as neural stem cell proliferation and differentiation, pain, and cerebral ischemia [17-19,33]. In primary murine microglia or N9 microglial cells, expression of ALX2/FPR2 has been identified and is up-regulated by inflammatory stimuli [20,21]. In the present study, the expression of ALX2/FPR2 and another murine high-affinity ALX1/FPR-rs1 were confirmed in BV-2 microglial cells. These findings suggest that ATL could work as a modulator of the inflammatory reaction of the brain immune system, eventually acting as a microglial activation repressor.

NO and pro-inflammatory cytokines such as IL-1 β and TNF- α are known to be important mediators in the process of inflammation. These proinflammatory mediators are thought to be responsible for some of the harmful effects of brain injuries and diseases, including ischemia, Alzheimer's disease, Parkinson's disease and multiple sclerosis [34]. Under various pathological conditions associated with inflammation, large amounts of NO are produced in the brain as a result of the induced expression of iNOS in glial cells [35]. High levels of NO exert their toxic effects through multiple mechanisms, including lipid peroxidation, mitochondrial damage,

protein nitration and oxidation, depletion of antioxidant reserves, activation or inhibition of various signaling pathways, and DNA damage [35]. Therefore, the effect of ATL on NO production and iNOS expression in LPS-stimulated microglia cells was examined. As shown in previous research [36,37], NO is produced at low levels in unstimulated microglia. Stimulation of BV-2 microglial cells with LPS induced strong NO production and iNOS expression. The magnitude of the NO/iNOS response to LPS in BV-2 microglial cells is different in different studies with different concentrations as well as durations of LPS treatment. In the present study, ATL markedly reduced NO production and mRNA and protein expression of iNOS in dose-dependent manners without significant cytotoxicity. This indicates that inhibition of NO production by ATL is a result of inhibition of iNOS gene expression. Previous studies also have shown that LXA₄ and ATL analogues inhibit LPS-induced NO production and peroxynitrite formation in human leukocytes [38] and in mouse lung [39].

Pro-inflammatory cytokines produced by activated microglia, including IL-1 β and TNF- α , play an important role in the process of neuroinflammatory diseases [34]. IL-1 β is a potent pro-inflammatory cytokine that

acts through IL-1 receptors found on numerous cell types, including neurons and microglia. TNF- α can cause cell death directly by binding to neuronal TNF receptors linked to death domains that activate caspase-dependent apoptosis [40] or by potentiating glutamate release, thereby enhancing excitotoxicity [41]. IL-1 β and TNF- α also drive self-propagating cycles of microglial activation and neuroinflammation by inducing activation of NF- κ B, cytokine generation and further activation of NF- κ B. Thus, inhibition of cytokine production or function serves as a key mechanism in the control of neurodegeneration. Our results showed that ATL markedly attenuates the production of IL-1 β and TNF- α , and their mRNA expressions; induced by LPS in BV-2 cells. Consistent with our findings, similar results have shown that LXA₄ and ATL inhibit LPS-induced production of IL-1 β and TNF- α in uvea and in macrophages and endothelial cells [42-44].

In subsequent studies, we found that ATL has a strong inhibitory effect on inflammatory signaling pathways that include NF- κ B and MAPK/AP-1. NF- κ B activity increases in acute neurodegenerative disorders such as stroke, severe epileptic seizures, and traumatic brain injury; and in chronic neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, Huntington disease, and amyotrophic lateral sclerosis [45]. In general, activation of NF- κ B in microglia contributes to neuronal injury and promotes the development of neurodegenerative disorders [45]. NF- κ B is known as a pleiotropic regulator of various genes involved in the production of many proinflammatory cytokines and enzymes. NF- κ B is also a central regulator of microglial responses to activating stimuli, including LPS and cytokines [46]. In this study, ATL was able to inhibit the LPS-evoked degradation of I κ B- α , nuclear translocation of NF- κ B p65 and the DNA-binding activities of NF- κ B in BV-2 cells. Previous studies have shown that LXs reduce nuclear translocation of NF- κ B in human neutrophils, mononuclear leukocytes [38] and macrophages [43]. It has also been reported that ATLs reduce NF- κ B-mediated transcriptional activation in an ALX-dependent manner, and inhibit the degradation of I κ B [47]. Therefore, induction of anti-inflammatory responses by LXs may be dependent on the NF- κ B signaling pathway.

In addition, LPS also activates MAPK pathways which lead to the induction of another transcription factor, AP-1. MAPKs are a group of signaling molecules that appear to play key roles in inflammatory processes [48]. We found that phosphorylation of ERK and p38 MAPK in response to LPS is decreased by ATL treatment. Our results also show that ATL treatment of BV-2 microglia results in decreased DNA-binding activities of AP-1 following LPS stimulation. This observation is in line with

studies in mesangial cells, endothelial cells, neutrophils, fibroblasts and T cells, which have shown that ERK and/or p38 MAPK activation is attenuated in the presence of LXs [42,49-51]. In the present study, ATL failed to inhibit LPS-induced phosphorylation of JNK. A previous study in primary astrocytes found that an ATL analogue prevents ATP-evoked JNK phosphorylation, but has no effect on TNF- α -induced JNK phosphorylation [33]. Strikingly, our results show that ATL induces JNK phosphorylation, but has no effect on ERK and p38 MAPK activity. In another study, LXA₄ attenuated microvascular fluid leaks caused by LPS partly mediated by the JNK signaling pathway [52]. LXA₄ and ATL analogues could promote ERK phosphorylation in macrophages and monocytes [53,54]. The reasons for these discrepancies are mainly due to differences in experimental models, cell types and stimulators.

Conclusions

In summary, our results show that ATL inhibits release of NO and pro-inflammatory cytokines in a concentration-dependent manner. Moreover, ATL acts at the level of transcription in LPS-stimulated microglia. A possible mechanism for this effect involves ATL's ability to activate a signaling cascade that results in repression of NF- κ B, ERK and p38 MAPK activation in activated microglia. Given the fact that microglial activation contributes to the pathogenesis of neurodegenerative diseases, ATL may be considered as a potential therapeutic agent for neurodegenerative diseases involving neuroinflammation.

Abbreviations

ALX: lipoxin A₄ receptor; AP-1: activator protein-1; ATL: aspirin-triggered lipoxin A₄; CNS: central nervous system; EMSA: Electrophoretic mobility shift assay; ERK: extracellular signal-regulated kinase; IL: interleukin; iNOS: inducible nitric oxide synthase; I κ B: inhibitor of κ B; JNK: c-jun N-terminal kinase; LPS: lipopolysaccharide; LX: lipoxin; LXA₄: lipoxin A₄; MAPK: mitogen-activated protein kinase; NF- κ B: nuclear factor- κ B; RIPA: radioimmune precipitation assay buffer

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Authors' contributions

JPW, YW and LYL performed the experiments and analyzed the data. JZ, RGL, and JPZ provided useful advice and reviewed the manuscript. YS conceived the study, participated in its design and coordination, and wrote the manuscript. SYY and SLY oversaw the experimental design and edited

the manuscript. All authors of this paper have read and approved the final version the manuscript.

Competing interests

The authors declare that they have no competing interests.

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