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Anti-inflammatory effects of garenoxacin on IL-8 production and ERK1/2 activation induced by lipopolysaccharides in A549 and THP-1 cells

Shintaro Hara [a], Yuji Ishimatsu [a,*], Hiroshi Mukae [b], Noriho Sakamoto [a], Tomoyuki Kakugawa [a], Hanako Fujita [a], Atsuko Hara [a], Shigeru Kohno [a]

[a] Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
[b] Department of Respiratory Disease, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahata-nishi-ku, Kitakyusyu, Fukuoka 807-8555, Japan

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ABSTRACT

The anti-inflammatory properties of macrolides have been applied to the treatment of inflammatory airway diseases. Although the anti-inflammatory properties of fluoroquinolones have been reported, no reports are available regarding a newly developed fluoroquinolone, garenoxacin (GRNX). To examine the immunomodulatory effect of GRNX, we examined the transcription and secretion of inflammatory cytokines by human airway epithelial cells and monocytes stimulated with lipopolysaccharide (LPS). A human lung epithelial cell line (A549) and a human monocyte cell line (THP-1) were stimulated with LPS and exposed to different concentrations of GRNX. The transcription and secretion of interleukin 8 (IL-8) in both A549 and THP-1 cells was measured by real-time PCR and an enzyme-linked immunosorbent assay, respectively. Treatment with GRNX significantly inhibited the transcription and secretion of IL-8 induced by LPS-stimulated cells through inhibitory ERK1/2 phosphorylation. GRNX has anti-inflammatory activity through its capacity to alter the secretion of IL-8 from A549 and THP-1 cell lines. Our findings suggest that GRNX is suitable for the treatment of LPS-induced respiratory infection and inflammatory airway diseases.

1. Introduction

Diffuse panbronchiolitis is a clinicopathological disease entity that is characterized by chronic inflammation localized in the respiratory bronchioles (Homma et al., 1983). Since the effectiveness of low-dose, long-term erythromycin treatment was established, diffuse panbronchiolitis has become easy to control and is now curable (Kadota et al., 1993). After the discovery of this treatment, a number of macrolides have been found to have significant immunomodulatory properties beyond their antibacterial activities. Inflammatory reactions, phagocytosis, natural cytotoxicity, cytokine production, antibody responses and cellular immunity are defensive mechanisms that might be modulated by therapeutic doses of other antimicrobial agents as well as macrolides (Labro, 2000; Williams, 2001).

Fluoroquinolones are synthetic, broad-spectrum antimicrobial agents that are commonly used to treat a variety of infections, including systemic infections in immune-compromised patients (Hooper and Wolfson, 1991). In addition to their antimicrobial properties, certain quinolones have significant immunomodulatory activities in vitro and in vivo (Dalhoff, 2005). A newly developed fluoroquinolone, garenoxacin mesilate hydrate (GRNX), has a low mutant prevention concentration (the concentration that prohibits the growth of mutants from a susceptible population of more than 10^{10} cells) and a narrow mutant selection window (defined as the range between the minimum inhibitory concentration and the mutant prevention concentration, provides a means for defining the ability of an antibiotic to prevent the emergence of mutants) and is useful for minimizing the selection of quinolone-resistant mutants of pneumococcal strains (Yamamoto et al., 2009). However, the immunomodulatory properties of GRNX in human lung cells have not previously been examined.

To confirm that GRNX has anti-inflammatory properties, we examined the transcription and production of IL-8 in A549 and THP-1 cells. In addition, we also examined the phosphorylation of ERK1/2 to clarify the immunomodulatory mechanism.

2. Materials and methods

2.1. Reagents

Clarithromycin (CAM) and GRNX were provided by Taisho-Toyama Pharmaceutical Co., Ltd., (Tokyo, Japan). Telithromycin (TEL) was provided by Sanofi-Aventis (Paris, France). Aminobenzyl penicillin (ABPC) was provided by Meiji Seika Pharma, Ltd., (Tokyo, Japan). Lipopolysaccharide (LPS; Pseudomonas aeruginosa serotype 10) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

* Corresponding author. Tel.: +81 95 819 7273; fax: +81 95 849 7285.
E-mail address: yuji-i@nagasaki-u.ac.jp (Y. Ishimatsu).

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2.2. Cell culture

The human alveolar epithelial cell line A549 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown as monolayers in tissue culture flasks (BD Falcon™; Franklin Lakes, NJ, USA) at 37 °C in a 5% CO_2-humidified atmosphere. The cells were maintained in Dulbecco's modified minimal essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). The cells were passaged weekly after exposure to 0.25% (w/v) trypsin. Subconfluent A549 monolayers (5.0×10^5 cells) were grown in 60-mm dishes in DMEM without FBS for 24 h before starting experiments. As a negative control, the A549 cells were incubated with DMEM alone (without FBS). The human monocytic cell line THP-1 (ATCC TIB 202) was purchased from ATCC and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS and 10 mM 6-melcaptoethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37 °C in a 5% CO_2-humidified atmosphere. Assays with THP-1 cells were performed at a density of 10^6 cells/ml in 6-well plates (Corning Incorporated, Corning, NY, USA). In the subsequent IL-8 production studies, THP-1 cells were stimulated with LPS while cultured in RPMI 1640 with 2% heat-inactivated FBS.

2.3. Real-time PCR

The IL-8 mRNA expression was measured by real-time PCR according to a procedure described previously (Amenomori et al., 2010; Hara et al., 2008). To determine the appropriate concentration of LPS, subconfluent A549 cells grown in 60-mm cell culture dishes (5.0×10^5/dish) and THP-1 cells (10^6 cells/ml) in 6-well plates were incubated for 3 h with medium alone (control) or 0.1, 1, 10, or 100 ng/ml of LPS. After the appropriate concentration of LPS was determined from the preliminary experiments, cells were incubated for 3 h with medium alone (control) or LPS (1 ng/ml) in the absence or presence of antibiotics (1.0 to 10 μg/ml of CAM, TEL, GRNX, or ABPC). Total RNA was isolated from cultured A549 cells and THP-1 cells using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A volume of pooled RNA (2 μl) was reverse-transcribed in a total reaction volume of 21 μl containing 0.5 μg random hexamer primer, 20 units RNasin ribonuclease inhibitor, and SuperScript III First-Strand Synthesis System (Invitrogen), according to the supplier's instructions. The resulting cDNA was subjected to quantitative real-time polymerase chain reaction (QRT-PCR). The QRT-PCR amplification was performed with TaqMan Gene Expression Assays for the IL-8 gene and human beta-actin as an endogenous control (Applied Biosystems, Foster City, CA, USA) in combination with the TaqMan Universal PCR Master Mix (Applied Biosystems). Cycle-to-cycle fluorescence emission readings were monitored and analyzed by the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Quantitation of IL-8 mRNA expression was calculated using the comparative C_T method.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The IL-8 protein production was measured by ELISA according to a procedure described previously (Amenomori et al., 2010; Hara et al., 2008). To determine the appropriate concentration of LPS, subconfluent A549 cells in 60-mm cell culture dishes (5.0×10^5/dish) and THP-1 cells in 6-well plates (10^6 cells/ml) were incubated for 24 h (A549 cells) or 9 h (THP-1 cells) with medium alone (control) or 1, 10, or 100 ng/ml of LPS. After the appropriate concentrations of LPS were determined from the preliminary experiments, cells were incubated for 24 h (A549 cells) or 9 h (THP-1 cells) with medium alone (control) or LPS (10 ng/ml for A549 cells or 1 ng/ml for THP-1 cells) in the absence or presence of antibiotics (1.0 to 10 μg/ml of CAM, TEL, GRNX, or ABPC). Cell-free supernatants were recovered by centrifugation and stored at −80 °C until they were assayed. IL-8 levels were measured using a Quantikine human CXCL8/IL-8 ELISA kit according to the supplier's instructions (R&D Systems Inc., Minneapolis, MN, USA).

2.5. Western blotting

ERK1/2 phosphorylation was measured by Western blotting according to a procedure described previously (Ishimoto et al., 2009). To determine the appropriate concentration of LPS for this assay, A549 cells and THP-1 cells were preincubated in serum-free medium at 37 °C for 3 h prior to the addition of 10 ng/ml, 100 ng/ml, 1 μg/ml, or 10 μg/ml of LPS (for A549 cells) or 1, 10, or 100 ng/ml of LPS (for THP-1 cells). Cells were incubated with LPS for 5 min. To determine the appropriate incubation time, cells were preincubated in serum-free medium for 3 h prior to the addition of 10 μg/ml of LPS for A549 cells or 100 ng/ml of LPS for THP-1 cells. Cells were incubated with LPS for 5, 15, 30 or 60 min. After the LPS concentration and exposure time were determined from the preliminary assays, A549 cells and THP-1 cells were preincubated in serum-free medium at 37 °C for 3 h in the absence or presence of 10 μg/ml of CAM or GRNX prior to the addition of 10 μg/ml LPS for A549 cells or 100 ng/ml of LPS for TPH-1 cells. For Western blotting analysis of ERK1/2, cells were incubated with LPS for 15 min (for A549 cells) or 5 min (for THP-1 cells). Then, the cells were washed twice with cold PBS and suspended in 600 μl (for A549 cells) or 40 μl (for THP-1 cells) of cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) that contained a 1:25 dilution of protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan). After being kept on ice for 15 min, the lysates were subjected to centrifugation ($20{,}000\times g$) at 4 °C for 15 min to obtain a cytosolic fraction. The protein concentration was determined by a Bradford assay (Bio-Rad, Munich, Germany) before storage at −70 °C. An aliquot of the cytosol fraction containing 20 μg of protein was resolved by 10% SDS-PAGE. After electrophoresis and electrophoretic transfer of proteins to Immun-Blot PVDF membranes (Bio-Rad), the membranes were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween (TBST) for 1 h. Membranes were incubated at room temperature with a 1:1000 dilution of rabbit anti-human ERK 1/2 or phosphorylated ERK 1/2 antibodies (Cell Signaling Technology Inc.) overnight at 4 °C. Subsequently, the membranes were incubated with a secondary antibody, horseradish peroxidase-linked anti-rabbit IgG (GE Healthcare UK Ltd., Buckinghamshire, UK), for 1 h at room temperature. The blots were washed three times in TBST and incubated in enhanced chemiluminescence reagent (Amersham ECL Plus Western Blot detecting system; GE Healthcare). The specific bands were photographed by FluorChem (Cell Biosciences, Inc., Santa Clara, CA, USA). Relative density values of ERK1/2 were determined by densitometric analysis (NIH Image J software, version 1.14o, NIH, Bethesda, MD, USA).

2.6. Statistical analysis

Values are expressed as mean ± standard error of mean (S.E.M.) for continuous parameters. All statistical analyses were performed with StatMate III for Macintosh (version 3.14, ATMS Co., Ltd., Tokyo, Japan). Results were analyzed by the Student's *t*-test for comparison between two groups and by the non-parametric equivalents of analysis of variance (ANOVA) with Newman-Keuls's multiple comparison test using either vehicle control or LPS treatment as references. Significance was assumed at $P<0.05$.

3. Results

3.1. Effect of CAM, TEL, GRNX, and ABPC on IL-8 mRNA expression in A549 and THP-1 cells stimulated with LPS

To determine the effect of LPS concentration on IL-8 mRNA expression, both cell lines were incubated with various concentrations