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Inhibition of Lipopolysaccharide-stimulated Inflammatory Cytokine Production by LY303511 in Human Macrophagic THP-1 Cells

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We have previously shown that the specific phosphatidylinositol 3-kinase inhibitor LY294002 (LY29), and its inactive analog LY303511 (LY30), inhibit a monocyte chemoattractant protein-1 (MCP-1) expression in human umbilical vein endothelial cells; these results suggest the potential of LY30 as an anti-inflammatory drug. In this study, we determined the effects of LY30 on the production of various inflammatory cytokines in human macrophagic THP-1 cells which were stimulated with lipopolysaccharide (LPS). LY30 selectively suppressed the mRNA expression of IL-12 p40, TNF-α, and MCP-1 without affecting the expression of IL-1α, IL-6, and IL-8. Inhibition of the production of IL-12 and TNF-α by LY30 was also demonstrated using ELISA assays. In order to elucidate the mechanisms of the action of LY30, we examined the role played by the mitogen-activated protein kinases and the key transcription factors, AP-1 and NF-κB in LPS-stimulated THP-1 cells. The results revealed that LY30 inhibited LPS-induced activation of ERK, but not p38 or JNK. Furthermore, the AP-1 DNA binding activity was suppressed by LY30 based upon the dosage, whereas NF-κB DNA binding was not affected. These results suggest that LY30 selectively inhibits cytokine production in the LPS-stimulated macrophagic THP-1 cells by down-regulating the activation of ERK and AP-1.

Key words: LY303511, THP-1, cytokine, mitogen-activated protein kinase, AP-1, NF-κB

Introduction

LY294002 (LY29) and wortmannin (WM) are the two widely used specific inhibitors of phosphatidylinositol 3-kinase (PI3K) and they are structurally distinct compounds. Meanwhile, LY303511 (LY30), a kinase inactive analog of LY29, is used as controls for LY29 in experiments looking at PI3K effects in cultured cells [1-3]. Interestingly, LY30 has been reported to have noteworthy effects on cellular activities. We showed that not only LY29 but also LY30 inhibits monocyte chemoattractant protein-1 (MCP-1) expression by interleukin (IL)-1 in human umbilical vein endothelial cells [4]. Other investigators reported that LY30 sensitizes tumor cells to drug-induced apoptosis and inhibits cell proliferation in vitro and in vivo [5,6]. One previous study reported that LY29 inhibited LPS-induced IL-10 expression by RAW264.7 macrophages, in a PI3K-independent mechanism [7].

Cytokines are important as therapeutic agents and as targets for specific antagonists in numerous immune and inflammatory

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diseases [8]. It is apparent that excessive or unrestricted activity of proinflammatory cytokines is detrimental. Macrophages are the principal source of proinflammatory cytokines. When macrophages are exposed to inflammatory stimuli, they secrete cytokines such as TNF, IL-1, IL-6, IL-8, and IL-12 [9]. Over the years, many efforts have been made to develop small molecules that can inhibit the production of pro-inflammatory cytokines and the resulting inflammation.

THP-1 is a human monocytic leukemia cell line. After treatment with phorbol esters, THP-1 cells differentiate into macrophage-like cells [10]. Using THP-1 macrophagic cells, we investigated the effect of LY30 on the production of various inflammatory cytokines.

Materials and Methods

Reagents

LY29, LY30, and WM were purchased from Sigma (St. Louis, MO, USA). *Escherichia coli* LPS was from Sigma. LY29 and WM were dissolved in a 1:1 mixture of dimethylsulfoxide and ethanol. LY30 was dissolved in water.

THP-1 culture

THP-1 cells were purchased from American Type Culture Collection (Manassas, VA, USA). THP-1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μg/ ml gentamicin at 37℃ in a humidified atmosphere containing 5% CO_2. The mature macrophage-like state was induced by treating THP-1 cells for 24 h with 100 ng/ml PMA in culture plates. Differentiated, plastic-adherent cells were washed twice with medium and incubated with fresh medium lacking PMA.

RT-PCR

Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer and was quantified spectctrophotometrically. First-strand cDNA was synthesized from 1 μg of RNA using random primers (Promega, Madison, WI, USA) and Molony murine leukemia virus reverse transcriptase (Invitrogen). 2 μl of cDNA products were amplified in 25 μl volumes under a layer of mineral oil using a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Each PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 U *Taq* DNA polymerase, and 0.5 μM of each primer. Each cycle consisted of denaturation at 94℃ (30 s), annealing at 55℃ (30 s), and extension at 72℃ (60 s). The sequences of primers were 5'-CAGCCAGATGCAATCAATGC-3', 5'-GTGGTCCATGGAATCCTGAA-3' for MCP-1 (198 bp); 5'-TCAATGAGGAGACTTGCCTG-3', 5'-GATGAGTTGTCATGTCCTGC-3' for IL-6 (260 bp); 5'-TGTGCTCTCCAAATTTTTTTTACTG-3', 5'-CTCTCTTTCCTCTTTAATGTCCAGC-3' for IL-8 (408 bp); 5'-CTTCTGCCTGCTGCACTTTGGA-3', 5'-TCCCAAAGTAGACCTGCCCAGA-3' for TNF-α (547 bp); 5'-GTCTCTGAATCAGAAATCCTTCTATC-3', 5'-CATGTCAAATTTCACTGCTTCATCC-3' for IL-1α (421 bp); 5'-CAGAAGCTAACCATCTCCTGGTTTG-3', 5'-TCCGGAGTAATTTGGTGCTTCACAC-3' for IL-12 p40 (394 bp); and 5'-AGCGGGAAATCGTGCG TG-3', 5'-CAGGGTACATGGTGGTGCC-3' for β-actin (300 bp). The PCR products of 10 μl were fractionated on 1.2% (w/v) agarose gels containing ethidium bromide, visualized by UV transillumination, and photographed.

ELISA

The THP-1 culture supernatants were sampled and centrifuged at 100 × g for 5 min for clarification of debris. The levels of cytokines were quantified using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions.

Western blot

THP-1 cells in 12-well plates were harvested and lysed with 100 μl of Cell Lysis Buffer (Cell Signaling Technology). 30 μg of each boiled sample was resolved by SDS-PAGE (10%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with rabbit anti-phospho-ERK, p38, or JNK polyclonal antibody (1:1000, Cell Signaling Technology) and a 1:1500 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology). Immunoreactive proteins were detected by enhanced chemiluminescence (LumiGLO, Cell Signaling Technology). The same membrane was stripped and reprobed with anti-β-actin (1:5000).

Gel shift assay

For the preparation of nuclear extracts, cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9 at 4℃, 0.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF) and

incubated for 10 min on ice, then the cells were lysed by addition of 10% IGEPAL CA-630, followed by vigorous vortex for 10 s. Nuclei were pelleted and resuspended in low-salt buffer (20 mM HEPES, pH 7.9 at 4℃, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and added high-salt buffer (20 mM HEPES, pH 7.9 at 4℃, 1.5 mM $MgCl_2$, 0.8 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) in a drop-wise fashion. After 30 min incubation at 4℃, the lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. Protein concentrations of the nuclear extracts were measured with DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). An oligonucleotide containing the NF-κB-binding site (5'-CCGGTTAACAGAGGGGG CTTTCCGAG-3') or AP-1-binding site (5'-AAGGCGCTTGAT GACTCAGCC GGAA-3') was synthesized (TaKaRa Korea, Sungnam, Korea) as a probe for the gel retardation assay. The probes were labeled with [α-^{32}P]dCTP and DNA polymerase I (Klenow fragment, Invitrogen). About 10 μg of nuclear extracts were incubated with 10,000 cpm of probe in 20 μl of reaction buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, and 200 ng of poly(dI·dC) for 30 min at room temperature. The DNA- protein complexes were separated on 4% polyacrylamide gels. The gels were dried and subjected to autoradiography.

Statistical analysis

Our experiments were conducted in three independent experiments to confirm the reproducibility of the results. The data are presented as means with standard deviations (SD). Statistical analysis of one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test was performed using GraphPad InStat (GraphPad Software, La Jolla, CA, USA). A p-value <0.05 was considered statistically significant.

Results

LY30 inhibited mRNA expression of various cytokines

We first investigated the effect of LY30 on the mRNA expression of various cytokines in macrophagic THP-1 cells using RT-PCR. LY30 as well as LY29 inhibited the LPS-induced mRNA expression of MCP-1, IL-12 p40, and TNF-α. The mRNA expression of IL-6 and IL-8 was not affected by LY29 or LY30. Meanwhile, IL-1α expression was inhibited by LY29 but not LY30. WM did not inhibit mRNA expression of any cytokines tested (Fig. 1).

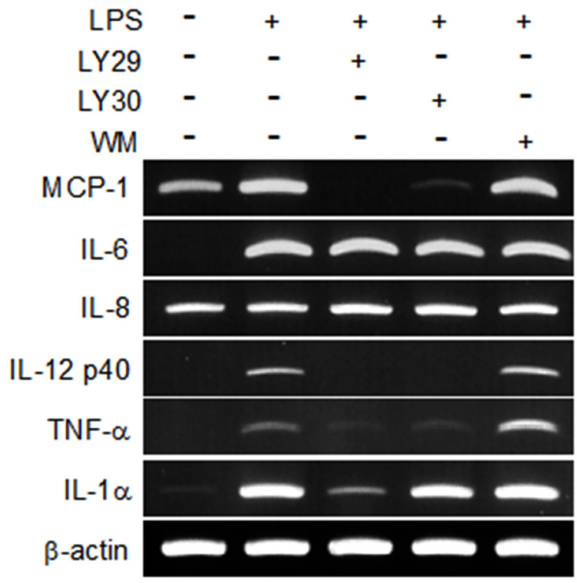

Fig. 1. Effect of LY30 on the mRNA expression of various cytokines in LPS-stimulated THP-1 cells. PMA-differentiated THP-1 cells were cultured in 12-well plates (1×10^6 cells/well in 1-ml volume). LY29 (50 μM), LY30 (50 μM), or WM (1 μM) were added 30 min before LPS stimulation (0.1 μg/ml). After 6 h, total RNA was isolated and the relative levels of mRNA expression of MCP-1, IL-6, IL-8, IL-12 p40, TNF-α, and IL-1α were determined by RT-PCR.

LY30 inhibited production of IL-12 and TNF-α

Next, we examined the effect of LY30 on the production of IL-12, IL-6, and TNF-α in macrophagic THP-1 cells using ELISA. In accordance with the results of RT-PCR, both LY30 and LY29 inhibited production of IL-12 and TNF-α. Interestingly, WM increased production of IL-12 and TNF-α, compared to LPS alone. As for IL-6, LY30 did not significantly inhibit IL-6 production (Fig. 2).

LY30 inhibited phosphorylation of ERK

Since mitogen-activated protein kinases (MAPKs) play central roles in the cytokine induction [11], we examined the effect of LY30 on the phosphorylation of the three members of MAPKs, JNK, p38, and ERK, using Western blot analysis. LPS induced phosphorylation of JNK and p38, but ERK phosphorylation was not additionally increased by LPS. LY30 decreased the levels of phospho-ERK at both time points of 30 min and 60 min (Fig. 3).

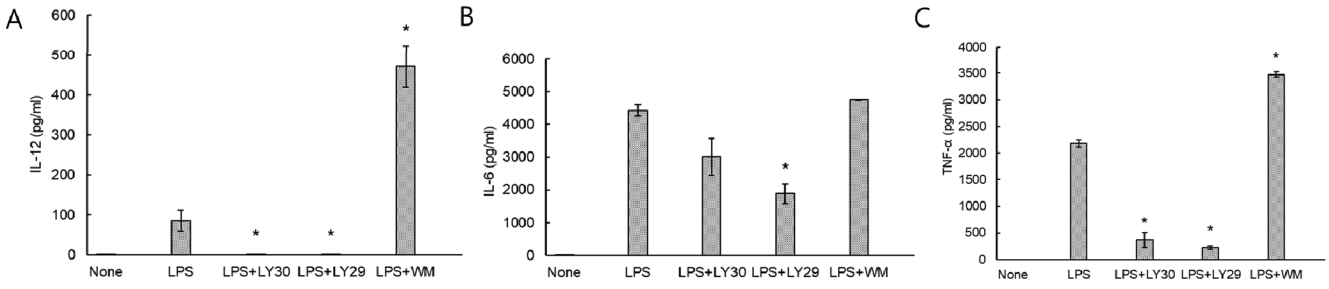

Fig. 2. Effect of LY30 on the production of IL-12, IL-6, and TNF-α in LPS-stimulated THP-1 cells. PMA-differentiated THP-1 cells were cultured in 12-well plates (1×10^6 cells/well in 1-ml volume). LY29 (50 μM), LY30 (50 μM), or WM (1 μM) were added 30 min before LPS stimulation (0.1 μg/ml). After 18 h, culture supernatants were collected, and concentrations of IL-12 (A), IL-6 (B), and TNF-α (C) were determined by ELISA. Statistical significance between LPS only and LY30, LY29, or WM was evaluated. The asterisks indicate significant differences.

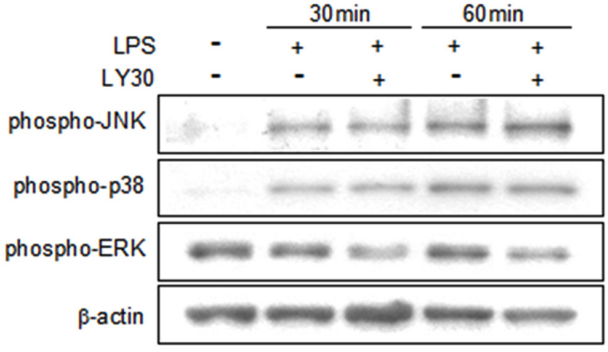

Fig. 3. Effect of LY30 on the activation of mitogen-activated protein kinases in LPS-stimulated THP-1 cells. PMA-differentiated THP-1 cells were cultured in 12-well plates (1×10^6 cells/well in 1-ml volume). LY30 (50 μM) was added 30 min before LPS stimulation (0.1 μg/ml). At 30 min and 60 min after LPS stimulation, cell lysates were prepared and Western blot was performed for phospho-JNK, p38, or ERK.

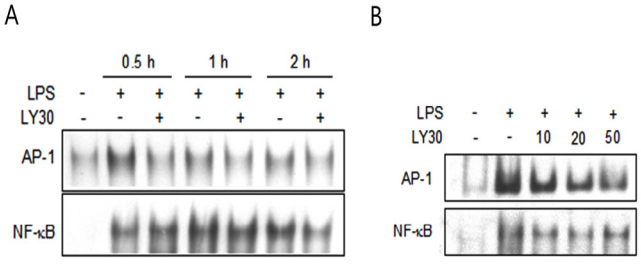

Fig. 4. Effect of LY30 on the activation of AP-1 and NF-κB transcription factors in LPS-stimulated THP-1 cells. PMA-differentiated THP-1 cells were cultured in 12-well plates (1×10^6 cells/well in 1-ml volume). (A) LY30 (50 μM) was added 30 min before LPS stimulation (0.1 μg/ml). At 0.5 h, 1 h, and 2 h after LPS stimulation, nuclear extracts were prepared and activation of AP-1 and NF-κB was determined by gel shift assays. (B) Various concentrations of LY30 were added 30 min before LPS stimulation (0.1 μg/ml). At 30 min after LPS stimulation, nuclear extracts were prepared and activation of AP-1 and NF-κB was determined by gel shift assays.

LY30 inhibited activation of AP−1

Since NF-κB and AP-1 are two important transcription factors mediating gene expression of various cytokines [12,13], we investigated the effect of LY30 on the activation of NF-κB and AP-1 through gel shift assays. LY30 inhibited LPS-stimulated activation of AP-1. Moreover, the inhibitory action of LY30 on AP-1 activation occurred in a dose-dependent manner. In contrast, the LPS-stimulated activation of NF-κB was not inhibited by LY30 (Fig. 4).

Discussion

This study showed that LY30 inhibited production of several inflammatory cytokines in THP-1 macrophagic cells. This inhibitory effect of LY30 on cytokine production was demonstrated at both mRNA and protein levels (Figs. 1 and 2). However, it appeared that production of certain cytokines are insensitive to the inhibitory action of LY30. In the present study, LY30 inhibited production of MCP-1, IL-12, and TNF-α, but it did not decrease that of IL-6, IL-8, and IL-1α. As PI3K inhibitors, LY29 and WM have been used at working concentrations of about 50 μM and 1 μM, respectively. 1 μM WM did not inhibit production of cytokines tested, which supports that the inhibitory action of LY30 and LY29 is PI3K-independent. Rather surprisingly, WM increased production of IL-12 and TNF-α (Fig. 2). It seems that PI3K acts as a negative regulator for the production IL-12 and TNF-α in THP-1 macrophagic cells [14].

MAPKs are a series of protein kinases that become phosphorylated and activated on cellular stimulation and lead to new gene expression. There are three well-known members

of MAPKs − ERK, p38, and JNK. Our Western blot analyses showed that LY30 inhibited activation of ERK without affecting activation of p38 and JNK in LPS-stimulated THP-1 macrophagic cells (Fig. 3). The activated ERK translocates to the nucleus and phosphorylates a protein called Elk, and phosphorylated Elk stimulates transcription of c-Fos, a component of the AP-1 transcription factor [15]. The gel shift assays showed that LY30 inhibits activation of AP-1 (Fig. 4). AP-1 is involved in transcriptional regulation of many cytokine genes. Along with AP-1, another transcription factor NF-κB also mediates induction of many cytokine genes. However, activation of NF-κB was not inhibited by LY30. Taken together, it appeared that LY30 inhibits activation of ERK, leading to reduced expression of c-Fos and reduced activation of AP-1.

As many efforts have been being made to develop useful anti-inflammatory drugs, our results are meaningful in terms of new drug development. Although LY29 inhibited production of various inflammatory cytokines, it is a PI3K inhibitor and unsuitable for the purpose of anti-inflammatory drugs. Despite the limitations of the present study, it suggests that LY30 may be used as a novel template for the development of new anti-inflammatory drugs.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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