BIOCHEMICAL PHARMACOLOGY 76 (2008) 507–519

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Inhibition of nitric oxide production by the carbazole compound LCY-2-CHO via blockade of activator protein-1 and CCAAT/enhancer-binding protein activation in microglia

Ling-Chu Chang [a], *Lo-Ti Tsao* [b], *Chi-Sen Chang* [c], *Chun-Jung Chen* [b], *Li-Jiau Huang* [d], *Sheng-Chu Kuo* [d], *Ruey-Hseng Lin* [a,e,1], *Jih-Pyang Wang* [b,d,1,*]

[a] *Institute of Medicine, Chung Shan Medical University, Taichung 403, Taiwan, ROC*
[b] *Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan, ROC*
[c] *Division of Gastroenterology and Hepatology, Taichung Veterans General Hospital, Taichung 407, Taiwan, ROC*
[d] *Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung 404, Taiwan, ROC*
[e] *Department of Pharmacology, Chung Shan Medical University, Taichung 403, Taiwan, ROC*

ARTICLE INFO

Article history:
Received 13 April 2008
Accepted 2 June 2008

Keywords:
LCY-2-CHO
Microglial cells
Nitric oxide
Inducible nitric oxide synthase
Activator protein-1
CCAAT/enhancer-binding protein

ABSTRACT

Excessive nitric oxide (NO) production by activated microglia plays a critical role in neurodegenerative disorders. In this study, we found that 9-(2-chlorobenyl)-9H-carbazole-3-carbaldehyde (LCY-2-CHO) suppressed the NO production in lipopolysaccharide (LPS)/interferon-γ (IFNγ)-stimulated murine microglial N9 and BV-2 cells and in LPS-stimulated N9 cells and rat primary microglia. LCY-2-CHO had no cytotoxic effect on microglia. In activated N9 cells, LCY-2-CHO abolished the expression of inducible nitric oxide synthase (iNOS) protein and mRNA but failed to alter the stability of expressed iNOS mRNA and the enzymatic activity of expressed iNOS protein. LCY-2-CHO did not block DNA-binding activity of nuclear factor-κB (NF-κB) or cyclic AMP response element-binding protein (CREB), but abolished that of activator protein-1 (AP-1), CCAAT/enhancer-binding protein (C/EBP) and nuclear factor IL6 (NF-IL6). LCY-2-CHO attenuated the nuclear levels of c-Jun and C/EBPβ, but not those of p65, p50, C/EBPδ, signal transducer and activator of transcription-1 (STAT-1) or the nuclear expression of IFN regulatory factor-1 (IRF-1). LCY-2-CHO had no effect on the phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), MAPK-activated protein kinase-2 (MAPKAPK-2), STAT-1, CREB or c-Jun in LPS/IFNγ-stimulated N9 cells, whereas it attenuated the phosphorylation of C/EBPβ at Ser105 and Thr235 residues, which occurred concomitantly with LCY-2-CHO inhibition of C/EBPβ expression and phosphorylation. Taken together, these results suggest that LCY-2-CHO inhibits NO production in microglia through the blockade of AP-1 and C/EBP activation.

* *Corresponding author at*: Department of Education and Research, Taichung Veterans General Hospital, 160, Sec. 3, Chung-Kang Road, Taichung 407, Taiwan, ROC. Tel.: +886 4 23592525x4023; fax: +886 4 23592705.
E-mail address: w1994@vghtc.gov.tw (J.-P. Wang).

[1] These authors contributed equally to this work.

Abbreviations: AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; CREB, cyclic AMP response element-binding protein; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; IRF-1, IFN regulatory factor-1; JAK, Janus tyrosine kinase; JNK, c-Jun NH_2-terminal kinase; LCY-2-CHO, 9-(2-chlorobenyl)-9H-carbazole-3-carbaldehyde; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase-2; NF-IL6, nuclear factor IL-6; NF-κB, nuclear factor-κB; NO, nitric oxide; STAT, signal transducer and activator of transcription.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.06.002

1. Introduction

Microglial cells, the resident macrophages in the central nervous system, play a pivotal role in the first line of host defense by removing pathogens. However, over-reactive microglial cells contribute to inflammatory damage by releasing various proinflammatory mediators, including nitric oxide (NO) [1]. The overproduction of NO in the central nervous system is believed to contribute to progressive damage in neurodegenerative diseases such as multiple sclerosis, Parkinson's disease, and Alzheimer's disease [1]. Thus, pharmacological interference with the NO production from microglia presents a reasonable strategy to control the potentially harmful proinflammatory activity of these cells in NO-mediated neurodegenerative damage.

NO is a product of the oxidation of L-arginine to L-citrulline catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS have been identified: two constitutive NOSs, endothelial NOS (eNOS) and neuronal NOS (nNOS), which are regulated by Ca^{2+}, and one inducible NOS (iNOS), which is Ca^{2+}-independent and is regulated mainly at the transcriptional level [2]. Bacterial lipopolysaccharide (LPS), widely used in brain inflammatory studies, activates microglial cells and induces the expression of iNOS [3], which is responsible for the prolonged and profound production of NO. A number of binding sites for transcription factors, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), interferon regulatory factor-1 (IRF-1), CCAAT/enhancer-binding protein (C/EBP), nuclear factor IL-6 (NF-IL6), cyclic AMP response element-binding protein (CREB), and signal transducer and activator of transcription (STAT), have been identified in iNOS promoter region in glial cells [4].

In studies of the anti-inflammatory activities of chemically synthetic carbazole compounds, 9-(2-chlorobenyl)-9*H*-carbazole-3-carbaldehyde (LCY-2-CHO) has been shown to inhibit platelet aggregation, neutrophil degranulation, superoxide anion generation [5,6] and NO production in macrophages and smooth muscle cells [7–9], and to induce malignant hematopoietic cells apoptosis [10]. Because NO derived from microglia is critical to brain damage, we extended this study by examining the effect of LCY-2-CHO on NO production in microglial cells and attempted to clarify the underlying mechanisms. The results demonstrated that inhibition of NO production by LCY-2-CHO in microglial cells could be attributed to the down-regulation of AP-1- and C/EBP-dependent pathways.

2. Materials and methods

2.1. Drugs and chemicals

Iscove's modified Dulbecco's medium (IMDM), Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride membrane and Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate were obtained from Millipore (Bedford, MA, USA). Hybond-N^+ nylon membrane was purchased from Amersham Bioscience (Buckinghamshire, UK). REzol^{TM} C&T reagent was obtained from Protech Technology (Taiwan). Express Hyb hybridization solution and the antibodies against iNOS were obtained from BD Biosciences (San Diego, CA, USA). Random primer fluorescein labeling kit was obtained from PerkinElmer (Hong Kong). DIG gel shift kit, positive charge nylon membrane and Cytotoxicity Detection Kit (LDH) were purchased from Roche Applied Science (Mannheim, Germany). Antibodies against p50, p65, p38 MAPK, phospho-STAT-1 (Tyr701), STAT-1, C/EBPβ, C/EBPδ, IRF-1 and proliferating cell nuclear antigen (PCNA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-p38 MAPK, extracellular signal-regulated kinase (ERK), phospho-ERK, phospho-CREB (Ser133), CREB, phospho-c-Jun (Ser73), c-Jun, c-Jun NH_2-terminal kinase (JNK), phospho-JNK, phospho-C/EBPβ (Ser105) and phospho-C/EBPβ (Thr235) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against β-actin was obtained from Chemicon (Temecula, CA, USA). Mouse recombinant IFNγ was obtained from R&D Systems (Minneapolis, MN, USA). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The final volume of DMSO in the culture medium was $\leq$0.1% (v/v).

2.2. Synthesis of LCY-2-CHO

Carbazole (3.34 g, 20 mmol) was dissolved in dry THF (50 ml), and KOH (2.24 g, 40 mmol) was added with stirring at room temperature. 2-Chlorobenzyl chloride (6.44 g, 40 mmol) in THF (10 ml) was added portionwise to the mixture and refluxed for 16 h. The reaction mixture was quenched with ice water (100 ml) and extracted with CH_2Cl_2, The CH_2Cl_2 layer was washed with aqueous $NaHCO_3$, dried over anhydrous $MgSO_4$ and the residue was purified by chromatography on silica gel. Elution with *n*-hexane/ethyl acetate (9:1) and recrystallization from *n*-hexane/CH_2Cl_2 to yield 9-(2-chlorobenzyl)-carbazole (2.26 g, 45%) which was then dissolved in DMF (20 ml) and $POCl_3$ (1.4 ml, 15 mmol) for 1 h at 5 °C followed by stirring at room temperature overnight, then heated at 60 °C for 2 h. The reaction was quenched with ice water (100 ml) and extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness. The residue was purified by silica gel chromatography and eluted with *n*-hexane/ethyl acetate (7:3) to yield LCY-2-CHO (Fig. 1) (1.52 g, 53%) as colorless powder; m.p.: 129–131 °C. ^{1}H NMR ($CDCl_3$) δ 5.54 (2H, s, N-CH_2), 7.11-7.55 (8H, *m*, H-1, H-6, H-7, H-8, H-3′, H-4′, H-5′, H-6′), 7.98 (1H, *dd*, *J* = 1.6, 8.6 Hz, H-2), 8.19 (1H, *d*, *J* = 1.6, 8.6 Hz, H-5), 8.65 (1H, *d*, *J* = 1.6 Hz, H-4),

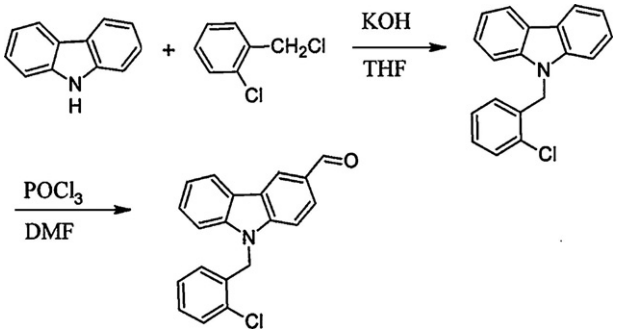

Fig. 1 – Synthesis of LCY-2-CHO compound.

10.10 (1H, s, -CHO). Anal. calcd. for $C_{20}H_{14}NOCl$: C, 75.12; H, 4.41; N, 4.38. Found: C, 75.06; H, 4.40; N, 4.37. LCY-2-CHO (purity > 99%) was dissolved in DMSO.

2.3. Cell culture

Murine microglial cell line N9 (kindly provided by Dr. P. Ricciardi-Castagnoli, CNR Cellular and Molecular Pharmacology Center, Italy), obtained by immortalization of E13 mouse embryonic cultures with the 3RV retrovirus carrying an activated v-myc oncogene that is similar to primary microglia in producing substantial amounts of NO and various cytokines after stimulation [11], was cultured in IMDM, supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol at 37 °C in humidified incubator with 5% CO_2. Stock cells were passaged 2–3 times/week with 1:10 split ratio and used within 8 passages. Murine microglial cell line BV-2 (kindly provided by Dr. J.-S. Hong, Neuropharmacology Section, NIEHS, Research Triangle Park, NC, USA), obtained by infecting primary microglia with a v-raf/v-myc oncogene-carrying retrovirus that exhibits both the phenotypic and functional properties of reactive microglia cells [12], was cultured in DMEM supplemented with 10% FCS, penicillin and streptomycin.

Rat primary microglial cultures were prepared as previously described [13]. Briefly, the cerebral cortices of 1-day-old rats (Sprague-Dawley) were dissected. The tissue chunks were incubated with papain solution (100 U/ml of papain, 0.5 mM EDTA, 0.2 mg/ml of cysteine, 1.5 mM $CaCl_2$, and 0.21% DNase I) at 37 °C for 20 min to dissociate the cells. The cells were suspended in the DMEM supplemented with 10% horse serum and plated onto poly-D-lysine-coated 75-mm^2 flask in humidified incubator with 5% CO_2 for 24 h. The medium was replaced by the DMEM supplemented with 10% FCS, penicillin and streptomycin, and the medium was changed every 4–5 days thereafter till 10–14 days after plating. Flasks were then shaken (150 rpm for 20 min) in a rotary shaker, and detached cells (as microglia) were plated into 96-well plate at a density of 8×10^4 cells/well with purity ≥98% (assessed using ED-1 antibody). All experiments in the present study were performed under the guidelines of the Institutional Experimental Laboratory Animal Committee and were in strict accordance with the principles and guidelines contained in the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

2.4. Determination of NO production and iNOS activity assay

N9 cells (8×10^4 cells/well), BV-2 cells (8×10^4 cells/well) or primary microglia (8×10^4 cells/well) were seeded onto 96-well plates and allowed to adhere overnight. Cells were then treated with the test drug at 37 °C for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for 18 h (for N9 cells) or with LPS (1 μg/ml) for 24 h (for BV-2 cells and rat primary microglia) in a final volume of 0.2 ml. The production of NO was determined by measuring the accumulation of nitrite, the stable metabolite of NO, in culture medium based on the Griess reaction. A standard nitrite curve was generated in the same fashion using $NaNO_2$. In the iNOS activity assay, N9 cells were stimulated with LPS/IFNγ for 12 h and then washed twice with PBS. Test drugs were added for an additional 12 h in the absence of LPS/IFNγ. The nitrite content of culture media was analyzed as above.

2.5. Preparation of cell lysates and nuclear extracts

Cells were washed twice with PBS and harvested in Laemmli SDS sample buffer as cell lysate preparation. Nuclear extracts were prepared as previously described [14]. Briefly, cells were suspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 1 mM Na_3VO_4) and incubated on ice for 15 min. Cells were then lysed by the addition of 0.5% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation (12,000 × *g* for 2 min at 4 °C) and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, and 1 mM Na_3VO_4). After 15 min on ice, lysates were centrifuged (12,000 × *g* for 10 min at 4 °C), and the supernatants were removed and stored at −70 °C.

2.6. Western blot analysis

Cell lysates (30 μg protein) or nuclear extracts (5 μg protein) were separated by 7.5% SDS-PAGE (for iNOS and β-actin), 10% SDS-PAGE (for c-Jun, MAPKs, STAT-1, CREB, and IRF-1), or 13% SDS-PAGE (for C/EBPβ and C/EBPδ) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated with 5% non-fat milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h, and blotted with various antibodies. After washing with TBST buffer, HRP-labeled IgG was added at room temperature for 1 h. The blots were developed using Immobilon Western chemiluminescent HRP substrate. The band intensity was detected and quantitated by Luminescent Image Analyzer (Fujifilm LAS-3000) using MultiGauge software.

2.7. RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis

The total RNA (20 μg), isolated from N9 cells using REzol™ C&T reagent according to the instructions of the manufacturer, was subjected to electrophoresis on 1% agarose gels, then transferred onto Hybond-N^+ nylon membranes by electroblotting. After UV cross-linking, the membranes were prehybridized, and then hybridized with a cDNA probe [7]. The iNOS and GAPDH cDNA probes were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using published iNOS and GAPDH primers. The primers used are as follows: iNOS, sense primer: 5′-TCA TTG TAC TCT GAG GGC TGA CAC A-3′, antisense primer: 5′-GCC TTC AAC ACC AAG GTT GTC TGC A-3′; and GAPDH, sense primer: 5′-TAT GAC AAC TCC CTC AAG AT-3′, antisense primer: 5′-AGA TCC ACA ACG GAT ACA TT-3′ [7]. The PCR products were labeled with a random primer fluorescein labeling kit. After hybridization, the membranes were washed and subsequently probed with antifluorescein-HPR conjugated antibody before visualization with the nucleic acid chemiluminescence reagent. To ensure equal loading of RNA, a GAPDH probe was used as an internal control to normalize iNOS mRNA expression. The densities of

the bands were detected and quantitated by Luminescent Image Analyzer (Fujifilm LAS-3000) using MultiGauge software. For mRNA degradation assay, cells were stimulated with LPS/IFNγ for 12 h. After washing, cells were incubated with vehicle or test drug in the presence of actinomycin D (5 μg/ml) for various time periods, and then the iNOS mRNA was determined.

2.8. Electrophoretic mobility shift assay (EMSA)

The consensus sequence of DNA probe used to detect the DNA-binding activities is as follows: NF-κB, 5′-TCG ACC AAC TGG GGA CTC TCC CTT TGG GAA CA-3′ [15]; AP-1, 5′-TCG AGA TCC GCT TGA TGA CTC AGC CGG AA-3′ [16]; CREB, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; C/EBP, 5′-TGC AGA TTG CGC AAT CTG CA-3 [17]; NF-IL6, 5′-CCA CAG AGT GAT GTA ATC A-3′ [18]. Nuclear extract (5 μg) was used for EMSA after end labeling of the DNA probes with DIG gel shift kit according to the protocol of the manufacturer. Samples were loaded on to 6% native polyacrylamide gels. The gels were then transferred onto positive charge nylon membrane for chemiluminescent detection. The specificity of binding was determined by the addition of 20-fold unlabeled oligonucleotide. Before the binding reaction, supershift assays were carried out by the addition of 2 μg of specific antibody to the nuclear extract at 4 °C overnight.

2.9. Statistical analysis

Statistical analyses were performed using Student's *t* test for two group comparison and the Bonferroni *t* test method after ANOVA for multigroup comparison; $P < 0.05$ was considered statistically significant. Analysis of the regression line test was used to calculate IC_{50} values.

3. Results

3.1. Effect of LCY-2-CHO on NO production in microglial cells

Treatment of N9 cells with either LPS or IFNγ evoked NO production in a concentration-dependent manner. The NO production was markedly stimulated when LPS (10 ng/ml) and IFNγ (10 U/ml) were given in combination (Fig. 2A). This result is in accordance with that of a previous report [19]; however

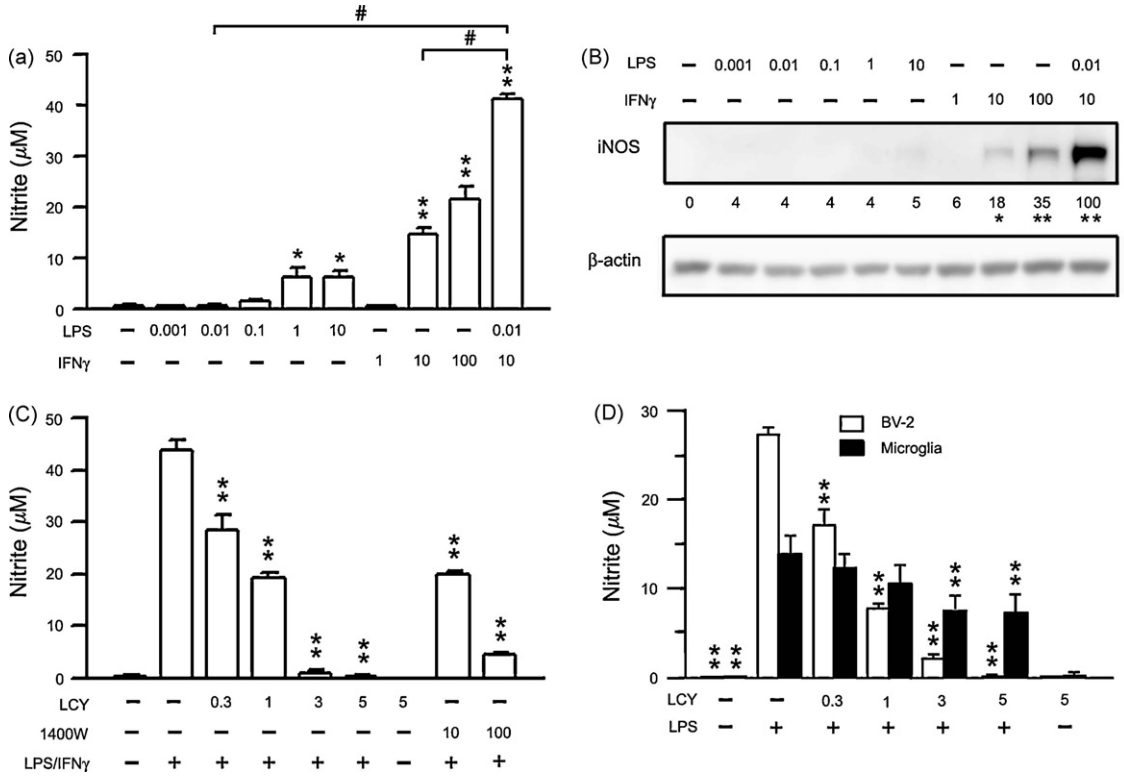

Fig. 2 – LPS/IFNγ induced NO production and iNOS protein expression in N9 cells, and effect of LCY-2-CHO (LCY) on NO production in microglial cells. N9 cells were treated with the indicated concentrations of LPS (μg/ml)/IFNγ (U/ml) for 18 h before the measurement of (A) nitrite in medium and (B) iNOS protein expression in cells. Nitrite values are means ± S.D. of 4 independent experiments. The iNOS protein and β-actin (as loading control) in cell lysates were determined by Western blot analysis and the mean of relative densitometric value from 4 independent experiments is shown below the blots. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the corresponding resting values (1st column for (A) and 1st lane for (B)). $^{\#}P < 0.001$. (C) N9 cells, (D) BV-2 cells or rat primary microglia were treated with the indicated concentrations (μM) of LCY or 1400 W for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for 18 h (for N9 cells) or with LPS (1 μg/ml) for 24 h (for BV-2 cells and primary microglia), and then the nitrite in the medium was determined. Values are means ± S.D. of 4 independent experiments. $^{**}P < 0.01$, compared with the corresponding control values (2nd group columns).

less then a 10-fold concentration was required in the present study to release a similar amount of NO. It is believed that iNOS plays a major role in the NO production in activated inflammatory cells. The parallel changes in NO production and iNOS protein expression (Fig. 2B) support the potentiating effect of LPS in the presence of IFNγ, therefore LPS (10 ng/ml) in combination with IFNγ (10 U/ml) (LPS/IFNγ) was used as stimulant in the following experiments. Pretreatment of cells with LCY-2-CHO attenuated the LPS/IFNγ-induced NO production in a concentration-dependent manner (Fig. 2C) with an IC_{50} value of 1.8 ± 0.1 μM. Under the same experimental conditions, addition of 10 and 100 μM 1400 W, a widely used iNOS inhibitor [20], resulted in 50% and 90% inhibition, respectively, of NO production. Cell viability, assessed in lactate dehydorgenase (LDH) release and 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, was >95% within the reaction intervals (data not shown). The inhibition by LCY-2-CHO of NO production was also observed in LPS-stimulated BV-2 microglial cells and rat primary microglia (Fig. 2D) with the IC_{50} values of 0.8 ± 0.1 and 3.2 ± 0.8 μM, respectively.

3.2. *Effects of LCY-2-CHO on iNOS activity and the expression of iNOS protein and mRNA*

We next examined whether the expression of iNOS protein and iNOS activity can be influenced by LCY-2-CHO in N9 cells. Stimulation with LPS/IFNγ for 12 h significantly increased the cellular iNOS activity (Fig. 3A). Subsequent LCY-2-CHO (up to 5 μM) addition had no appreciable effect on the expressed iNOS activity, whereas the NOS inhibitor L-NAME (0.3 mM) attenuated the iNOS activity to $11.0 \pm 2.0\%$ of the control value. Western blot analysis demonstrated that LCY-2-CHO down-regulated iNOS protein expression in N9 cells (with an IC_{50} value of 0.8 ± 0.5 μM) (Fig. 3B) as well as in LPS-stimulated rat primary microglia (with an IC_{50}

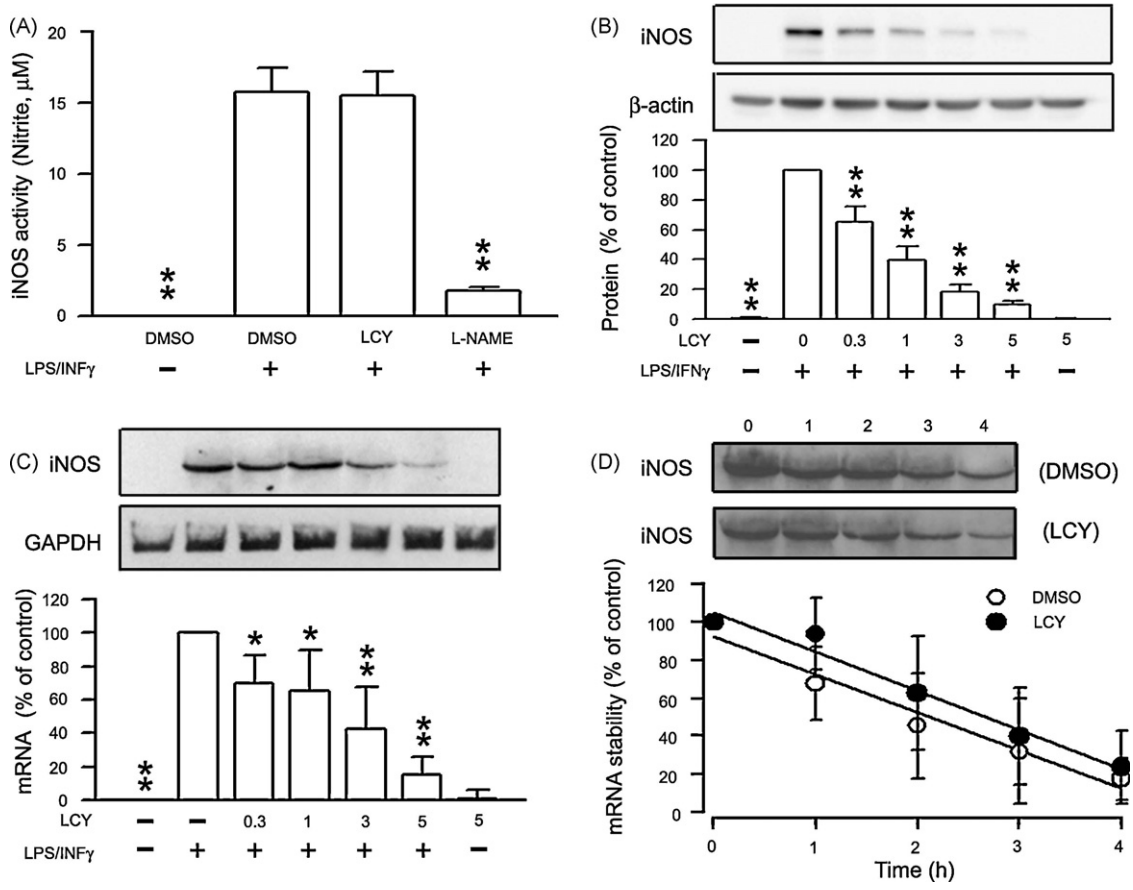

Fig. 3 – Effects of LCY-2-CHO (LCY) on the expression of iNOS protein and mRNA and on iNOS activity in N9 cells. (A) Cells were stimulated with LPS (10 ng/ml)/IFNγ (10 U/ml) for 12 h, then washed with PBS and treated with DMSO, 5 μM LCY or 0.3 mM L-NAME for additional 12 h in the absence of LPS/IFNγ before the measurement of nitrite in the medium. Values are means ± S.D. of 4 independent experiments. $^{**}P < 0.01$, compared with the control value (2nd column). Cells were also treated with the indicated concentrations (μM) of LCY for 1 h before stimulation with LPS/IFNγ for an additional 18 and 6 h, respectively, for measuring the expression of (B) iNOS protein by means of Western blot analysis (β-actin as loading control) and (C) iNOS mRNA by means of Northern blot analysis (GAPDH as internal standard). Values are means ± S.D. of 4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the corresponding control values (2nd column). (D) Cells were stimulated with LPS/IFNγ for 12 h, and then washed with PBS and treated with DMSO or 5 μM LCY for the indicated time periods (h) in a fresh medium containing 5 μg/ml of actinomycin D before the measurement of iNOS mRNA. Values are means ± S.D. of 3 independent experiments.

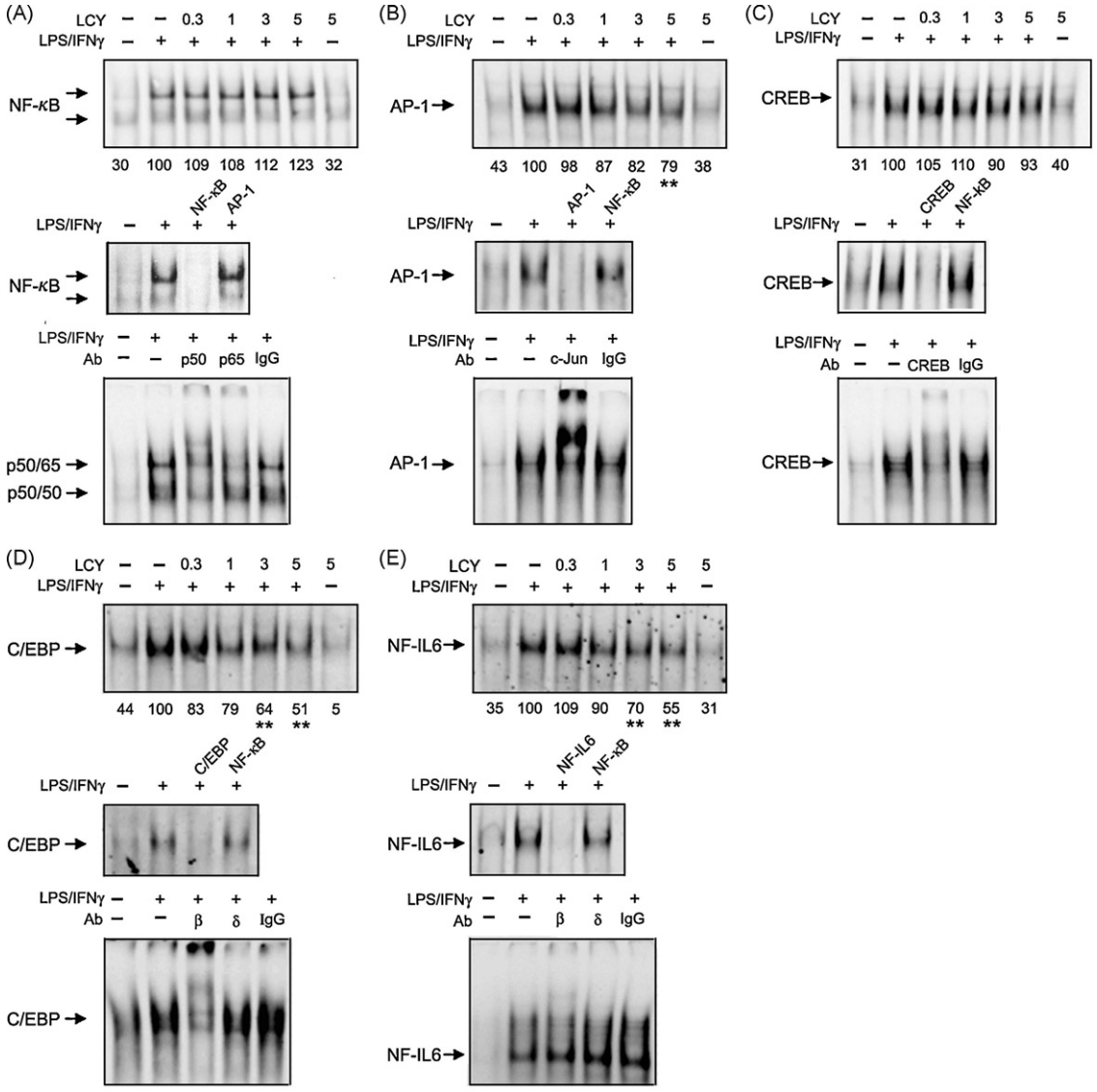

Fig. 4 – Effects of LCY-2-CHO (LCY) on the binding activation of NF-κB, AP-1, CREB, C/EBP and NF-IL6 in N9 cells. Cells were treated with the indicated concentrations (μM) of LCY for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for additional 1.5 h. The nuclear extracts were prepared and EMSA was performed by incubation with a DIG-labeled probe containing sequences for promoter (A) NF-κB, (B) AP-1, (C) CREB, (D) C/EBP and (E) NF-IL6 binding. The mean of relative densitometric value from 4 independent experiments is shown below the graphs. $^{**}P < 0.01$, compared with the corresponding control values (2nd lane). Specificity was conducted by the addition of 20-fold excess of unlabeled oligonucleotide competitors to nuclear extracts (the middle graph of each panel). The supershift experiments were carried out by incubation of nuclear extract with specific antibody before the binding reaction (the lower graph of each panel).

value of 1.9 ± 0.8 μM) (data not shown). Therefore, we undertook experiments to assess whether LCY-2-CHO can affect the *iNOS* gene expression. LCY-2-CHO decreased iNOS mRNA expression in N9 cells with an IC_{50} value of 2.5 ± 0.9 μM as evidenced by Northern blot analysis (Fig. 3C). Moreover, 12 h after stimulation of cells with LPS/IFNγ, the degradation rate of the expressed iNOS mRNA was not changed by subsequent addition of LCY-2-CHO ($t_{1/2}$ value of 2.2 ± 0.8 h vs. 2.8 ± 1.1 h for control, $P > 0.05$) (Fig. 3D) in the presence of actinomycin D to blockade the synthesis of new iNOS mRNA.

3.3. *Effect of LCY-2-CHO on DNA-binding activity of transcription factors in the iNOS promoter*

To determine whether LCY-2-CHO influences the activation of transcription factors, which have binding sites in iNOS promoter, we examined its effects on the DNA-binding activity of NF-κB, AP-1, CREB, C/EBP and NF-IL6 by using EMSA. After stimulation with LPS/IFNγ, the DNA-binding activity of these transcription factors was detectable at 0.5 h and peaked at 1.5–2 h before declining (data not shown). The time of maximal response was used in the following EMSA experiments. LCY-2-

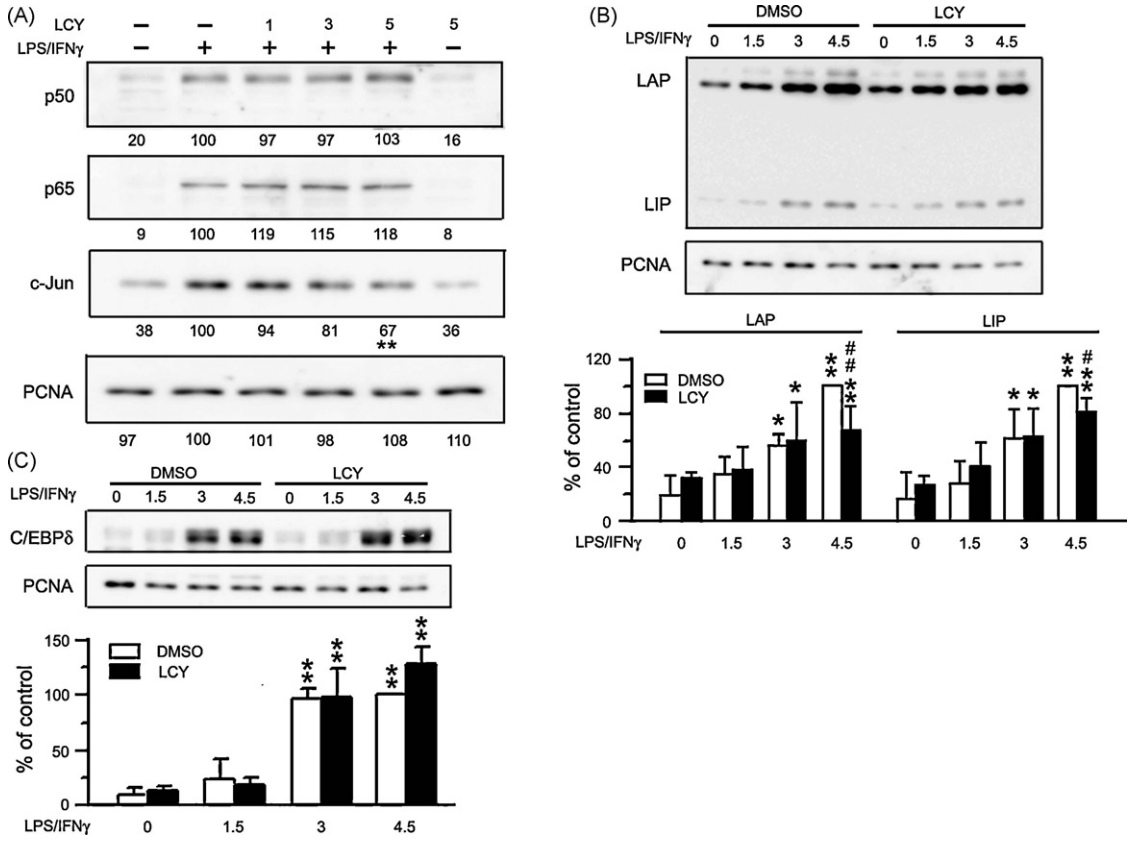

Fig. 5 – Effect of LCY-2-CHO (LCY) on nuclear translocation of transcription factors in N9 cells. (A) Cells were treated with the indicated concentrations (μM) of LCY for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for additional 1.5 h, and then the nuclear extracts were prepared for Western blot analysis by using antibodies against NF-κB (p50 and p65), c-Jun or PCNA (as loading control). The mean of relative densitometric value from 3 independent experiments is shown below the blots. $^{**}P < 0.01$, compared with the corresponding control value (2nd lane). Cells were also treated with DMSO or 5 μM LCY for 1 h before stimulation with LPS/IFNγ for the indicated time periods (h), and then nuclear extracts were prepared for Western blot analysis using antibodies against (B) C/EBPβ (LAP and LIP) or (C) C/EBPδ. The densitometric values of C/EBPs are expressed as means ± S.D. of 4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the corresponding resting values (1st group columns); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the corresponding DMSO-treated values.

CHO attenuated the LPS/IFNγ-induced DNA-binding activity of AP-1 (about 40% inhibition at 5 μM LCY-2-CHO) (Fig. 4B), C/EBP and NF-IL6 (IC_{50} values of 1.9 ± 1.2 and 3.7 ± 0.3 μM, respectively) (Fig. 4D and E), whereas it had no effect on NF-κB (Fig. 4A) or CREB (Fig. 4C). The specificity of DNA-binding was confirmed by using an excessive oligonucleotide competitor. The supershift experiments were assessed by incubation of nuclear extract with specific antibody before the binding reaction. These results suggest the involvement of AP-1, C/EBP and NF-IL6 signalings in LCY-2-CHO-mediated inhibition of iNOS expression.

3.4. *Effects of LCY-2-CHO on the levels of nuclear transcription factors*

It is believed that the recruitment of transcription factors to nucleus is essential for transcriptional activation. LCY-2-CHO did not affect the levels of nuclear NF-κB as assessed by immunoblot analysis of p65 and p50, two subunits of NF-κB heterodimer in microglia [21], in nuclear extracts of LPS/IFNγ-stimulated N9 cells, whereas it attenuated the levels of nuclear c-Jun, a major AP-1 component (about 50% inhibition at 5 μM LCY-2-CHO) (Fig. 5A).

Six members of C/EBP transcription factor family have been identified, among them, C/EBPβ and C/EBPδ have been implicated in glial iNOS induction [4]. NF-IL6 is a member of the C/EBP family and is composed of C/EBPβ and C/EBPδ in transcription activation by LPS. Three C/EBPβ isoforms including two full-length liver-enriched activating proteins (LAPs), 38- and 35-kDa, and a 21-kDa truncated liver-enriched inhibitory protein (LIP) have been identified. Only LAPs have been reported to function as transcriptional activators, whereas LIP lacks a transactivation domain in a dominant negative fashion [22]. All three C/EBPβ isoforms as well as C/EBPδ were evident in N9 cells, which showed a time-dependent increase in the nuclear levels of these isoforms in response to LPS/IFNγ (Fig. 5B and C). Pretreatment of cells with 5 μM LCY-2-CHO attenuated the levels of nuclear C/EBPβ (about 40% and 30% inhibition for LAPs and LIP, respectively, at 4.5 h after LPS/IFNγ stimulation), whereas it had no effect on the levels of nuclear C/EBPδ.

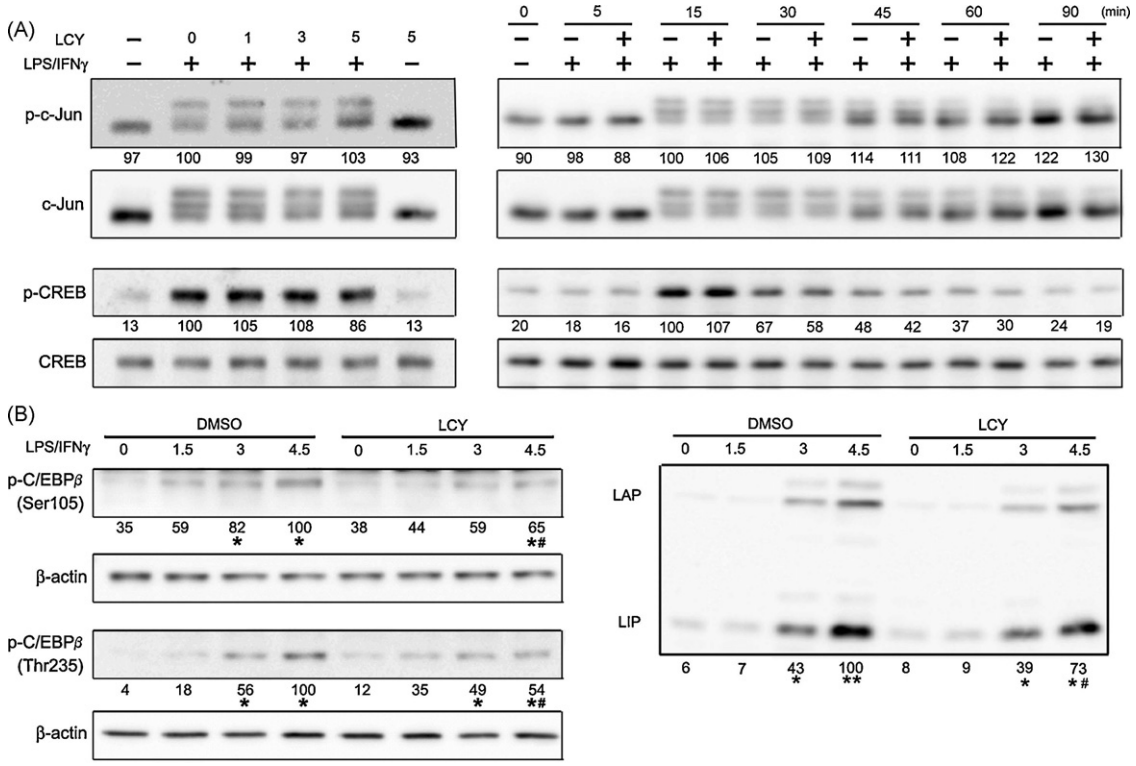

Fig. 6 – Effect of LCY-2-CHO (LCY) on phosphorylation of transcription factors in N9 cells. (A) Cells were treated with the indicated concentrations (μM) of LCY for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for additional 15 min, then cell lysates were prepared for Western blot analysis using antibodies against phospho-c-Jun, c-Jun (as loading control), phospho-CREB or CREB (as loading control) (left panel). Cells were also incubated with 5 μM LCY for 1 h before stimulation with LPS/IFNγ for the indicated time periods before Western blot analysis (right panel). The mean of relative densitometric value from 3–4 independent experiments is shown below the blots. Cells were treated with DMSO or 5 μM LCY for 1 h before stimulation with LPS/IFNγ for the indicated time periods (h), and then cell lysates were prepared for Western blot analysis using antibodies against (B) phospho-C/EBPβ (Ser105), phospho-C/EBPβ (Thr235), C/EBPβ (LAP and LIP) or β-actin (as loading control). The ratio of immunointensity between the phospho-c-Jun, phospho-CREB, phospho-C/EBPβ and the corresponding loading controls was calculated. The mean of relative ratio value from 3 independent experiments is shown below the blots. *P < 0.01, compared with the corresponding resting values (1st lane of each group); #P < 0.01, compared with the corresponding DMSO-treated values.

3.5. *Effects of LCY-2-CHO on phosphorylation of transcription factors*

Both c-Jun and CREB were activated through phosphorylation and translocated into nucleus for transcription of target genes [23,24]. LPS/IFNγ stimulation evoked the phosphorylation of c-Jun and CREB in N9 cells, and these responses were not changed in the presence of LCY-2-CHO (Fig. 6A) even with time up to 1.5 h after LPS/IFNγ stimulation. To standardize protein loading in each lane, blots were stripped and reprobed with the antibody against c-Jun or CREB. There was no difference in the total cellular levels of c-Jun between the LCY-2-CHO-treated and control cells.

In addition to the regulation by nuclear translocation, phosphorylation has been also proposed to regulate C/EBPβ activity [25]. Fig. 6B shows a time-dependent manner of the phosphorylation of C/EBPβ at Thr235 and Ser105 residues in N9 cells by Western blot analysis using anti-phospho-C/EBPβ (Thr235) and anti-phospho-C/EBPβ (Ser105) antibodies, respectively. Phosphorylation of C/EBPβ was detectable at 3 h and the immunointensity continued to rise for at least 4.5 h after exposure to LPS/IFNγ. A significant attenuation of C/EBPβ phosphorylation and the total level of C/EBPβ by LCY-2-CHO was observed at 4.5 h after LPS/IFNγ stimulation.

3.6. *Effect of LCY-2-CHO on the protein expression of C/EBPβ and C/EBPδ*

It has been reported that the inflammatory stimulation leads to alteration of C/EBPs expression in microglia [26]. Therefore, we undertook experiments to assess whether the decrease in the levels of nuclear C/EBP is mediated by LCY-2-CHO inhibition of C/EBP expression. Western blot analysis of cell lysates indicated that the cellular levels of both C/EBPβ and C/EBPδ were increased in a time-dependent manner in LPS/IFNγ-stimulated N9 cells, with immunoreactive bands being detectable at 3 h (Fig. 7A and B). C/EBPβ continued to rise for at least 18 h, whereas C/EBPδ continued to rise for 6 h and declined thereafter (data not shown). A similar time-dependent increase in C/EBPβ expression was also demonstrated in

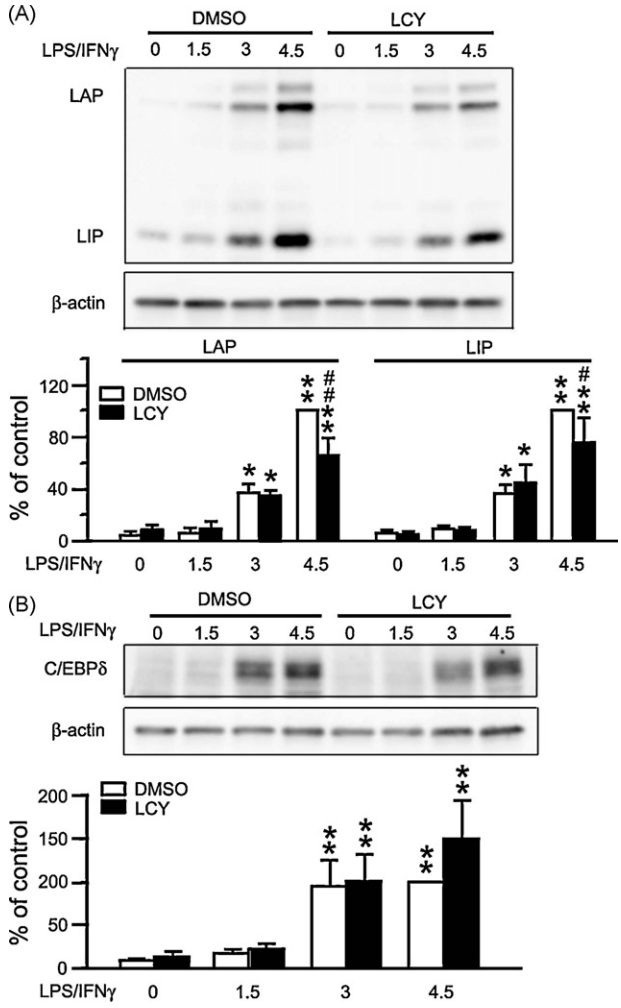

Fig. 7 – Effects of LCY-2-CHO (LCY) on C/EBPβ and C/EBPδ protein expression in N9 cells. Cells were treated with DMSO or 5 μM LCY for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for the indicated time periods (h), and then cell lysate were prepared for Western blot analysis using antibodies against (A) C/EBPβ and (B) C/EBPδ or β-actin (as loading control). The densitometric values of C/EBPs are expressed as means ± S.D. of 4 independent experiments $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the corresponding resting values (1st group columns); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the corresponding DMSO-treated values.

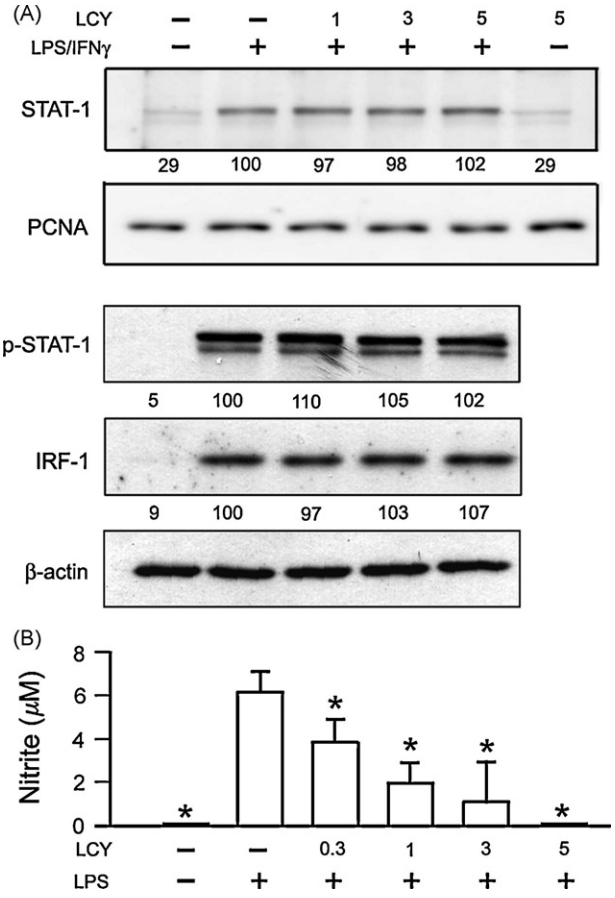

Fig. 8 – Effect of LCY-2-CHO (LCY) on STAT signaling pathway in N9 cells. (A) Cells were treated with the indicated concentrations (μM) of LCY for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for an additional 1 h. The STAT-1 and PCNA (as loading control), phospho-STAT-1, IRF-1 and β-actin (as loading control) in the nuclear extracts were determined by Western blot analysis. The result shown is representative of 4 independent experiments. (B) Cells were treated with the indicated concentrations (μM) of LCY for 1 h before stimulation with 2 μg/ml of LPS for another 24 h. The nitrite content of culture media was analyzed. Values are means ± S.D. of 4 independent experiments. $^{*}P < 0.01$, compared with the control value (2nd column).

macrophages [27]. LCY-2-CHO attenuated the expression of all three isoforms of C/EBPβ at 4.5 h after LPS/IFNγ stimulation, whereas it did not affect the cellular levels of C/EBPδ.

3.7. *Effect of LCY-2-CHO on STAT signaling pathway*

Since IFNγ activates transcription factors IRF-1 and STAT-1 [4], we therefore examined the effect of LCY-2-CHO on activation of these factors in response to LPS/IFNγ. The results displayed in Fig. 8A indicate that stimulation of N9 cells with LPS/IFNγ elicited a prominent phosphorylation of STAT-1 and increased the nuclear levels of both IRF-1 and STAT-1. LCY-2-CHO (up to 5 μM) failed to change these LPS/IFNγ-induced responses. In the absence of IFNγ, LPS alone evoked NO production in N9 cells at higher concentrations (2 μg/ml of LPS) (Fig. 8B). LCY-2-CHO exhibited an inhibitory effect on LPS-induced NO production with an IC_{50} value of 1.2 ± 0.2 μM.

3.8. *Effect of LCY-2-CHO on phosphorylation of mitogen-activated protein kinases (MAPKs)*

MAPKs cascades have been implicated in the regulation of iNOS expression at the transcriptional level in LPS-stimulated glial cells [28]. It is conceivable that the dual-phosphorylation of a conserved domain (TXY) in the activation loop of MAPKs

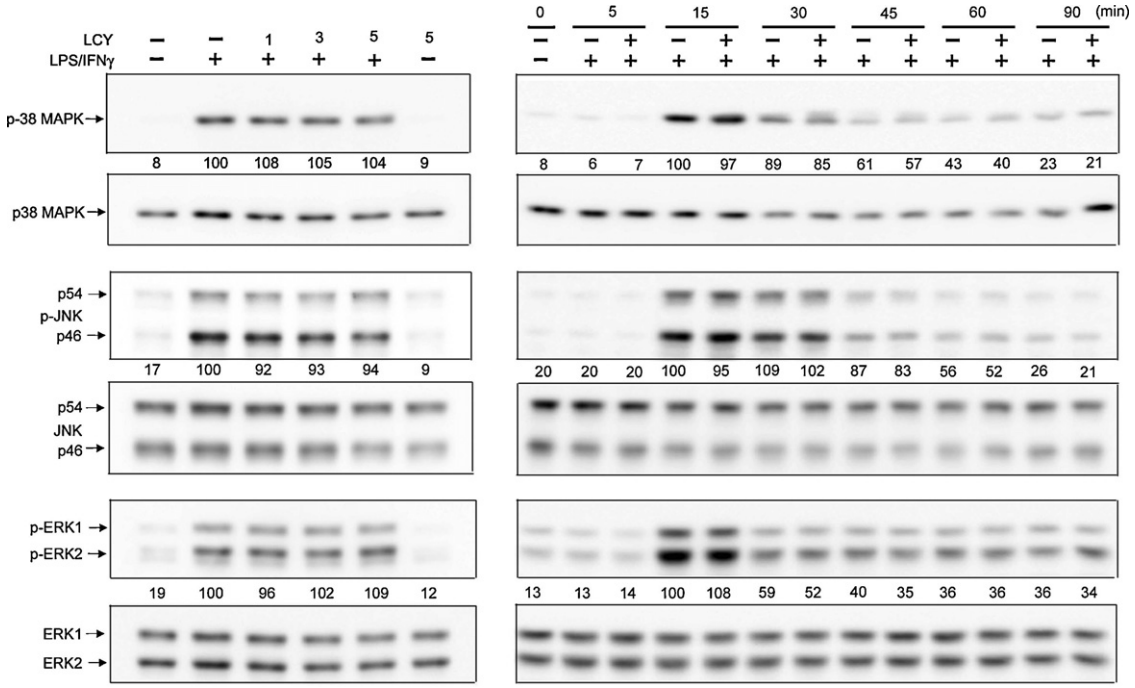

Fig. 9 – Effects of LCY-2-CHO (LCY) on MAPKs phosphorylation in N9 cells. Cells were treated with the indicated concentrations (μM) of LCY for 1 h before stimulation with LPS (10 ng/ml)/IFNγ (10 U/ml) for additional 15 min, and then cell lysate were prepared for Western blot analysis using antibodies against phospho-p38 MAPK, p38 MAPK (as loading control), phospho-ERK1/2, pan-ERK (as loading control), phospho-JNK or JNK (as loading control) (left panel). Cells were also incubated with 5 μM LCY for 1 h before stimulation with LPS/IFNγ for the indicated time periods before Western blot analysis (right panel). The ratio of immunointensity between the phospho-MAPKs, phospho-JNK and the corresponding loading controls was calculated. The mean of relative ratio value from 4 independent experiments is shown below the blots.

by the upstream MEKs leads to activation of ERK, p38 MAPK and JNK [29]. LPS/IFNγ stimulation increased the phosphorylation of p38 MAPK, ERK and JNK (both p46 and p54 isoforms) in N9 cells as assessed by immunoblot analysis of dually phosphorylated forms of MAPKs (Fig. 9), but LCY-2-CHO had no appreciable inhibitory effect on MAPKs phosphorylation even with time up to 1.5 h of exposure of cells to LPS/IFNγ. To standardize protein loading in each lane, blots were stripped and reprobed with the antibodies against p38 MAPK, pan-ERK or JNK. p38 MAPK activation was also assessed in monitoring the phosphorylation of downstream target MAPK-activated protein kinase-2 (MAPKAPK-2) by immunoblotting. LCY-2-CHO had no appreciable effect on LPS/IFNγ-stimulated MAPKAPK-2 phosphorylation (data not shown).

4. Discussion

In the present study, we demonstrated that a synthetic carbazole compound LCY-2-CHO inhibited NO production in LPS/IFNγ-stimulated N9 microglial cells and in LPS-stimulated BV-2 microglial cells and rat primary microglial cells. Therefore, LCY-2-CHO inhibition of NO production is not restricted to macrophages, as indicated in previous reports [7,8] and to immortalized microglial-like cells. This inhibitory effect is not mediated by cytotoxic effect on microglia. LCY-2-CHO had no appreciable inhibitory effect on the enzymatic activity of expressed iNOS. It is plausible that the blockade of transcription step plays an important role as evident from the parallelism of the inhibition of both iNOS protein and mRNA expression by LCY-2-CHO, which was carried out without affecting the half-life of the expressed iNOS mRNA. The attenuation of nuclear levels of transcription factors AP-1 and C/EBP and their binding to the DNA consensus sequence of the iNOS promoter reinforces the critical role for transcriptional regulation in LCY-2-CHO inhibition of microglial NO production.

NF-κB and AP-1 have been the most commonly studied transcription factors that modulate iNOS expression. NF-κB is composed of p65 and p50 heterodimer in microglia [21], and c-Jun/c-Fos heterodimer is the prominent form of AP-1 [30]. The results that LCY-2-CHO effectively inhibited DNA-binding activity of AP-1 and attenuated the nuclear levels of c-Jun, whereas it did not have any appreciable effect on NF-κB activation as assessed in DNA-binding activity of NF-κB and in nuclear levels of p50 and p65 in N9 microglial cells, are compatible with the results of earlier reports on RAW 264.7 murine macrophages [7,8]. Many of the LPS effects on glial cells are mediated through activation of the MAPKs [28]. Three major subfamilies of MAPK cascades, i.e., ras-raf1-MEK1/2-ERK1/2, rac-MEKK1-MEK4/7-JNK1/2 and MEK3/6-p38 MAPK, have been defined [29]. MAPKs transmit signals in the form of sequential phosphorylation events. The MAPKs are activated

upon dual-phosphorylation of Tyr and Thr residues in the activation loop of the kinase domain. Stimulation of N9 cells with LPS/IFNγ resulted in the dual-phosphorylation of all three MAPKs (ERK, p38 MAPK and JNK). The downstream targets of ERK and JNK include AP-1 [31]. Activation of JNK, in turn, phosphorylates the N-terminus (Ser63 and Ser73) of c-Jun protein leading to increases in activity [32]. However, LCY-2-CHO did not affect the MEK-JNK-c-Jun cascades as evidenced from the failure of LCY-2-CHO to alter the phosphorylation states of JNK, ERK and c-Jun. The loss of nuclear AP-1 likely results from decreased import or enhanced export by LCY-2-CHO, since (1) there was no difference in the total cellular levels of c-Jun between the LCY-2-CHO-treated and control cells, precluding the increased degradation of AP-1; and (2) both the DNA-binding and nuclear levels of AP-1 were attenuated by LCY-2-CHO with the same efficacy. It has been reported that c-Jun binds specifically to the nuclear import receptor importin in a leucine zipper-dependent manner to promote efficient import of transcription factor c-Jun into the nucleus [33]. The import complex is then dissociated in the nucleus upon the interaction of Ran-GTP with importin, before recycling of the latter into the cytosol. Further study will be required to clarify the mechanism of LCY-2-CHO inhibition of AP-1 nuclear recruitment. Since p38 MAPK is required for NF-κB-dependent gene expression [34], the lack of effect on p38 MAPK activation as assessed by Western blot analysis of the phosphorylation of both p38 MAPK and its downstream MAPKAPK-2 is in line with the notion that LCY-2-CHO inhibition of iNOS expression does not implicate a NF-κB signaling.

The synergistic NO production after combined treatment with LPS and IFNγ indicates that they act through complementary signaling pathways that are required for optimal activation of iNOS/NO system. The Janus tyrosine kinase (JAK)-STAT pathway has been implicated in IFNγ-induced iNOS expression in glial cells [35]. Phosphorylated STAT-1 translocates to the nucleus and induces transcription of IRF-1 via binding to the γ-interferon-activated site (GAS) [36], and IRF-1 plays a role in the expression of iNOS via binding to the interferon-stimulated responsive element (ISRE) of the iNOS promoter. The results that LCY-2-CHO did not affect the phosphorylation of STAT-1 and the nuclear levels of STAT-1 and IRF-1 preclude the involvement of IFN-JAK-STAT-IRF signaling pathway, and the finding that similar IC_{50} values were obtained in LCY-2-CHO inhibition of LPS-induced NO production whether in the presence or absence of IFNγ reaffirms this interpretation. Thus, LCY-2-CHO primarily affects the LPS-mediated signaling pathways.

In the present study, we found that a 2–3-fold lower concentration of LCY-2-CHO was required to diminish C/EBP and NF-IL6 binding to the DNA consensus sequence of the iNOS promoter than to inhibit DNA-binding activity of AP-1. It is likely that the transcriptional activity of C/EBP and NF-IL6 also plays an important role. Six members of the C/EBPs, a family of leucine zipper transcription factors, including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and growth arrest DNA damage/C/EBP homologous protein (CHOP/Gadd153), have been identified. Among them, C/EBPβ and C/EBPδ have been implicated in induction of glial iNOS [4]. NF-IL6, also known as C/EBPβ, was shown to form heterodimer with C/EBPδ (or NF-IL6β), resulting in a synergistic transcriptional effect [4] which recognizes the same nucleotide sequences as C/EBP. We, therefore, focused here on examining the effect of LCY-2-CHO on C/EBPβ and C/EBPδ activation. A previous report demonstrated that the transcriptional activity of C/EBP correlated with its phosphorylation, DNA-binding ability and nuclear localization [37]. C/EBPβ contains an intramolecular autoinhibitory element that hinders its binding to DNA, and this autoinhibition is relieved upon phosphorylation at several discrete Thr or Ser residues by various protein kinases [25]. Phosphorylation of C/EBPβ at Ser105 by 90 kDa ribosomal S6 kinase (RSK), the main MAPKAPK of the ERK cascade, and at Thr235 by ERK, results in recruitment of C/EBPβ to nucleus for the gene transactivation [38,39]. Although the phosphorylation of C/EBPβ at Ser105 and Thr235 residues as well as the nuclear levels of C/EBPβ was attenuated by LCY-2-CHO, this inhibitory effect may result from LCY-2-CHO-decreased C/EBPβ expression in LPS/IFNγ-stimulated cells as evidenced from (1) the closely matched inhibitory profiles, including the time dependency and inhibitory intensity, of all three responses; and (2) the result that ERK activation was not affected by LCY-2-CHO. This is in accordance with the findings of a previous report that the most frequent mode of regulation of C/EBP is by increased de novo synthesis [27]. All three MAPKs (ERK, p38 MAPK and JNK) pathways which mediated the LPS activation of AP-1 and CREB have been implicated in the induction of *C/EBPβ* gene transcription in LPS-stimulated microglia [26]. Based on current data, the blockade of AP-1 transactivation seems to be the most likely mechanism of attenuation of C/EBPβ upregulation by LCY-2-CHO in LPS/IFNγ-stimulated N9 cells. Although the participation of other transcription factors has not been excluded, further studies are warranted to clarify this mechanism. A recent report has demonstrated that the murine *C/EBPδ* gene promoter is regulated by STAT-3 and Sp1 transcriptional activators [40]. LCY-2-CHO failed to modulate the expression and the nuclear levels of C/EBPδ implying a lack of effect on these transcription factors. However, it is worth noting that LCY-2-CHO inhibition of C/EBP binding to the DNA consensus sequence of the iNOS promoter could play a major role during the initial stage of iNOS transcriptional activation, since LCY-2-CHO inhibition of C/EBP DNA-binding activity (at 1.5 h) before C/EBPβ and C/EBPδ concentrations increased (at 3 h) in LPS/IFNγ-stimulated microglia, while effective attenuation of C/EBPβ expression (at 4.5 h) by LCY-2-CHO could contribute to maintenance of the inhibition of iNOS transcription. This proposition is in line with the finding of a previous report that the specific C/EBP induction mechanism may not be required for the rapid activation of some endogenous target genes [27].

The second messenger cAMP mediates the transcriptional induction of numerous genes through protein kinase A (PKA)-dependent phosphorylation of transcription factors, including C/EBPβ (Ser299) and CREB (Ser133) [41,42], which is essential for nuclear translocation and transcriptional activation. Although a number of studies have reported the effects of cAMP on NO production, the role of cAMP in the microglia and macrophages remains controversial [43,44]. In the present study, it is unlikely that LCY-2-CHO inhibition of NO production in N9 cells was via the cAMP-PKA signaling pathway because it had no appreciable effect on phosphorylation of CREB at Ser133 residue.

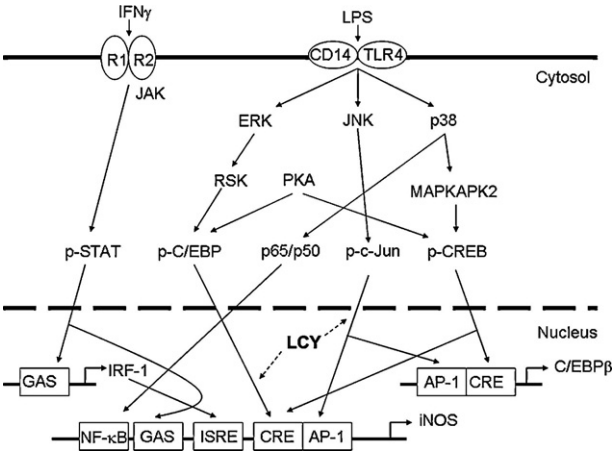

Fig. 10 – Schematic diagram showing the mechanisms underlying the inhibition by LCY-2-CHO (LCY). The signaling molecules generally employed in mediating activation of the transcription factors for iNOS expression in response to LPS/IFNγ are indicated. Some additional signaling molecules in these pathways have been omitted for the sake of clarity. The broken line arrow indicates the possible sites of action of LCY.

In summary, we have demonstrated that inhibition of LPS/IFNγ-induced NO production by LCY-2-CHO in microglia is attributed to the down-regulation of AP-1 activity probably through the interference with nuclear translocation, and to the suppression of C/EBP activity probably through the blockade of DNA-binding and C/EBPβ expression (Fig. 10). Further investigation is required to identify the molecular target(s) of LCY-2-CHO in the AP-1 and C/EBP signaling pathways. It is worth noting that C/EBPβ contributes to the stimulation of transcription in conjunction with transcription factors of other families [45]. It is reasonable to propose that inhibition of both C/EBP and AP-1 by LCY-2-CHO represents a potential therapeutic strategy for the treatment of neurological disorders in which activated microglia contributes to neurodegeneration.

Acknowledgements

This work was supported in part by grants from the National Science Council (NSC-89-2320-B-075A-010) and Taichung Veterans General Hospital (TCVGH-877305D, TCVGH-887304D, and TCVGH-897315D), Taiwan, Republic of China.

REFERENCES

[1] Liu B, Gao HM, Wang JY, Jeohn GH, Cooper CL, Hong JS. Role of nitric oxide in inflammation-mediated neurodegeneration. Annal N Y Acad Sci 2002;962:318–31.
[2] Marletta MA. Nitric oxide synthase structure and mechanism. J Biol Chem 1993;268:12231–4.
[3] Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J Immunol 1992;149:2736–41.
[4] Saha RN, Pahan K. Regulation of inducible nitric oxide synthase gene in glial cells. Antioxid Redox Signal 2006;8:929–47.
[5] Lee CY, Kuo SC, Teng CM, Huang LJ. Synthesis antiplatelet activity of 9-benzyl-3-hydroxymethylcarbazoles. Chin Pharm J 2002;54:25–34.
[6] Lee CY, Huang LJ, Wang JP, Kuo SC. Anti-inflammatory activity of 9-substituted benzyl-3-substituted carbazole derivatives. Chin Pharm J 2002;54:35–40.
[7] Tsao LT, Lee CY, Huang LJ, Kuo SC, Wang JP. Inhibition of lipopolysaccharide-stimulated nitric oxide production in RAW 264.7 macrophages by a synthetic carbazole, LCY-2-CHO. Biochem Pharmacol 2002;63:1961–8.
[8] Ho FM, Lai CC, Huang LJ, Kuo TC, Chao CM, Lin WW. The anti-inflammatory carbazole, LCY-2-CHO, inhibits lipopolysaccharide-induced inflammatory mediator expression through inhibition of the p38 mitogen-activated protein kinase signaling pathway in macrophages. Br J Pharmacol 2004;141:1037–47.
[9] Ho FM, Kang HC, Lee ST, Chao Y, Chen YC, Huang LJ, et al. The anti-inflammatory actions of LCY-2-CHO, a carbazole analogue, in vascular smooth muscle cells. Biochem Pharmacol 2007;74:298–308.
[10] Hsu MJ, Chao Y, Chang YH, Ho FM, Huang LJ, Huang YL, et al. Cell apoptosis induced by a synthetic carbazole compound LCY-2-CHO is mediated through activation of caspase and mitochondrial pathways. Biochem Pharmacol 2005;70:102–12.
[11] Corradin SB, Manuel J, Donini SD, Quattrocchi E, Ricciardi-Castagnoli P. Inducible nitric oxide synthase activity of cloned murine microglial cells. Glia 1993;7:255–62.
[12] Bocchini V, Mazzolla R, Barluzzi R, Blasi E, Sick P, Kettenmann H. An immortalized cell line expresses properties of activated microglial cells. J Neurosci Res 1992;31:616–21.
[13] Wang MJ, Huang HM, Hsieh SJ, Jeng KC, Kuo JS. Resveratrol inhibits interleukin-6 production in cortical mixed glial cells under hypoxia/hypoglycemia followed by reoxygenation. J Neuroimmunol 2001;112:28–34.
[14] Chen CC, Wang JK, Lin SB. Antisense oligonucleotides targeting protein kinase C-α, -βI, or -δ but not -η inhibit lipopolysaccharide-induced nitric oxide synthase expression in RAW 264.7 macrophages: involvement of a nuclear factor κB-dependent mechanism. J Immunol 1998;161:6206–14.
[15] Velasco M, Diaz-Guerra MJ, Diaz-Achirica P, Rivas L, Bosca L. Macrophage triggering with cecropin A and melittin-derived peptides induces type II nitric oxide synthase expression. J Immunol 1997;158:4437–43.
[16] Tsi CJ, Chao Y, Chen CW, Lin WW. Aurintricarboxylic acid protects against cell death caused by lipopolysaccharide in macrophages by decreasing inducible nitric-oxide synthase induction via IκB kinase, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase inhibition. Mol Pharmacol 2002;62:90–101.
[17] Woo KJ, Lim JH, Suh SI, Kwon YK, Shin SW, Kim SC, et al. Differential inhibitory effects of baicalein and baicalin on LPS-induced cycloxoxygenase-2 expression through inhibition of C/EBPβ DNA-binding activity. Immunobiology 2006;221:359–68.
[18] Chen CJ, Rung SL, Liao SL, Chen SY. Inhibition of inducible nitric oxide synthase expression by baicalein in endotoxin-cytokine-stimulated microglia. Biochem Pharmacol 2004;67:957–65.
[19] Lockhart BP, Cressey KC, Lepagnol JM. Suppression of nitric oxide formation by tyrosine kinase inhibitors in murine N9 microglia. Br J Pharmacol 1998;123:879–89.
[20] Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, et al. 1400 W is a slow, tight binding, and highly selective

inhibitor of inducible nitric-oxide synthase in vitro and in vivo. J Biol Chem 1997;272:4959–63.
[21] Kim MO, Si Q, Zhou JN, Pestell RG, Brosnan CF, Locker J, et al. Interferon-β activates multiple signaling cascades in primary human microglia. J Neurochem 2002;81:1361–71.
[22] Descombes P, Schibler U. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 1991;67:569–79.
[23] Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997;9:240–6.
[24] Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 1993;365:855–9.
[25] Williams SC, Baer M, Dillner AJ, Johnson PF. CRP2 (C/EBPβ) contains a bipartite regulatory domain that controls transcriptional activation, DNA-binding and cell specificity. EMBO J 1995;14:3170–83.
[26] Ejarque-Ortiz A, Mediana MG, Tusell JM, Perez-Gonzalez AP, Serratosa J, Saura J. Upregulation of CCAAT/enhancer-binding protein β in activated asrocytes and microglia. Glia 2007;55:178–88.
[27] Bradley MN, Zhou L, Smale ST. C/EBPβ regulation in lipopolysaccharide-stimulated macrophages. Mol Cell Biol 2003;23:4841–58.
[28] Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-α gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 1998;18: 1633–41.
[29] Krens SFG, Spaink HP, Snaar-Jagalska BE. Functions of the MAPK family in vertebrate-development. FEBS Lett 2006;580:4984–90.
[30] Hambleton J, Weinstein SL, Lem L, DeFranco AL. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. Proc Natl Acad Sci USA 1996;93:2774–8.
[31] Cano E, Mahadevan LC. Parallel signal processing among mammalian MAPKs. Trends Biochem Sci 1995;20:117–22.
[32] Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, et al. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 1994;76:1025–37.
[33] Waldmann I, Walde S, Kehlenbach RH. Nuclear import of c-Jun is mediated by multiple transport receptors. J Biol Chem 2007;282:27685–92.
[34] Carter AB, Knudtson KL, Monick MM, Hunninghake GW. The p38 mitogen-activated protein kinase is required for NF-κB-dependent gene expression. The role of TATA-binding protein (TBP). J Biol Chem 1999;274:30858–63.
[35] Kitamura Y, Takahashi H, Normura Y, Taniguchi T. Possible involvement of Janus kinase Jak2 in interferon-γ induction of nitric oxide synthase in rat glial cells. Eur J Pharmacol 1996;306:297–306.
[36] Gao J, Morrison DC, Parmely TJ, Russell SW, Murphy WJ. An interferon-γ-activated site (GSA) is necessary for full expression of the mouse iNOS gene in response to interferon-γ and lipopolysaccharide. J Biol Chem 1997;272:1226–30.
[37] Sprott KM, Chumley MJ, Hanson JM, Dobrowsky RT. Decreased activity and enhanced nuclear export of CCAAT-enhancer-binding protein β during inhibition of adipogenesis by ceramide. Biochem J 2002;365:181–91.
[38] Lee SJ, Kim SG. Role of p90 ribosomal S6-kinase-1 in oltipraz-induced specific phosphorylation of CCAAT/enhancer-binding protein-β for *GSTA2* gene transactivation. Mol Pharmacol 2006;69:385–96.
[39] Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T, et al. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc Natl Acad Sci USA 1993;90:2207–11.
[40] Zhang Y, Sif S, DeWille J. The mouse C/EBPδ gene promoter is regulated by STAT3 and Sp1 transcriptional activators, chromatin remodeling and c-Myc repression. J Cell Biochem 2007;102:1256–70.
[41] Chinery R, Brockman JA, Dransfield DT, Coffey RJ. Antioxidant-induced nuclear translocation of CCAAT/Enhancer-binding protein β. A critical role for protein kinase A-mediated phosphorylation of Ser299. J Biol Chem 1997;272:30356–61.
[42] Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, et al. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. Mol Cell Biol 1993;13:4852–9.
[43] Nakamura Y, Si QS, Kataoka K. Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide. Neurosci Res 1999;35:95–100.
[44] Zhang B, Yang L, Konishi Y, Maeda N, Sakanaka M, Tanaka J. Suppressive effects of phosphodiesterase type IV inhibitors in rat cultured microglial cells: comparison with other types of cAMP-elevating agents. Neuropharmacology 2002;42:262–9.
[45] Akira S. IL-6-regulated transcription factors. Int J Biochem Cell Biol 1997;29:1401–18.