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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

[1]Feng-Ming Ho, [1]Chih-Chang Lai, [2]Li-Jiau Huang, [3]Tsun Cheng Kuo, [4]Chien M. Chao & *,[5]Wan-Wan Lin

[1]Department of Internal Medicine, Tao-Yuan General Hospital, Department of Health, the Executive Yuan, Taiwan; [2]Graduate Institute of Pharmaceutical Chemistry, China Medical College, Taichung, Taiwan; [3]Department of Cosmetic Science, Chia-Nan University of Pharmacy, Tainan, Taiwan; [4]Department of Orthopedics, National Taiwan University College of Medicine, Taipei, Taiwan and [5]Department of Pharmacology, College of Medicine, National Taiwan University, Taipei 100, Taiwan

1 The present study was undertaken to investigate the anti-inflammatory effects of a synthetic compound, LCY-2-CHO, on the expression of inducible nitric oxide synthase (iNOS), COX-2, and TNF-α in murine RAW264.7 macrophages.

2 Within 1–30 μM, LCY-2-CHO concentration-dependently inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor-α (TNF-α) formation, with IC_{50} values of 2.3, 1, and 0.8 μM, respectively. Accompanying inhibition of LPS-induced iNOS, cyclooxygenase-2 (COX-2), and pro-TNF-α proteins was observed.

3 Reverse transcription-polymerase chain reaction (RT–PCR) and promoter analyses indicated that iNOS expression was inhibited at the transcriptional level ($IC_{50} = 2.3\,\mu M$), that inhibition of COX-2 expression only partially depended on gene transcription ($IC_{50} = 7.6\,\mu M$), and that TNF-α transcription was unaffected.

4 Transcriptional assays revealed that activation of AP-1, but not NF-κB, was concomitantly blocked by LCY-2-CHO. Our results showed that LCY-2-CHO was capable of interfering with post-transcriptional regulation, altering the stability of COX-2 and TNF-α mRNAs.

5 Since the 3′-untranslated region (3′ UTR) of both COX-2 and TNF-α mRNA contains a p38 mitogen-activated protein kinase (MAPK)-regulated element involved in mRNA stability, we assessed the effect of LCY-2-CHO on p38 MAPK. Our data clearly indicated an inhibition ($IC_{50} = 1.7\,\mu M$) of LPS-mediated p38 MAPK activity, but not of extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK) activity. However, kinase assays ruled out a direct inhibition of p38 MAPK action. The selective p38 MAPK inhibitor, SB203580, inhibited the promoter activities of iNOS and COX-2 rather than that of TNF-α.

6 In conclusion, LCY-2-CHO downregulates inflammatory iNOS, COX-2, and TNF-α gene expression in macrophages through interfering with p38 MAPK and AP-1 activation.
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Keywords: LCY-2-CHO; p38 MAPK; iNOS; COX-2; TNF-α; mRNA stability; macrophage

Abbreviations: AP-1, activator protein-1; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolidum bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; PGE_2, prostaglandin E_2; RT–PCR, reverse-transcription–polymerase chain reaction; TNF-α, tumor necrosis factor-α

Introduction

Chronic inflammation leads to destruction of normal tissue integrity. Production of inflammatory mediators through upregulation of several inducible genes, such as inducible nitric oxide (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor-α (TNF-α), contributes to inflammatory responses and tissue damage. Transcriptional induction of these genes is mediated by intracellular signaling cascades regulated by kinase enzymes. The mitogen-activated protein kinases (MAPKs) are part of such signaling cascades through which diverse extracellular stimuli converge to initiate inflammatory cellular responses. Among MAPK subgroups, p38 MAPK, which is activated by many different stimuli, positively regulates a variety of genes involved in inflammation, such as TNF-α, interleukin (IL)-1, IL-6, IL-8, COX-2, and iNOS (Bhat *et al*., 1998; Guan *et al*., 1998b; Chen *et al*., 1999; Herlaar & Brown, 1999; Nick *et al*., 2000; Ono & Han, 2000).

*Author for correspondence: E-mail: wwl@ha.mc.ntu.edu.tw
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In a program to develop anti-inflammatory drugs, we previously synthesized a series of compounds with carbazole structures. Among them, LCY-2-CHO ([9-(2-chlorobenzyl)-9H-carbazole-3-carbaldehyde]) has been shown to possess anti-inflammatory properties by directly downregulating leukocyte functions. These include attenuation of neutrophil degranulation and superoxide anion (O_2^-) generation (Lee *et al*., 2002a,b), and inhibition of lipopolysaccharide (LPS)-induced NO production in murine macrophages (Tsao *et al*., 2002). Although iNOS promoter activity was attenuated, LCY-2-CHO had no effect on nuclear factor-κB (NF-κB) activation. Therefore, the mechanism by which LCY-2-CHO decreased transcription of iNOS mRNA was still unclear. The aim of the present study was to evaluate the effect of LCY-2-CHO on lipopolysaccharide (LPS)-induced COX-2 and TNF-α expression, and to determine the molecular mechanisms involved.

Methods

Cell culture

Murine RAW264.7 macrophages, obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), were grown at 37°C in 5% CO_2 using DMEM containing 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. When examining the effects of LCY-2-CHO, cells were treated with vehicle (DMSO) as a control.

Nitrite measurement

Nitrite production was measured in RAW264.7. macrophage supernatants. Briefly, cells were cultured in 24-well plates in 500 μl of culture medium until confluence. Cells were treated with LPS for 24 h, and then the culture media were collected. Nitrite was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100-μl samples of culture medium. The optical density at 550 nm (OD_{550}) was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the OD_{550} produced using standard solutions of sodium nitrite in the culture medium.

Prostaglandin E_2 (PGE_2) and TNF-α assays

RAW264.7. macrophages cultured in 24-well plates were stimulated with the indicated agents and cultured for 24 h. PGE_2 and TNF-α productions were, respectively, measured by a commercial kit from Cayman Chemical Company and R&D Systems, according to the manufacturer's instructions.

Measurement of cell viability

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolidum bromide MTT assay. Cells plated in 24-well plates were treated with different concentrations of LCY-2-CHO. After 24 h of incubation, MTT (1 mg ml^{-1}) was added for 60 min, then the culture medium was removed, and the cells were dissolved in DMSO and shaken for 10 min. OD values at 550 and 630 nm were measured using a microplate reader. The net absorbance ($OD_{550}-OD_{630}$) indicates the enzymatic activity of mitochondria and provides information on cell viability.

Propidium iodide staining for DNA content

Propidium iodide staining was used to measure the extent of cell death. Cells were initially seeded at 5×10^6 cells in six-well plates and incubated with the indicated concentration of LCY-2-CHO or 0.1% DMSO for selected time periods. Following treatment, all cells were collected. Cells were then centrifuged, washed once in phosphate-buffered saline (PBS), resuspended in ice-cold 70% v v^{-1} EtOH with vortexing, and stored at 0°C until analysis. Fixed cells were collected by centrifugation, washed once in PBS, incubated in 400 μl phosphate–citric acid buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.8) for 10 min at room temperature, and stained with 0.5 ml propidium iodide staining buffer (1% Triton X-100, 1 mg ml^{-1} RNase A, 80 μg ml^{-1} propidium iodide) for more than 30 min in the dark. Before the cytometric analysis, cells were filtered onto a nylon mesh filter. The cell cycle distribution was also analyzed by flow cytometry using FACScan and the Cellquest program (Becton Dickinson, NJ, U.S.A.).

Immunoblot analysis

After stimulation, the cell medium was aspirated. Cells were rinsed twice with ice-cold PBS, and 100 μl of cell lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 10 μg ml^{-1} leupeptin, and 10 μg ml^{-1} aprotinin) was then added to each well. After harvesting cells, cell lysates were centrifuged, and equal protein amounts of the soluble protein, as determined by the Bradford protein assay, were denatured, subjected to SDS–PAGE, and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% Tween 20) containing 5% nonfat milk for 1 h at room temperature. After immunoblotting with the first specific antibodies (1 : 1000 dilution), membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1 : 2000 dilution) for 1 h. After three washes with TBST, the protein bands were detected with enhanced chemiluminescence detection reagent, and quantified using a densitometer.

Immunoprecipitation and kinase assay

To investigate the direct effect of LCY-2-CHO on p38 MAPK activity, cells were stimulated with 1 μg ml^{-1} LPS for 30 min. After cell extraction, anti-p38 MAPK with protein A/G agarose beads was used to precipitate kinase. Immunoprecipitation proceeded at 4°C overnight. The precipitated beads were washed three times with 1 ml of ice-cold cell lysis buffer and twice with kinase buffer (25 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, and 2 mM dithiothreitol). The immunecomplex kinase assay was performed in the presence of LCY-2-CHO or SB203580 at 30°C for 30 min in 20 μl of kinase reaction buffer containing 1 μg of myelin basic protein, 25 μM ATP, and 3 μCi of [γ-^{32}P]ATP. The reaction was terminated with 5 × Laemmli sample buffer, and the products were resolved by 12% SDS–PAGE.

RT-PCR

Macrophages were homogenized with 1 ml of RNAzol B reagent Gibco/Invitrogen (Carlsbad, CA, U.S.A.); total RNA was extracted by acid guanidinium thiocyanate–phenol–chloroform extraction; and RT was performed using a StrataScript RT–PCR kit. The oligonucleotide primers used corresponded to mouse iNOS (5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′ and 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′), COX-2 (5′-CAG CAA ATC CTT GCT GTT CC-3′ and 5′-TGG GCA AAG AAT GCA AAC ATC-3′), TNF-α (5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3′ and 5′-ACA TTC GAG GCT CCA GTG AAT TCG G-3′), and β-actin (5′-GAC TAC CTC ATG AAG ATC CT-3′ and 5′-CCA CAT CTG CTG GAA GGT GG-3′). PCR was performed using an initial step of denaturation (1 min at 94°C), 35 cycles of amplification (94°C for 1 min, 58°C for 30 s, and 72°C for 30 s), and an extension (72°C for 7 min). PCR products were analyzed on 2% agarose gels. The mRNA of β-actin served as the internal control for sample loading and mRNA integrity.

Transient transfection and luciferase assay

All reporter genes were prepared using endotoxin-free plasmid preparation kits (Qiagen). Using electroporation (280 V, 1070 μF, 30-ms time constant), RAW264.7 cells (2×10^7 cells cuvette^{-1}) were cotransfected with 1 μg of expression vector or promoter construct, and 1 μg of the β-galactosidase expression vector (pCR3lacZ; Pharmacia, Uppsala, Sweden). After electroporation, transfected cells were cultured in 24-well plates at 2×10^6 cells well^{-1} and incubated in 10% FBS DMEM for 24 h. Subsequently, cells were treated with agents for 24 h, and using a Promega kit, luciferase activity was assayed with a microplate luminometer (Packard, Meriden, CT, U.S.A.). Luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression, and presented as the percentages of luciferase activity measured without LPS or LCY-2-CHO treatment.

Materials

LCY-2-CHO (purity >99%) was synthesized as we previously described (Lee *et al*., 2002b) and was dissolved in DMSO. DMEM, FBS, penicillin, and streptomycin were obtained from Gibco/Invitrogen (Carlsbad, CA, U.S.A.). Polyclonal antibodies against iNOS, COX-2, TNF-α, and p38 MAPK; the monoclonal antibody against phosphorylated Tyr-182 of p38 MAPK; and the horseradish peroxidase-coupled anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies specific to the phosphorylated ERK and phosphorylated JNK were purchased from New England Biolabs (Beverly, MA, U.S.A.). The ECL detection agents were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). SB203580 was purchased from Calbiochem (San Diego, CA, U.S.A.). LPS (L8274) from *Escherichia coli* and other chemicals were purchased from Sigma Aldrich (St Louis, MO, U.S.A.). All materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The iNOS promoter-luciferase reporter plasmid containing binding sites for activator protein-1 (AP-1) and NF-κB, which are required for maximal responses to LPS, was provided by Dr C. K. Glass (Department of Medicine, University of California, San Diego, CA, U.S.A.) (Ricote *et al*., 1998). The murine COX-2 promoter with a wild-type construct (native −966/+23) was kindly provided by Dr Byron Wingerd (Michigan State University, East Lansing, MI, U.S.A.). The TNF-α-luciferase reporter plasmid (−200 WT) was a gift from Dr Anne E. Goldfeld (Center for Blood Research, Harvard Medical School, Boston, MA, U.S.A.) (Tsytsykova & Goldfeld, 2002). The pGL2-ELAM-luciferase construct (κB-Luc) under the control of one NF-κB binding site was constructed. The AP-1-luciferase construct was provided by Dr G. Haegeman (Flanders Interuniversity Institute for Biotechnology and University of Gent, Gent, Belgium).

Statistical evaluation

The values are expressed as the mean ±s.e.m. of at least three experiments, which were performed in duplicate. Student's *t*-test was used to assess the statistical significance of the differences, and a *P*-value of less than 0.05 was considered statistically significant.

Results

LCY-2-CHO inhibits LPS-induced NO, PGE_2, and TNF-α release

To assess the effect of LCY-2-CHO on LPS-induced production in RAW264. 7 macrophages, cell culture medium and cell lysates were harvested. Measuring nitrite as the index of nitric oxide (NO) production by the Griess method, we found that in mouse RAW264.7 macrophages, LPS (1 μg ml^{-1}) treatment for 24 h resulted in a large amount of NO release, from a basal level of 6±2 to 41±7 μM. The increased NO release was accompanied by the induction of iNOS protein upon exposure to LPS for 24 h. Coaddition of LCY-2-CHO with LPS inhibited the formation of NO in a concentration-dependent manner, with an IC_{50} value of 2.3±0.3 μM (Figure 1a). Expression of iNOS protein was also decreased in the presence of LCY-2-CHO, with an IC_{50} value of 2.2±0.3 μM (Figure 1b).

In addition to inhibiting LPS-induced NO production, coaddition of LCY-2-CHO with LPS inhibited the formation of PGE_2 and TNF-α accompanied by the induction of COX-2 and pro-TNF-α (MW of 26 kDa) (Figures 2 and 3). The IC_{50} values were 1.0±0.5 and 0.8±0.1 μM for LPS-induced PGE_2 and TNF-α release, respectively, and were 1.3±0.3 and 1.0±0.3 μM for LPS-induced COX-2 and pro-TNF-α expression, respectively.

LCY-2-CHO at concentrations of up to 30 μM by itself did not affect the release of NO, PGE_2, or TNF-α above levels seen in medium-treated macrophages. LCY-2-CHO at concentrations ranging from 1 to 30 μM failed to affect cell viability. Mitochondrial activity as assessed by the MTT assay indicated cell viability of 95±3% in the LCY-2-CHO (30 μM)-treated group. DNA integrity, assessed by propidium iodide staining, also indicated that the percentage of apoptotic cells defined in the sub-G1 fraction of the cell cycle did not change (5±1% of LCY-2-CHO-treated cells as compared to 4±1% of the control group).

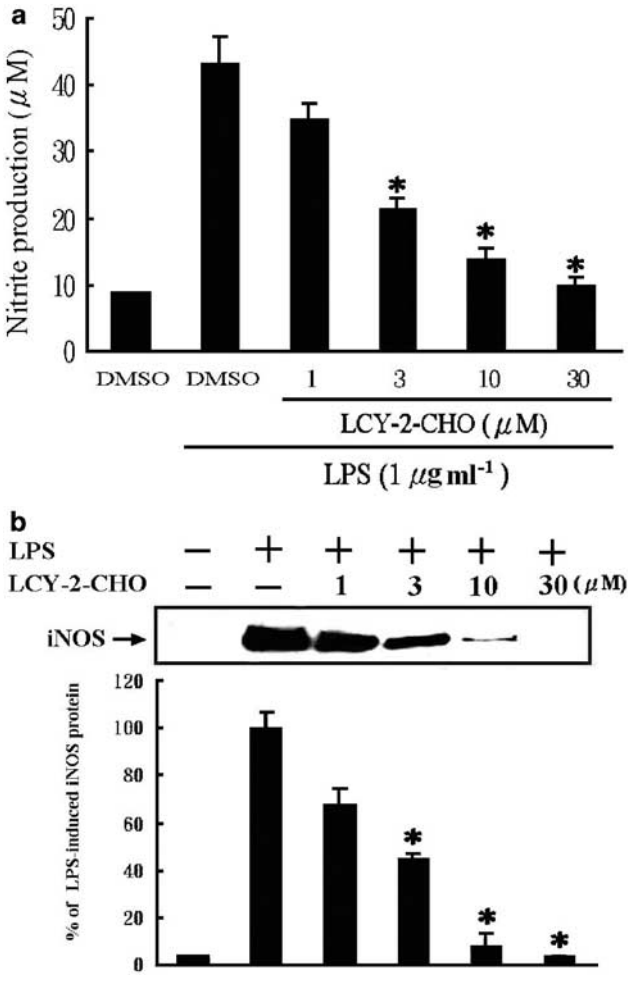

Figure 1 Concentration-dependent inhibition of LPS-induced NO release and iNOS expression by LCY-2-CHO. RAW264.7 macrophages were treated with LPS, LCY-2-CHO, and/or vehicle (DMSO) at the concentrations indicated for 24 h. After incubation, the culture medium was collected for nitrite assay (a), and cell lysates were subjected to SDS–PAGE for iNOS measurement (b). Data in (a) represent the mean ± s.e.m. from at least three independent experiments. Traces shown in (b) are representative of three separate experiments. $*P<0.05$ indicates significant inhibition by LCY-2-CHO.

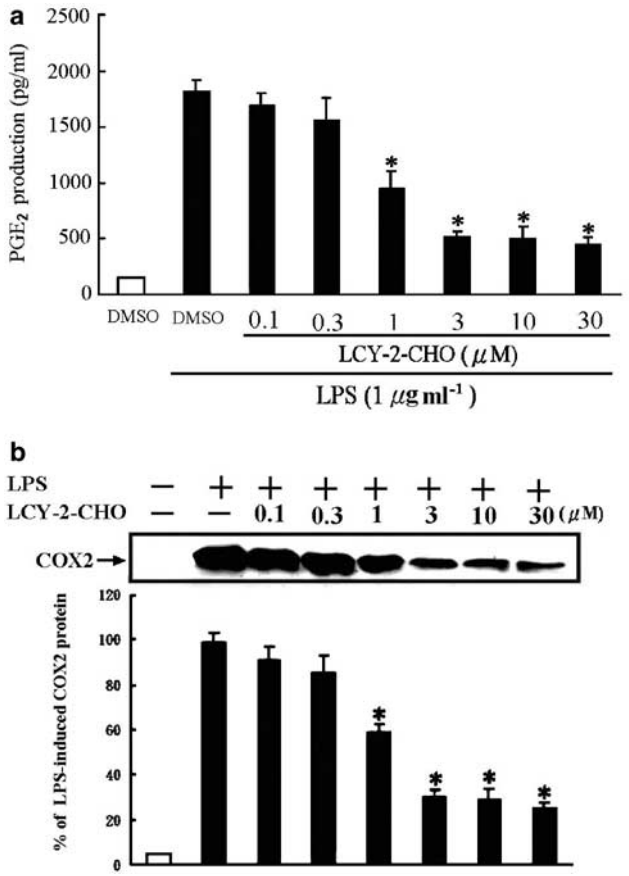

Figure 2 Concentration-dependent inhibition of LPS-induced PGE_2 release and COX-2 expression by LCY-2-CHO. RAW264.7 macrophages were treated with LPS, LCY-2-CHO, and/or vehicle (DMSO) at the concentrations indicated for 24 h. After incubation, the culture medium was collected for PGE_2 assay (a), and cell lysates were subjected to SDS–PAGE for COX-2 measurement (b). Data in (a) represent the mean ± s.e.m. from at least three independent experiments. Traces shown in (b) are representative of three separate experiments. $*P<0.05$ indicates significant inhibition by LCY-2-CHO.

Effects of LCY-2-CHO on iNOS, COX-2, and TNF-α promoter activity

Two methods were used to elucidate the effect of LCY-2-CHO on gene expression. First, RAW264.7 macrophages were transiently transfected with reporter plasmids containing the promoters for iNOS, COX-2, and TNF-α. Second, the steady-state level of mRNA following drug treatment was measured by RT–PCR. As shown in Figure 4, we found that LCY-2-CHO decreased LPS-induced iNOS promoter activity and mRNA. The respective IC_{50} values of 2.3 ± 0.5 and $2.2\pm0.7\,\mu$M were correlated with those for NO production, suggesting that NO reduction by LCY-2-CHO is related to transcriptional inhibition. Figure 5 showed that both COX-2 promoter activity and mRNA expression were also inhibited, while the IC_{50} value of promoter activation ($7.6\pm2.4\,\mu$M) was higher than those of PGE_2 ($1.0\pm0.5\,\mu$M) and mRNA ($3.5\pm0.9\,\mu$M) reduction. These data suggest that in addition to transcriptional inhibition, LCY-2-CHO may exert an additional action on COX-2 induction. Investigating the effect on TNF-α expression, we surprisingly observed that neither promoter activation nor mRNA induction was altered by LCY-2-CHO at concentrations of up to 10 μM (Figure 6). Since transfection makes cells more susceptible to toxic effects, we did not examine effects of 30 μM LCY-2-CHO treatment on promoter activity.

Effects of LCY-2-CHO on NF-κB and AP-1 activation

Considering the inhibition of iNOS and COX-2 transcription, we next determined the effects of LCY-2-CHO on two essential transcription factors, NF-κB and AP-1, which have been demonstrated to play essential roles in the expression of both genes. Figure 7 shows that LPS-elicited NF-κB activation, as assessed by the transactivation index, was not changed by LCY-2-CHO, while that stimulated through AP-1 binding was concentration-dependently reduced by LCY-2-CHO with an IC_{50} value of $2.5\pm0.4\,\mu$M.

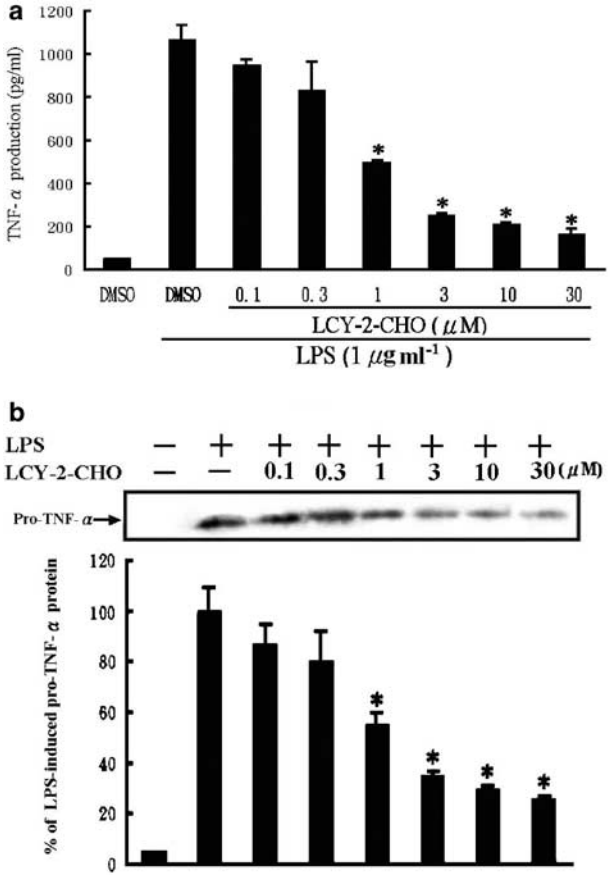

Figure 3 Concentration-dependent inhibition of LPS-induced TNF-α release and pro-TNF-α expression by LCY-2-CHO. RAW264.7 macrophages were treated with LPS, LCY-2-CHO, and/or vehicle (DMSO) at the concentrations indicated for 24 h. After incubation, the culture medium was collected for TNF-α assay (a), and cell lysates were subjected to SDS–PAGE for pro-TNF-α measurement (b). Data in (a) represent the mean ± s.e.m. from at least three independent experiments. Traces shown in (b) are representative of three separate experiments. $*P<0.05$ indicates significant inhibition by LCY-2-CHO.

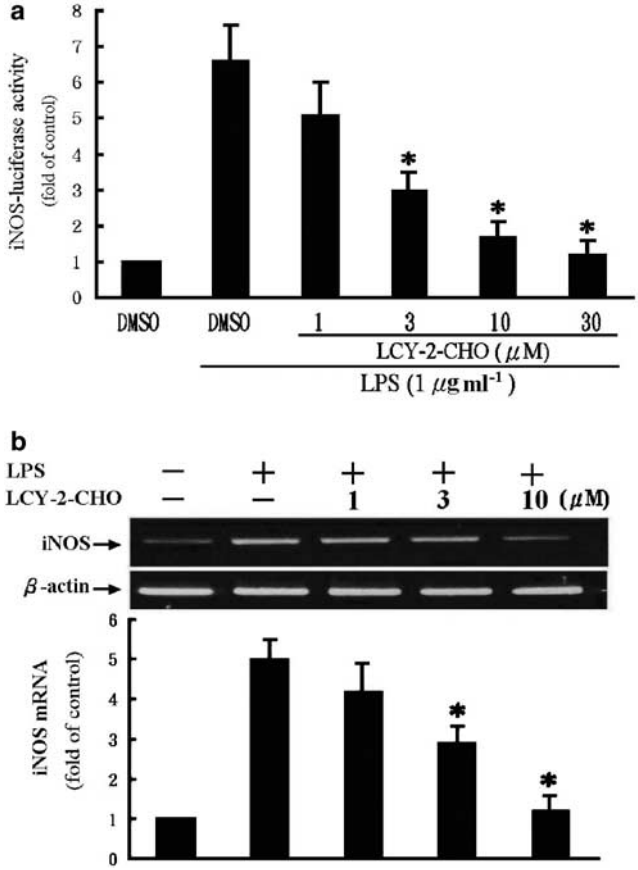

Figure 4 Transcriptional inhibition of LPS-induced iNOS gene expression by LCY-2-CHO. (a) Cells transfected with the iNOS-luciferase construct (1 μg) and lacZ plasmid (1 μg) for 24 h were stimulated with LPS (1 μg ml^{-1}), either in the presence or absence of LCY-2-CHO at the concentrations indicated for 24 h. Cell lysates were then prepared for the luciferase assay, which was normalized by lacZ transfection efficiency, and the results are expressed as a percentage of the control response without stimulus treatment. (b) After stimulation with the indicated agents for 12 h, total RNA was prepared and subjected to RT–PCR analysis for the iNOS mRNA level. The β-actin mRNA level was considered an internal control. Data on iNOS mRNA levels were measured by densitometry, normalized to the level of β-actin mRNA, and calculated as percentages of the basal response. Data are presented as the mean ± s.e.m. from at least three independent experiments. $*P<0.05$ as compared with the control LPS response.

Effects of LCY-2-CHO on induced mRNA stability

In addition to the transcriptional inhibition of iNOS and COX-2 gene expression, we also assessed whether LCY-2-CHO influences the post-transcriptional stability of upregulated mRNA. To answer this question, cells were treated with LPS for 12 h, followed by rapid washout and incubation with actinomycin D (an inhibitor of gene transcription) in the absence or presence of LCY-2-CHO for another hour. Figure 8 indicates that within 1 h, the iNOS, COX-2, and TNF-α mRNAs had decayed by 23, 20, and 25%, respectively. After incubation with LCY-2-CHO, the 1 h decay of iNOS mRNA remained unchanged (21%), while that of COX-2 and TNF-α were increased to 80 and 82%, respectively.

LCY-2-CHO inhibits LPS-induced p38 MAPK activation

Given that p38 MAPK is a key regulator of gene transcription and mRNA stability, we assessed the action of LCY-2-CHO on p38 MAPK-mediated gene regulation. We first determined the effect of an established p38 MAPK inhibitor, SB203580, on the induction of these genes. Our results indicated that SB203580 was effective in inhibiting iNOS and COX-2 promoter activity, while TNF-α transcription was unaffected (Figure 9). The IC_{50} values were $1.0\pm0.2\,\mu M$ for iNOS inhibition, and $2.4\pm0.5\,\mu M$ for COX-2 inhibition. Secondly, we determined p38 MAPK phosphorylation, an index of p38 MAPK activation, in macrophages stimulated with LPS in the presence of increasing concentrations of LCY-2-CHO. Figure 10a shows that LCY-2-CHO was able to inhibit p38 MAPK phosphorylation induced by LPS, in a concentration-related fashion, with an IC_{50} value of $1.7\pm0.5\,\mu M$. In contrast, LCY-2-CHO did not affect phosphorylation of ERK or JNK induced by LPS (Figure 10b, c). In an *in vitro* kinase assay to determine the direct effect of LCY-2-CHO on p38 MAPK, we found that, unlike SB203580, which can inhibit p38 MAPK over 1–10 μM, LCY-2-CHO did not affect the kinase activity of p38 MAPK (Figure 10d).

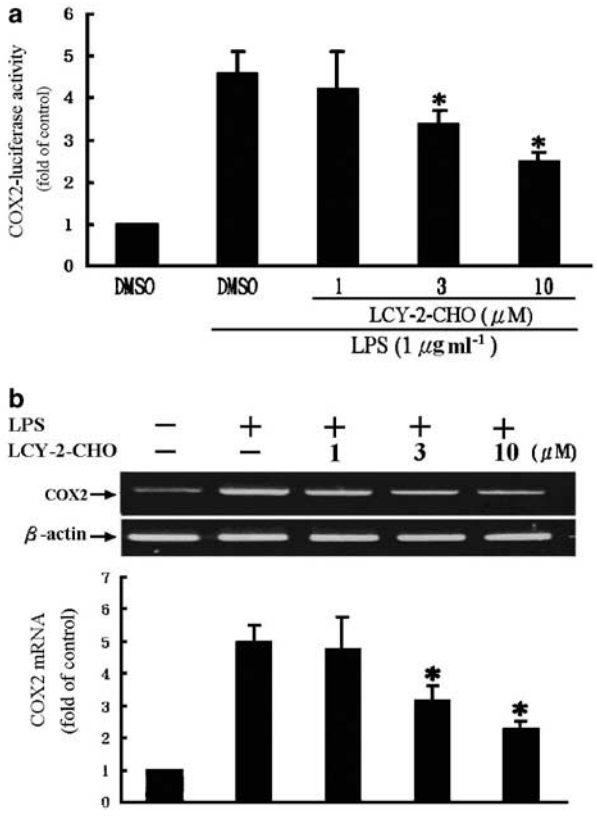

Figure 5 Transcriptional inhibition of LPS-induced COX-2 gene expression by LCY-2-CHO. (a) Cells transfected with COX-2-luciferase construct (1 μg) and the lacZ plasmid (1 μg) for 24 h were stimulated with LPS (1 μg ml^{-1}), either in the presence or absence of LCY-2-CHO at the concentrations indicated for 24 h. Cell lysates were then prepared for luciferase assay, which was normalized by lacZ transfection efficiency, and expressed as a percentage of the control response without stimulus treatment. (b) After stimulation with the indicated agents for 12 h, total RNA was prepared and subjected to RT–PCR analysis for the COX-2 mRNA level. The β-actin mRNA level was considered an internal control. Data on COX-2 mRNA levels were measured by densitometry, normalized to the level of β-actin mRNA, and calculated as percentages of the basal response. Data are presented as the mean $\pm$ s.e.m. from at least three independent experiments. $^*P<0.05$ as compared with the control LPS response.

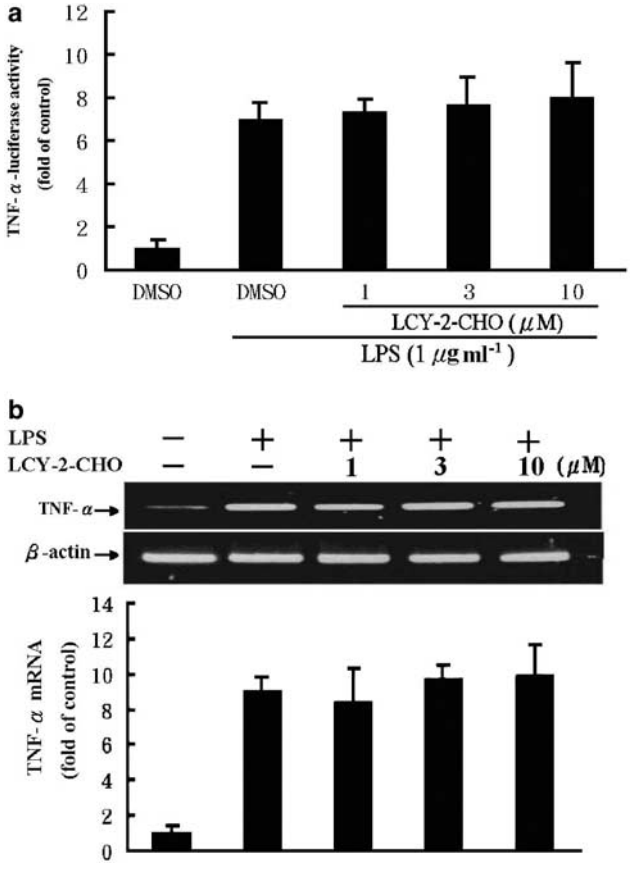

Figure 6 LCY-2-CHO did not change LPS-induced TNF-α gene expression. (a) Cells transfected with the TNF-α-luciferase construct (1 μg) and lacZ plasmid (1 μg) for 24 h were stimulated with LPS (1 μg ml^{-1}), either in the presence or absence of LCY-2-CHO, at the concentrations indicated for 24 h. Cell lysates were then prepared for the luciferase assay, which was normalized by lacZ transfection efficiency, and expressed as a percentage of the control response without stimulus treatment. (b) After stimulation with the indicated agents for 12 h, total RNA was prepared and subjected to RT–PCR analysis for the TNF-α 2 mRNA level. The β-actin mRNA level was considered an internal control. Data on TNF-α mRNA levels were measured by densitometry, normalized to the level of β-actin mRNA, and calculated as percentages of the basal response. Data are presented as the mean $\pm$ s.e.m. from at least three independent experiments.

Discussion

The present investigation examined the effects and mechanisms of action of LCY-2-CHO on LPS-induced production of three inflammatory mediators. In our previous investigation of antiplatelet agents, 9-benzyl-3-hydroxymethyl carbazole was used as a lead compound to prepare a series of derivatives. Among them, LCY-2-CHO significantly inhibited platelet aggregation (Lee *et al*., 2002b), and exhibited anti-inflammatory properties (Lee *et al*., 2002a). Our present results suggest that the inhibition of the p38 MAPK signaling pathway by LCY-2-CHO contributes to its anti-inflammatory action in reducing the induction of iNOS, COX-2, and TNF-α protein. In contrast, LCY-2-CHO did not affect the other two MAPK signaling cascades, ERK and JNK, elicited by LPS.

LPS-induced activation of p38 MAPK in macrophages has been widely demonstrated to correspond to the *in vitro* and *in vivo* effects of LPS in positive regulation of a variety of genes involved in inflammation. Therefore, p38 MAPK signaling may form the basis of a new strategy for treatment of inflammatory diseases (Lee *et al*., 2000; Ono & Han, 2000; Branger *et al*., 2002). Accordingly, several potent p38 MAPK inhibitors have been developed and evaluated in animal models of inflammatory diseases (Badger *et al*., 1996; 2000; Jeon *et al*., 2000; Nick *et al*., 2000; Underwood *et al*., 2000). Moreover, recent data have established an anti-inflammatory effect of a p38 MAPK inhibitor during human endotoxemia, thus providing hope for the future use of p38 MAPK inhibitors in patients with inflammatory diseases (Branger *et al*., 2002).

Our present data obtained with the synthetic compound LCY-2-CHO are consistent with the previous findings identifying p38 MAPK as the principal signaling molecule in LPS-induced TNF-α (Badger *et al*., 1996; Bhat *et al*., 1998; Nick *et al*., 2000; Branger *et al*., 2002), COX-2 (Hwang *et al*., 1997; Guan *et al*., 1998a, b; Chen *et al*., 1999; von Knethen

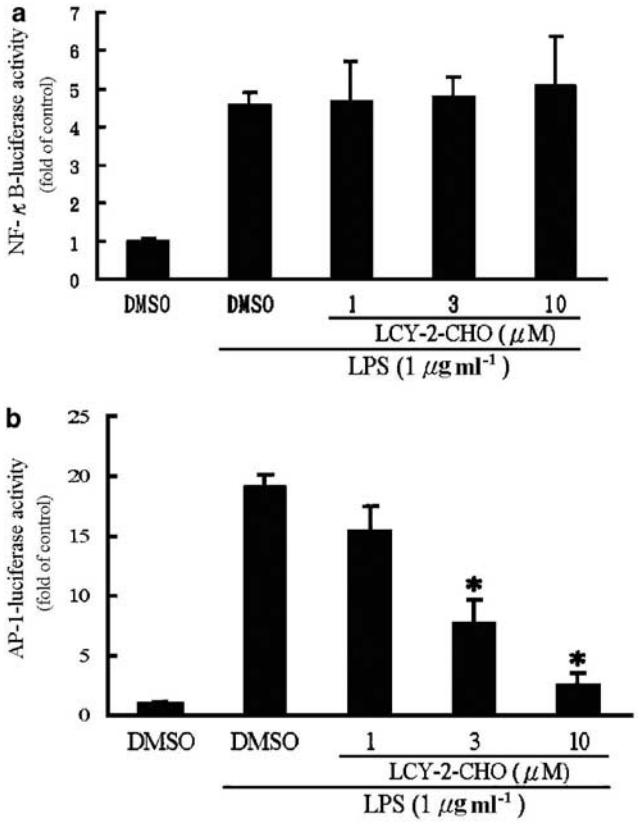

Figure 7 Effects of LCY-2-CHO on LPS-induced κB and AP-1 activation. Cells transfected with a vector, the κB-luciferase reporter gene, the AP-1-luciferase reporter gene, and β-gal-lacZ (each at 1 μg) were treated with LPS and/or LCY-2-CHO at the concentrations indicated. Luciferase activity was normalized to the transfection efficiency with β-gal-lacZ. The data represent the mean ± s.e.m. from at least three independent experiments. $*P<0.05$, indicating the ability of LCY-2-CHO to inhibit the LPS response.

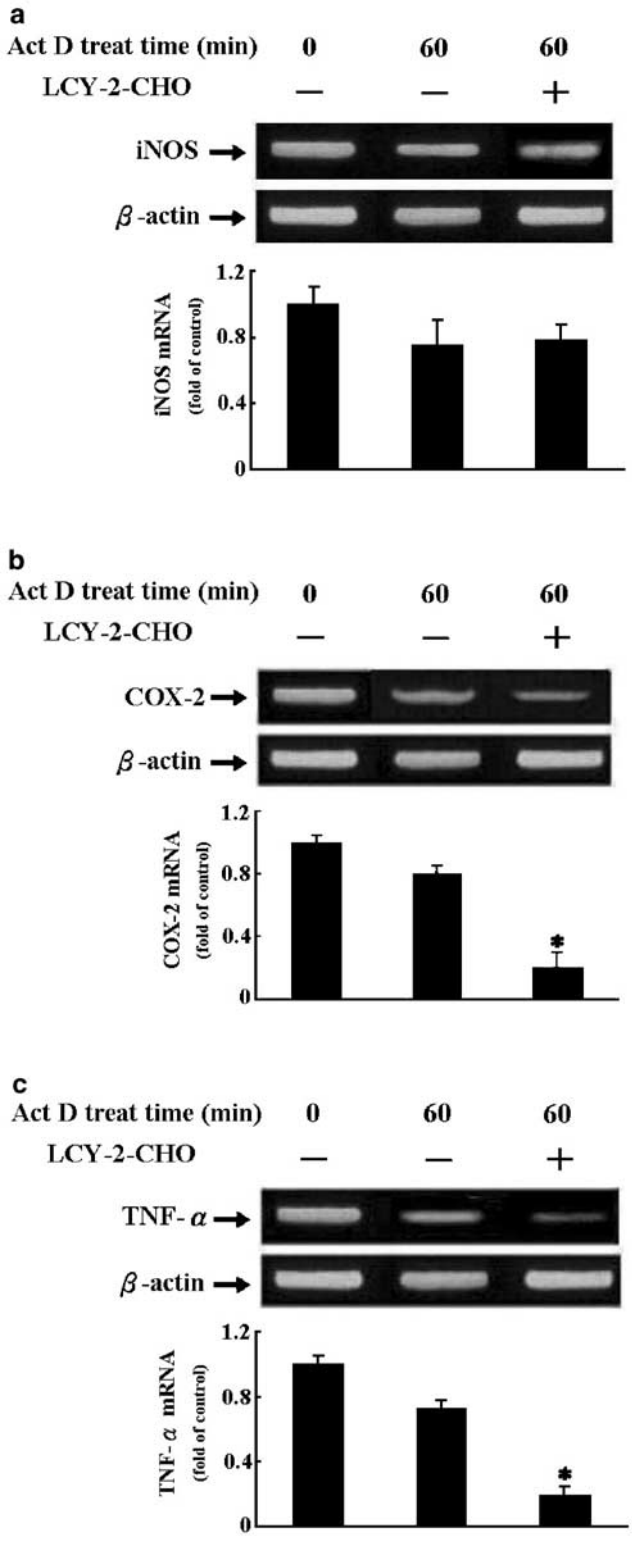

Figure 8 Effects of LCY-2-CHO on the mRNA stability of iNOS, COX-2, and TNF-α. Cells were stimulated with LPS (1 μg ml^{-1}) for 12 h, and then replaced with fresh medium containing actinomycin D (10 μM) and/or LCY-2-CHO (10 μM). After 60 min, the mRNA levels of iNOS (a), COX-2 (b), and TNF-α (c) were determined by RT–PCR, measured by densitometry, normalized to the level of β-actin mRNA, and calculated as percentages of the response following 12-h induction by LPS prior to the addition of actinomycin D. Data are presented as the mean ± s.e.m. from three independent experiments.

et al., 1999), and iNOS (Chen *et al*., 1998; 1999; Hsu *et al*., 2001; Kristof *et al*., 2001; Tsi *et al*., 2002) expression. Thus, p38 MAPK signaling is implicated in LPS activation of AP-1 (Karin, 1995), which binds to the AP-1 site in the iNOS promoter and activates the iNOS promoter (Marks-Konzalik *et al*., 1998; Kristof *et al*., 2001), whose results are compatible with our observation of LCY-2-CHO inhibition of iNOS transcription. Similarly, the role of AP-1 in COX-2 induction has been established (Xie *et al*., 1994; von Knethen *et al*., 1999), most probably achieved through its binding to CRE (Xie *et al*., 1994; Xie & Herschman, 1995) and NF-IL-6 sites (Mestre *et al*., 2001). Activation of both elements exerts cooperative action on COX-2 promoter activity (Kirtikara *et al*., 2000; Wadleigh *et al*., 2000; Mestre *et al*., 2001). Our results showing inhibition of LPS-induced COX-2 transcription by LCY-2-CHO are also consistent with these findings.

The similar potencies of LCY-2-CHO as an inhibitor of induced NO production, of iNOS protein, iNOS mRNA, and iNOS promoter activity suggest that transcriptional inhibition contributes importantly to the overall effect. In contrast, as its IC_{50} for inhibition of the COX-2 promoter was higher than that of PGE_2 production and of COX-2 protein expression, we suggest that both the transcriptional and post-transcriptional mechanisms are involved in the regulation of COX-2 expression. Using a similar analysis, our results further suggested

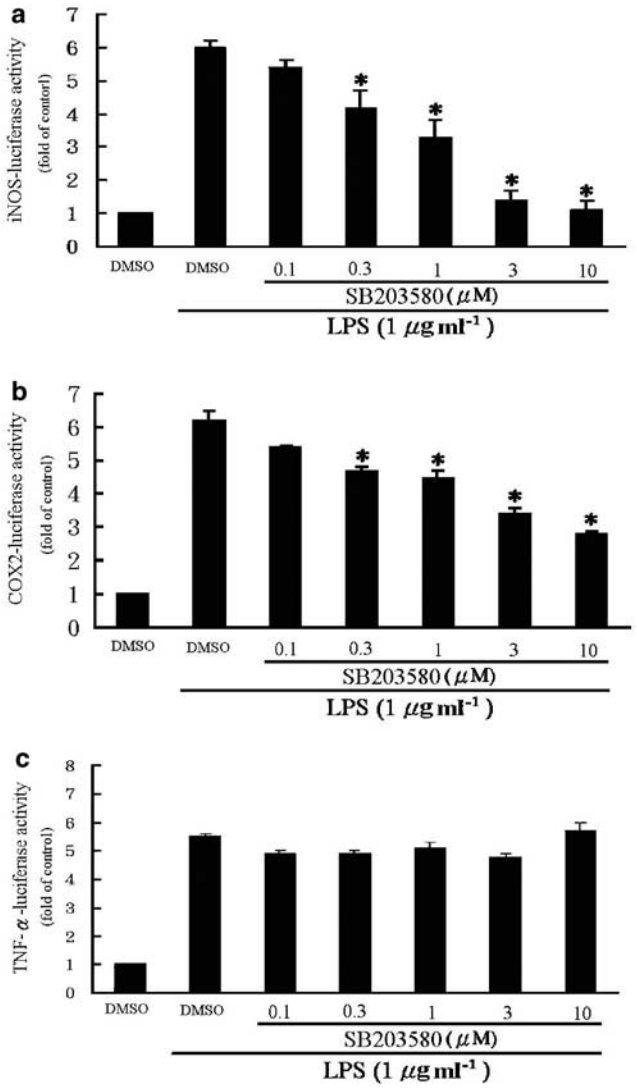

Figure 9 Role of p38 MAPK in LPS-mediated gene transcription. Cells transfected with a vector, each reporter gene of iNOS (a), COX-2 (b), and TNF-α (c), and β-gal-lacZ (each at 1 μg) were treated with LPS and/or SB203580 at the concentrations indicated. Luciferase activity was normalized to the transfection efficiency with β-gal-lacZ. Data represent the mean ± s.e.m. from at least three independent experiments. $*P<0.05$, indicating the ability of SB203580 to inhibit LPS responses.

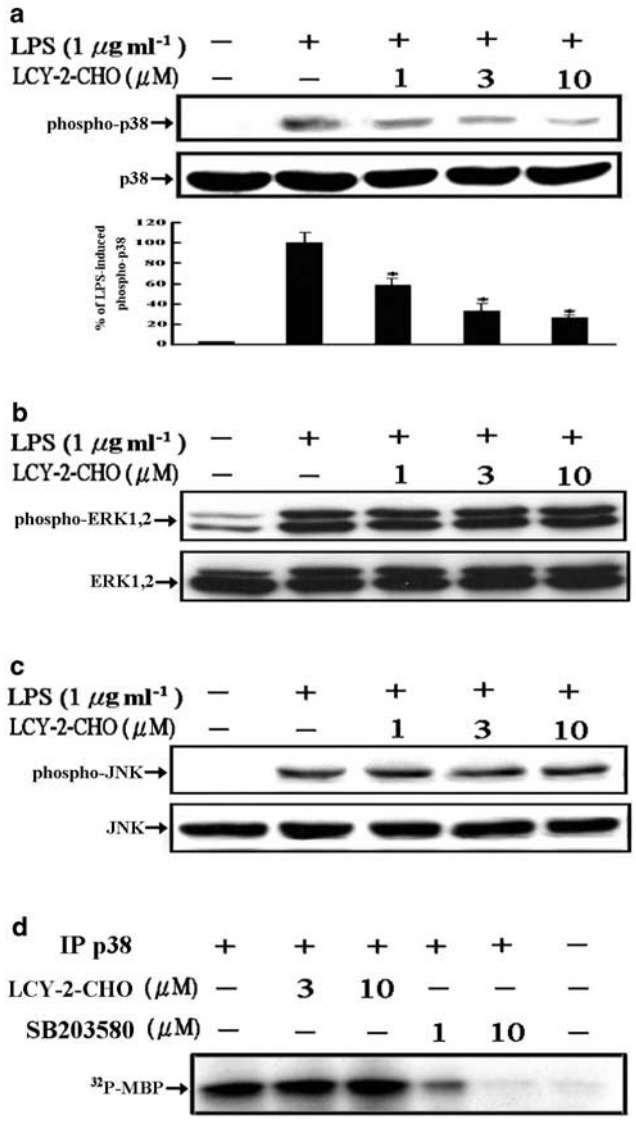

Figure 10 LCY-2-CHO inhibition of LPS-induced p38 MAPK activation. Cells were stimulated with the indicated concentrations of LPS and/or LCY-2-CHO for 30 min. Immunoblotting analyses of the levels of total and phosphorylated p38 MAPK (a), ERK (b), and JNK (c) were carried out. Quantitative data in (a) represent the mean ± s.e.m. from three independent experiments. In (d), p38 MAPK was immunoprecipitated from cell lysates prepared from cells treated with 1 μg ml^{-1} LPS for 30 min. Equal aliquots of immune complexes were used for testing the effects of LCY-2-CHO and SB203580 on kinase activities. The results are representative of three different experiments.

that a post-transcriptional mechanism is responsible for TNF-α regulation by LCY-2-CHO. Thus, although TNF-α production and procytokine formation were both inhibited, the promoter activity of TNF-α was not affected by LCY-2-CHO. This shows a striking difference for p38 MAPK involvement in transcription between these three genes and suggests that, compared to iNOS and COX-2, the p38 MAPK pathway might not play an essential role in mRNA transcription of TNF-α.

Although in monocytes/macrophages cooperation between the AP-1/CRE-like site and the adjacent NF-κB site is required for strong LPS responsiveness (Yao *et al*., 1997; O'Donnell & Taffet, 2002), the cooperation with multiple enhancers renders this gene regulation more complex (Tsai *et al*., 2000). In addition, our present results are in line with previous reports showing the redundancy of the four MAPK pathways activated by LPS in the regulation of TNF expression (Zhu *et al*., 2000). Thus, p38 inhibition alone cannot lead to significant attenuation of this high transcriptional activity (Brook *et al*., 2000). Moreover, the ineffectiveness of SB203580, a selective p38 MAPK inhibitor, on TNF-α promoter activity, as demonstrated in this study, further strengthens this notion. However, another key event regulating the production of this cytokine is the control of TNF-α mRNA degradation (see discussion below).

In addition to AP-1 being under the control of p38 MAPK activation, NF-κB can be activated by stimulation with LPS or cytokines, and controls iNOS (Lowenstein *et al*., 1993;

Kleinert *et al*., 1996; Kristof *et al*., 2001), COX-2 (Yamamoto *et al*., 1997; von Knethen *et al*., 1999; Kirtikara *et al*., 2000), and TNF-α (Collart *et al*., 1990; Shakhov *et al*., 1990; Yao *et al*., 1997; Paludan *et al*., 2001; O'Donnell & Taffet, 2002) gene transcription. Indeed, the coordinated interaction of both transcription factors seems to be required for full expression of these genes. The promoter activity assay performed in the present study and that performed by Tsao *et al*. (2002) have consistently ruled out interference with the upstream signaling regulators for NF-κB activation by LCY-2CHO.

Multiple mechanisms have been proposed for the regulation of gene expression by p38 MAPK. Mediation by p38 MAPK is not only involved in gene transcription as mentioned above but also in post-transcriptional events, that is, mRNA stabilization and translation. The role of p38 MAPK in LPS- or cytokine-mediated COX-2 and TNF-α gene expression is an example of this (Raabe *et al*., 1998; Brook *et al*., 2000; Jang *et al*., 2000; Lasa *et al*., 2000). Translational activation of both genes is dependent on AU-rich elements (AREs) found within the 3′-UTRs). These elements, which confer repression of mRNA stability and translational activation, must be depressed in order for COX-2 (Lasa *et al*., 2000) and TNF-α (Han *et al*., 1990; Kontoyiannis *et al*., 1999; Brook *et al*., 2000) translation to proceed. Accordingly, the activity of p38 MAPK is necessary and sufficient for regulating both events (Dean *et al*., 1999; Kontoyiannis *et al*., 1999; 2001; Mahtani *et al*., 2001). Blocking the p38 pathway reduces the stability of mRNA in full-length genes, but does not affect the stability of mRNAs lacking the ARE sequence, indicating that p38 MAPK may act *via* AREs to modulate mRNA stability. In parallel, studies have shown that the zinc-finger protein, tristetraprolin (TTP), through binding to AREs in the 3′-TNF mRNA, promotes destabilization of mRNA (Carballo *et al*., 1998; Lai *et al*., 1999). The suppressive effect of TTP can be removed by p38 phosphorylation of TTP (Mahtani *et al*., 2001; Zhu *et al*., 2001). Studies on downstream signals further found that p38 MAPK-regulated MAPKAPK2 (MK2) mediates stabilization of TNF-α mRNA and its translation (Kotlyarov *et al*., 1999, 2002; Mahtani *et al*., 2001; Neininger *et al*., 2002). In addition to mediating the TNF-ARE-dependent translation process, MK2 also stabilizes p38 activation (Kotlyarov *et al*., 2002). Taken together, post-transcriptional regulation (the control of mRNA stability and translation) by p38 MAPK appears to play a critical role in regulating TNF-α expression.

In the context of the inhibition of p38 MAPK signaling induced by LPS, we further showed that LCY-2-CHO is not a direct inhibitor of p38 MAPK's catalytic function. This finding demonstrates that the targets of SB203580 and LCY-2-CHO are clearly distinct. Structurally SB203580 is a pyridinylimidazole compound, which has been identified as being a selective p38 MAPK inhibitor (Lee *et al*., 1999). The different structure of LCY-2-CHO taken together with our present results suggests that LCY-2-CHO might antagonize the pathways leading to p38 MAPK activation, upstream of its action. The exact target of LCY-2-CHO in this context is currently being investigated.

In conclusion, the findings obtained with the synthetic compound LCY-2-CHO provide strong pharmacological evidence of the involvement of p38 MAPK signaling in the transcriptional and post-transcriptional regulation of inflammatory gene expression induced by LPS. Blocking the upstream events required for p38 MAPK activation by LCY-2-CHO may thus provide a new therapeutic option in the treatment of human inflammatory diseases.

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