Macelignan Attenuates Activations of Mitogen-Activated Protein Kinases and Nuclear Factor kappa B Induced by Lipopolysaccharide in Microglial Cells

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A previous study showed that macelignan extracted from *Myristica fragrans* has anti-inflammatory properties using hippocampal neuronal and primary microglial cells. Subsequently, a study using animals with chronic lipopolysaccharide (LPS) infusion into the brain showed that oral treatments of macelignan reduced the hippocampal microglial activation and hippocampal-dependent spatial memory impairments induced by LPS. However, the molecular mechanisms responsible for the anti-inflammatory activity of macelignan have not been elucidated in the microglia. Therefore, the present study was conducted to determine if mitogen-activated protein kinase (MAPK) signaling and nuclear factor-kappa B (NF- κ B) activities are related to the anti-inflammatory effects of macelignan on LPS-stimulated BV-2 microglial cells. The results show that macelignan suppresses both the phosphorylations of MAPKs and the degradation of inhibitory-kappa B ($I\kappa B\alpha$) and increases of nuclear NF- κ B in LPS-stimulated BV-2 microglial cells. These results suggest that macelignan has an anti-inflammatory effect on the affected brain through regulation of the inflammation through the MAPK signal pathway.

Key words macelignan; anti-inflammation; microglia; alzheimer's disease; mitogen-activated protein kinase; nuclear factor-kappa B

Chronic inflammatory processes play an important role in the pathogenesis of Alzheimer's disease (AD) and other neurodegenerative diseases.¹⁾ In particular, AD is characterized by neuroinflammatory changes and increased free radicals, as well as classic neuropathological features such as amyloid plaques, neuronal loss, and neurofibrillary tangles.²⁾ For example, clumps of activated microglia and reactive astrocytes appear in the vicinity of senile plaques.²⁾

Microglial cells play major roles in host defense and tissue repair in the central nervous system (CNS).³⁾ Microglial cells are activated in response to brain injury and exposure to lipopolysaccharide (LPS), interferon (IFN)- γ , or β -amyloid.^{4,5)} Once chronically activated, microglial cells produce a variety of proinflammatory mediators and potentially neurotoxic compounds such as interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , reactive oxygen species (ROS) and nitric oxide (NO). These have the deleterious effects on brain injuries and neurodegenerative diseases.^{6,7)}

Macelignan, isolated from *Myristica fragrans* HOUTT, has been reported to exhibit free-radical scavenging and prostaglandin inhibitory properties.⁷⁾ Studies using neuronal cells and primary microglial cells have demonstrated that macelignan suppresses NO production by inhibiting inducible nitric oxide (iNOS) expression at the transcriptional level; it also significantly suppresses the production of proinflammatory cytokine TNF- α and IL-6.⁸⁾ The anti-inflammatory effects of macelignan were also evaluated using animal model with a chronic infusion of LPS, one of well-characterized animal models incorporating important neuropathological features seen in AD. Oral administration of macelignan reduces hippocampal microglial activation and the impairments of spatial memory that are induced by

chronic infusions of LPS into the fourth ventricle in rat brains.⁹⁾ These results indicate that macelignan possesses therapeutic potential against neurodegenerative diseases that involve neuroinflammation.^{8,9)} However, the protecting molecular mechanisms of its actions have not yet been studied.

It has recently been suggested that mitogen-activated protein kinases (MAPKs) play a role in the inflammation associated with the formation of plaques, eventually leading to AD.¹⁰⁾ The present study investigates whether macelignan has anti-inflammatory effects through the suppression of the LPS-induced activation of nuclear factor kappa B (NF- κ B) by blocking the degradation of inhibitory-kappa B ($I\kappa B\alpha$) and the phosphorylations of MAPK. To determine the involvement of the MAPK signal pathway and NF- κ B in mediating the anti-inflammatory effects of macelignan, the activities of MAPKs such as p38, extracellular-signal-regulated kinase1/2 (ERK1/2), and c-Jun NH₂-terminal kinase (JNK) were measured in response to treatments of macelignan in LPS-stimulated BV-2 microglial cells.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL. The culture dishes and flasks were acquired from Becton Dickinson and p38 MAP kinase antibody, Phospho-p38 MAP kinase (Thr180/Tyr182) antibody (p-p38), p44/42 MAP kinase antibody (ERK), phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (p-ERK), SAPK/JNK antibody (JNK), Phospho-SAPK/JNK (Thr183/Tyr185) antibody (p-JNK) and anti-rabbit labeled with horseradish peroxidase (HRP)-conjugated secondary antibody were sourced from

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Cell Signaling (Beverly, MA, U.S.A.). Anti-NF- κ B p65 was obtained from Upstate (Temecula, CA, U.S.A.) and all inhibitors were from Calbiochem. Anti-mouse labeled with horseradish peroxidase (HRP)-conjugated secondary antibody, the ECL system, and Hyperfilm were acquired from Amersham (Piscataway, NJ, U.S.A.). Nuclear and cytoplasmic extraction reagents NE-PER was purchased from Pierce Biotechnology (Rockford, IL, U.S.A.). LPS, monoclonal anti β -actin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) powder, and all reagents were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.), unless otherwise stated.

Plant Material and Isolation of Macelignan from *Myristica fragrans* *Myristica fragrans* HOUTT (Myristicaceae) was collected from the Biofarmaka Research Center of Bogor Agricultural University (Indonesia). The plant material was shade-dried and ground to powder. One hundred grams of dried seed kernels of *Myristica fragrans* was ground and extracted twice with 75% aqueous methanol (400 ml, v/v) for 24 h at room temperature. The methanol extract was concentrated, frozen, lyophilized (7 g), and further fractionated successively with ethyl acetate, *n*-butanol, and water. The ethyl acetate fraction (4.2 g) was separated using silica gel column chromatography (Merck Kieselgel 60, 70—230 mesh) by eluting with an *n*-hexane:ethyl acetate solution (10:1, v/v), and 10 ml of the eluent was collected in test tubes. The collected tubes were divided into six fractions (Fr. I to Fr. VI), and Fr. III was further separated through a silica gel column with *n*-hexane:ethyl acetate (20:1, v/v), yielding Fr. III-B (0.52 g). Fr. III-B was eluted with 80% methanol by means of Rp-18 column chromatography (Merck LiChro-prep, 25—40 μ m), and Fr. III-B-2 (0.5 g) was finally obtained as a single compound. This compound was isolated previously by Woo *et al.*,¹¹ and reference to this literature confirmed that the compound was macelignan.⁸

BV-2 Cell Culture The BV-2 immortalized murine microglial cell line was received from Dr. E. Joe at Ajou University.¹² Briefly, the BV-2 microglia cells were cultured in DMEM and supplemented with 10% FBS containing 1% penicillin and streptomycin. Cells were maintained in a humidified incubator with 5% CO₂. First, cells were cultured in a 100 mm dish for 2—3 d. They were then washed twice with media to remove unattached cells. The healthy cells were detached from the dish by mild shaking with a small amount of media. 5×10^5 cells/dish were planted in 60 mm dishes and cultured for 36—48 h before treatment. In experiments that investigated the activities of MAPKs, the cells were pretreated with macelignan (1, 2.5, 5 μ M), U0126 (ERK inhibitor, 2.5 μ M) or SP600125 (JNK inhibitor, 20 μ M) for 2.5 h before the addition of LPS (100 ng/ml). In experiments involving NF- κ B, the cells were pretreated with macelignan (1 μ M) for 23.5 h before the addition of LPS (100 ng/ml). The cells were processed for Western blot analyses 30 min later.

Cell Cytotoxicity Test The cells were seeded in a 24-well dish (1×10^5 cells/ml) for 16 h before being exposed to macelignan alone (1, 2.5, 5 μ M) or macelignan with LPS (100 ng/ml) for 24 h. MTT solution (0.5 mg/ml) was then added to each well and the cells were incubated for 2 h at 37 °C and in 5% CO₂. Subsequently, the supernatant was removed and the formation of formazan was solubilized with dimethyl sulfoxide (DMSO) and measured at 540 nm with a

microplate reader.

BV-2 Whole Cell Protein Extraction The cells were washed twice with cold PBS, collected within 1 ml PBS and centrifuged for 10 min at 2700 rpm at 4 °C. The precipitate was then lysed in 30 μ l of ice-cold lysis buffer (50 mM Tris, pH 7.5, 5% Glycerol, 0.5 mM DTT, 40 mM KCl, 1.5 mM EDTA, 1% TritonX-100, 1 mM Na₃VO₄, 1 mM NaF and 1 mM PMSF) containing protease inhibitors (Cat. No. 539131, Calbiochem). The lysate was placed on ice for 30 min and mixed with a vortex every 5 min. The supernatant was then collected by centrifugation for 30 min at 14000 rpm at 4 °C.

Nuclear and Cytoplasmic Extract of BV-2 Microglial Cells Nuclei and cytoplasm were isolated from BV-2 cells with NE-PER according to the protocol of the manufacturer.¹³ Specifically, a 10 μ l packed cell volume (*ca.* 20 mg) of sunken cells were isolated in a 1.5 ml microcentrifuge tube by centrifugation at 500 $\times g$ for 2—3 min. The supernatant (PBS) was then removed and discarded, leaving the cell pellets as dry as possible. Following the addition of 100 μ l of ice-cold CER I to the cell pellet, the tubes were vortexed vigorously for 15 s to resuspend the cell pellet fully, and the cells were then incubated on ice for 10 min. 5.5 μ l of ice-cold CER II was added to the tube and was vortexed for 5 s and this was then incubated on ice for 1 min. Next, they were centrifuged for 5 min at maximum speed (*ca.* 16000 $\times g$) in a microcentrifuge (Eppendorf), which was followed by 5 s of vortexing. The supernatant (cytoplasmic extract) fraction was transferred to a clean pre-chilled tube immediately and was placed on ice until use. The insoluble fraction was resuspended in 30 μ l of ice-cold NER and vortexed for 15 s, tubes were returned to ice and successively vortexed for 15 s every 10 min for a total of 40 min, and they were then centrifuged at maximum speed (*ca.* 16000 $\times g$) in a microcentrifuge for 10 min. The supernatant (nuclear extract) fraction was immediately transferred to a clean pre-chilled tube.

Protein Quantification of BV-2 Microglial Cells Bovine serum albumin (BSA, 1 mg/ml) was used as standard protein. The protein in nuclear and cytoplasmic extracts or whole cell extraction was mixed with Bradford Reagent (Sigma), and the absorbance at 595 nm was recorded using a SPECTRA MAX 190 device (Molecular Devices). The protein concentration was determined in a comparison to a standard curve. All of these steps were performed at room temperature. Afterward, Laemmli sample buffer 5x (250 mM Tris-HCl, pH 6.8, 50% Glycerol, 10% SDS, 500 mM β -mercaptoethanol, 0.1% Bromophenol blue) was added and the samples were boiled for 3—5 min. They were then chilled immediately in ice until use.

Western Blot Analysis of BV-2 Microglial Cells Protein samples (10—20 μ g for each) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with 70—100 V and transferred onto a Hybond-P PVDF membrane (Amersham Pharmacia Biotech) for 1 h at 100 V with cooling. The membrane was blocked with 5% skim milk dissolved in Tris-buffered saline Tween-20 (TBST) (20 mM Tris, pH 7.6, 136 mM NaCl, 0.1% Tween 20) for 3 h at room temperature. The blots were incubated with antibodies against p38 (1:1000), p-p38 (1:1000), ERK (1:1000), p-ERK (1:1000), JNK (1:1000), p-JNK (1:1000), NF- κ B (1:2000), and actin (1:5000) overnight at 4 °C, washed with TBST for 10 min three times, and were then in-

cubated with HRP-conjugated secondary antibody (1 : 1000 or 1 : 2000 or 1 : 5000) for 1 h at room temperature. Protein bands were visualized using an ECL Plus system and developed onto Hyperfilm (Amersham) as described above. The relative expression levels of protein were determined by densitometry and were normalized by actin.

Statistical Analyses All experiment and replications always included a vehicle-treated control (control) and only LPS-treated conditions (LPS). First, the changes of MAPKs and NF- κ B induced by LPS treatments were compared to the controls and these were statistically examined with a *t*-test. Next, a one-way ANOVA was conducted to determine the effects of macelignan by itself on abundances of MAPKs and NF- κ B in microglial cells and to determine the effects of macelignan on MAPKs and NF- κ B in LPS-stimulated microglial cells as compared to abundances of those induced by only a LPS treatment. Following this, *post hoc* analyses (Duncan) were subsequently conducted to observe the minimum effective doses of macelignan. Any *p* values that were less than 0.05 were considered significant, unless otherwise specified. All data were expressed as the mean $\pm$ S.E.M.

RESULTS

No Effects of Macelignan on the Viabilities of BV-2 Microglial Cells Neither treatment of macelignan alone nor macelignan with LPS treatments had an effect on the viabilities of BV-2 cells (see Fig. 1).

LPS Treatment Increased Phosphorylation of MAPKs Compared to Those of the Control Exposure of BV-2 cells to LPS for 30 min did not change the abundance of

Fig. 1. Effects of Macelignan on the Cell Viabilities of BV-2 Microglial Cells

BV-2 cells were treated with 1, 2.5, and 5 μ M of macelignan without LPS treatments or with LPS treatments. BV-2 cell viabilities were measured and expressed as means $\pm$ S.E.M. for three independent experiments.

MAPKs (p38, ERK, and JNK) but significantly induced the phosphorylation of MAPKs in both of the experiments that were conducted to determine the effects of macelignan itself (p-p38 ($t(4) = -3.20$, $p < 0.05$), p-ERK ($t(6) = -3.43$, $p < 0.05$), p-JNK ($t(4) = -3.91$, $p < 0.05$) as well as in the experiments to determine the effects of macelignan on the activities of MAPKs in LPS-stimulated microglial cells (p-p38 ($t(4) = -3.94$, $p < 0.01$), p-ERK ($t(10) = -8.03$, $p < 0.01$), and p-JNK ($t(4) = -7.13$, $p < 0.05$) (see Table 1).

Effects of Macelignan Itself on Activities of MAPK and NF- κ B To observe if macelignan alone has an effect on

Fig. 2. Macelignan Suppresses p38 Phosphorylation in LPS-Stimulated BV-2 Microglial Cells

(A) A representative Western blot of p38, (B) A representative Western blot of p-p38, (C) LPS increased the phosphorylation of p38 compared with those of the control (*). One micromolar treatment of macelignan decreased the abundance of p38 phosphorylation in LPS-stimulated BV-2 microglial cells ($p < 0.05$), whereas 2.5 μ M and 5 μ M did not. These results are representative of those obtained from three independent experiments. Values are expressed as means $\pm$ S.E.M.

Table 1. The Effect of Macelignan on MAPKs in Microglial Cells

	Control	MACE 1 μ M	MACE 2.5 μ M	MACE 5 μ M	LPS 100 ng/ μ l
p38/ACTIN	1.26 $\pm$ 0.30 (n=3)	1.21 $\pm$ 0.21 (n=3)	1.23 $\pm$ 0.24 (n=3)	1.25 $\pm$ 0.27 (n=3)	1.30 $\pm$ 0.25 (n=3)
p-p38/ACTIN	0.19 $\pm$ 0.09 (n=3)	0.24 $\pm$ 0.11 (n=3)	0.27 $\pm$ 0.14 (n=3)	0.27 $\pm$ 0.10 (n=3)	0.87 $\pm$ 0.19 (n=3)
ERK/ACTIN	1.15 $\pm$ 0.09 (n=3)	1.15 $\pm$ 0.08 (n=3)	1.13 $\pm$ 0.07 (n=3)	1.11 $\pm$ 0.06 (n=3)	1.07 $\pm$ 0.04 (n=3)
p-ERK/ACTIN	0.16 $\pm$ 0.11 (n=4)	0.17 $\pm$ 0.05 (n=4)	0.22 $\pm$ 0.08 (n=4)	0.42 $\pm$ 0.08 (n=4)	0.73 $\pm$ 0.13 (n=4)
JNK/ACTIN	1.05 $\pm$ 0.03 (n=3)	1.01 $\pm$ 0.03 (n=3)	1.01 $\pm$ 0.03 (n=3)	1.07 $\pm$ 0.05 (n=3)	1.08 $\pm$ 0.06 (n=3)
p-JNK/ACTIN	0.28 $\pm$ 0.16 (n=3)	0.20 $\pm$ 0.12 (n=3)	0.13 $\pm$ 0.04 (n=3)	0.23 $\pm$ 0.12 (n=3)	0.54 $\pm$ 0.16 (n=3)
Cytosolic I κ B	0.68 $\pm$ 0.02 (n=3)	0.49 $\pm$ 0.03 (n=3)*	—	—	0.33 $\pm$ 0.02 (n=3)**
Nuclear NF- κ B	0.67 $\pm$ 0.10 (n=3)	0.80 $\pm$ 0.10 (n=3)*	—	—	1.16 $\pm$ 0.10 (n=3)**

* $p < 0.05$, ** $p < 0.01$, compared with control; mean $\pm$ S.E.M. MACE: macelignan

MAPKs, the phosphorylation of MAPKs, cytosolic inhibitory- κ B, and nuclear NF- κ B, we examined the effects of macelignan on p38, ERK, JNK expression and these phosphorylation forms in microglial BV-2 cells and on the abundance of cytosolic I κ B and nuclear NF- κ B. Macelignan did not affect the abundance of MAPKs or the phosphorylation of MAPKs; however, it slightly degraded cytosolic I κ B and increased nuclear NF- κ B in comparison with controls ($p < 0.05$) (see Table 1). This result suggests that macelignan itself did not affect the abundance of MAPKs or the phosphorylation of MAPKs, although macelignan had a minor effect on the degradation of cytosolic I κ B and on the translocation of nuclear NF- κ B.

Effects of Macelignan on Activities of MAPKs in LPS-Stimulated Microglial Cells The effects of macelignan on the activities of the MAPKs of LPS-stimulated microglial cells were investigated. Macelignan in LPS-stimulated BV-2 cells significantly suppressed the abundance of p38 phosphorylation ($F(3,8)=5.14$, $p < 0.05$) but did not affect the abundance of p38. *Post hoc* analyses revealed that treatment with 1 μ M of macelignan decreased the abundance of p38

phosphorylation in LPS-stimulated BV-2 cells, whereas the amounts of 2.5 μ M and 5 μ M did not (Fig. 2). Macelignan and U0126 (the ERK inhibitor) completely suppressed the abundance of ERK phosphorylation ($F(4,25)=10.01$, $p < 0.01$) but did not affect the abundance of ERK in LPS-stimulated BV-2 cells. *Post hoc* analyses revealed that at all concentrations used, macelignan decreased the abundance of ERK phosphorylation ($p < 0.05$) and U0126 completely suppressed phosphorylation of ERK ($p < 0.05$) (Fig. 3). Macelignan and SP600125 (the JNK inhibitor) significantly suppressed the abundance of JNK phosphorylation ($F(4,10)=7.41$, $p < 0.05$) but did not affect the abundance of JNK in LPS-stimulated BV-2 cells. *Post hoc* analyses revealed that, at all concentrations used, macelignan decreased the abundance of JNK phosphorylation ($p < 0.05$) while SP600125 significantly suppressed the phosphorylation of ERK ($p < 0.05$) (Fig. 4).

Effects of Macelignan on Activities of NF- κ B in LPS-Stimulated BV-2 Microglial Cells NF- κ B, one of the Rel family transcription factors, is implicated in the regulation of iNOS.¹⁴ In a previous study, LPS stimulation was shown to increase NF- κ B activation through I κ B α degradation.¹⁵ Therefore, it was determined to be inhibited by pretreatment

Fig. 3. Macelignan Suppresses ERK Phosphorylation in LPS-Stimulated BV-2 Microglial Cells

(A) A representative Western blot of ERK. (B) A representative Western blot of p-ERK. (C) LPS increased the phosphorylation of ERK compared to those of controls (*). Macelignan, at any concentrations used, completely decreased the abundance of ERK phosphorylation ($\#p < 0.05$) and U0126, the ERK inhibitor, completely suppressed phosphorylation of ERK ($\#p < 0.05$). These results are representative of those obtained from three (ERK) or six (p-ERK) independent experiments. Values are expressed as means $\pm$ S.E.M.

Fig. 4. Macelignan Suppresses JNK Phosphorylation in LPS-Stimulated BV-2 Microglial Cells

(A) A representative Western blot of JNK. (B) A representative Western blot of p-JNK. (C) LPS increased the phosphorylation of JNK compared to those of controls (*). Macelignan, at any concentrations used, decreased the abundance of JNK phosphorylation ($\#p < 0.05$) and SP600125, the JNK inhibitor, significantly suppressed the phosphorylation of ERK ($\#p < 0.05$). These results are representative of those obtained from three independent experiments. Values are expressed as means $\pm$ S.E.M.

Fig. 5. Effect of Macelignan on Cytosolic IκB Degradation and Nuclear NF-κB Activation in LPS-Stimulated BV-2 Microglial Cells

Macelignan suppresses cytosolic IκB degradation and nuclear NF-κB activation induced by LPS. (A) A representative Western blot of cytosolic IκB. (B) LPS decreased the microglial cytosolic IκB abundances compared to those of controls (*). Macelignan (1 μM) increased the abundance of cytosolic IκB (# $p < 0.05$) in LPS-stimulated BV-2 microglial cells. (C) A representative Western blot of nuclear NF-κB. (D) LPS increased nuclear NF-κB abundances compared to those of controls (*). Macelignan decreased the abundance of nuclear NF-κB (# $p < 0.05$). These results are representative of those obtained from three independent experiments. Values are expressed as means $\pm$ S.E.M.

with macelignan in NF-κB activation induced by LPS. LPS stimulation significantly enhanced NF-κB activities.¹⁶⁾ According to the MAPKs results, macelignan 1 μM significantly suppressed the phosphorylation of MAPKs in LPS-stimulated BV-2 microglial cells. Thus 1 μM concentration of macelignan was used in LPS-stimulated BV-2 microglial cells. The cytosolic IκB was degraded ($t(4)=8.63$, $p < 0.01$) and significant levels of NF-κB p65 were localized to the nucleus 30 min ($t(4)=-9.73$, $p < 0.01$) after the LPS treatment. As shown in Table 1, 1 μM of macelignan treatment slightly affected the abundance of cytosolic IκB and nuclear NF-κB, and macelignan (1 μM) pretreatment significantly attenuated this nuclear translocation ($t(4)=5.21$, $p < 0.01$), blocking the degradation of IκBα ($t(4)=-3.24$, $p < 0.05$) in LPS-stimulated BV-2 microglial cells (Fig. 5).

DISCUSSION

To expand our understanding of the anti-inflammatory actions of macelignan, these experiments were conducted to determine its effects in murine BV-2 microglial cell stimulated with LPS. LPS acts through Toll-like receptor-4 and in turn enhances the expression of some inflammatory genes, such as cyclooxygenase-2 (COX-2) and iNOS, by the activation of transcription factors that include AP-1, NF-κB, and the cyclic AMP responses element. These transcription factors are regulated by MAPKs and IκBα. Therefore, the effects of macelignan on this signaling pathway were examined to attempt to pinpoint the mechanism of action based on the results reported earlier.⁸⁾

As demonstrated in the aforementioned report,⁸⁾ macelignan suppressed the expression of COX-2 in LPS-stimulated primary microglial cells and reduced the generation of intracellular ROS in glutamate-stimulated neuronal hippocampal cells. The present study demonstrated that macelignan suppresses the degradation of IκBα and increases nuclear NF-κB in LPS-stimulated BV-2 microglia cells. Therefore, macelignan may suppress the generation of intracellular ROS induced by LPS, which accordingly may not activate NF-κB and induce the expression of COX-2; otherwise, these would have been activated and induced by LPS.

Their animal models indicate that p38 and ERK1/2 most likely play a significant role in initiating and sustaining inflammatory reactions.^{17,18)} *In vitro* studies have reported that p38 and ERK1/2 regulate the production of iNOS and NO in LPS-stimulated microglia.^{19,20)} These responses likely involve the activation of transcription factors that positively regulate the induction of inflammatory genes.²¹⁾ In this study, macelignan at a concentration of 1 μM slightly suppressed the phosphorylation of p38 but was not effective at other concentrations in LPS-stimulated BV-2 microglial cells. On the other hand, macelignan at any of the concentrations used in these experiments inhibited the phosphorylation of ERK1/2 nearly half as much as U0126, the ERK inhibitor, inhibited those in LPS-stimulated BV2 microglial cells. However, these results may be replicated with other microglia cells such as the primary microglial cells and with other inflammation-inducing stimuli, as the MAPK signaling pathway related to iNOS gene regulation depends on the cell type and on the stimulus.

JNK is activated in microglial cells in response to various immunogenic or inflammatory stimuli, such as LPS, TNF- α , and thrombin.²²⁾ JNK is involved in morphological enlargement and cytokine transcription.²³⁾ Therefore, it has been suggested that JNK is a relevant co-mediator in mediating the activation of microglia.²⁴⁾ Consequently, an agent with the inhibitory properties of JNK phosphorylation may protect against activated pro-inflammatory microglia. Macelignan at any of the concentration used in these experiments inhibited the phosphorylation of JNK almost half as much as SP600125, the JNK inhibitor, inhibited those in LPS-stimulated BV2 microglial cells.

NF- κ B is an important transcription factor in mediating proinflammatory responses.^{25,26)} The blockage of NF- κ B transcriptional activity can also suppress the expression of iNOS, COX-2, and proinflammatory cytokines.^{27,28)} The promoter regions of the murine gene encoding iNOS and COX-2 contain NF- κ B and AP-1 binding sites.^{29,30)} The inhibitory effect on inflammatory gene expression is also considered to be related to the inhibition of the DNA binding activity of NF- κ B.³¹⁾ Therefore, the effect of macelignan on NF- κ B activation and I κ B degradation was investigated in LPS-stimulated BV-2 microglial cells. Macelignan significantly inhibited NF- κ B activation *via* the regulation of I κ B in LPS-stimulated microglia, and these actions lead to the suppression of proinflammatory cytokine including TNF- α and IL-6 in LPS-stimulated microglia cells, as shown in a previous study by the authors.⁸⁾ These results suggest that the inhibition of NF- κ B nuclear translocation by macelignan is responsible for the suppression of NO, COX2, and proinflammatory cytokines.

Recent studies showing a protective effect of macelignan in animal models of diabetes mellitus and hepatotoxicity indicate that macelignan is a peroxisome proliferator-activated receptor- α/γ agonist and that it is related to the MAPK signaling pathway, especially JNK.^{32,33)} It is well known that PPAR γ agonists elicit anti-inflammatory and anti-amyloidogenic effects and that MAPK has emerged as a key factor in the regulation of *Tau* and β -amyloid precursor proteins.^{10,34)}

This study explored possible anti-inflammatory mechanisms related to MAPKs by macelignan in LPS-stimulated BV-2 microglial cells. The study showed that macelignan significantly inhibits the activation of MAPKs, though phosphorylation of p38 was significantly but slightly inhibited in LPS-stimulated BV2 microglial cells. As the microglial activation and the activation of MAPKs occur in most chronic neurodegenerative diseases including AD, macelignan is considered to be a promising neuroprotective reagent, performing its action through the MAPK pathway as well as the downregulation of NF- κ B activation *via* the regulation of the I κ B α .

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