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Lancemaside A inhibits microglial activation via modulation of JNK signaling pathway

Yeon-Hui Jeong [a], Ji-Sun Jung [a], Thi Kim Van Le [b], Dong-Hyun Kim [b], Hee-Sun Kim [a,*]

[a] Department of Molecular Medicine, Tissue Injury Defense Research Center, Ewha Womans University Medical School, Seoul, Republic of Korea
[b] Department of Life and Nanopharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Seoul, Republic of Korea

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ABSTRACT

Microglial activation plays an important role in neurodegenerative diseases. Thus, controlling microglial activation is considered to be a promising therapeutic target for neurodegenerative diseases. In the present study, we found that lancemaside A, a triterpenoid saponin isolated from *Codonopsis lanceolata*, inhibited iNOS and proinflammatory cytokines in LPS-stimulated BV2 microglial cells. By analyzing molecular mechanisms underlying the anti-inflammatory effects of lancemaside A, we found that lancemaside A selectively inhibited LPS-induced JNK phosphorylation among the three types of MAP kinases. A JNK-specific inhibitor, SP600125, like lancemaside A, significantly inhibited not only NO, TNF-α, and IL-6 productions, but also NF-κB and AP-1 activities, suggesting that JNK inhibition is largely involved in the anti-inflammatory mechanism of lancemaside A. Interestingly, both the lancemaside A and SP600125 inhibited ROS production by suppressing the expression and/or phosphorylation of NADPH oxidase subunit proteins, such as $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$. The antioxidant effects of lancemaside A and SP600125 appear to be related with an increase of hemeoxygenase-1 expression by both agents. Finally, we demonstrated the neuroprotective effects of lancemaside A and SP600125 in microglia-neuron coculture systems. Collectively, our data suggest that JNK pathway plays a key role mediating anti-inflammatory effects of lancemaside A in LPS-stimulated microglia.

1. Introduction

Microglia are the resident macrophages in the brain, which constitute an immune surveillance system by spreading over the brain parenchyma. In the healthy brain, microglia play an important role in the normal brain development and adult physiology. They have highly motile processes and directly contacts synaptic elements and exchange molecular signals. In addition, microglia are involved in phagocytosis of synaptic elements and newborn cells, contributing to the reconstruction of neural circuits [1]. The microglia are activated by various stress and brain injury, and provide support signals leading to neuronal recovery. However, overactivation of microglia results in neuronal death, which is associated with neurodegenerative diseases such as multiple sclerosis, Parkinson's disease and Alzheimer's disease [2,3]. Thus, the development of agents that can control microglial activation has been suggested as one of the important strategies as a treatment for neurodegenerative diseases.

Lancemaside A, a triterpenioid saponin, is the main constituent of *Codonopsis lanceolata* (family Campanulaceae). The rhizome of *C. lanceolata* has been used in herbal medicines in Asian countries for inflammatory disease, such as bronchitis and cough [4]. Lancemaside A, after orally administered into mice, is metabolized to echinocystic acid by intestinal microflora, so that echinocystic acid is usually detected in the blood [5]. Recent studies reported that lancemaside A potently inhibited colitis via toll like receptor (TLR)-linked NF-κB activation in mice [6]. Lancemaside A also inhibited an interaction between LPS and TLR4, as well as IRAK-4 in peritoneal macrophage [7]. Furthermore, lancemaside A and echinocystic acid ameliorated scopolamine-induced memory and learning deficits in mice [8].

However, the effects of lancemaside A in activated microglia have not been reported until now. Moreover, the detailed molecular mechanisms underlying the anti-inflammatory effects of lancemaside A are not completely understood. Therefore, in the present study, we investigated the effect of lancemaside A in LPS-stimulated BV2 microglial cells and analyzed detailed molecular mechanism. Here, we demonstrate that lancemaside A exerts

Abbreviations: AP-1, activator protein-1; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; HO-1, hemeoxygenase-1; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase.

* Corresponding author. Address: Department of Molecular Medicine, Ewha Womans University School of Medicine, Mok-6-dong 911-1, Yangchun-Ku, Seoul 158-710, Republic of Korea. Fax: +82 2 2653 8891.
E-mail address: hskimp@ewha.ac.kr (H.-S. Kim).

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anti-inflammatory, antioxidant and neuroprotective effects, mainly by modulating the JNK signaling pathway.

2. Materials and methods

2.1. Reagents and antibodies

Lancemaside A, a main constituent of *C. lanceolata*, was isolated according to the previous method [5]. All reagents used for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). LPS and Trizol reagent were obtained from Sigma–Aldrich (St. Louis, MO, USA). All the reagents and enzymes for RT-PCR were purchased from Promega (Madison, WI, USA). Antibodies against COX-2 and iNOS were obtained from BD Bioscience (San Diego, CA, USA). Antibodies against phospho-/total form of MAP kinases and AKT were purchased form Cell Signaling Technology (Beverley, MA, USA). Antibody for p-p47^{phox} [anti-phospho-(Ser345)] was purchased from Assay Biothechnology Company Inc. (Sunnyvale, CA, USA).

2.2. Microglia, neuronal cell cultures and cell viability test

The immortalized murine BV2 microglial and Neuro-2a mouse neuroblastoma cell lines [9] were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, streptomycin (10 μg/ml), and penicillin (10 U/ml) at 37 °C. Co-culture of microglia and neuronal cells were performed, using Transwell cell culture inserts (Nunc, Naperville, IL, USA). In brief, BV2 microglial cells on the microporous membrane of the Transwell cell culture inserts were treated with LPS (100 ng/ml) for 6 h, then placed into wells containing the Neuro-2a cells. After 24–48 h of incubation, the neuronal cell viability was checked. To examine the effect of lancemaside A or SP600125, microglia were treated with the agents for 30 min before stimulation with LPS. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay, as previously described [9].

2.3. Measurement of nitric oxide, ROS, and cytokines

Microglial cells (1×10^5 cells per well in a 24-well plate) were pre-treated with lancemaside A for 1 h and stimulated with LPS (100 ng/ml) for 16 h. Then, the supernatants of the cultured microglia were collected and the accumulated nitrite was measured using the Griess reagent (Promega). The intracellular accumulation of ROS was measured with H_2DCF-DA (Sigma), as previously described [9]. The concentrations of TNF-α and IL-6 in the supernatants were measured by ELISA, according to the procedure recommended by the supplier (PharMingen, San Diego, CA).

2.4. RT-PCR

BV2 cells (7.5×10^5 cells on a 6-cm dish) were treated with LPS in the presence or absence of lancemaside A, and total RNA was extracted with TRI reagent (Sigma). For RT-PCR, total RNA (1 μg) was reverse-transcribed in a reaction mixture that contains 1 U RNase inhibitor, 500 ng random primers, 3 mM MgCl2, 0.5 mM dNTP, and 10 U reverse transcriptase (Promega). The synthesized cDNA was used as a template for PCR reaction using GoTaq polymerase (Promega) and primers, as below (Table 1).

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from treated microglia were prepared, as described previously [9]. The double-stranded DNA oligomers containing consensus sequences of NF-κB or AP-1 were end-labeled, using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) in the presence of [γ-^{32}P] ATP. Five micrograms of the nuclear proteins were incubated with ^{32}P-labled probes on ice for 30 min and resolved on a 5% acrylamide gel and visualized by autoradiography.

2.6. Western blot analysis

Cells were appropriately treated and total cell lysates were prepared, as described previously [9]. Proteins (20–100 μg) were heated with 4× SDS sample buffer and separated by SDS–PAGE gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies (1:1000) and then, horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution in TBST; New England Biolabs, Ipswich, MA) were applied and the blots were developed using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA).

2.7. Statistical analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as mean ± S.E.M. and statistical comparisons between the groups were performed using one-way ANOVA, followed by Student's *t*-test. A p value < 0.05 was considered significant.

3. Results

3.1. Lancemaside A suppresses iNOS, TNF-α, IL-6, and COX-2 expressions in LPS-stimulated microglia

To investigate the anti-inflammatory effects of lancemaside A, BV2 microglial cells were stimulated with LPS (100 ng/ml) in the

Table 1
Primers used in RT-PCR experiments.

	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size
iNOS	CAAGAGTTTGACCAGAGGACC	TGGAACCACTCGTACTTGGGA	450 bp
TNF-α	CCTATGTCTCAGCCTCTTCT	CCTGGTATGAGATAGCAAAT	354 bp
IL-6	CCACTTCACAAGTCGGAGGCTT	CCAGCTTATCTGTTAGGAGA	395 bp
COX-2	TTCAAAAGAAGTGCTGGAAAAGGT	GATCATCTCTACCTGAGTGTCTTT	304 bp
HO-1	TGTCACCCTGTGCTTGACCT	ATACCCGCTACCTGGGTGAC	209 bp
p47phox	CGATGGATTGTCCTTTGTGC	ATCACCGGCTATTTCCCATC	256 bp
p67phox	CCCTTGGTGGAAGTCCAAAT	ATCCTGGATTCCCATCTCCA	242 bp
p22phox	AAAGAGGAAAAAGGGGTCCA	TAGGCTCAATGGGAGTCCAC	239 bp
gp91phox	ACTGCGGAGAGTTTGGAAGA	GGTGATGACCACCTTTTGCT	201 bp
GAPDH	ATGTACGTAGCCATCCAGGC	AGGAAGGAAGGCTGGAAGAG	420 bp

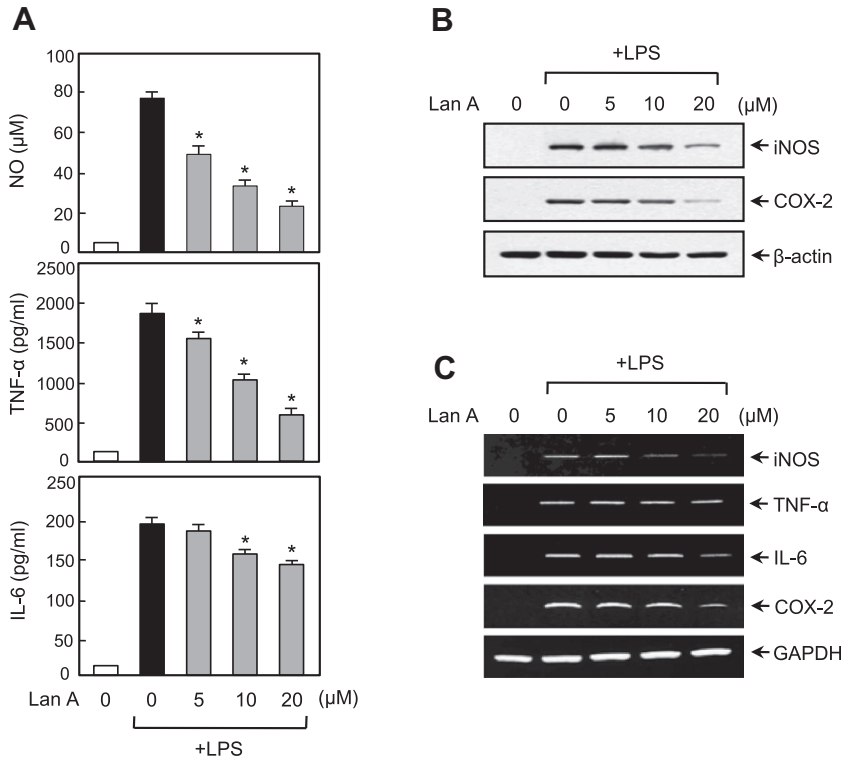

Fig. 1. Effect of lancemaside A on iNOS, COX-2 and pro-inflammatory cytokines in LPS-stimulated BV2 microglia. (A) The BV2 cells were pre-treated with the indicated concentration of lancemaside A for 1 h, followed by treatment of LPS (100 ng/ml) for 16 h. The amounts of NO, TNF-α and IL-6 released into media were measured as described in the method section. The data are expressed as the mean ± S.E.M. of three independent experiments. *P < 0.05, significantly different from LPS-treated sample. (B) Western blot for iNOS and COX-2 protein expression. (C) RT-PCR data for iNOS, COX-2 and cytokine mRNA expressions. The data are representative of at least three independent experiments.

presence or absence of lancemaside A. As shown in Fig. 1A, lancemaside A significantly inhibited NO, TNF-α, and IL-6 production in LPS-stimulated BV2 cells. In addition, lancemaside A suppressed LPS-induced iNOS and COX-2 protein expression (Fig. 1B). Subsequently RT-PCR analysis revealed that lancemaside A significantly inhibited the mRNA expressions of iNOS, IL-6 and COX-2 (Fig. 1C). However, TNF-α mRNA level was not altered by lancemaside A. The data suggest that lancemaside A modulates iNOS, IL-6 and COX-2 expression mainly at the transcriptional level, and TNF-α at the post-transcriptional level.

3.2. Lancemaside A inhibited the phosphorylation of JNK, which is associated with inhibition of proinflammatory molecules

To analyze the molecular mechanism underlying the anti-inflammatory effect of lancemaside A, we examined the effect of lancemaside A on PI3 K/Akt and MAP kinases, which are upstream signaling molecules in inflammatory responses [10]. Western blot analysis revealed that lancemaside A remarkably inhibited LPS-induced JNK phosphorylation. However, lancemaside A did not affect the phosphorylations of p38 MAPK, ERK or Akt (Fig. 2A). Next, to investigate a possible involvement of JNK pathway in the anti-inflammatory effect of lancemaside A, we examined the effect of a JNK-specific inhibitor, SP600125, on microglial activation. SP600125, like lancemaside A, significantly inhibited NO, TNF-α, and IL-6 production (Fig. 2B). Furthermore, both the lancemaside A and SP600125 inhibited NF-κB and AP-1 DNA binding activities, which are important transcription factors for inflammatory gene expression (Fig. 2C). Thus, the data collectively suggest that JNK pathway plays a key role mediating the anti-inflammatory effect of lancemaside A in LPS-stimulated BV2 microglia.

3.3. Lancemaside A and SP600125 suppressed ROS production, expression and/or phosphorylation of NADPH oxidase subunits, while they enhanced antioxidant enzyme HO-1 expression

ROS plays a role as the second messengers in inflammatory reactions and excessive ROS generation by microglia contributes to the neuronal cell death and neurodegeneration [11,12]. In this study, we found that lancemaside A and SP600125 significantly inhibited LPS-induced ROS production in the BV2 cells (Fig. 3A–B). Since NADPH oxidase is a major enzyme for microglial ROS release, we examined the effect of lancemaside A on membrane ($gp91^{phox}$, $p22^{phox}$) and cytosolic components ($p47^{phox}$, $p67^{phox}$) of NADPH oxidase. Lancemaside A and SP600125 suppressed the expression and phosphorylation of $p47^{phox}$ (Fig. 3C–D). Moreover, both agents inhibited the mRNA expressions of $p67^{phox}$ and $gp91^{phox}$, but not $p22^{phox}$ (Fig. 3C). The inhibition of $gp91^{phox}$ by lancemaside A was rather modest compared with the inhibition of $p47^{phox}$ and $p67^{phox}$, which was confirmed by real-time PCR analysis (data not shown).

We next examined the effects of lancemaside A on HO-1, which mediates anti-inflammatory and antioxidant effects in the activated microglia [9,13]. Lancemaside A and SP600125 increased HO-1 expression at mRNA and protein levels (Fig. 3E–F). Thus, the antioxidant effect of lancemaside A may attribute to JNK-mediated inhibition of NADPH oxidase activity and upregulation of HO-1.

3.4. Neuronal cell viability was recovered by treatment of lancemaside A or SP600125 in microglia-neuronal cell coculture system

To determine whether the anti-inflammatory effects of lancemaside A affect the viability of neighboring neuronal cells, we

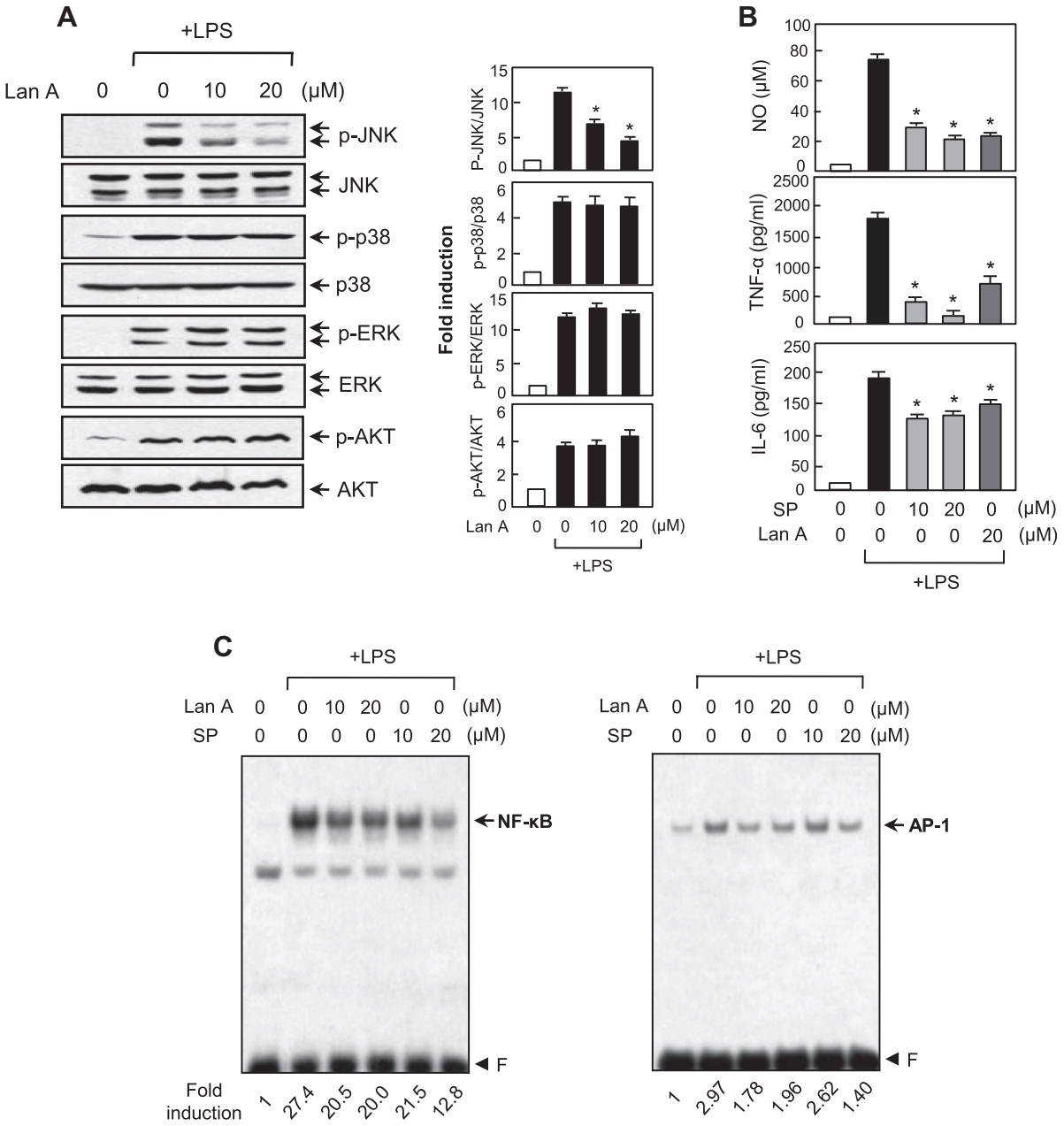

Fig. 2. JNK pathway is involved in anti-inflammatory mechanism of lancemaside A. (A) Cell extracts were prepared from BV2 cells treated with LPS for 30 min in the absence or presence of lancemaside A and then subjected to immunoblot analysis using antibodies against the phospho- or total-forms of the three MAP kinases and Akt. Quantification of Western blot data (right panel). Levels of the active forms of MAPKs were normalized with the total form, and are expressed as fold changes versus untreated control samples, which were arbitrarily set to 1.0. (B) Comparison of the effects of SP600125 and lancemaside A on LPS-induced NO, TNF-α and IL-6 production. Values are the mean ± S.E.M. of three independent experiments. *P < 0.05; significantly different from LPS-treated cells. (C) EMSA data. Effect of lancemaside A and SP600125 on LPS-induced NF-κB and AP-1 DNA binding activities. The data are representative of at least three independent experiments. Lan A and SP indicate lancemaside A and SP600125, respectively.

performed microglia/neuronal cell co-culture assays, using Transwell membrane inserts. The BV2 cells on the microporous membrane were treated with LPS in the absence or presence of lancemaside A for 6 h and then placed into wells containing Neuro-2a neuronal cells. After incubation for 36 h, neuronal cell viability was determined by MTT assay. We found that neuronal cell viability was markedly recovered in the lancemaside A or SP600125 treated-groups compared with that of the LPS-treated groups (Fig. 4). However, either lancemaside A or SP600125 did not directly affect the viability of neuronal cells (data not shown). Thus, our data collectively suggest that the neuroprotective effects of lancemaside A and SP600125 were due to the reduced secretion of proinflammatory/neurotoxic molecules from microglia rather than direct effect of the agents on neuronal cells.

4. Discussion

In the present study, we report for the first time the anti-inflammatory effect of lancemaside A in LPS-stimulated microglia. Inhibition of JNK signaling pathway was suggested to be the main mechanism responsible for the anti-inflammatory action of lancemaside A. We demonstrated that lancemaside A and SP600125 suppressed LPS-induced expression of iNOS, COX-2, TNF-α and IL-6 via inhibition of NF-κB and AP-1 activities. The RT-PCR data

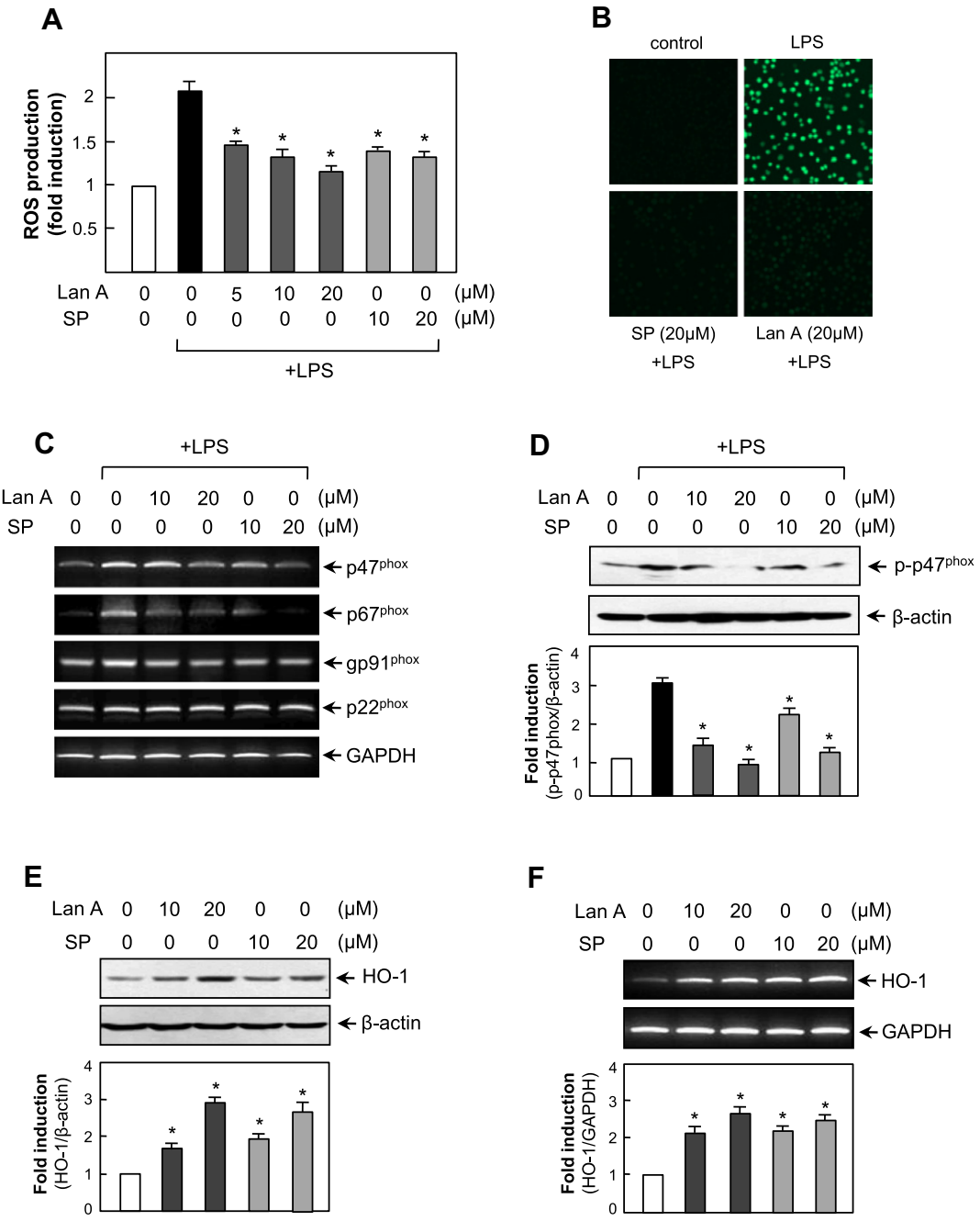

Fig. 3. Lancemaside A and SP600125 inhibited ROS production via suppression of NADPH oxidase subunits and increased HO-1 expression. (A) The BV2 cells were pre-treated with lancemaside A for 1 h, followed by treatment with LPS (0.1 μg/ml) for 16 h. The intracellular ROS levels were then measured by the DCF-DA method as described in the Materials and methods section. The data are expressed as the mean ± S.E.M. of three independent experiments. *P < 0.05, significantly different from LPS-treated sample. (B) The representative image of DCF-derived fluorescence in BV2 cells (n = 3). (C) RT-PCR for mRNA expression of NADPH oxdiase subunits ($p47^{phox}$, $p67^{phox}$, $gp91^{phox}$, $p22^{phox}$) in the BV2 cells. (D) Western blot analysis for phosphorylation of the $p47^{phox}$ subunit. (E) Western blot analysis shows the effect of lancemaside A and SP600125 on HO-1 protein expression. The BV2 cells were treated with lancemaside A or SP600125 for 24 h and cell lysates were obtained. *P < 0.05, significantly different from the control sample. (F) RT-PCR was performed to determine the HO-1 mRNA expression. Quantification data are shown in the graph (n = 3).

suggest that lancemaside A modulates iNOS, IL-6 and COX-2 expression mainly at the transcriptional level, and TNF-α at the post-transcriptional level. Previous studies reported that JNK pathway controls translation initiation and mRNA stability of TNF-α [14,15]. Therefore, lancemaside A-mediated post-transcriptional control of TNF-α is thought to be closely related with its JNK inhibitory effect. In addition, lancemaside A and SP600125 inhibited ROS production via modulation of cytosolic and membrane components of NADPH oxidase (i.e. $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$). Upregulation of HO-1 expression also contributes to anti-inflammatory and antioxidant effects of lancemaside A and SP600125. Finally, the anti-inflammatory effect of lancemaside A seems to increase the viability of neighboring neuronal cells.

JNK is one of three types of MAP kinases, which are critical mediators of inflammation in response to extracellular stimuli and control gene expression via phosphorylation and regulation of transcription factors [16–18]. Thus, MAPK inhibitors emerge as promising anti-inflammatory agents because they are able to suppress various inflammatory mediators at transcriptional, post-transcriptional and translational level [17]. In particular, p38 MAPK

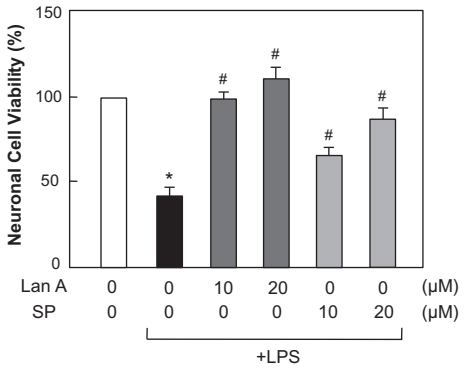

Fig. 4. Effect of lancemaside A and SP600125 on neuronal cell viability. The BV2 microglial cells were cocultured with mouse neuroblastoma Neuro-2a cell using Transwell membrane inserts. The BV2 cells on the microporous membrane were treated with LPS in the absence or presence of lancemaside A or SP600125 for 6 h and then placed into cells containing the Neuro-2a cells. After 36 h of incubation, transwell insert were removed and MTT assay was performed using the Neuro-2a cells. Values are the mean ± S.E.M. of three independent experiments. *$P < 0.05$, significantly different from control samples. #$P < 0.05$, significantly different from LPS-treated samples.

and JNK signaling inhibitors have been suggested as potential neuroprotective drugs. Inhibitors of p38 MAPK (e.g. SB203580, SB239036) reduced the infarct volume and neurological deficits in rats and gerbil ischemia models [19,20]. In addition, the p38 MAPK inhibitors decreased the expressions of iNOS, TNF-α and IL-1β in ischemic brain and Aβ or LPS-stimulated microglia [21,22]. The neuroprotective effects of JNK inhibitors have been reported in animal models of Parkinson's disease and cerebral ischemia [23,24]. In addition, our present study and others demonstrated that SP600125 suppressed LPS-induced proinflammatory cytokines and iNOS in activated microglia [16,25]. Furthermore, both SB203580 and SP600125 inhibited ROS production in various cell types [26,27]. Interestingly, SB203580 abrogated TNF-α-induced phosphorylation of Ser345 on $p47^{phox}$, and thus, inhibited NADPH oxidase activity in neutrophils [28]. In the present study, we demonstrated that JNK inhibitor SP600125 inhibits LPS-induced $p47^{phox}$ phosphorylation in microglia.

Although small molecule inhibitors against p38 MAPK and JNK pathway have therapeutic benefits in animal models of neurological disorders, they are usually recommended for treatment of acute neurologic disorders due to their side effects caused by long term use [17]. Thus, development of safer MAPK inhibitors are necessary that can be applied to chronic neurodegenerative diseases. In this perspective, lancemaside A may be a good therapeutic candidate because lancemaside A has nearly equivalent anti-inflammatory efficacy to SP600125 and has fewer side effects.

Lancemaside A is metabolized to echinocystic acid by intestinal microflora, after oral administration to mice, so echinocystic acid is usually detected in the blood and brain [6,8]. We observed that treatment of echinocystic acid suppressed NO, TNF-α and ROS production in the LPS-stimulated BV2 cells (unpublished data). However, approximately 2.5-fold higher concentration of echinocystic acid was required to obtain the equivalent effect of lancemaside A. This may be due to some stabilizing effect of sugar attached to lancemaside A, which is absent in echinocystic acid structure.

A recent study reported that lancemaside A ameliorate scopolamine-induced memory and learning deficits by inhibiting acetylcholine esterase activity and inducing BDNF and p-CREB expressions in the mouse brain [8]. The results suggest the effectiveness of lancemaside A and/or echinocystic acid in the central nervous system. Therefore, the strong anti-inflammatory effects of lancemaside A in microglia may provide therapeutic potential for various neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

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