Newly Synthesized ‘Hidabeni’ Chalcone Derivatives Potently Suppress LPS-Induced NO Production *via* Inhibition of STAT1, but Not NF- κ B, JNK, and p38, Pathways in Microglia

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Chalcones are open-chain flavonoids that are biosynthesized in various plants. Some of them possess anti-inflammatory activity. We previously found that chalcone glycosides from *Brassica rapa* L. ‘hidabeni’ suppress lipopolysaccharide (LPS)-induced nitric oxide (NO) production in rat microglia highly aggressively proliferating immortalized (HAPI) cells. In this study, to explore chalcone derivatives with potent NO inhibitory activity, we synthesized ten compounds based on ‘hidabeni’ chalcone and examined their effects on LPS-triggered inducible NO synthase (iNOS) expression and NO production. Compounds C4 and C10 potently inhibited NO production (IC₅₀: 4.19, 2.88 μ M, respectively). C4 and C10 suppressed LPS-induced iNOS expression *via* the inhibition of the signal transduction and activator of transcription 1 (STAT1), but not nuclear factor-kappa B (NF- κ B), c-Jun N terminal kinase (JNK), and p38, pathways. C10, but not C4, inhibited activation of the MEK/extracellular signal-regulated kinase (ERK) pathway. C4 and C10 also suppressed LPS-induced expression of interferon regulatory factor 1 (IRF-1), which is an important transcription factor involved in iNOS expression. Our findings indicate that these chalcone derivatives are candidate compounds for preventing microglia-mediated neuroinflammation.

Key words microglia; nitric oxide (NO); inducible NO synthase; lipopolysaccharide; interferon β ; signal transduction and activator of transcription 1 (STAT1)

There is growing evidence that inflammation is involved in the pathogenesis of neurodegeneration in many central nervous system (CNS) disorders, such as cerebral ischemia and Alzheimer’s disease.^{1–3} Microglia, resident immune cells in the CNS, are activated in response to neuronal injury. Activated microglia produce inflammatory mediators including nitric oxide (NO), superoxide, and proinflammatory cytokines, leading to a robust neuroinflammatory response.^{4,5} NO produced by inducible NO synthase (iNOS) rapidly reacts with superoxide to form a highly toxic product, peroxynitrite. This product has been shown to exaggerate neuropathological injury.⁶ Therefore, suppression of iNOS expression is thought to ameliorate microglia-mediated neurodegeneration.

The induction of iNOS has been reported to be mediated *via* activation of toll-like receptor 4 (TLR4) in microglia/macrophages.⁷ Lipopolysaccharide (LPS), an exogenous TLR4 ligand, stimulates TLR4 and provokes several signaling pathways including nuclear factor- κ B (NF- κ B) and the mitogen-activated protein kinase (MAPK) family (c-Jun N terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)).^{8,9} In addition, activation of the interferon- β (IFN- β) autocrine loop caused by LPS also plays a key role in LPS-induced iNOS expression.¹⁰ Previous reports have demonstrated that TLR4 participates in the exacerbation of ischemic brain injury.^{7,11} It has been demonstrated that damaged or dying cells after cerebral ischemia release damage-associated molecular pattern molecules such as high mobility group box 1, heat shock proteins, and peroxiredoxin, which are known as endogenous TLR4 ligands, and promote neuroinflammation.^{2,12}

Chalcones are open-chain flavonoids that are biosynthesized in various plants. Many studies have demonstrated that chalcone derivatives have a variety of biological functions including anti-inflammatory, anti-allergic, and anti-oxidant activities.^{13–15} Some of them have been reported to inhibit the expression of iNOS and proinflammatory cytokines caused by LPS. Previously, we isolated chalcone glycosides from *Brassica rapa* L. ‘hidabeni,’ a popular Japanese turnip, which is mainly cultivated and consumed as a traditional vegetable in Gifu.¹⁶ The ‘hidabeni’ chalcones are 4'-glycosidized-3'-oxychalcones, but their structures have rarely been reported. We found that an isolated ‘hidabeni’ chalcone glycoside has inhibitory effects on LPS-induced NO production in rat microglia highly aggressively proliferating immortalized (HAPI) cells.¹⁷

In this study, to explore compounds with more potent inhibitory activity toward LPS-induced iNOS expression and NO production, we newly synthesized ten ‘hidabeni’ chalcone derivatives and performed a structure activity relationship analysis. Moreover, we also addressed the mechanism by which these compounds inhibit LPS-induced iNOS expression.

MATERIALS AND METHODS

Chemistry Chalcones C1–C10 were synthesized through the boron trifluoride–etherate (BF₃·Et₂O)-mediated Claisen–Schmidt reaction of acetophenones and acetylated benzaldehydes, as previously described.¹⁷ BF₃·Et₂O (6.0 mmol) was added to a stirred solution of acetophenones (1.2 mmol) and benzaldehydes (2.4 mmol) in 1,4-dioxane (10 mL) at room temperature. After stirring for 19 h, the resultant solution was partitioned with EtOAc, washed with 10% HCl aq., dis-

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Fig. 1. Synthesis and Chemical Structures of 'Hidabeni' Chalcone Derivatives

tilled water, and brine, dried over anhydrous Na_2SO_4 , and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (CC) eluted with *n*-hexane/ CHCl_3 (2/1) to yield chalcones. Acetylated chalcones were stirred in 10 mg/mL sodium methoxide/methanol solution (10 mL) for 10 min. The reaction mixture was neutralized using a Dowex Marathon C (H^+ form), filtrated, and concentrated *in vacuo*. The residue was purified by silica gel CC eluted with CHCl_3 /MeOH (10/1) to yield deprotected chalcones **C5–C7**. Their chemical structures are depicted in Fig. 1.

Materials LPS was purchased from Sigma (St. Louis, MO, U.S.A.). Anti-signal transduction and activator of transcription 1 (STAT1), anti-phospho-JNK (Thr183/Thr185), anti-JNK, anti-phospho ERK, anti-ERK, anti-phospho p38, and anti-p38 antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-phospho-STAT1 (Thr701) antibody was purchased from Signalway Antibody (Pearland, TX, U.S.A.). Anti-NF- κB p65 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-actin antibody was purchased from Millipore (Billerica, MA, U.S.A.).

Cell Culture and Treatment Rat immortalized microglia HAPI cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 4 mM glutamine, 100 units/mL penicillin G, and 0.1 mg/mL streptomycin in a humidified 5% CO_2 /95% air incubator at 37°C. HAPI cells were seeded in a 6-cm-diameter dish at densities of 8.0×10^5 cells/dish in DMEM containing 1% heat-inactivated FCS. The next day, cells were treated with LPS at a concentration of 100 ng/mL for the time indicated in the Figure legends. Compounds were added to the cultures 30 min prior to treatment with LPS.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from the treated cells with TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). First-strand

cDNA was synthesized from 4 μg of total RNA. Aliquots of transcription reaction mixture (1 μL) were amplified with primers specific for iNOS (forward primer, 5'-TTGCTTCTG TGCTAA TGCGG-3'; reverse primer, 5'-CAGAACTGAGGG TACATGCT-3'), IFN- β (forward primer, 5'-ATCGACTAC AAGCAGCTCCA-3'; reverse primer, 5'-ACCTTTGTACCC TCCAGTAA-3'), interferon regulatory factor 1 (IRF-1; forward primer, 5'-CAACAA GGA TGCCTG TCTGT-3'; reverse primer, 5'-GCTGTG TAA CTGCTG TGGTC-3'), tumor necrosis factor- α (TNF- α ; forward primer, 5'-AAAGCATGATCC GAGATGTG-3'; reverse primer, 5'-ATCTGCTGGTACCAC CAGTT-3'), interleukin-1 β (IL-1 β ; forward primer, 5'-AGT GTCTGA AGCAGCTATGG-3'; reverse primer, 5'-TCA TCA TCCACGAG TCA CA-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH: forward primer, 5'-ACCACA GTC CATGCCATCAC-3'; reverse primer, 5'-TCCACCACCCTG TTGCTGTA-3'). For amplification of iNOS and TNF- α , PCR was carried out using EX Taq polymerase (TaKaRa Bio, Otsu, Japan) as follows: 2 min at 94°C, one cycle; 40 s at 94°C, 40 s at 58°C, 1 min at 72°C, 27 and 32 cycles, respectively. For amplification of IFN- β , PCR was carried out using Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) as follows: 2 min at 94°C, one cycle; 40 s at 94°C, 40 s at 54°C, 1 min at 72°C, 35 cycles. For amplification of IRF-1, IL-1 β , and GAPDH, PCR was carried out using Taq DNA polymerase as follows: 2 min at 94°C, one cycle; 40 s at 94°C, 40 s at 58°C, 1 min at 72°C, 25, 30, and 18 cycles, respectively. Aliquots of the PCR mixtures were separated on 2% agarose gel and stained with ethidium bromide. Densitometric analyses were performed using the Multi Gauge software (FUJIFILM, Tokyo, Japan). The mRNA levels were normalized relative to the GAPDH mRNA level in each sample.

Measurement of Nitrite Production HAPI cells were seeded in a 24-well plate at a density of 1.0×10^5 cells in DMEM containing 1% FCS. The next day, the cells were

treated with 100 ng/mL LPS in the presence or absence of compounds for 24 h. The nitrite concentrations in the medium were measured by the modified Griess method. Briefly, the medium (50 μ L) was mixed with 50 μ L of 1% sulfanilamide in 5% phosphoric acid. Five minutes later, it was mixed with 50 μ L of 0.1% *N*-1-naphthylethylenediamine dihydrochloride in water, followed by incubation for 5 min. The absorbance was measured at 570 nm. A standard curve was prepared with solutions of sodium nitrite.

Preparation of Whole-Cell Lysates and Nuclear Extracts

After the treatment, cells were washed twice with ice-cold PBS. For the preparation of whole-cell extracts, the cells were collected using 150 μ L of lysis buffer (20 mM Tris-HCl, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1% Triton X-100, 10 mM NaF, 1 mM Na₃VO₄,

20 mM β -glycerophosphate, 5 μ g/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT)), and were lysed on ice for 30 min. The lysates were centrifuged at 14000 rpm for 10 min at 4°C to remove cellular debris. For preparation of nuclear extracts, the cells were collected using buffer A (20 mM *N*-(2-hydroxyethyl)perazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.8, containing 15 mM KCl, 2 mM MgCl₂, 5 μ g/mL leupeptin, 0.5 mM PMSF, and 2 mM DTT) and centrifuged. The cells were lysed in buffer B (buffer A containing 0.2% Nonidet P-40) for 5 min on ice and centrifuged. Finally, the pellets were suspended in buffer C (20 mM HEPES-NaOH, pH 7.8, containing 0.4 M NaCl, 10% glycerol, 5 μ g/mL leupeptin, 0.5 mM PMSF, and 2 mM DTT) and stood on ice for 30 min. The nuclear extracts were centrifuged at 14000 rpm for 10 min at 4°C to remove cellular debris. The protein content of the supernatants was

Fig. 2. Effects of Compounds on LPS-Induced iNOS Expression and NO Production

(A) HAPI cells were treated with LPS (100 ng/mL) for 6 h in the presence or absence of compounds (C1–C10 and A11, 10 μ M). After the treatment, total RNA was extracted, and then RT-PCR was performed. Values (mean $\pm$ S.E.M., $n=3$) are expressed as a percentage relative to the mRNA expression level in cells treated with LPS alone. * $p<0.05$ and ** $p<0.01$, respectively (vs. cells treated with LPS alone). (B) HAPI cells were treated with LPS (100 ng/mL) for 24 h in the presence or absence of compounds (C1–C10 and A11, 10 μ M). After the treatment, nitrite level in culture medium was measured. Values (mean $\pm$ S.D., $n=3$) are expressed as a percentage relative to the nitrite level in cells treated with LPS alone. * $p<0.05$ and ** $p<0.01$, respectively (vs. cells treated with LPS alone). (C) Correlation between inhibitory effects of compounds on iNOS expression and NO production. (D) Dose-dependent study of C4 and C10 on NO production. HAPI cells were treated with LPS (100 ng/mL) for 24 h at the indicated concentrations of C4 or C10. After the treatment, nitrite levels in culture medium were measured. Values (mean $\pm$ S.D., $n=3$) are expressed as a percentage relative to the nitrite level in cells treated with LPS alone.

determined using Bio-Rad protein assay reagent.

Western Blotting Whole-cell lysates (40 μ g) or nuclear extracts (10 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% (w/v) polyacrylamide gels. After being transferred onto a polyvinylidene difluoride (PVDF) membrane, the blotted membrane was blocked using PBS containing 1% BSA. The membrane was sequentially incubated with each primary antibody (1:3000), biotin-conjugated second antibody (1:3000), and ABC reagents (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) (1:5000), and then visualized using the Super-signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.).

Statistical Analysis Data were analyzed using ANOVA followed by *post hoc* Bonferroni tests or Student's *t*-test. A *p* value less than 0.05 was considered significant.

RESULTS

Chemistry We previously demonstrated that 'hidabeni' chalcone glycoside derivatives prevent LPS-induced iNOS expression in HAPI cells.¹⁷ To explore compounds with more potent inhibitory effects on LPS-induced iNOS expression, we synthesized ten compounds based on the 'hidabeni' chalcone skeleton (Fig. 1). Chalcones **C1**–**C10** were synthesized

through the $\text{BF}_3 \cdot \text{Et}_2\text{O}$ -mediated Claisen–Schmidt reaction of acetophenones and acetylated benzaldehydes. Acetylated chalcones were deprotected using sodium methoxide in methanol.

Effects of Synthesized Compounds on LPS-Induced iNOS Expression To determine whether the newly synthesized compounds inhibit LPS-induced iNOS mRNA expression, HAPI cells were treated with LPS (100 ng/mL) for 6 h in the presence of compounds (10 μ M). After the treatment, the expression levels of iNOS mRNA were detected using RT-PCR. As shown in Fig. 2A, almost all compounds suppressed LPS-induced iNOS expression, and especially **C4** and **C10** had the most potent inhibitory effects. **A11**, which was previously reported to inhibit iNOS expression at a concentration of 50 μ M, had no effect at a concentration of 10 μ M. We also examined the effects of these compounds on LPS-induced NO production. HAPI cells were treated with LPS (100 ng/mL) for 24 h in the presence of compounds (10 μ M), and then nitrite concentrations were measured in the medium of treated cells. As shown in Fig. 2B, these compounds suppressed LPS-induced NO production. There was a good correlation ($R^2=0.7775$) between the inhibitory effects of these compounds on LPS-induced iNOS mRNA expression and NO production (Fig. 2C). To determine the IC_{50} values of **C4** and **C10** toward NO production, we carried out dose-dependent experiments. The IC_{50} values of **C4** and **C10** were 4.19 μ M and

Fig. 3. Effects of **C4** and **C10** on NF- κ B and MAPK Pathways

(A) Effects of **C4** and **C10** on LPS-induced nuclear translocation of NF- κ B. HAPI cells were treated with LPS (100 ng/mL) for 3 h in the presence or absence of **C4** and **C10** (10 μ M). Nuclear extracts were prepared from the treated cells, and then subjected to Western blot analysis. A representative blot from three independent experiments is shown. Values (mean $\pm$ S.E.M., $n=3$) are expressed as a percentage relative to nuclear translocation in cells treated with LPS alone. (B) Effects of **C4** and **C10** on phosphorylation of MAPKs (JNK, p38, and ERK) and MEK. HAPI cells were treated with LPS (100 ng/mL) for 1 h (JNK, p38, and ERK) or 30 min (MEK) in the presence or absence of **C4** and **C10** (10 μ M). Whole-cell lysates were prepared from the treated cells, and then subjected to Western blot analysis. A representative blot from three independent experiments is shown. Values (mean $\pm$ S.E.M., $n=3$) are expressed as fold change relative to the ratio of phospho-MAPK or MEK to total MAPK or MEK in untreated cells. ^a $p<0.05$ and ^b $p<0.01$, respectively (vs. cells treated with LPS alone).

2.88 μM , respectively (Fig. 2D).

Effects of C4 and C10 on LPS-Induced NF- κ B Nuclear Translocation and MAPK Activation Since C4 and C10 potentially suppressed LPS-induced iNOS expression, we addressed the inhibitory mechanism of these compounds. LPS regulates iNOS expression through NF- κ B and MAPK pathways. Therefore, we investigated whether C4 and C10 inhibit activation of these pathways caused by LPS. First, to determine the effects of C4 and C10 on LPS-induced translocation of NF- κ B into the nucleus, HAPI cells were treated with LPS (100 ng/mL) for 3 h in the presence of these compounds (10 μM) and nuclear extracts prepared from the treated cells were subjected to Western blot analysis. As shown in Fig. 3A, however, C4 and C10 had no effect on NF- κ B nuclear translocation.

Next, to examine the effects of C4 and C10 on LPS-induced activation of MAPKs, HAPI cells were treated with LPS (100 ng/mL) for 1 h in the presence of these compounds (10 μM) and then phosphorylation of JNK, p38, and ERK was detected by Western blotting. As shown in Fig. 3B, LPS provoked phosphorylation of JNK and p38. Although the co-treatment with LPS and these compounds tended to increase the phosphorylation levels of JNK compared with the treatment with LPS alone, this alteration was not significant. Likewise, C4 and C10 did not affect the phosphorylation state of p38. LPS only slightly phosphorylated ERK. Interestingly, however, C10, but not C4, suppressed ERK phosphorylation below the basal level (Fig. 3B). Therefore, we examined the effect of C10 on the phosphorylation of MEK, which is an upstream effector of ERK. HAPI cells were treated with LPS (100 ng/mL) for 30 min in the presence of C4 and C10 (10 μM), and then phosphorylation of MEK was detected. As expected, C10, but not C4, inhibited LPS-induced MEK phosphorylation (Fig. 3B).

C4 and C10 Suppress LPS-Induced Activation of IFN- β Signal and Expression of IRF-1 LPS induces the production of IFN- β and stimulates the IFN- β autocrine loop, leading to the expression of iNOS.¹⁰ Secreted IFN- β in LPS-activated macrophages stimulates phosphorylation of STAT1 *via* the type I interferon receptor. Since LPS-induced iNOS expression has been shown to be suppressed in macrophages prepared from *STAT1*^{-/-} mice, STAT1 is known to be indispensable for this phenomenon.¹⁸

Since the IFN- β signal plays an important role in LPS-induced iNOS expression, we examined the effects of C4 and C10 on LPS-induced IFN- β mRNA expression. HAPI cells were treated with LPS (100 ng/mL) for 6 h in the presence of compounds (10 μM) and expression levels of IFN- β mRNA were detected using RT-PCR. As shown in Fig. 4A, C4 and C10 significantly suppressed LPS-induced IFN- β mRNA expression. Next, to examine whether C4 and C10 influence LPS-induced STAT1 activation, HAPI cells were treated with LPS (100 ng/mL) for 3 h in the presence of these compounds (10 μM) and the phosphorylation of STAT1 in the treated cells was detected by Western blotting. As shown in Fig. 4B, C4 and C10 inhibited LPS-induced STAT1 phosphorylation.

In addition to IFN- β /STAT1 signaling, IRF-1 has been shown to play a critical role in LPS-induced iNOS expression.¹⁹ We also examined the effects of C4 and C10 on LPS-induced IRF-1 mRNA expression. As shown in Fig. 4C, these compounds significantly suppressed the induction of IRF-1 mRNA caused by LPS.

Fig. 4. Involvement of IFN- β /STAT1 Signaling and IRF-1 in the Inhibitory Effects of C4 and C10

(A) Effects of C4 and C10 on LPS-induced IFN- β mRNA expression. HAPI cells were treated with LPS (100 ng/mL) for 6 h in the presence or absence of C4 and C10 (10 μM). After the treatment, total RNA was extracted, and then RT-PCR was performed. Values (mean $\pm$ S.E.M., $n=3$) are expressed as a percentage relative to the mRNA expression level in cells treated with LPS alone. ** $p<0.01$ (vs. cells treated with LPS alone). (B) Effects of C4 and C10 on LPS-induced phosphorylation of STAT1. HAPI cells were treated with LPS (100 ng/mL) for 3 h in the presence or absence of C4 and C10 (10 μM). After the treatment, whole-cell lysates were prepared from the treated cells, and then subjected to Western blot analysis. A representative blot from four independent experiments is shown. Values (mean $\pm$ S.E.M., $n=4$) are expressed as fold change relative to the ratio of phospho-STAT1 to total STAT1 in untreated cells. a and b, $p<0.05$ and $p<0.01$, respectively (vs. cells treated with LPS alone). (C) Effects of C4 and C10 on LPS-induced IRF-1 expression. HAPI cells were treated with LPS (100 ng/mL) for 6 h in the presence or absence of C4 and C10 (10 μM). After the treatment, total RNA was extracted, and then RT-PCR was performed. Values (mean $\pm$ S.E.M., $n=3$) are expressed as a fold change relative to the mRNA expression level in untreated cells. ** $p<0.01$ (vs. cells treated with LPS alone).

Effects of Compounds on LPS-Induced Expression of Proinflammatory Cytokines LPS has been shown to induce various inflammatory cytokines such as TNF- α and IL-1 β .^{18,20} We examined the effects of these compounds on the LPS-induced expression of TNF- α and IL-1 β mRNAs. As shown in Fig. 5A, although these compounds suppressed the induction of TNF- α mRNA, they showed little difference in the extent of suppression of TNF- α mRNA expression. Interestingly, however, C10 had a potent inhibitory effect compared with

Fig. 5. Effects of Compounds on LPS-Induced TNF- α (A) and IL-1 β (B) mRNA Expression

HAPI cells were treated with LPS (100 ng/mL) for 6 h in the presence or absence of compounds (C1–C10, A11, 10 μ M). After the treatment, total RNA was extracted, and then RT-PCR was performed. Values (mean $\pm$ S.E.M., $n=3-6$) are expressed as a percentage relative to the mRNA expression level in cells treated with LPS alone. * $p<0.05$ and ** $p<0.01$, respectively (vs. cells treated with LPS alone).

the other compounds. In contrast, both **C4** and **C10** potentially inhibited LPS-induced IL-1 β mRNA expression (Fig. 5B).

DISCUSSION

Previously, we reported that 4'-*O*- β -D-glucopyranosyl-3',4-dimethoxychalcone from the aerial parts of *Brassica rapa* L. 'hidabeni' and its synthesized derivative 4'-*O*- β -D-glucopyranosyl-3'-methoxychalcone (**A11**) prevent LPS-induced iNOS expression and NO production in HAPI cells.¹⁷⁾ In this study, to explore compounds with a more potent inhibitory effect on LPS-induced iNOS expression, we newly synthesized ten chalcones. Almost all chalcone derivatives tested in this study had inhibitory activity toward LPS-induced NO production. The chalcones having monohydroxy and monomethoxy (**C7**), dimethoxy (**C3**), monohydroxy and dimethoxy (**C6**), or trimethoxy (**C4**, **C8**–**C10**) groups on the B ring led to augmentation of inhibitory effects compared with the chalcones having a monohydroxy (**C5**) or monomethoxy (**C2**) group or no functional group (**C1**) on the B ring. Additionally, the 3,4,5-trimethoxy-substituted chalcone (**C4**) more potently inhibited LPS-induced iNOS expression and NO production than 4-monohydroxy and 3,5-dimethoxy-substituted chalcone (**C6**). Moreover, the chalcone with no functional group on the A ring (**C8**) was less effective in terms of NO inhibition than those with 4'-monohydroxy (**C9**) and 3'-monomethoxy (**C10**) groups. These results indicate that the substitutions with a methoxy group at the 3'- and/or a hydroxy group at the 4'-position on the A ring and methoxy, but not hydroxy, groups at 3,4-positions or 3,4,5-positions on the B ring are

required to acquire potent inhibitory effects by chalcones on LPS-induced NO production.

It has been reported that chalcones prevent LPS-induced proinflammatory cytokines and iNOS expression by the inhibition of NF- κ B, JNK, and p38 pathways.^{13,21–24)} We demonstrated here that **C4** and **C10** potentially suppressed iNOS induction and NO production caused by LPS, but did not affect NF- κ B, JNK, and p38 pathways. Interestingly, **C10**, but not **C4**, potently inhibited the MEK/ERK pathway. However, since both **C4** and **C10** inhibited NO production to a similar extent, the inhibition of the MEK/ERK pathway might slightly contribute to the inhibitory effect of **C10** on LPS-induced iNOS expression. The structure of **C10** is identical to that of **C4**, except for the substitution of one hydroxy group at the 4'-position on the A ring. Thus, these results indicate that the difference in substitution at the position on the A ring confers specificity in the MEK-ERK pathway. We found that the suppressive effect of **C10** on LPS-induced TNF- α mRNA expression was stronger than that of **C4**. Since LPS-induced TNF- α expression is reported to be involved in ERK,^{25,26)} the inhibitory effect of **C10** on the expression of TNF- α may be attributable to inhibition of the MEK/ERK pathway by **C10**. Compounds other than **C10** partially prevented induction of TNF- α by LPS. At present, we do not know the details of how these compounds suppress TNF- α induction. However, we have previously found that several inhibitors, such as LY294002 (phosphatidylinositol 3-kinase), diphenyl-eneiodonium (NADPH oxidase), and PP2 (Src family kinase), prevent LPS-induced TNF- α mRNA expression in this cell line (unpublished data). Therefore, it is likely that inhibitory

action of these compounds on other pathway(s) contributes to prevention of TNF- α induction.

IFN- β /STAT1 signaling plays a key role in LPS-induced iNOS expression.¹⁰⁾ We found that **C4** and **C10** suppressed LPS-induced IFN- β expression in HAPI cells (Fig. 4A). In addition, these compounds inhibited STAT1 phosphorylation caused by LPS (Fig. 4B). Therefore, it is likely that the suppression of IFN- β production by these compounds is due to the prevention of STAT1 activation. Previously, however, we reported that the synthesized chalcone glycoside **A11** hardly affects IFN- β production but decreases STAT1 activation by LPS.¹⁷⁾ In contrast, it has been shown that other tyrosine protein kinases such as EGF receptor and c-Src mediate phosphorylation of STAT1.^{27,28)} Thus, we cannot rule out the possibility that **C4** and **C10** inhibit STAT1 phosphorylation independently of type I IFN receptor activation by IFN- β .

Likewise, IRF-1 has been shown to be an essential transcription factor for LPS-induced iNOS expression because LPS fails to provoke iNOS expression in IRF-1-deficient macrophages.¹⁹⁾ **C4** and **C10** suppressed LPS-induced IRF-1 mRNA expression in HAPI cells (Fig. 4C). Therefore, the inhibitory effect of **C4** and **C10** on iNOS induction is likely to be attributable, at least in part, to suppression of IRF-1 expression by these compounds. In addition, IRF-1 is reported to bind to the promoter region of IFN- β and up-regulates its expression.²⁹⁾ Thus, it is possible that suppression of LPS-induced IRF-1 expression by **C4** and **C10** inhibits IFN- β production and leads to prevention of induction of the IFN- β /STAT1 pathway. Besides IFN- β and iNOS, IRF-1 has been shown to mediate the expression of various inflammatory factors.³⁰⁾ Therefore, inhibition of IRF-1 induction caused by **C4** and **C10** might decrease LPS-induced IL-1 β expression in HAPI cells.

The blood–brain barrier (BBB) permeability of compounds is critical to develop drugs for the CNS diseases. At present, whether compounds tested in this study penetrate the BBB is unknown. However, flavonoids have been shown to be able to traverse the BBB in *in vitro* and *in situ* models.³¹⁾ Additionally, *in vivo* studies using middle cerebral artery occlusion model have demonstrated that flavonoids and chalcones protect against brain injury caused by cerebral ischemia.^{32,33)} Therefore, the ‘hidabeni’ chalcone derivatives **C4** and **C10** could penetrate the BBB.

In conclusion, we found that 3',3,4,5-tetramethoxy-4'-hydroxychalcone (**C4**) and 3',3,4,5-tetramethoxychalcone (**C10**) have potent inhibitory activity on LPS-induced NO production. Although several chalcone derivatives have been reported to inhibit LPS-induced activation of NF- κ B and JNK pathways, **C4** and **C10** did not affect these pathways. Our results suggest that the mechanism by which the compounds suppress LPS-induced iNOS expression differs from that of previously reported chalcones. Therefore, we think that **C4** and **C10** may be good candidates to develop novel mechanism-based therapeutic drugs for neurodegenerative diseases associated with microglia.

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