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Biphasic regulation of levofloxacin on lipopolysaccharide-induced IL-1β production

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Abstract

Fluoroquinolones have been known to exert modulatory activity on immune responses to microbial infection. However, the mechanism of this immunomodulation has not been well elucidated. In this study, we investigated the effect of levofloxacin on lipopolysaccharide (LPS)-induced production of interleukin-1beta (IL-1β) in RAW264.7 cells. We showed that LPS-stimulated release of pre-synthesized IL-1β was promoted by levofloxacin, in part via the p38 mitogen-activated protein kinase (MAPK) pathway. On the other hand, newly synthesized IL-1β production was inhibited by levofloxacin. This immunoregulatory function of levofloxacin in the later phase as well as promotion of pre-synthesized IL-1β release by levofloxacin in the early phase might be advantageous in the host defense to microbial pathogens.

Keywords: Levofloxacin; LPS; Interleukin-1β; Immunoregulatory function; Fluoroquinolones

Introduction

Macrophages and monocytes are activated in response to components of pathogenic bacteria invading the host, and they initiate many intracellular cascades of cytokines and chemokines (Baumann and Gauldie, 1994). Inflammatory cytokines, including interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and nitric oxide are produced and released from macrophages after stimulation by lipopolysaccharide (LPS), a component of Gram-negative bacteria. Inflammatory cytokines induce a phagocytic response against pathogenic bacteria, and nitric oxide (NO) exerts cytotoxic and bacteriocidal activity.

Fluoroquinolones, which target bacterial DNA gyrase and topoisomerase II, are widely used antibiotics because of their broad spectrum efficacy against bacteria and their safety for the host (Hooper, 2000). In addition to bacteriocidal activity, fluoroquinolones are known to have immunomodulatory activity on immune systems. In vivo and in vitro studies have revealed that several fluoroquinolones inhibit LPS-induced production of cytokines including IL-1β and TNF-α (Bailly et al., 1990, 1991; Stunkel et al., 1991; Khan et al., 1998; Shalit et al., 2001; Araujo et al., 2002). Levofloxacin is the L-form of ofloxacin, and also has an immunomodulatory action on cytokine production (Yoshimura et al., 1996). However, effects on immunomodulatory activities vary widely among fluoroquinolones and their mechanism of immunomodulation has not been well elucidated. In this study, we examined the effect and mechanism of levofloxacin on LPS-induced production of cytokines in a macrophage-like cell line, RAW264.7, focusing in particular on production and release of IL-1β.

Materials and methods

Reagents and antibodies

Levofloxacin (Daiichi Co. Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO; Dojin Chemical Co. Kumamoto, Japan). LPS from *Escherichia coli* (O55:B5) was purchased from Sigma (St. Louis, MO). Specific antibodies for phospho-ERK, phospho-p38, and phospho-JNK were purchased from Cell Signaling Technology (Beverly, MA). β-Actin antibody

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was obtained from Abcam (UK). HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were obtained from Dako (Denmark). SB203580, a specific inhibitor of p38 MAPK, was purchased from Calbiochem (San Diego, CA).

Cell culture, treatment of levofloxacin and stimulation by LPS

RAW264.7, a murine macrophage-like cell line, was obtained from the ATCC (Manassas, VA), and was maintained in Eagle's modified minimal essential medium (EMEM) supplemented with 2 mM glutamine (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin (ICN, Aurora, OH), and 10% FBS (Sigma). Cells were stimulated with LPS at 37 °C.

Analysis of cytokine production

Production of IL-1β, TNF-α or GM-CSF was measured essentially as previously reported (Okugawa et al., 2003). Briefly, RAW264.7 cells were seeded at a density of 1×10^5 cells/ml in 24-well plates; supernatants were collected 24 h after LPS stimulation. The concentrations of IL-1β, TNF-α or GM-CSF were measured by ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA).

Analysis of nitrite production

RAW264.7 cells were seeded at a density of 1×10^5 cells/ml in 24-well plates and supernatants collected 24 h after LPS stimulation. Nitrite concentration was measured by Griess assay according to the manufacturer's instructions (Biosource International).

Immunoblotting

Immunoblotting was performed as previously described (Nakayama et al., 2004). Briefly, after LPS stimulation, ice-cold PBS was added and cells were then lysed with lysis buffer. Total cell lysates were separated by SDS–PAGE under reducing conditions and were electrically transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 1% bovine serum albumin in TBS and was incubated with the corresponding antibody. Reactive bands were visualized with HRP-coupled secondary antibody via an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, UK) according to the manufacturer's procedures.

Transfection and reporter assays

Transfection and reporter assays were performed as previously described (Nakayama et al., 2003). Briefly, in order to normalize transfection efficiency, we employed the dual-luciferase reporter assay system (Promega), in which the pRL-thymidine kinase (TK) plasmid containing the Renilla luciferase gene under control of the TK promoter was co-transfected as an internal control. The same number of cells was plated in a 96-well plate 18 h before transfection. They were then transiently transfected with NF-κB luciferase reporter construct and pRL-TK (Promega) using LipofectAMINE Reagent (Invitrogen). At 24 h after transfection, cells were treated with graded concentrations of levofloxacin, and then stimulated with LPS. At 6 h after LPS stimulation cells were washed and harvested. The activities of control Renilla luciferase and firefly luciferase (experimental) were measured in triplicate. After normalization for Renilla luciferase activity, promoter activity was calculated as increase relative to control.

Isolation of ribonucleic acid and reverse transcription

Ribonucleic acid (RNA) was isolated from RAW264.7 cells using RNeasy Mini kit (Qiagen, Valencia, CA). Each total RNA (0.5 μg) was converted to cDNA, and PCR was performed with the amplification sequence protocol: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s for 30 cycles. The following primers were used in the experiments: IL-1β (sense) 5′-TTGACGGACCCCAAAA-GATG-3′, (antisense) 5′-AGAAGGTGCTCATGTCCTCA-3′ (Zhou et al., 1997); β-actin (sense) 5′-TCATGAAGTGTGAC-GTTGACATCCGT-3′, (antisense) 5′-CTTAGAAGCATTTGC-GGTGCACGATG-3′ (Promega, Madison, WI). PCR products were separated by electrophoresis on 2% agarose gels and visualized with ethidium bromide staining.

Statistical analysis

All data are expressed as mean ± S.E.M. Differences between groups were analyzed by Fisher's Least Significant Difference test using SPSS software (SPSS Inc. USA). Values of $p < 0.05$ were considered to represent a statistically significant difference.

Results

Levofloxacin inhibited IL-1β production during the 8 h after LPS stimulation

We first examined whether levofloxacin regulates production of cytokines during the 8 h after LPS stimulation. RAW264.7 cells were pretreated with graded concentrations of levofloxacin, and then stimulated with LPS. LPS-induced production of IL-1β was decreased in cells pretreated with levofloxacin in a dose-dependent manner, as compared with that in control cells (Fig. 1A). In contrast, levofloxacin had little effect on LPS-induced production of TNF-α, GM-CSF or NO (Fig. 1B, 1C, 1D). These results indicate that levofloxacin inhibits the 8-h production of IL-1β, but does not affect TNF-α, GM-CSF or NO.

Levofloxacin promoted IL-1β production during the 3 h after LPS stimulation

We then examined whether levofloxacin was involved in regulating production of IL-1β during the 3 h after stimulation by LPS. RAW264.7 cells were pretreated with graded concentrations of levofloxacin, and then stimulated with LPS. LPS-induced production of IL-1β was enhanced in cells pretreated with higher concentrations (>30 mg/l) of levofloxacin,