ORIGINAL PAPER

Aspirin-Triggered Lipoxin A$_4$ Attenuates Lipopolysaccharide-Induced Intracellular ROS in BV2 Microglia Cells by Inhibiting the Function of NADPH Oxidase

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Abstract Lipoxins have emerged as mediators of key events in endogenous anti-inflammation and resolution. However, the implication of these novel lipid mediators on neuroinflammation has not been investigated. Microglia is the major cells involved in brain tissue damage during infection and neurodegenerative diseases. One of the major features shared by neuroinflammation conditions is the increased production of reactive oxygen species (ROS) generated by NADPH oxidase activation. In this study, we have examined whether aspirin-triggered lipoxin A$_4$ (ATL) modulates ROS generation in BV2 cells. Pre-treatment of BV2 cells with ATL blocked ROS production triggered by LPS in the time-dependent and concentration-dependent manner. ATL inhibited the translocation of the cytoplasmic NADPH oxidase subunit p47^{phox} to the cell membrane as well as NADPH oxidase activity. Taken together, these results demonstrate that ATL suppresses NADPH oxidase-mediated ROS generation in BV2 microglia cells, strongly indicating that ATL may play an important role against the development and progression of neuroinflammtion.

Keywords Aspirin-triggered · Lipoxin A$_4$ · Microglia · Reactive oxygen species · NADPH oxidase

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Introduction

Microglial cells are the resident immune cell population in the central nervous system. They are commonly activated in response to variety of neurodegenerative and neuroinflammatory conditions in conjunction with the release of proinflammatory factors, reactive nitrogen species, and reactive oxygen species (ROS). ROS has been characterized as an important secondary messenger and modulator for various mammalian intracellular signaling pathways [1, 2], including neuroinflammatory processes [3, 4]. Therefore, both ROS generation and its respective neutralizing system may modulate the molecular mechanisms governing microglial function.

Lipoxins (LXs) are groups of recently discovered endogenous anti-inflammatory lipid-based autacoids and generate from arachidonic acid via lipoxygenase-mediated transcellular biosynthesis. An additional route of LX biosynthesis is triggered by aspirin and generates 15-epi-LX or aspirin-triggered-lipoxins (ATL). In the presence of aspirin, acetylated COX-2 loses activity required to form prostaglandin H$_2$ but retains oxygenase activity to produce 15R-hydroxyeicosatetraenoic acid from arachidonate. This intermediate is transformed via 5-lipoxygenase to generate ATL [5, 6]. These ATL share many of the biological functions of native LX, albeit with greater potency and efficacy. ATL binds to its specific G protein-coupled receptor termed ALX [6], which was cloned in leukocytes, monocytes, macrophage, as well as resident cells such as astrocytes, microglia, and neural stem cells in the central nervous system. This implies that these cells may be targets for LXs action in the brain [7–10]. In our previous research, we evaluated the anti-inflammatory activity of an LXA$_4$ analogue, 5(S), 6(R)-LXA$_4$ methyl ester, in a rat model of permanent focal cerebral ischemia and focal

cerebral ischemia reperfusion [11–13], and 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 [14]. Our results showed that these LXs could attenuate inflammatory responses and inhibit activation of microglia in vivo and in vitro. However, the mechanism by which ATL regulates the function of microglia in neuroinflammation remains to be determined.

We demonstrate for the first time that ATL inhibits ROS production in BV2 microglia cells, an effect that may have therapeutic benefits by ameliorating the brain oxidative stress. These findings corroborate to the statement of ATL research as a novel frame of opportunity for new therapeutic strategies against neurodegenerative diseases.

Materials and Methods

Materials

ATL was purchased from Cayman Chemical (Ann Arbor, MI). LPS (*Escherichia coli* strain O111:B4) was obtained from Calbiochem (San Diego, CA, USA). Boc-2 (Boc-Phe-Leu-Phe-Leu-Phe) was obtained from Phoenix Pharmaceuticals, Inc. The immortalized murine microglia cell line BV-2 was purchased from Cell Resource Centre of Peking Union Medical College (Beijing, China). Cell culture ingredients were obtained from Gibco-BRL-Life Technologies (Gaithersburg, MD). Dichlorodihydrofluorscein diacetate (DCFH-DA), NADPH, lucigenin, were obtained from Sigma-Aldrich (St Louis, MO, USA). The polyclonal anti-P47[phox] was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse anti-gp91[phox] was purchased from BD Transduction Laboratories (San Jose, CA). FITC-conjugated goat anti-rabbit IgG were obtained from Pierce (Rockford, IL). The biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). Bicinchoninic Acid Kit for protein determination (BCA kit), Enhanced chemiluminescence (ECL) kit was purchased from Pierce Chemical Company (Rockford, IL, USA).

Cell Culture

The immortalized murine microglia cell line BV-2 was maintained in Dulbecco's modified Eagle's medium with F12 supplement (DMEM/F12), which was supplemented with 10 % fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95 % air, 5 % CO_2. Confluent cultures were passaged by trypsinization. Before each experiment, cells were serum-starved for 12 h. In all experiments, cells were

treated with ATL or vehicle (0.035 % ethanol) for indicated time before addition of 100 ng/ml LPS under serum-free conditions. To investigate the involvement of ALXs in the anti-inflammatory effects of ATL, the cells were treated with 10 μM Boc-2, a specific receptor antagonist, prior to the treatment with ATL for 30 min.

Analysis of ROS Production

Intracellular ROS levels were determined by using a DCFH-DA (2′,7′-dichlorofluorescin diacetate) assay as our previous report [15]. DCFH-DA enters cells passively and is deacetylated by esterase to form nonfluorescent DCFH, and DCFH reacts with ROS to form the fluorescent product DCF. BV2 cells (1×10^5 cells/ml) were treated with LPS in the presence or absence of indicated concentration ATL for indicated time periods at 37 °C. Boc-2 was added to naive BV2 cells preconditioning 30 min if necessary. Then the cells were incubated with 1 mM DCFH-DA for 1 h at 37 °C and were washed three times with PBS. After centrifugation at 1,000g for 5 min, the supernatants were removed and the pellets were resolved with 1 % Triton X-100, and fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 540 nm using a fluorescence microplate reader.

Immunofluorescence Confocal Microscopy

For the detection of membrane location of p47[phox], BV-2 cells were cultured on sterile glass cover slips in 24 well plates and treated with ATL and LPS as described above. After treatment, cells were fixed with 4 % paraformaldehyde in PBS for 30 min. After rinsing, cells were blocked with 3 % BSA in PBS for 1 h and incubated with anti-p47[phox] antibody overnight at 4 °C. After washing, cells were incubated with FITC-conjugated IgG for 1 h. After washing with PBS, the cover slips were mounted on slides, and the cells were observed with a confocal microscope Olympus Fluoview FV500.

NADPH Oxidase Activity Assay

The lucigenin-derived enhanced chemiluminescence assay was used to determine NADPH oxidase activity in BV2 cells. BV2 cells were treated as indicated, washed twice with ice-cold PBS (pH 7.4) and centrifuged at 2,000g for 5 min. The pellet was resuspended in ice-cold buffer (pH 7.0) containing 1 mmol/L ethylene glycol tetraacetic acid (EGTA), protease inhibitors, and 150 mmol/L sucrose. Then, the cells were lysed. The total protein concentration was determined and adjusted to 1 mg/ml. Two hundred microliters of protein sample, including 5 μmol/l lucigenin, were measured over 6 min in quadruplicate using NADPH

(100 μmol/L) as a substrate in a luminometer counter (Berthold luminometer centro LB 960, Germany). Data were collected at 2-min intervals in order to measure relative changes in NADPH oxidase activity.

Subcellular Fractionation

For making whole cell lysates, the cells were lysed in radioimmune precipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (Roche). Membrane and cytoplasmic were performed with Proteo JET™ membrane and cytoplasmic extraction kit (Fermentas life science) according to manufacturer's protocol.

Western Blot Analysis

Equal amounts of membrane or cytoplasmic 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 antibodies at 4 °C overnight. The membrane was washed three times with Tris-bufffered saline containing 0.05 % Tween 20 (TBST) for 10 min and incubated with anti-rabbit or anti-mouse IgG-horseradish peroxidase at room temperature for 1 h. The Supersignal West Pico chemiluminescent substrate system 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.

Statistical Analysis

The data were presented as the means ± SEM. Statistical significance was assessed by ANOVA, followed by Bonferroni's t test, and $P < 0.05$ was taken as statistically significant.

Results

ATL Prevented LPS-Induced ROS Generation in BV2 Cells in a Time-Dependent and Concentration-Dependent Manner.

In order to determine the time course of ATL effect upon ROS generation, BV2 cells were incubated with the ATL (100 nM) for different periods of time (0–3 h) before exposure to LPS. As shown in Fig. 1, inhibitory effect of ATL was already seen after 15 min of pre-treatment, reaching maximal inhibition after 30 min, and remaining significant until 3 h.
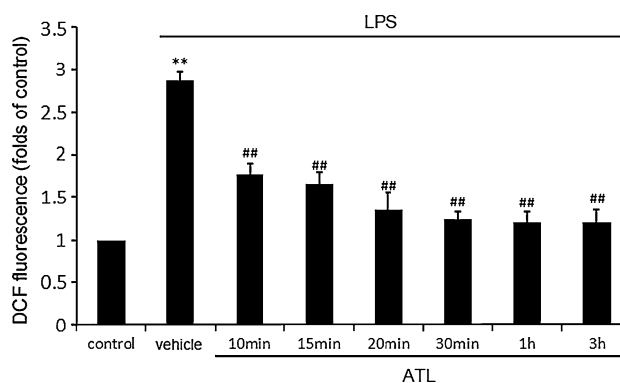

Fig. 1 ATL inhibited LPS-induced production of ROS in the time-dependent manner. BV2 cells were pre-incubated with ATL (100 nM) for different periods of time (0–3 h). BV2 cells were then exposed or not (control) to LPS (100 ng/ml) for 30 min. ROS generation was assessed as described in Materials and Methods. The quantification of cellular fluorescence intensity is expressed in arbitrary units as mean ± SEM from three independent experiments. **$P < 0.01$ compared with control (untreated group). ##$P < 0.01$, compared with vehicle (treated by LPS alone)
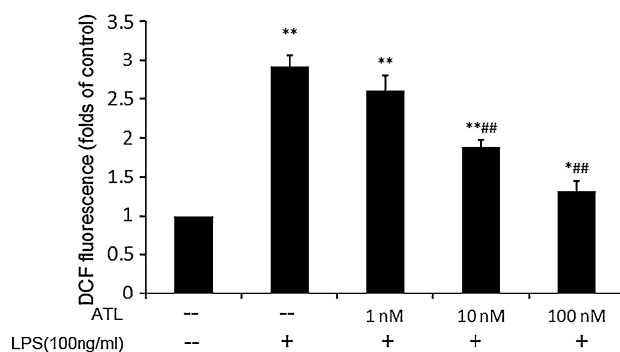

Fig. 2 ATL reduced LPS-induced accumulation of ROS in the concentration-dependent manner. BV2 cells were pre-incubated in the absence or in the presence of different concentrations of ATL (1–100 nM) for 30 min. BV2 cells were then incubated or not with LPS (100 ng/ml) for 30 min. ROS generation was assessed as described in "Materials and Methods". Data are expressed as percent of values in untreated control cultures, and are means ± SEM of 4 replicate values in 3 separate experiments. *$P < 0.05$, **$P < 0.01$, compared with control, ##$P < 0.01$, compared with LPS alone

Pre-incubation of BV2 cells with different concentrations of ATL (1–100 nM) for 30 min caused a visible reduction in LPS-induced ROS generation, with a more marked effect at higher concentrations (Fig. 2).

ATL Suppressed ROS Generation Requires Lipoxin-Dependent Signaling

The bioactions of ATL is transduced by the high-affinity G protein coupled receptor ALX, which has been identified in several cell types [6]. Exposure of the cells to Boc-2 (10 μM), a selective ALX inhibitor significantly reverted
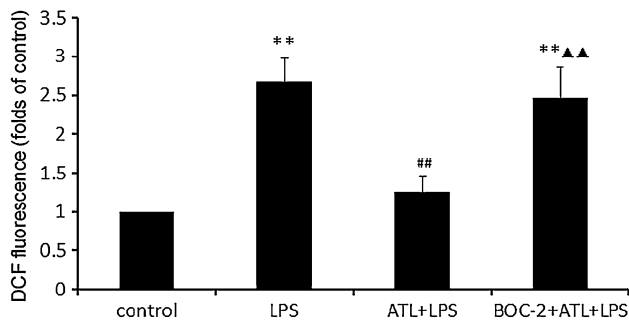
Fig. 3 ATL suppressed LPS-induced generation of ROS is receptor-mediated effect. BV2 cells were pre-incubated with vehicle or Boc-2 (100 μM) for 30 min. The cells were then incubated in the absence or in the presence of ATL (100 nM) for 30 min and exposed to LPS (100 ng/ml) for 30 min. Each value represents the mean ± SEM for three independent experiments. $**P < 0.01$ compared with control. $\#\#P < 0.01$ in comparison with LPS-stimulated cells. $\blacktriangle\blacktriangle P < 0.01$ in comparison with ATL pretreatment and then LPS-stimulated cells

ATL impairment of ROS generation, indicating that ATL effect relies on ALX signaling (Fig. 3).

ATL Impaired NADPH Oxidase Activity

In order to confirm the inhibition of NADPH oxidase activation by ATL, we measured NADPH oxidase activity with lucigenin-enhanced chemiluminescence. BV2 cells were pre-treated with or without ATL (100 nM) for 30 min and exposed or not to LPS (100 ng/ml) for 20 min. Following 20 min treatment of BV2 cells with 100 ng/ml LPS, we detected an increase of NADPH oxidase activity compared with the control cells. In contrast, BV2 cells which were pre-treated with ATL (100 nM) for 30 min showed a significant decrease in NADPH oxidase activity compared with the LPS-treated cells. The results show that pretreatment with ATL resulted in the inhibition of LPS-induced NADPH oxidase activity (Fig. 4).
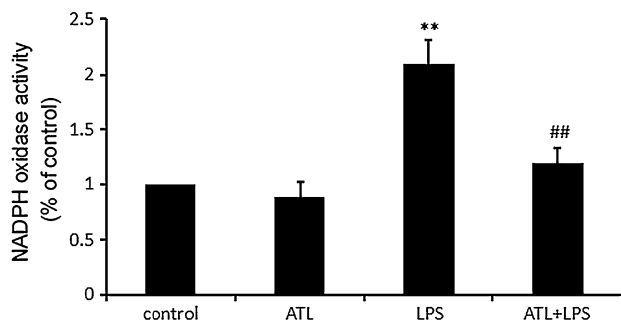

Fig. 4 ATL inhibited LPS-induced increase in NADPH oxidase activity. BV2 cells were pre-treated with or without ATL (100 nM) for 30 min and exposed or not to LPS (100 ng/ml) for 20 min. Cells were then lysed and NADPH oxidase activity was assessed as described in "Materials and Methods". The results are representative of three independent experiments performed in triplicate. $**P < 0.01$ in comparison with control. $\#\#P < 0.01$ in comparison with LPS-stimulated cells

ATL Inhibited LPS-Induced Translocation of the Cytosolic Subunits p47phox to the Cellular Membrane

It has previously been shown that activation of the NADPH oxidase requires that the cytosolic component p47phox be phosphorylated, and subsequently translocated along with the p67phox component to the plasma membrane, where they associate with cytochrome b558 to assemble into an active enzyme complex [16].

Because ATL showed potent effects on extracellular ROS production, we sought to determine whether ATL inhibits NADPH oxidase activation by preventing the phosphorylation and translocation of the cytoplasmic subunit p47phox from cytosol to membrane in the BV2 microglial cell line following LPS stimulation. Therefore, we first examined the levels of LPS-induced p47phox phosphorylation using the anti-phospho-Ser345-p47phox antibody. As shown in Fig. 5a, the level of phosphorylated p47phox in the cellular membrane fraction was low in BV2 cells under unchallenged condition. Enhanced levels of p47phox were detected 10 min after LPS was added to the culture, and levels remained elevated for at least 60 min. The maximal level immunoreactivity of p47phox in the cellular membrane fraction of BV2 cells occurred at 20 min after LPS treatment.

In the absence of LPS, weak basal phosphorylation of p47phox in membrane was detected, and an increase in the immunoreactivity of p47phox was observed 20 min following LPS stimulation. LPS-induced phosphorylation of p47phox was significantly inhibited by ATL pretreatment 30 min (Fig. 5b). Figure 5c showed the decrease expression of p47phox in the cellular cytosolic fraction of BV2 cells within 20 min after LPS treatment, while following ATL pretreatment, we see p47phox remaining mostly within the cytosolic fraction.

Consistent with the results of the Western blot assay, confocal study clearly showed that LPS initiated the translocation of cytosolic p47phox, and that within 20 min after LPS stimulation the majority of the p47phox-FITC fluorescence signal was found clustered on the membrane. The pretreatment of ATL at 100 nM for 30 min significantly prevented the translocation of p47phox. In cells treated with vehicle or ATL alone, p47phox were found localized mainly in the cytosol (Fig. 6). Therefore, it is likely that ATL-mediated inhibition of superoxide production by LPS is primarily through the inhibition of p47phox translocation to the cellular membrane.

Discussion

Microglias are the resident immune cells in the brain and serve the role of immune surveillance. Once exposed to
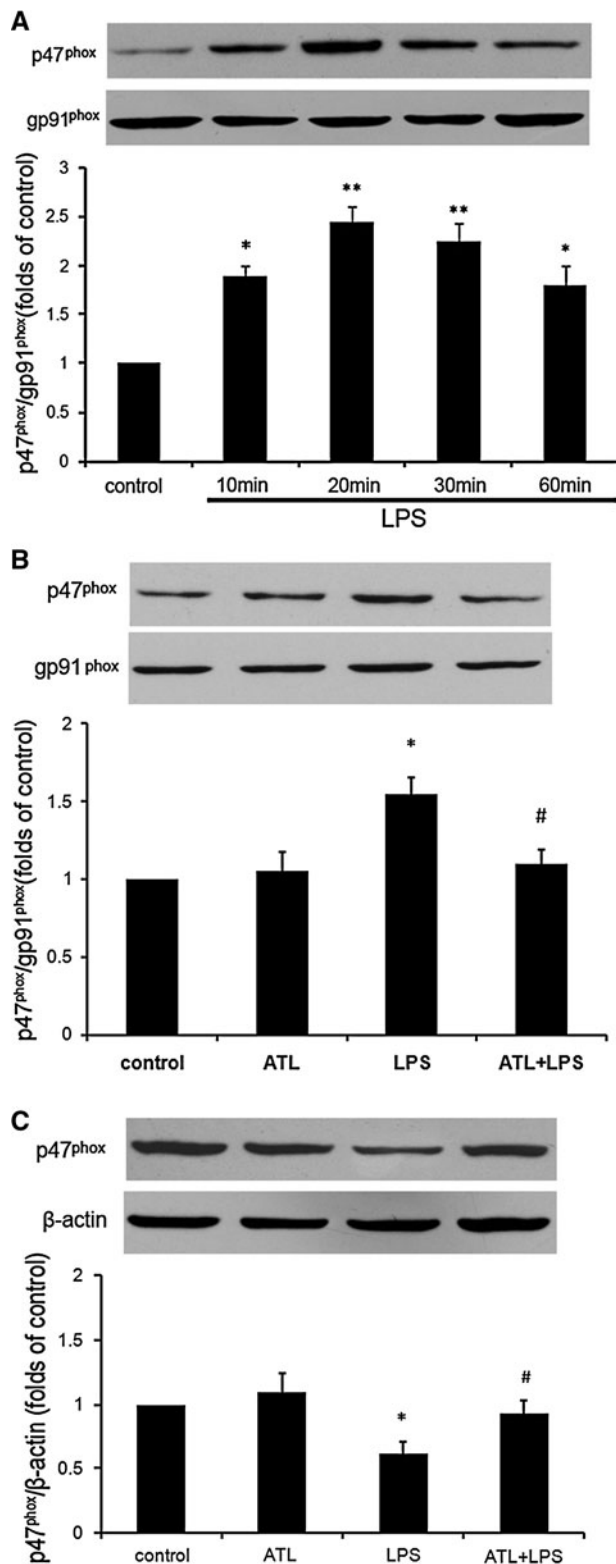
◀ **Fig. 5** Effect of ATL on p47^{phox} subunit expression in cytosolic and membrane subcellular fractions. (**a**) Representative Western blots showing the protein levels of phosphorylated p47^{phox} in membrane of BV2 cells treated with LPS at serial time points. BV2 cells were pre-incubated with vehicle or ATL (100 nM) for 30 min followed by LPS (100 ng/ml) treatment or not for 20 min. Membrane protein (**b**) and cytosolic protein (**c**) were isolated to perform Western blot analysis. GAPDH is as an internal cytosolic control, and gp91^{phox} is as an internal membrane control. Each experiment has been performed three times

microglia also produces a large number of proinflammatory factors including cytokines, reactive nitrogen species, and ROS. Among these proinflammatory factors, ROS play a key role in neurodegeneration by serving dual functions. First, high concentrations of ROS released extracellularly may exert direct toxicity to neurons, and second, the increase in intracellular concentrations of ROS may modify the signaling events leading to the activation of microglia.

A growing body of evidence suggests oxidative stress involvement in neurodegenerative diseases [17]. Understanding the mechanisms that control microglial expression of neurotoxic effectors is crucial for the development of new therapeutic approaches to neuroinflammatory in neurodegenerative diseases. Previous studies proved that antioxidants inhibit NF-κB activation and block the expression of inflammatory cytokines by inhibiting the generation of ROS [18].

The present study demonstrates that the increased ROS production in BV2 cells stimulated by LPS was inhibited by the pre-treatment with ATL. In agreement with our data, it has been shown that LXA4 significantly inhibits porphyromonas gingivalis-induced aggregation and ROS generation in whole blood [19]. Furthermore, ATL also down-regulates ROS production, attenuating NADPH oxidase activity in endothelial cells [20].

The primary mediator of the oxidative stress response in microglial cells is the enzyme NADPH oxidase (PHOX), and upon LPS stimulation, microglia cells are known to produce ROS via PHOX activation [21]. PHOX is a multicomponent enzyme consisting of a membrane-associated cytochrome b558 (composed of two subunits: gp91^{phox} and p22^{phox}) and the cytosolic components: p47^{phox}, p67^{phox}, p40^{phox}, and a small GTPase rac2 [16]. Previous studies have shown that activation of PHOX activity requires p47^{phox} phosphorylation, a protein that has an important role in translocation of cytosolic components to cytochrome b558, as well as in the assembly and activation of PHOX [22].

Our data show that ATL impairs LPS-induced p47^{phox} translocation to the BV2 cell membrane, affording strong evidence that ATL impairment of ROS production depends on the NADPH oxidase inhibition, more specifically interfering on NADPH oxidase assembly, modulating the
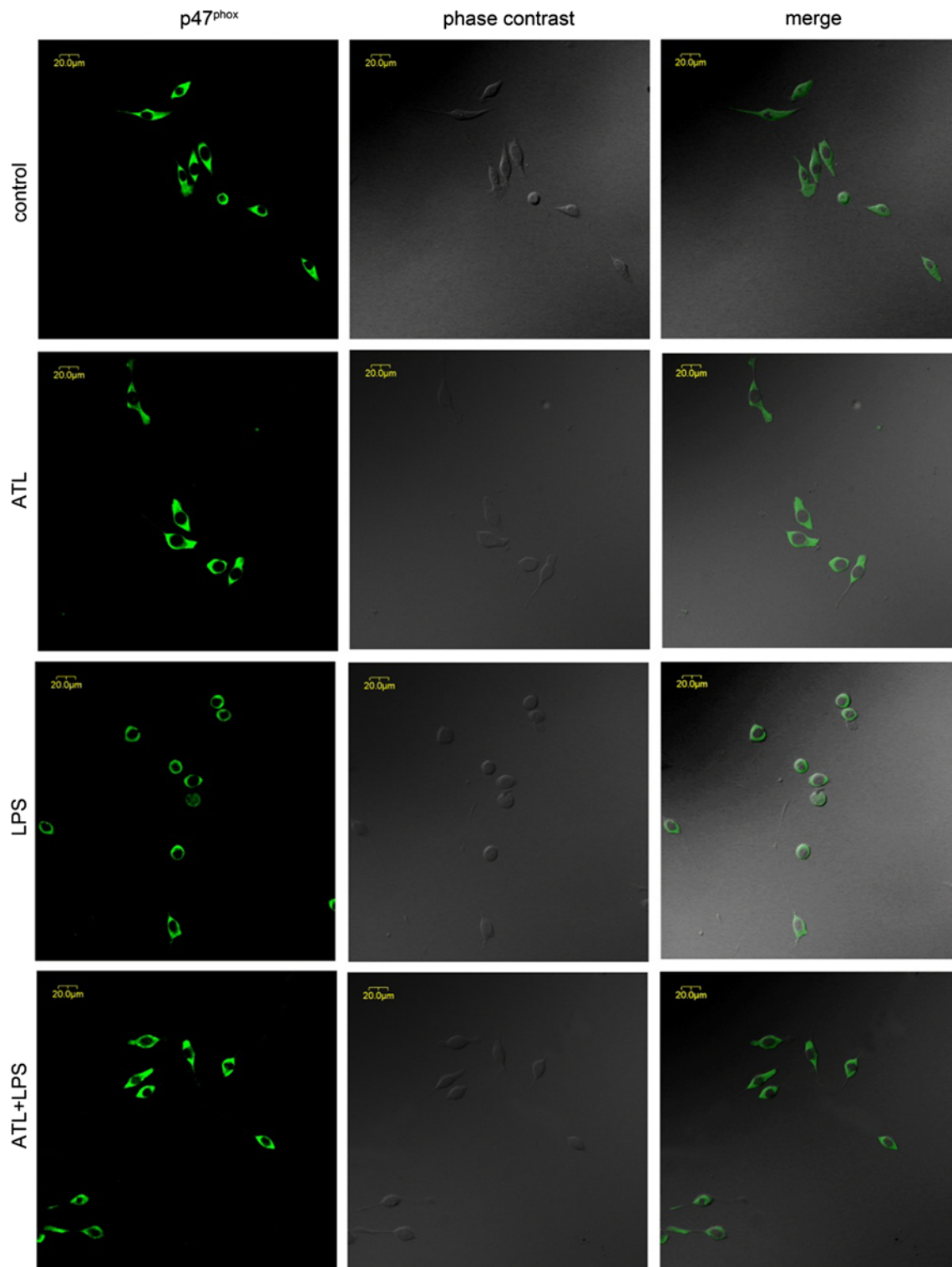
immunological challenges such as invading pathogens and neuronal injuries, microglia readily becomes activated and undergoes changes in morphology (hypertrophy), number (proliferation), and function (phagocytosis). Activated

Fig. 6 Effect of ATL on cytosolic p47^{phox} protein translocation. Immunofluorescence and confocal microscopy analysis of p47^{phox} localization in LPS-stimulated BV2 cells. BV2 cells were treated with LPS for 20 min in the absence or presence of ATL pretreatment for 30 min. Cells were stained with rabbit polyclonal antibody against p47^{phox} and then washed and incubated with FITC-conjugated antibody. The signal of p47^{phox} (FITC-p47^{phox}; *on left*), the phase contrast (*in the middle*) and the merge view of fluorescence and cell morphology (FITC-p47^{phox} plus phase, *on right*) are shown

p47[phox] phosphorylation status. This result is supported by the direct assessment of NADPH oxidase activity, which revealed that ATL significantly inhibits LPS induced NADPH oxidase activity in BV2 cells.

Together with the present work, ATL abrogates NADPH oxidase-dependent ROS generation on BV2 cells, inhibiting p47[phox] translocation to the cell membrane, and subsequent NADPH oxidase activation. We hope that this seminal observation that ATL blocks NADPH oxidase activation in BV2 cells, allied to the understanding of this event, may enable to devise therapeutic strategies in order to target specific cellular events contributing to neurodegenerative disease.

References

1. Finkel T (1999) Signal transduction by reactive oxygen species in non-phagocytic cells. J Leukoc Biol 65:337–340

2. Maulik N (2002) Redox signaling of angiogenesis. Antioxid Redox Signal 4:805–815

3. Floyd RA (1999) Antioxidants, oxidative stress, and degenerative neurological disorders. Proc Soc Exp Biol Med 222:236–245

4. Block ML, Hong JS (2007) Chronic microglial activation and progressive dopaminergic neurotoxicity. Biochem Soc Trans 35:1127–1132

5. Clària J, Serhan CN (1995) Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. Proc Natl Acad Sci USA 92:9475–9479

6. Chiang N, Arita M, Serhan CN (2005) Anti-inflammatory circultry: lipoxin, aspirin- triggered lipoxins and their receptor ALX. Prostaglandins Leukot Essent Fat Acids 73:163–177

7. Maddox JF, Hachicha M, Takano T et al (1997) Lipoxin A4 stable analogues are potent mimetics that stimulate human monocytes and THP-1 cell via a G-protein linked lipoxin A4 receptor. J Biol Chem 272:6972–6978

8. Sodin-Semrl S, Spagnolo A, Mikus R et al (2004) Opposing regulation of interleukin-8 and NF-kappaB responses by lipoxinA4 and serumamyloid Avia the common lipoxin A receptor. Int J Immunopathol Pharmacol 17:145–156

9. Wada K, Arita M, Nakajima A et al (2006) Leukotriene B4 and lipoxin A4 are regulatory signals for neural stem cell proliferation and differentiation. FASEB J 20:1785–1792

10. Svensson CI, Zattoni M, Serhan CN (2007) Lipoxins and aspirin triggered lipoxin inhibit inflammatory pain processing. J Exp Med 204:245–252

11. Wu Y, Ye XH, Guo PP et al (2010) Neuroprotective effect of lipoxin A(4) methyl ester in a rat model of permanent focal cerebral ischemia. J Mol Neurosci 42: 226–234

12. Ye XH, Wu Y, Guo PP et al (2010) Lipoxin A4 analogue protects brain and reduces inflammation in a rat model of focal cerebral ischemia reperfusion. Brain Res 1323:174–183

13. Wu Y, Wang YP, Guo P et al (2011) A Lipoxin A(4) analog ameliorates blood-brain barrier dysfunction and reduces MMP-9 expression in a rat Model of focal cerebral ischemia-reperfusion injury. J Mol Neurosci 46(3):483–491

14. Wang YP, Wu Y, Li LY et al (2011) Aspirin-triggered lipoxin A4 attenuates LPS-induced pro-inflammatory responses by inhibiting activation of NF-κB and MAPKs in BV-2 microglial cells. J Neuroinflammation 8:95

15. Wu Y, Shang Y, Sun S et al (2007) Antioxidant effect of erythropoietin on 1-methyl-4-phenylpyridinium-induced neurotoxicity in PC12 cells. Eur J Pharmacol 564:47–56

16. Liva SM, Kahn MA, Dopp JM et al (1999) Signal transduction pathways induced by GM-CSF in microglia: significance in the control of proliferation. Glia 26:344–352

17. Emerit J, Edeas M, Bricaire F (2004) Neurodegenerative diseases and oxidative stress. Biomed Pharmacother 58:39–46

18. Wang JY, Wen LL, Huang YN et al (2006) Dual effects of antioxidants in neurodegeneration: direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation. Curr Pharm Des 12:3521–3533

19. Börgeson E, Lönn J, Bergström I et al (2011) Lipoxin A$_4$ inhibits porphyromonas gingivalis-induced aggregation and reactive oxygen species production by modulating neutrophil-platelet interaction and CD11b expression. Infect Immun 79:1489–1497

20. Nascimento-Silva V, Arruda MA, Barja-Fidalgo C et al (2007) Aspirin-triggered lipoxin A4 blocks reactive oxygen species generation in endothelial cells: a novel antioxidative mechanism. Thromb Haemost 97:88–98

21. Qin L, Liu Y, Wang T et al (2004) NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. J Biol Chem 279:1415–1421

22. Groemping Y, Rittinger K (2005) Activation and assembly of the NADPH oxidase: a structural perspective. Biochem J 386:401–416