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Anti-inflammatory effects of lipoic acid through inhibition of GSK-3 β in lipopolysaccharide-induced BV-2 microglial cells

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Abbreviations:

GSK-3 β : glycogen synthase kinase-3 β

iNOS: inducible nitric oxide synthase

IL-1 β : interleukin-1 β

LA: lipoic acid

LiCl: lithium chloride

LPS: lipopolysaccharide

LY: LY294002

NF- κ B: nuclear factor-kappa B

TNF α : tumor necrosis factor α

Abstract

Activated microglial cells play an important role in immune and inflammatory responses in CNS and play a role in neurodegenerative diseases. We examined the effects of lipoic acid (LA) on inflammatory responses of BV-2 microglial cells activated by lipopolysaccharide (LPS), and explored the underlying mechanisms of action of LA. BV-2 cells treated with LPS showed an up-regulation of mRNA of the pro-inflammatory molecules, inducible nitric oxide synthase (iNOS). LA suppressed the expression of iNOS and furthermore, LPS-induced production of nitrite. Moreover, LA suppressed the nuclear translocation of RelA, a component of nuclear factor-kappa B (NF- κ B) that contains transcriptional activator domain for LPS. The mechanisms of LA-mediated anti-inflammatory effects on microglia remain unknown, and we suggested an involvement of Akt/glycogen synthase kinase-3 β (GSK-3 β) phosphorylation. The results showed that inhibitor of phosphatidylinositol 3-kinase prevented LA-mediated suppression of LPS induction of RelA and expression of iNOS. Furthermore, these inflammatory actions were prevented by GSK-3 β inhibitors. These data demonstrate a role for LA as a chemical modulator of inflammatory responses by microglia, and thus may be a therapeutic strategy for treating neurodegenerative diseases with an inflammatory component.

1. Introduction

Microglial cells are important players in immune and inflammatory responses in the CNS (Nam et al. 2010), and their activation is implicated in several chronic neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and brain ischemia (Kang et al. 2004). Activated microglial cells promote neuronal injury via the production of pro-inflammatory molecules such as the cytokines, tumor necrosis factor (TNF) α and interleukin (IL)-1 β , and other cytotoxic molecules, such as reactive oxygen species (ROS) (Dheen et al. 2007). The overexpression of pro-inflammatory cytokines has emerged as an important determinant of the cytotoxicity associated with inflammation in neurodegenerative diseases (Jin et al. 2007). Furthermore, excessive production of nitric oxide (NO) in activated glia, resulting from the production of inducible nitric oxide synthase (iNOS), is thought to play a role in neurodegenerative pathology. Therefore, promoting counter regulatory mechanisms is essential to avoid inflammation-mediated injury in the CNS (McCarty 2006), and would thus require therapeutic agents that possess anti-inflammatory actions, targeting over-activated microglia cells.

Lipoic acid (LA) was identified as a cofactor for mitochondrial α -ketoacid dehydrogenases in mammals (Smith et al. 2004). LA may easily cross the blood brain barrier because of its small molecular size (206 g/mol) and high water and lipid solubility (Goraca and Aślanowicz-Antkowiak 2009). A number of studies have reported on the beneficial effects of LA on various disorders, such as hypertension (Thirunavukkarasu et al. 2004) and diabetes mellitus (Kamenova 2006). Although the cellular and molecular mechanisms associated with the positive actions of LA are partially understood for these diseases, little is known about its mechanistic actions

underlying anti-inflammatory responses through GSK-3 β , including its effect on activated microglia (Zhang et al. 2007). Therefore, the present study aimed to investigate the effect of LA on LPS-activated BV-2 murine microglia cell lines and the effect of LA injection in LPS-exposed mice by examining their cortices. The results reported herein may thus provide critical information on the use of LA as a therapeutic agent via its suppression of microglial activation.

2. Materials and methods

2.1. Chemicals

LA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) containing 0.33% 5 M NaOH. LPS was obtained from Sigma-Aldrich (*Escherichia coli* 0111:B4, Sigma-Aldrich, Tokyo, Japan). The phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002 (LY), was purchased from Sigma-Aldrich. The glycogen synthase kinase-3 β (GSK-3 β) inhibitor, lithium chloride (LiCl), and GSK-3 β inhibitor VII were purchased from Wako (Osaka, Japan) and Calbiochem (San Diego, CA, USA), respectively.

2.2. Cell culture and treatment

The BV-2, murine cell line was purchased from Banca Biologica e Cell Factory (Genoa, Italy) and maintained in RPMI1640 (Wako, Osaka, Japan) and 10% fetal bovine serum (FBS, Gibco, Grand Island, USA). The cells were pretreated with the inhibitors for 10 min followed by LA. After a 30-min incubation of the inhibitors and LA, LPS was added.

2.3. RNA isolation and RT-PCR

Total RNA was isolated from BV-2 cells using Sepasol RNAI (Nacalai Tesque, Kyoto, Japan). cDNA fragments were obtained by RT-PCR. We used the RNA PCR kit (AMV) Version 3 (Takara, Kyoto, Japan) to obtain the PCR products following the specific primers for: iNOS (5'-ACGAGACGGATAGGCAGAGA-3'(forward) and 5'-AGCTGGAAGCCACTGACACT-3'(reverse)), GAPDH (5'-ACCACAGTCCATGCCATCAC -3'(forward) and 5'-TCCACCACCCTGTTGCTGTA -3'(reverse)). The PCR products underwent electrophoresis and were stained with ethidium bromide. The product bands were quantified using Scion Image software (Scion Corporation, Frederick, MD, USA) and were normalized against the corrected GAPDH mRNA data.

2.4. Determination of nitrite levels by the Griess assay

Nitrite levels were determined using the Griess method, as previously described by our group (Koriyama et al. 2009a). Briefly, the medium from treated cells cultured in 24-well plates was removed and placed into a 96-well plate (50 µl per well). The medium was mixed with Griess reagent (1% sulfanilamide solution and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride) for 10 min. Absorbance was measured at 550 nm using a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA). Sodium nitrite was used to construct the standard curve.

2.5. Subcellular fractionation of RelA protein

BV-2 cells were lysed with hypotonic buffer (10 mM HEPES-KOH [pH7.9], 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail [Sigma-Aldrich, Tokyo, Japan]) and centrifuged (10,000 ×g, 15 min at 4°C). The supernatants comprised of the cytoplasmic fractions. The pellets were incubated (30 min on ice) with a nuclear lysis buffer (20 mM HEPES-KOH

[pH7.9], 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 5% glycerol, and protease inhibitor cocktail). The generated lysates were centrifuged (18,000 ×g, 15 min at 4°C) and the resulting supernatants comprised of the nuclear fractions to be analyzed.

2.6. Western blot analysis

Cultured BV-2 cells or mouse cortical tissue were extracted, and sample aliquots (30 µg of protein) were subjected to polyacrylamide gel electrophoresis using a 12.5% gel, as previously described (Koriyama et al. 2013). The separated proteins were transferred to a nitrocellulose membrane and incubated with the primary antibodies (all at 1:500): anti-iNOS, anti-GSK-3β or Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-phospho-(p)-GSK-3β (Ser9), anti-p-Akt (Ser473) or anti-RelA (Cell Signaling Technology, Tokyo, Japan). The secondary antibody was alkaline phosphatase-labeled anti-rabbit IgG (1:500, Sigma-Aldrich). The immunoreactive protein bands were detected using the BCIP/NBT Kit (KPL, Gaithersburg, MD, USA). β-actin (1:500) (Gene Tex, San Antonio, TX) and Histone H4 (1:500) (Cell Signaling Technology) were used as an internal standard and a standard for nuclear proteins, respectively. The protein bands were analyzed via densitometry using Scion Image Software. All experiments were repeated at least three times.

2.7. Animals and surgery

All experiments on animals were performed in accordance with the guidelines for animal experiments of Kanazawa University. Male C57BL/6 mice (8–9 weeks old) were anesthetized by intraperitoneal injection of sodium pentobarbital (30–40 mg/kg body weight). Mice were assigned to 6 groups: (1) normal (vehicle), (2) 30 mg/kg LA only, (3) 60 mg/kg LA only, (4) LPS (5 mg/kg) only, (5) LPS+LA 30 mg/kg, or (6) LPS+LA 60 mg/kg. Solutions of LA were prepared immediately before use by dissolving 10

mg/ml LA in 120 mM Tris buffer, adjusting the pH to 7.4 followed by filter sterilization of the solution. LA was administered intraperitoneally once per day for 3 days. For the LPS-treated groups, LPS (in saline) was injected (5 mg/kg, intraperitoneally) 3 h after the final administration of LA, and tissue collected after 3 days, as previously described (Qin et al. 2007; Corona et al. 2012).

2.8. Tissue preparation and immunohistochemistry

Tissue fixation and cryosectioning were performed, as previously described (Koriyama et al. 2009b). Briefly, the brain was dissected and then fixed in 4% paraformaldehyde solution containing 0.1 M phosphate buffer (pH 7.4) followed by immersion in sucrose (at 4 °C) starting at a 5% concentration and gradually increasing to 30% over 2 h. The brain was then embedded in optimal cutting temperature compound (Tissue Tek; Miles, Eikhart, IN, USA) and cryosectioned (30- μ m-thick sections). Coronal sections of cortex at bregma -1.5 to -2.0 mm were used for immunohistochemistry. The frozen sections were mounted onto silane-coated glass slides and air-dried. After washing and blocking with Blocking One (Nacalai Tesque), brain sections were incubated with primary antibodies: anti-Iba1 (1:100) (Wako) and anti-iNOS (1:500) (Transduction Laboratories, Lexington KY, USA) at 4 °C, overnight. The sections were then incubated with fluorescent secondary antibodies (1:1000, Invitrogen) at room temperature.

2.9. Double-staining immunohistochemistry

To identify the cells which express iNOS protein, we used brain sections treated with LPS (5 mg/kg, 3 days). After identification of microglial cells by immunohistochemistry with using primary antibody, anti-Iba1 (1:100, WAKO) and

fluorescent antibody, anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen), samples were combined with anti-iNOS immunohistochemistry. We used anti-iNOS (1:500, Transduction Laboratories, Kexington KY, USA) as a primary antibody, and biotin-conjugated anti-mouse IgG (Santa Cruz Biotechnology, CA, USA) as a secondary antibody. Then, sections were treated with DyLight 594-conjugated NeutrAvidin (Thermo Scientific, Waltham, MA, USA).

2.10. Quantification of microglia in the mouse cortex

Stereological counts of Iba1-positive microglial cells were undertaken in 30 μm sections adjacent to those used for quantitative immunohistochemistry in the cortex from retrosplenial granular cortex to the secondary somatosensory cortex at Bregma \sim -1.6 (Paxinos and Franklin, 2001) from images taken by optical light microscope (at \times 200 magnification). To define the microglial cells, we used a signal/noise (S/N) ratio for each cell in each tissue. Cell bodies ($> 5 \mu\text{m}$) with a higher immunoreactivity (S/N ratio >3) were considered as Iba-1 positive microglia. To quantify the densities of immunoreactivity, we used ImageJ software (National Institutes of Health, Bethesda, Maryland). The values of microglia were presented as a percentage of those of the vehicle control group, and represented the average of 3 sections per mouse and a total of 5 mice per group.

2.11. Statistics

All results are reported as the mean \pm SEM of three to five experiments. Differences between groups were analyzed using ANOVA, followed by Dunnett's multi-comparison test with (PASW) Software (SPSS Inc., Chicago, IL, USA). Significance was reached at values of $P < 0.05$.

3. Results

3.1. LA decreased the LPS-induced expression of iNOS and production of nitrite in BV-2 cells.

RT-PCR was done to investigate the expression of mRNA of iNOS in LPS-exposed BV-2 cells. LPS treatment for 8 h significantly increased mRNA of iNOS in a concentration-dependent manner (50, 100, 200 ng/ml LPS) (data not shown). We used 200 ng/ml LPS in culture system hereafter. At 4, 6 and 8 h of LPS treatment, mRNA expression of iNOS was induced (without changes in the levels of GAPDH) (Fig. 1A). When compared with the control, an up-regulation of mRNA of iNOS (Fig. 1A) occurred at 4 h of LPS treatment. Furthermore, the mRNA levels of iNOS was dramatically increased (8.4-fold) at 8 h. A pre-treatment of LA (200 and 400 μ M; 30 min) followed by LPS (8 h) markedly, but not completely, inhibited the LPS-induced expression of iNOS (Fig. 1B) in a concentration-dependent manner. LA also suppressed LPS-induced mRNA synthesis of another inflammatory mediators, TNF α , IL-1 β (data not shown). We next focused on measuring the level of iNOS at the protein level to confirm the results obtained with the previous analyses regarding mRNA. Western blotting for iNOS revealed that LA (400 μ M) significantly inhibited the LPS-induced expression of this catalytic enzyme for nitric oxide (Fig. 1C). Therefore, we evaluated the amount of nitrite production in BV-2 cells at 8 h of LPS treatment with or without LA (200 μ M or 400 μ M). We found that LPS increased the production of nitrite while LA significantly suppressed it in a concentration-dependent manner (Fig.1D).

3.2 LA transiently activated Akt and inactivated GSK-3 β in BV-2 cells.

We recently reported that LA activates the phosphorylation of Akt in retinal precursor cell line of RGC-5 (Koriyama et al. 2013). GSK-3 β is one of the major substrates of Akt, and is involved in the promotion of inflammation (Green and Nolan 2012). Therefore, we evaluated the effect of LA on the phosphorylation of both Akt and GSK-3 β . Levels of p-Akt and p-GSK-3 β rapidly increased and peaked at 30-60 min, and then declined at 90 min after LA treatment (Fig. 2A and 2B, respectively). Next, we compared the ratio of p-GSK-3 β to total GSK-3 β under various culture conditions (Fig. 2B). LA (60 min) significantly increased the levels of p-GSK-3 β /GSK-3 β . LPS alone tended to increase the expression levels of GSK-3 β . In the data of p-GSK-3 β /GSK-3 β ratio, LPS significantly activated GSK-3 β . LA restored the levels of the ratio of p-GSK-3 β /GSK-3 β to vehicle control levels.

3.3 LA inhibited LPS-induced nuclear translocation of RelA in BV-2 cells.

The gene expression of inflammatory mediators (e.g. cytokines and iNOS) can be modulated by NF- κ B. The most abundant form of NF- κ B is a heterodimer of p50 and RelA (p65) subunits, of which the RelA subunit contains the transcriptional activator domain. Therefore, we investigated the effect of LA on LPS-induced nuclear translocation of RelA. RelA proteins localized in nuclei were significantly higher 1-2 h after LPS treatment (Fig. 3A). A pre-treatment of LA significantly attenuated the LPS-mediated nuclear translocation of RelA in a concentration-dependent manner (200–400 μ M, Fig. 3B). These results indicated that the inhibition of RelA nuclear translocation by LA may have been one of the mechanisms responsible for the suppression of LPS-stimulated production of the inflammatory mediators determined previously. Moreover, the PI3K inhibitor, (LY), prevented the LA-mediated inhibition of LPS-stimulated nuclear translocation of RelA (Fig. 3C). Similar effects were also

observed with the GSK-3 β inhibitors, LiCl and GSK-3 β inhibitor VII (Fig. 3E), although either LiCl or GSK-3 β inhibitor VII alone did not change the levels of RelA compared with vehicle control (Fig. 3D). These results suggest that LA mimics as GSK-3 β inhibitors against LPS-induced inflammatory actions of RelA.

3.4 LA attenuated LPS-induced iNOS expression through Akt activation/GSK-3 β inactivation signaling in BV-2 cells.

To determine the role of p-Akt and p-GSK-3 β on LA-induced anti-inflammatory action, we used LY and LiCl or GSK-3 β inhibitor VII, respectively. Pretreatment of LY prevented LA-mediated suppression of iNOS expression during LPS exposure (Fig. 4A). Similar results were also observed by inactivating GSK-3 β by using LiCl or GSK-3 β inhibitor VII (Fig. 4B). Neither LiCl nor GSK-3 β inhibitor VII alone had an effect on iNOS levels (data not shown). These results suggested that the anti-inflammatory effects of LA in BV-2 cells acted via the activation of Akt and inactivation of GSK-3 β .

3.5 LA reduced microglial activation in an in vivo model of neuroinflammation in the mouse cortex.

To evaluate the anti-inflammatory effect of LA in vivo, we induced neuroinflammation using LPS (5 mg/kg), and evaluated the effects of LA, administered 3 days before the LPS treatment. Iba1 antibody was used to detect activated microglial cells (Qin et al. 2007). Light microscopy indicated that LPS treatment increased the number of Iba1-positive cells in the cortex (Fig. 5B) compared with the vehicle treatment group (Fig. 5A), which suggests microglial activation. A decrease in Iba1-positive cells was seen with 30 mg/kg and 60 mg/kg LA (Fig. 5C and 5D, respectively), which suggests a decrease in the number of activated microglia. When

quantified, pretreatment of mice with 30 and 60 mg/kg LA significantly reduced microglial activation in the cortex by 42.9 and 61.9%, respectively (Fig. 5E).

3.6 LA suppressed the expression of iNOS in LPS-induced inflammation in the mouse cortex.

Our final objective was to evaluate the effect of LA on the presence of iNOS during LPS exposure in vivo. Fig 6A reveals that the majority of cells expressing iNOS by LPS treatment were also positively stained for anti-Iba1, indicating that these Iba1-positive cells were indeed activated. Light microscopy indicated that LPS treatment increased the number of iNOS-positive cells in the cortex (Fig. 6D) compared with the vehicle treatment group (Fig. 6B), and LA reduced this number (Fig. 6E). No change was seen with lipoic acid alone for 3 days (Fig. 6C). Western blotting and quantification of cortical iNOS confirmed these results. The expression of iNOS was significantly reduced by LA treatment during LPS exposure (Fig. 6F). LA alone did not change the levels of iNOS compared to vehicle control.

4. Discussion

This study has revealed several interesting findings. First, we found that LA attenuated LPS-stimulated expression of the inflammatory mediator of iNOS, and another mediators, such as TNF α and IL-1 β (data not shown) in BV-2 cells. In this model of inflammation, LA activated Akt and inactivated GSK-3 β . Furthermore, LA inhibited LPS-stimulated RelA nuclear translocation via a dependent pathway that inactivated PI3K and GSK-3 β . Finally, we found that LA prevented LPS-induced microglial activation and iNOS expression in the mouse cortex.

4.1 Anti-inflammatory effect of LA in LPS-stimulated BV-2 cells.

Inflammation is closely associated with neurodegenerative diseases, such as AD (McGeer and McGeer 2003), PD (Barcia et al. 2003), post traumatic brain injury and brain ischemia (Rock and Peterson 2006). Prolonged activation of microglia by LPS may be directly toxic to neuronal cells because of the significant release of various inflammatory mediators, such as ROS and NO (Banati et al. 1993). Moreover, the release of NO and pro-inflammatory cytokines (e.g. TNF α and IL-1 β) from activated microglia may be important factors for neuroinflammatory-mediated involvement in neurodegenerative diseases. Evidence to support this hypothesis has revealed that high levels of NO generated by iNOS are regarded to be key for inflammation and neurotoxicity after its conversion to peroxynitrite (via the superoxide anion). The expression of pro-inflammatory cytokines (TNF α and IL-1 β) and iNOS in BV-2 cells is regulated by NF- κ B (Jin et al. 2006). Additionally, up-regulation of LPS-stimulated iNOS-mediated production of NO and increased levels of TNF α and IL-1 β are attenuated by the inhibition of NF- κ B (Xie et al. 1994). Therefore, in the present study, we investigated the expression of pro-inflammatory genes and NF- κ B signaling pathways to clarify the mechanism of action of LA in LPS-induced inflammation by activated glia. As expected, LA significantly inhibited LPS-induced production of inflammatory mediators in BV-2 cells. Furthermore, LA blocked the nuclear translocation of the RelA subunit.

Elevated levels of oxidative stress play an important role in chronic inflammation. As a result, LA has been studied for its antioxidant properties, occurring via the inhibition of NF- κ B in cytokine-induced inflammation (Shay et al. 2009). Lee et al. reported that activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling followed by induced expression of antioxidative enzymes, such as heme oxygenase-1

(HO-1), showed anti-inflammatory activity (Lee and Chau 2002). We recently reported that LA stimulated Nrf2 signaling and promoted the expression of HO-1 in RGC-5 cells (Koriyama et al. 2013). In our data, LY partially cancelled the LA-induced anti-inflammatory activity (Fig. 3B and 4A). Therefore, the proposed link of the anti-inflammatory action of LA occurring via the antioxidative pathway may be involved. As a result, the mechanism by which LA induced an anti-inflammatory action may have been upstream of LPS-mediated nuclear translocation of RelA.

4.2 The anti-inflammatory activity of LA involves Akt activation/GSK-3 β inactivation in BV-2 cells.

We clearly showed that LA phosphorylated both Akt and GSK-3 β in BV-2 cells. Inflammatory cytokine production is promoted by GSK-3 β (Martin et al. 2005, Beurel and Jope 2009). GSK-3 β is a highly conserved Ser/Thr protein kinase that is constitutively active (Cohen and Frame 2001; Jope and Jhonson 2004). Akt is a key mediator for the phosphorylation of the PI3K pathway. Activation of Akt phosphorylates (Ser473) several downstream targets of the PI3K pathway, including GSK-3 β (Ser9). Phosphorylation of this target results in its inhibition (Cross et al. 1995). GSK-3 β can both positively and negatively affect a variety of transcription factors that are critical in regulating the production of pro- and anti-inflammatory cytokines (Hoefflich et al. 2000; Grimes and Jope 2001; Németh et al. 2002). Our present results demonstrate that inhibition of GSK-3 β in LPS-exposed BV-2 cells prevents the up-regulation of NF- κ B nuclear translocation and subsequent expression of iNOS. Therefore, these findings strongly suggest that LA-mediated inactivation of GSK-3 β (i.e. via Ser9 phosphorylation) plays a role in the anti-inflammatory response. Further evidence for such a role is shown by Huang et al. (2009) with their identification of

GSK-3 β activation in the BV-2 cells in response to LPS stimulation. Furthermore, they demonstrate that GSK-3 β inhibition blocks the production of NO and iNOS in LPS-exposed microglia.

NF- κ B activity is precisely regulated by its interaction with the inhibitor of kappa B (I κ B) (Baldwin 1996). NF- κ B stimulates the phosphorylation of I κ B by I κ B kinase (IKK) and ubiquitin-dependent proteolysis of I κ B, releasing NF- κ B for nuclear translocation and subsequent transcriptional activation of NF- κ B-regulated genes. Genetic depletion of GSK-3 β suppresses inflammation-induced activation of IKK, and I κ B phosphorylation and ubiquitination (Takada et al. 2004). In addition, LiCl-induced inhibition of GSK-3 β results in decreased NF- κ B activity (Grimes and Jope 2001). These reports confirm our hypothesis of an anti-inflammatory action of LA, via the inactivation-dependent effect on GSK-3 β during LPS exposure to BV-2 cells. Several studies have reported that LA phosphorylates Akt in various cellular models (Zhang et al. 2007; Wang et al. 2011; Yamada et al. 2011; Deslauriers et al., 2012, Koriyama et al. 2013). However, the mechanism of the anti-inflammatory action of LA via Akt and/or GSK-3 β in BV-2 cells had not been clarified. In the current study, we used iNOS as a representative inflammatory mediator, and furthermore showed that its production was inhibited by LA-mediated down-regulation of nuclear translocation of RelA during LPS exposure to BV-2 cells. Additionally, this effect by LA was reversed using PI3K inhibitor or mimicked with either LiCl or GSK-3 β inhibitor VII. Surprisingly however, LPS alone increased the levels of GSK-3 β , a result also found by Green et al. (2012). The regulation of transcriptional activation of GSK-3 β may be via genomic and non-genomic mechanisms (Rubio-Patiño et al. 2012). Our findings showed that LA

reversed LPS-activation of GSK-3 β and promoted anti-inflammatory action. One of the mechanisms of this effect was found to be via Akt activation and GSK-3 β inactivation.

4.3 Possibility of LA as an anti-inflammatory agent

Increased levels of inflammatory mediators, particularly cytokines and activated microglia have been reported in many neurodegenerative diseases (Beurel 2011). In addition, GSK-3 β is involved in the pathology of neurodegenerative diseases. Therefore, interventions using LA as an inhibitor of GSK-3 β may be beneficial, by reducing the potential detrimental effects of inflammation concomitantly occurring with neurodegenerative processes. Aging-related neurological diseases are associated with a wide range of pathophysiological changes eventually leading to compromised cognitive status (Farkas and Luiten 2001). Interestingly, endogenous levels of LA have been found to be diminished during aging (Palaniappan and Dai 2007) and thus, may play a role in cognitive decline.

Overall, our study provides new insights into the effects of LA during LPS-induced inflammation, in which its mechanism of anti-inflammatory action occurs via Akt activation and GSK-3 β inactivation in BV-2 cells.

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References

- Baldwin, A.S. Jr., 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Ann, Rev. Immunol.* 14, 649-683.
- Banati, R.B., Gehrmann, J., Schubert, P., Kreutzberg, G.W. 1993. Cytotoxicity of microglia. *Glia* 7, 111-118.
- Barcia, C., Fernández-Barreiro, A., Poza, M., Herrero, M.T. 2003. Parkinson's disease and inflammatory changes. *Neurotox. Res.* 5, 411-418.
- Beurel, E., Jope, R.S., 2009. Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *J. Neuroinflammation.* doi: 10.1186/1742-2094-6-9.
- Beurel, E. 2011. Regulation by glycogen synthase kinase-3 of inflammation and T cells in CNS diseases. *Front. Mol. Neurosci.* 4, 3-10.
- Cohen, P., Frame, S. 2001. The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* 2, 769-776.
- Corona, A.W., Norden, D.M., Skendelas, J.P., Huang, Y., O'Connor, J.C., Lawson, M., Dantzer, R., Kelley, K.W., Godbout, J.P, 2012. Indoleamine 2,3-dioxygenase inhibition attenuates lipopolysaccharide induced persistent microglial activation and depressive-like complications in fractalkine receptor (CX(3)CR1)-deficient mice. *Brain. Behav. Immun.* (in press)
- Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785-789.
- Deslauriers, J., Desmarais, C., Sarret, P., Grignon, S. 2012. α -Lipoic Acid Interaction with Dopamine D2 Receptor-Dependent Activation of the Akt/GSK-3 β Signaling Pathway Induced by Antipsychotics: Potential Relevance for the Treatment of

- Schizophrenia. *J. Mol. Neurosci.* (in press)
- Dheen, S.T., Kaur, C., Ling, E.A. 2007. Microglial activation and its implications in the brain diseases. *Curr. Med. Chem.* 14, 1189-1197.
- Farkas, E., Luiten, P.G. 2001. Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog. Neurobiol.* 64, 575-611.
- Goraca, A., Aslanowicz-Antkowiak, K. 2009. Prophylaxis with alpha-lipoic acid against lipopolysaccharide-induced brain injury in rats. *Arch. Immunol. Ther. Exp.* 57.141-146.
- Green, H.F., Nolan, Y.M. 2012. GSK-3 mediates the release of IL-1 β , TNF- α and IL-10 from cortical glia. *Neurochem. Int.* 61, 666-671.
- Grimes, C.A., Jope, R.S. 2001. CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. *J. Neurochem.* 78, 1219-1232.
- Grimes, C.A., Jope, R.S. 2001. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog. Neurobiol.* 65, 391-426.
- Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., Woodgett, J.R. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406, 86-90.
- Huang, W.C., Lin, Y.S., Wang, C.Y., Tsai, C.C., Tseng, H.C., Chen, C.L., Lu, P.J., Chen, P.S., Qian, L., Hong, J.S., Lin, C.F. 2009. Glycogen synthase kinase-3 negatively regulates anti-inflammatory interleukin-10 for lipopolysaccharide-induced iNOS/NO biosynthesis and RANTES production in microglial cells. *Immunology* 128, e275-286.
- Jin, C.Y., Moon, D.O., Lee, K.J., Kim, M.O., Lee, J.D., Choi, Y.H., Park, Y.M., Kim, G.Y. 2006. Piceatannol attenuates lipopolysaccharide-induced NF-kappaB activation

- and NF-kappaB-related proinflammatory mediators in BV2 microglia. *Pharmacol. Res.* 54, 461-467.
- Jin, C.Y., Lee, J.D., Park, C., Choi, Y.H., Kim, G.Y. 2007. Curcumin attenuates the release of pro-inflammatory cytokines in lipopolysaccharide-stimulated BV2 microglia. *Acta. Pharmacol. Sin.* 28, 1645-1651.
- Joep, R.S., Johnson, G.V. 2004. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem. Sci.* 29, 95-102.
- Kang, G., Kong, P.J., Yuh, Y.J., Lim, S.Y., Yim, S.V., Chun, W., Kim, S.S. 2004. Curcumin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression by inhibiting activator protein 1 and nuclear factor kappaB bindings in BV2 microglial cells. *J. Pharmacol. Sci.* 94. 325-328.
- Kamenova, P. 2006. Improvement of insulin sensitivity in patients with type 2 diabetes mellitus after oral administration of alpha-lipoic acid. *Hormones* 5. 251-258.
- Koriyama, Y., Yasuda, R., Homma, K., Mawatari, K., Nagashima, M., Sugitani, K., Matsukawa, T., Kato, S. 2009a. Nitric oxide-cGMP signaling regulates axonal elongation during optic nerve regeneration in the goldfish in vitro and in vivo. *J. Neurochem.* 110, 890-901.
- Koriyama, Y., Ohno, M., Kimura, T., Kato, S., 2009b. Neuroprotective effects of 5-S-GAD against oxidative stress-induced apoptosis in RGC-5 cells. *Brain Res.* 1296, 187-195.
- Koriyama, Y., Nakayama, Y., Matsugo, S., Kato, S. 2013. Protective effect of lipoic acid against oxidative stress is mediated by Keap1/Nrf2-dependent heme oxygenase-1 induction in the RGC-5 cell line. *Brain Res.* 1499, 145-147.
- Lee, I.S., Lim, J., Gal, J., Kang, J.C., Kim, H.J., Kang, B.Y., Choi, H.J. 2011.

- Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. *Neurochem. Int.* 58, 153-160.
- Lee, T.S., Chau, L.Y. 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat. Med.* 8, 240-246.
- Martin, M., Rehani, K., Jope, R.S., Michalek, S.M. 2005. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* 6, 777-784.
- McGeer, E.G., McGeer, P.L. 2003. Inflammatory processes in Alzheimer's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 741-749.
- McCarty, M.F. 2006. Down-regulation of microglial activation may represent a practical strategy for combating neurodegenerative disorders. *Med. Hypotheses* 67, 251-269.
- Nam, K.N., Choi, Y.S., Jung, H.J., Park, G.H., Park, J.M., Moon, S.K., Cho, K.H., Kang, C., Kang, I., Oh, M.S., Lee, E.H. 2010. Genipin inhibits the inflammatory response of rat brain microglial cells. *Int. Immunopharmacol.* 10, 493-499.
- Németh, Z.H., Deitch, E.A., Szabó, C., Fekete, Z., Hauser, C.J., Haskó, G. 2002. Lithium induces NF-kappa B activation and interleukin-8 production in human intestinal epithelial cells. *J. Biol. Chem.* 277, 7713-7719.
- Palaniappan, A.R., Dai, A. 2007. Mitochondrial ageing and the beneficial role of alpha-lipoic acid. *Neurochem. Res.* 32, 1552-1558.
- Paxinos, G. and Franklin, K.B.J., 2001. *The Mouse Brain in Stereotaxic Coordinates*, Second Edition, Academic Press, San Diego.
- Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.S., Knapp, D.J., Crews, F.T. 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55, 453-462.

- Rock, R.B., Peterson, P.K. 2006. Microglia as a pharmacological target in infectious and inflammatory diseases of the brain. *J. Neuroimmune Pharmacol.* 1, 117-126.
- Rubio-Patiño, C., Palmeri, C.M., Pérez-Perarnau, A., Cosiàlls, A.M., Moncunill-Massaguer, C., González-Gironès, D.M., Pons-Hernández, L., López, J.M., Ventura, F., Gil, J., Pons, G., Iglesias-Serret, D. 2012. Glycogen synthase kinase-3 β is involved in ligand-dependent activation of transcription and cellular localization of the glucocorticoid receptor. *Mol. Endocrinol.* 26, 1508-1520.
- Shay, K.P., Moreau, R.F., Smith, E.J., Smith, A.R., Hagen, T.M. 2009. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. *Biochim. Biophys. Acta.* 1790,1149-1160.
- Smith, A.R., Shenvi, S.V., Widlansky, M., Suh, J.H., Hagen, T.M. 2004. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr. Med. Chem.* 11. 1135-1146.
- Takada, Y., Fang, X., Jamaluddin, M.S., Boyd, D.D., Aggarwal, B.B. 2004. Genetic deletion of glycogen synthase kinase-3 β abrogates activation of IkappaB α kinase, JNK, Akt, and p44/p42 MAPK but potentiates apoptosis induced by tumor necrosis factor. *J. Biol. Chem.* 279, 39541-39554.
- Thirunavukkarasu, V., Anitha-Nandhini, A.T., Anuradha, C.V. 2004. Lipoic acid attenuates hypertension and improves insulin sensitivity, kallikrein activity and nitrite levels in high fructose-fed rats. *J. Comp. Physiol. B* 174, 587-592.
- Wang, X., Wang, Z., Yao, Y., Li, J., Zhang, X., Li, C., Cheng, Y., Ding, G., Liu, L., Ding, Z. 2011. Essential role of ERK activation in neurite outgrowth induced by α -lipoic acid. *Biochim. Biophys. Acta.* 1813, 827-838.
- Xie, Q.W., Kashiwabara, Y., Nathan, C. 1994. Role of transcription factor NF-kappa

B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* 269, 4705-4708.

Yamada, T., Hashida, K., Takarada-Iemata, M., Matsugo, S., Hori, O. 2011. α -Lipoic acid (LA) enantiomers protect SH-SY5Y cells against glutathione depletion. *Neurochem. Int.* 59, 1003-1009.

Zhang, W.J., Wei, H., Hagen, T., Frei, B. 2007. Alpha-lipoic acid attenuates LPS-induced inflammatory responses by activating the phosphoinositide 3-kinase/Akt signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4077-4082.

Figure legends

Fig. 1 LA decreases LPS-induced expression of iNOS and nitrite production in BV-2 cells. (A) Cells were treated for 8 h with 200 ng/ml of LPS, followed by RT-PCR analysis. LPS treatment at differing exposure times increased the mRNA of iNOS. (B) BV-2 cells were pretreated (30 min) with LA (200 μ M or 400 μ M) followed by LPS (200 ng/ml) for 8 h, and RT-PCR analysis was performed thereafter for iNOS. (C) Western blotting for iNOS and subsequent quantification. (D) Measurement of nitrite production by the Griess assay. * $p < 0.05$ vs vehicle control, ** $P < 0.01$ vs vehicle control, [†] $p < 0.01$ vs LPS alone, ^a $p < 0.01$ vs LA (200 μ M), ^b $p < 0.01$ vs LA (400 μ M) (n=3).

Fig. 2 LA activates Akt and inactivates GSK-3 β in LPS-exposed BV-2 cells. (A) Time course of p-Akt and p-GSK-3 β after LA treatment. Cells were treated with 400 μ M LA for various times until 90 min. Levels of activated Akt and inactivated GSK-3 β rapidly increased at 30 min, peaked at 30–60 min and then declined at 90 min after LA

treatment. (B) LA reversed LPS-mediated activation of GSK-3 β . Cells were pretreated with LA for 30 min and treated with or without LPS (30 min). *p < 0.01 vs vehicle control, ⁺p < 0.01 vs LPS alone, #P < 0.01 vs LA alone (n=3).

Fig. 3 Suppressive effect of LA on nuclear translocation of RelA induced by LPS occurs via PI3K- and GSK-3 β - dependent mechanisms in BV-2 cells. (A) Time course of RelA nuclear translocation by LPS (200 ng/ml). *p < 0.05 vs vehicle control, **p < 0.01 vs vehicle control. (B) Nuclear translocation of RelA protein in BV-2 cells after LPS (200 ng/ml) treatment with or without the LA pre-treatment (30 min). * p < 0.05 vs vehicle control, **p < 0.01 vs vehicle control, ⁺p < 0.01 vs LPS alone (n=3). (C) PI3K inhibitor prevented the inhibition of LPS-induced RelA nuclear translocation by LA. BV-2 cells were pretreated (30 min) with LA (400 μ M) or LY (10 μ M, 40 min) followed by LPS (2 h), and levels of translocated RelA were measured. * p < 0.01 vs vehicle control, ⁺p < 0.01 vs LPS alone, # p < 0.01 vs LPS plus LA, ^ap < 0.01 vs LA (400 μ M) (n=3). (D) GSK-3 β inhibitors had no effect on RelA levels of vehicle control. (E) GSK-3 β inhibitors prevented LPS-induced nuclear translocation of RelA. Cells were pretreated (30 min) with LiCl (10 mM) or GSK-3 β inhibitor VII (1 μ M) followed by LPS (2 h). Levels of nuclei-containing RelA were then measured. *p < 0.01 vs vehicle control, ⁺p < 0.01 vs LPS alone (n=3).

Fig. 4 LA prevents LPS-induced expression of iNOS via PI3K and by inhibitors of GSK-3 β in BV-2 cells. (A) PI3K inhibitor prevented the inhibition of LPS-induced iNOS expression by LA in BV-2 cells. BV-2 cells were pretreated (30 min) with LA (400 μ M) with or without LY (10 μ M, 40 min) followed by LPS treatment (8 h).

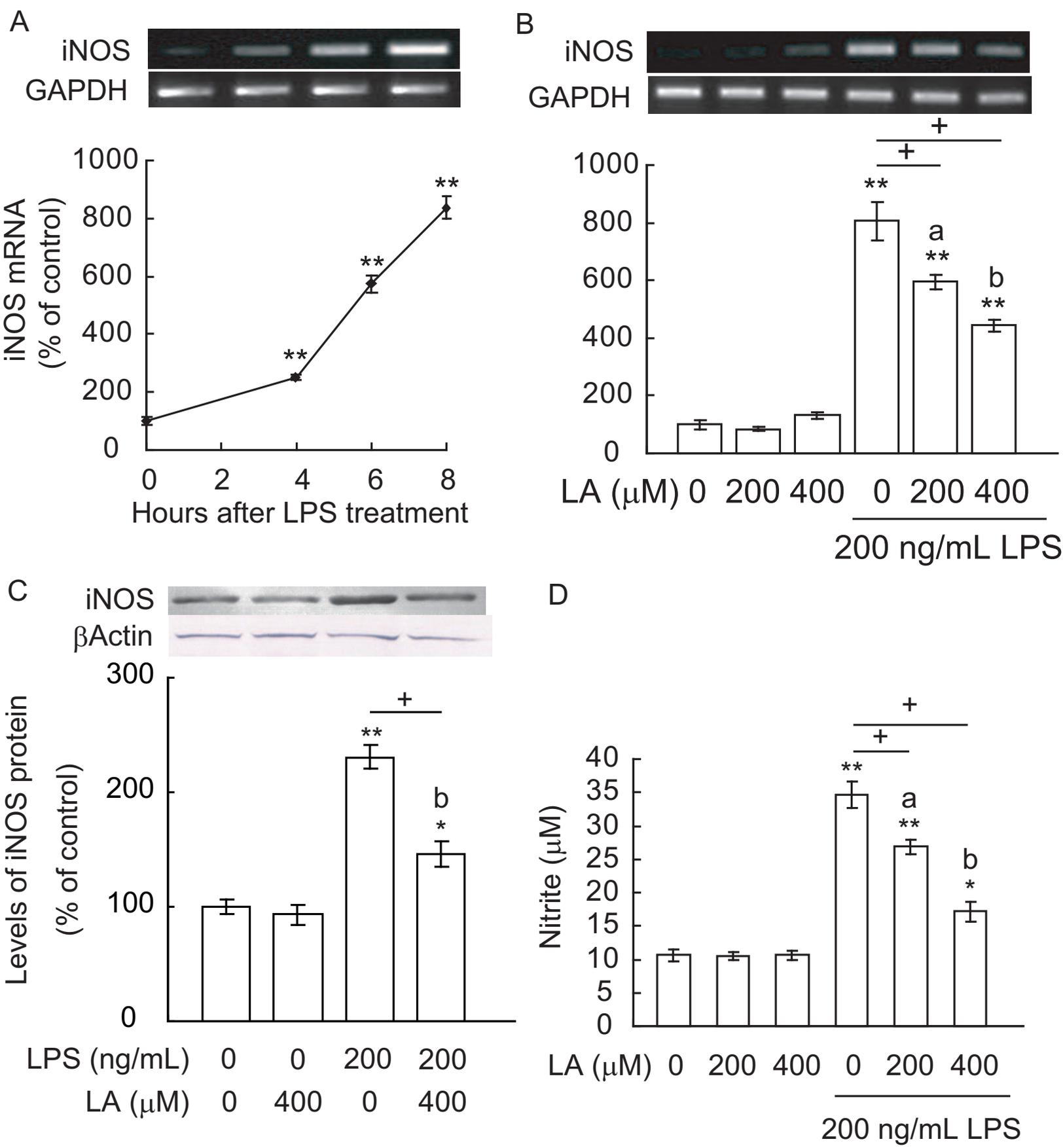
Western blot analysis was then performed. * $p < 0.05$ vs vehicle control, ** $p < 0.01$ vs vehicle control, ⁺ $p < 0.01$ vs LPS alone, [#] $p < 0.01$ vs LPS plus LA (n=3). (B) GSK-3 β inhibitors reversed LPS-induced expression of iNOS in BV-2 cells. Cells were pretreated (30 min) with LiCl (10 mM) or GSK-3 β inhibitor VII (1 μ M) for 30 min followed by LPS incubation (8 h). Western blot analysis was then performed. * $p < 0.05$ vs vehicle control, ** $p < 0.01$ vs vehicle control, ⁺ $p < 0.05$ vs LPS alone, ⁺⁺ $p < 0.01$ vs LPS plus LA (n=3).

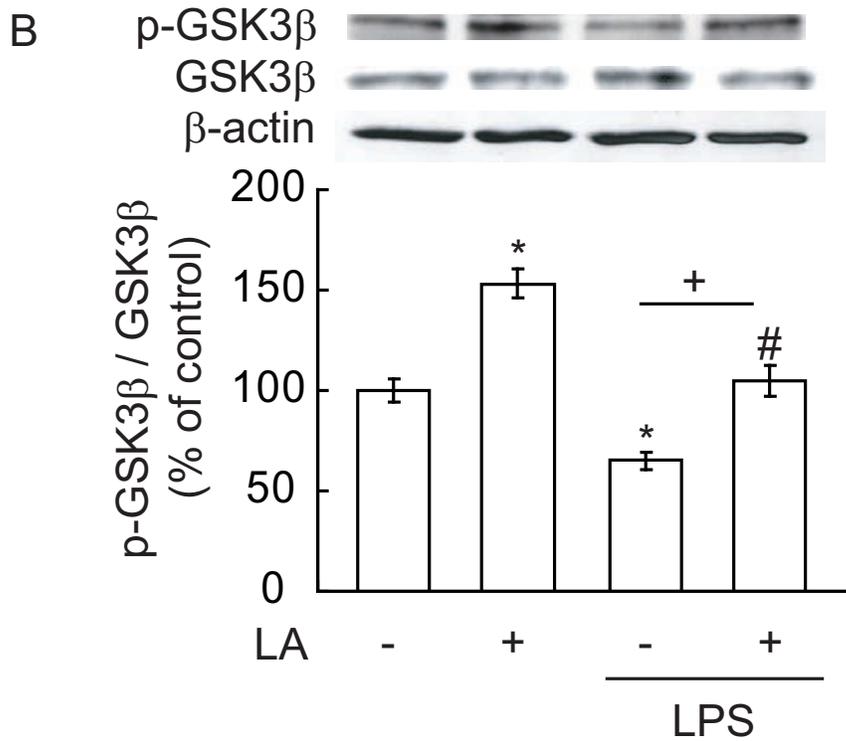
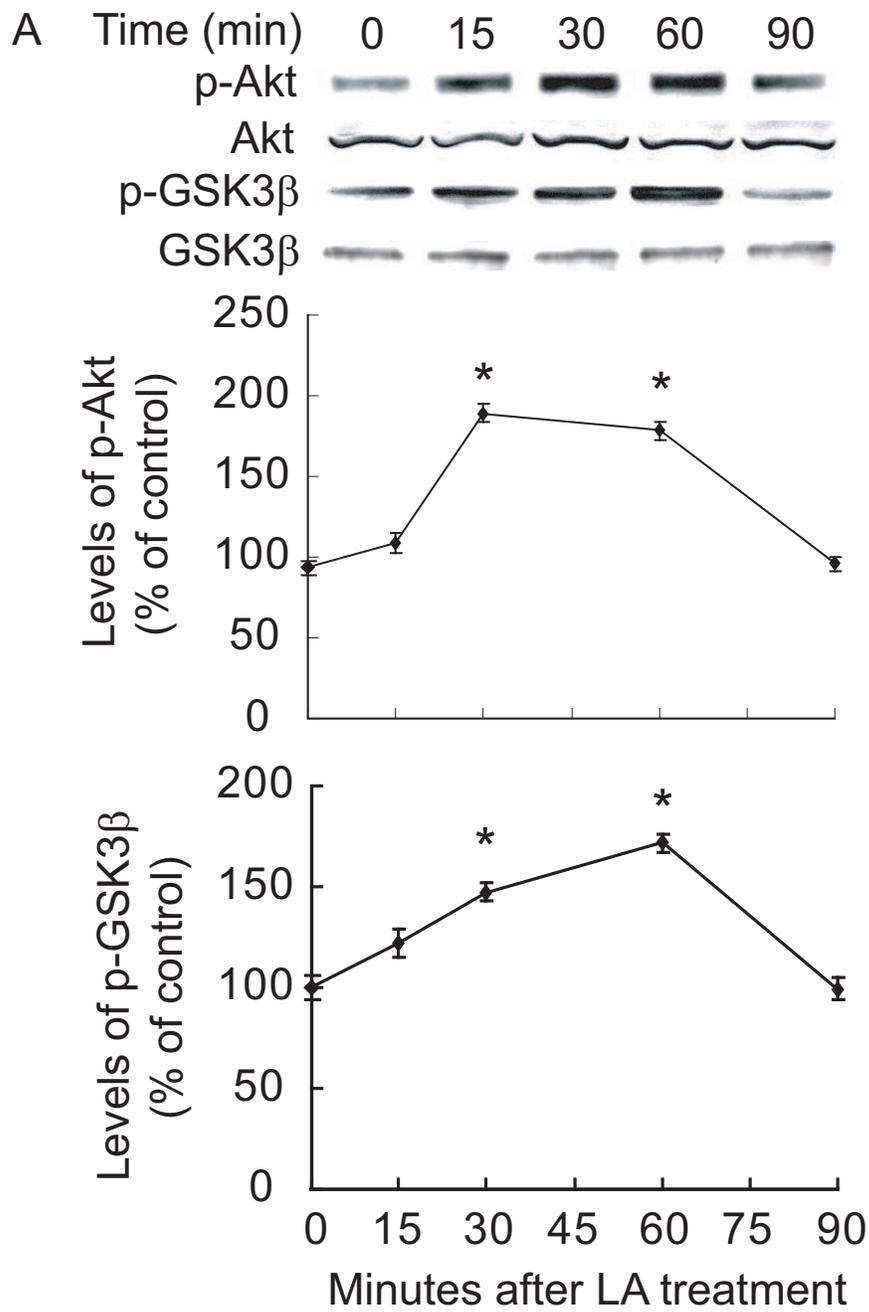
Fig. 5 Effect of LA on microglial activation in a mouse model of LPS-induced neuroinflammation. LA was administered intraperitoneally once per day for 3 days before LPS treatment (5 mg/kg, 3 days). Representative photomicrographs of microglial activation in the cortex of each group: (A) Vehicle control, (B) 5 mg/kg LPS, (C) LPS plus LA (30 mg/kg), (D) LPS (5 mg/kg) plus LA (60 mg/kg). (E) Quantification of microglial activation was performed by counting the number of Iba1-immunoreactive cells in the cortex. Data were presented as a percentage of the vehicle control group value. * $p < 0.01$ vs vehicle control, ⁺ $p < 0.05$ vs LPS alone, ^a $p < 0.01$ vs LA (30 mg/kg), ^b $p < 0.01$ vs LA (60 mg/kg) (n=3). (n=12). Scale= 50 μ m.

Fig. 6 LA inhibits LPS-induced iNOS expression in mouse cortex. (A) LPS-induced iNOS expression in Iba1-positive microglia in the cortex. The localization of iNOS expression in cortex was confirmed using the microglial marker, Iba1. LA was administered intraperitoneally once per day for 3 days before LPS treatment (5 mg/kg, 3 days). Representative photomicrographs iNOS expression in the cortex of each group: (B) Vehicle control, (C) LA (60 mg/kg), (D) LPS (5 mg/kg), (E) LPS plus LA (60

mg/kg). (F) Quantification of iNOS expression was performed by western blot of cortical tissue. *p < 0.05 vs vehicle control, **p < 0.01 vs vehicle control, ⁺p < 0.05 vs LPS alone, ^ap < 0.01 vs LA (n=3). Scale= 20 μm.

Fig.1





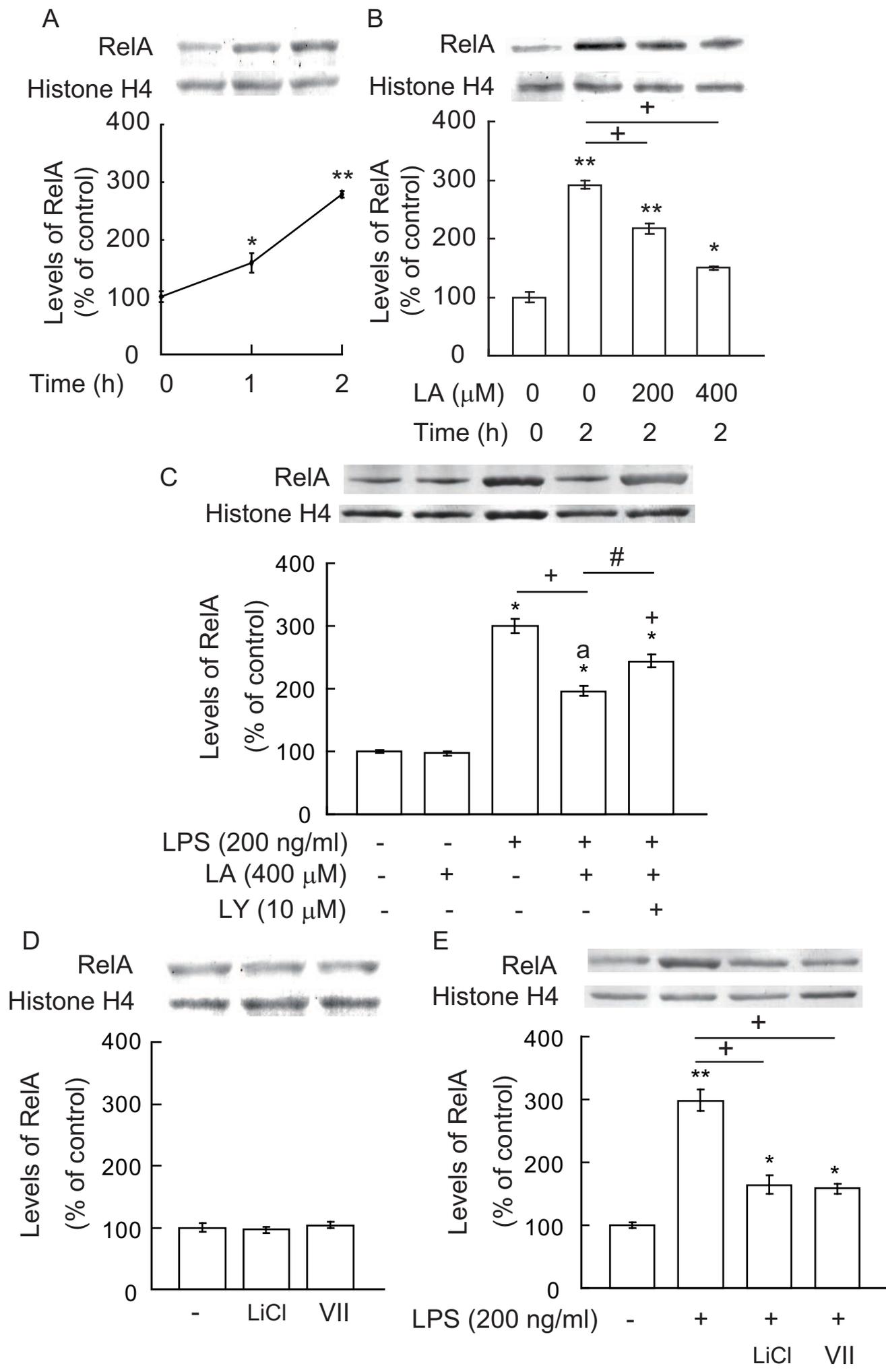
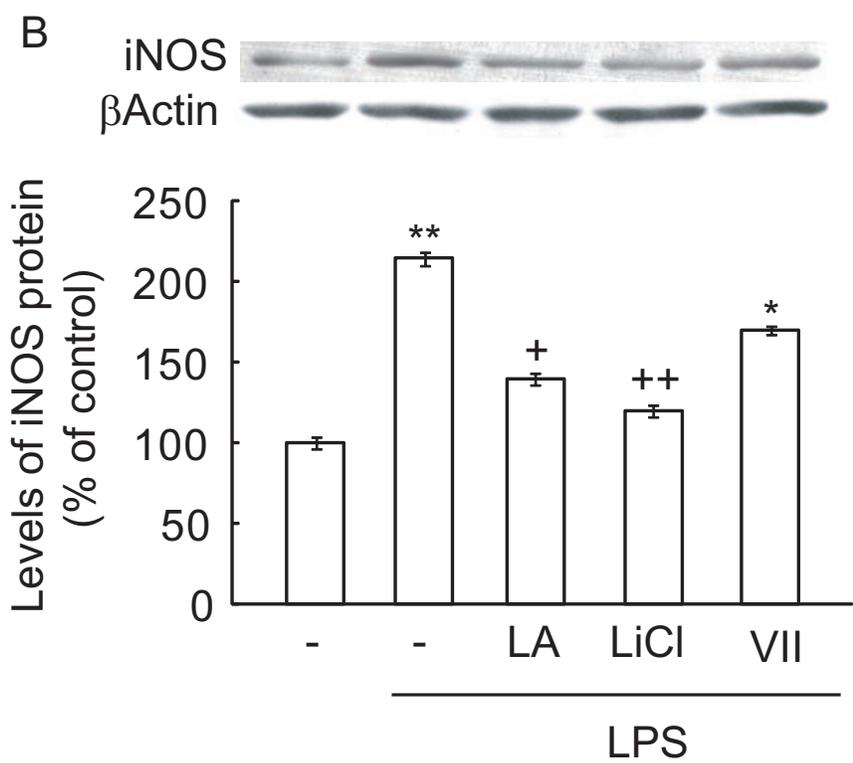
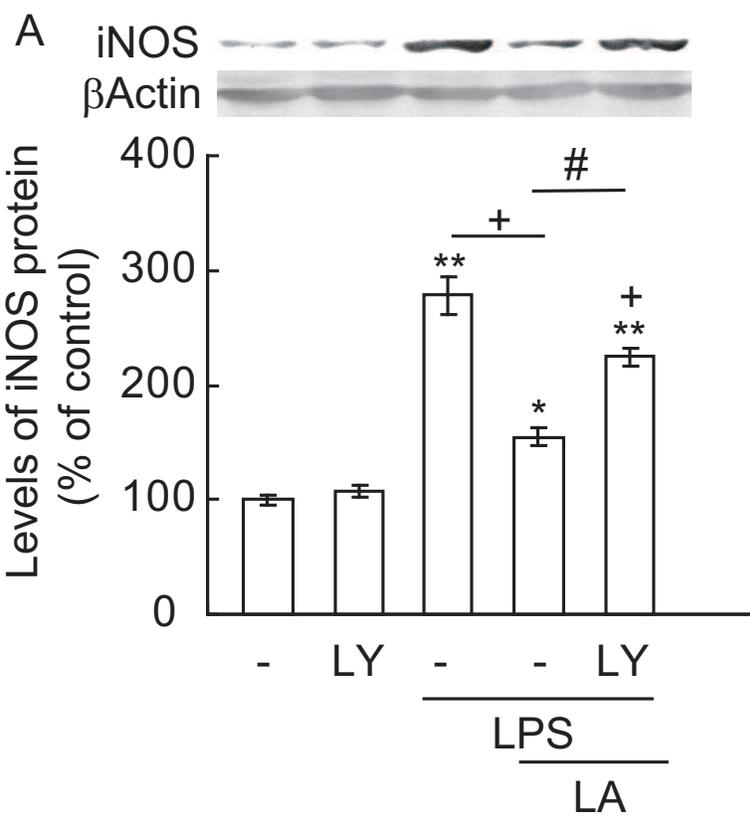


Fig. 4



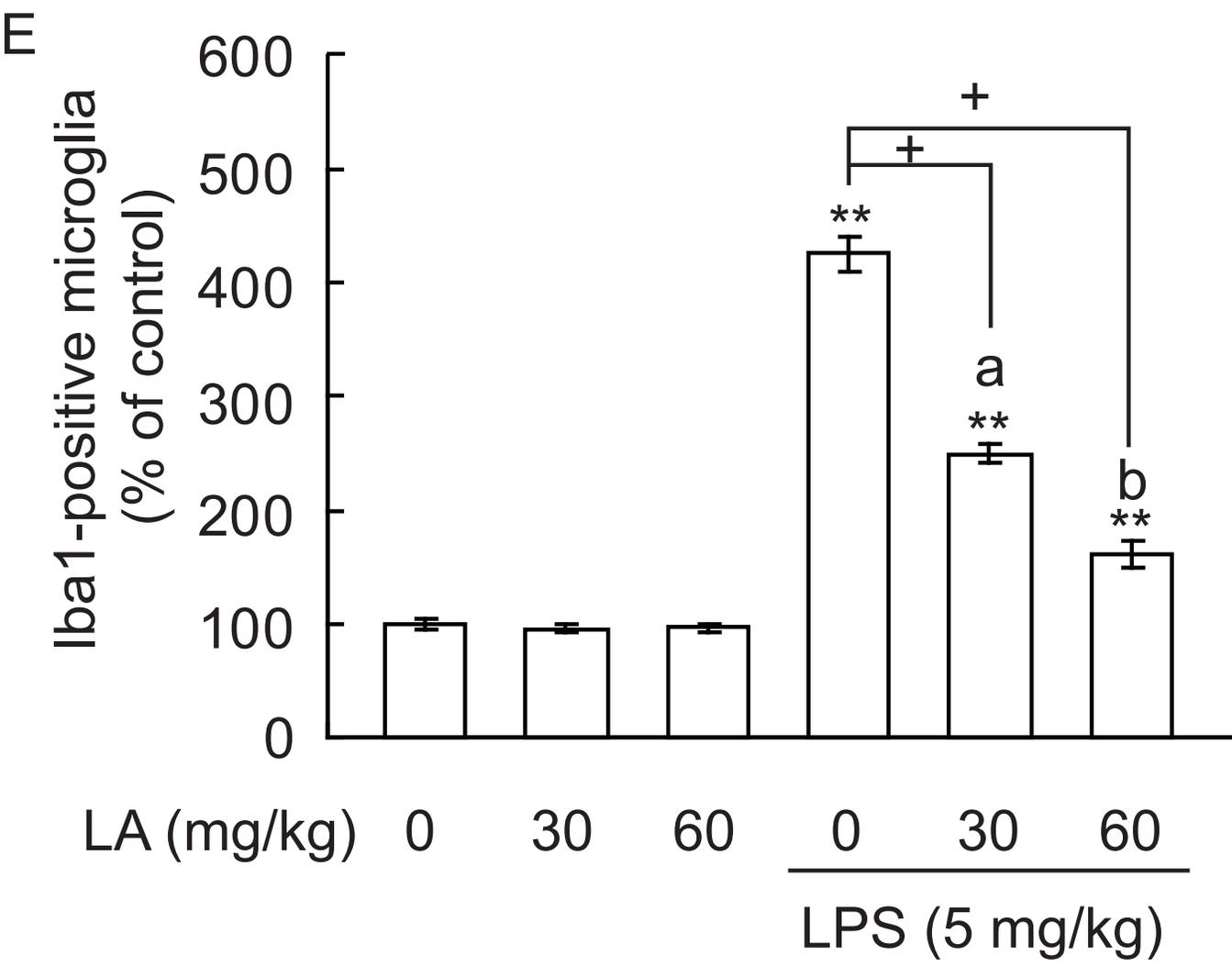
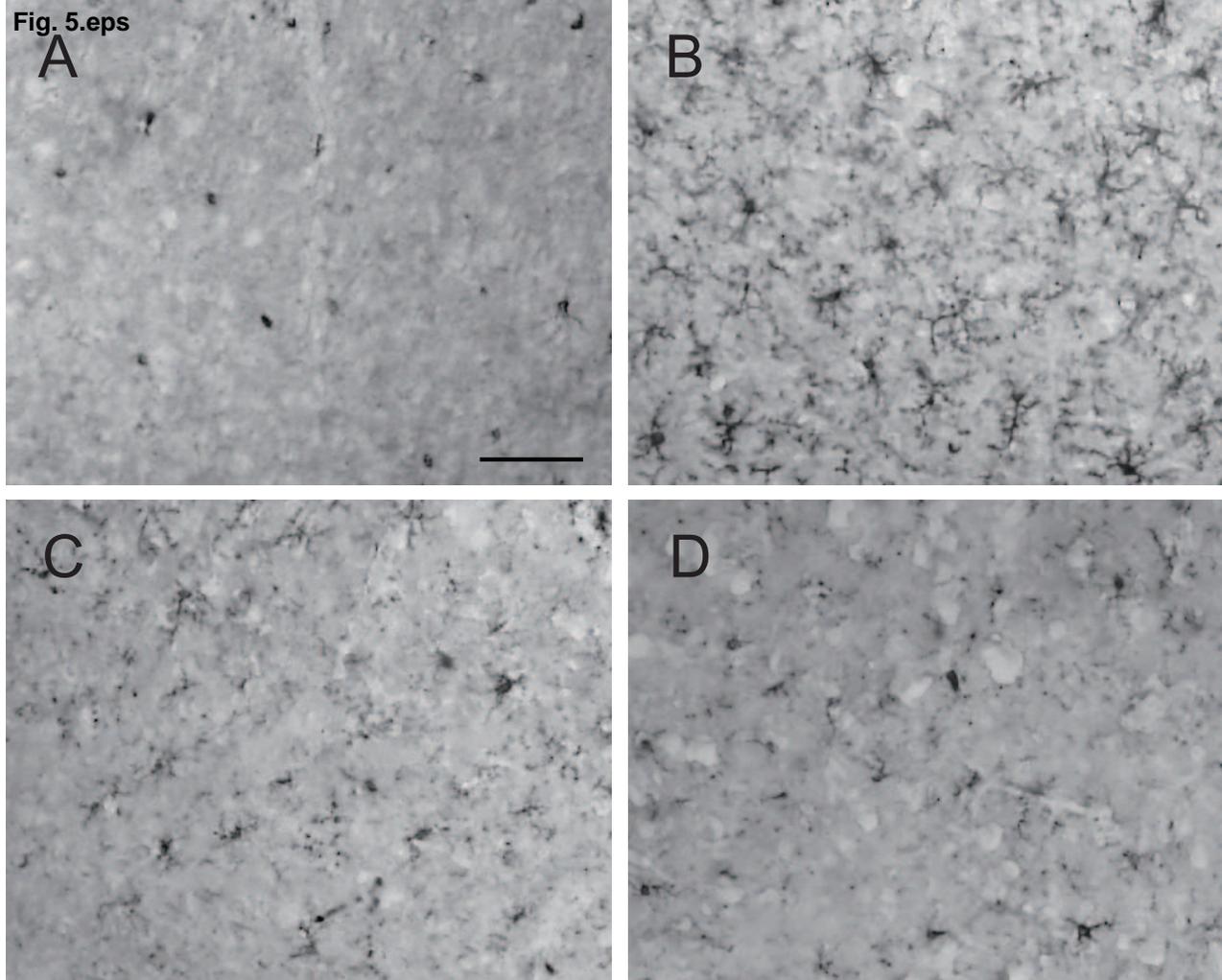


Fig. 6

