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Furaltadone suppresses IgE-mediated allergic response through the inhibition of Lyn/Syk pathway in mast cells

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ABSTRACT

Mast cells are critical cells that prompt various allergic response-inducing factors, contributing to allergic diseases. While used as an antibiotic for livestock, there is no study on the effect of furaltadone on allergic response. This study investigated the effect of furaltadone on mast cells and passive cutaneous anaphylaxis (PCA). Furaltadone inhibited the degranulation of mast cells stimulated by antigen (IC_{50}, ~ 3.9 μM), and also suppressed the production of tumor necrosis factor (TNF)-α and interleukin (IL)-4 in a concentration dependent manner. In addition, furaltadone inhibited allergic responses in an acute allergy animal model, PCA. Further investigation on the mechanism for these inhibitory effects of furaltadone found that the activities of Lyn/Syk and Syk-dependent downstream proteins such as mitogen-activated protein (MAP) kinases were inhibited by furaltadone in mast cells. Taken together, this study demonstrates that furaltadone inhibits the activation of mast cells by antigen via the suppression of the Lyn/Syk pathway and ameliorates allergic responses in vivo.

1. Introduction

Incidences of allergic diseases such as allergic rhinitis, asthma, eczema, and allergic anaphylaxis have increased in developed countries (World Allergy Organization, 2014), and approximately 25% of the population has been reported to have an allergic disease (Galli et al., 2008). Mast cells are well known as one of the critical effector cells responsible for allergic diseases (Gilfillan and Beaven, 2011). Once mast cells are activated by binding antigen to immunoglobulin (Ig) E that adheres to the IgE high affinity receptor, FcεRI, on the mast cell membrane, mast cells secrete histamine or protease at the early stage of the allergic response. They further secrete prostaglandin, leukotriene and inflammatory cytokines in the later stage, by which allergic responses are induced (Yamaguchi et al., 1999; Locksley, 2010). Thus, IgE-mediated activation of mast cells is a significant event for various allergic diseases and studies have been under way to treat allergic diseases through the suppression of mast cells (Holgate et al., 2005; Galli and Tsai, 2012).

The signaling pathway for activating IgE-mediated mast cells begins with antigen binding to the IgE bound to the FcεRI receptors of mast cells. Immunoreceptor tyrosine-based activating motifs (ITAMs) that are present in β-and γ-subunits of FcεRIs are phosphorylated by Lyn, a Src family kinase, following which Syk binds to the phosphorylated ITAMs for optimal activation (Siraganian, 2003). The activated Syk directly or indirectly induces activation of downstream signaling molecules including the Src homology 2 domain-containing leukocyte protein of 76 kD, a linker for activated T cells (LAT), phospholipase (PL) C-γ, and Gab2 (Gilfillan and Rivera, 2009). Thereafter, the phosphatidylinositol-3-kinases (PI3K)/Akt pathway, Ca^{2+} mobilization, and mitogen-activated protein kinase (MAPK) are activated, leading to degranulation and the secretion of inflammatory cytokines, which in the end results in allergic responses (Kawakami and Galli, 2002). There are several reports that a deficiency of Syk in mast cells leads to the reduction of the activation of mast cells (Zhang et al., 1996, 2010; Siraganian et al., 2010). As such, the Src family kinases and Syk, which are pivotal signaling proteins for the activation of mast cells, were recently proposed as targets in the treatment of allergic diseases (Holgate and Polosa, 2008).

Furaltadone, 5-morpholinomethyl-3-(5-nitrofurfurylideneamino)-2-oxazolidinone, is a nitrofuran family antibiotic having a 5-nitrofuran ring (Vass et al., 2008). Nitrofuran family antibiotics have been broadly used as veterinary drugs (Draisci et al., 1997) and also for scalded skin infection (Vasheghani et al., 2008), cholera (Roychowdhury et al., 2008), bacterial diarrhea (Petri, 2005), and urinary tract infection

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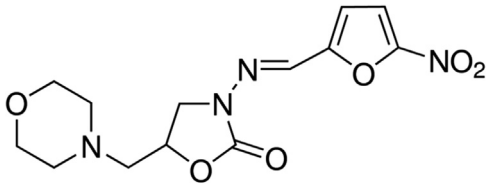

Fig. 1. The chemical structure of furaltadone.

(Guay, 2008) in humans. Although furaltadone is well known for its use as an antibiotic, there has been no study on its effect on allergic diseases. Thus, this study conducted a series of experiments at the cellular level and with animal models in order to investigate the effect of furaltadone on allergic responses. The results found that furaltadone suppressed the activation of mast cells through the inhibition of the Lyn/Syk pathway and significantly ameliorated antigen-induced allergic responses in mice.

2. Materials and methods

2.1. Reagents

Furaltadone (Fig. 1) was purchased from Selleckchem (Houston, TX). Cell culture medium was bought from GIBCO/Life Technologies, Inc. (Rockville, MD). Monoclonal dinitrophenol (DNP)-specific IgE, DNP-human serum albumin (DNP-HSA, antigen), Evans blue, cetirizine, and toluidine blue were obtained from Sigma (St. Louis, MO). 4-Amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine (PP2) was from Calbiochem (Lajolla, CA). Antibodies for phosphorylated proteins including Syk, LAT, Akt, Erk1/2, p38, JNK, and actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Syk and LAT antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Animals

Five to six week-old male BALB/c mice were obtained from Orient Bio, Inc. (Gyeonggi-do, Korea) and used for the preparation of bone marrow-derived mast cells (BMMCs) and PCA experiments. After approval by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University, animal experiments were conducted following institutional guidelines.

2.3. Preparation of mast cells and cell culture

Rat basophilic leukemia (RBL)-2H3 cells were obtained from the American Type Culture Collection, and cultured in a minimal essential medium supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15% fetal bovine serum. To prepare BMMCs, bone marrow cells were collected from the femoral region of 5 week-old male BALB/c, and cultured to differentiate into BMMCs for at least 4 weeks in Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES), 10% fetal bovine serum, and 10 ng/ml IL-3.

2.4. Measurement of β-hexosaminidase release in RBL-2H3 cells and BMMCs

RBL-2H3 cells were dispensed into 24 well-plates (1.8×10^5 cells/well), followed by sensitization with 20 ng/ml DNP-specific IgE for 12 h and 2 rounds of washing with 1,4-piperazinediethanesulfonic acid (PIPES) buffer (25 mM PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from a bovine serum). Thereafter, furaltadone was diluted in PIPES buffer and PP2 was also diluted separately as a control, followed by pretreatment for 30 min. After stimulating with 25 ng/ml DNP-HSA for 15 min, the supernatant and cell lysate were subjected to reaction with 1 mM 4-nitrophenyl-N-acetyl-β-D-glucosaminide at 37 °C for 1 h, followed by quenching with 0.1 M carbonate and the measurement of optical density (OD) at 405 nm in wavelength. BMMC was suspended in Tyrode buffer (20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA) for experimentation. The degranulation of mast cells was measured by the ratio of β-hexosaminidase secreted relative to total β-hexosaminidase, which is the sum of secreted β-hexosaminidase in culture medium and the remaining β-hexosaminidase inside the cells.

2.5. Measurement of cell viability

After the culture of RBL-2H3 cells (2×10^4 cells/well) in 96 well-plates for 12 h, the cells were incubated with furaltadone for 4 h, and followed by culturing with a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) for 1 h. Absorbance was measured at 450 nm.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from RBL-2H3 cells by using Easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Seongnam-si, Korea). The extracted RNA was subjected to reverse transcription using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). PCR conditions were 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s. Primer sequences were as follows: rat TNF-α forward, 5′-CACCA CGCTCTTCTGTCTACTGAAC-3′; rat TNF-α reverse, 5′-CCGGA CTCCGTGATGT CTAAGTACT-3′; rat IL-4 forward, 5′-ACCTTGCTGTCA CCCTGTTC-3′; rat IL-4 reverse, 5′-TTGTGAGCGTGGACTCATTC-3′; rat GAPDH forward, 5′-GTGGAGTC TACTGGCGTCTTC-3′; rat GAPDH reverse, 5′-CCAAGGCTGTGGGCAAGG TCA-3′.

2.7. Measurement of cytokines by enzyme-linked immunosorbent assay (ELISA)

IgE-primed RBL-2H3 cells (5×10^5 cells/well) were stimulated with 25 ng/ml DNP-HSA for 3 h with and without furaltadone or PP2. The amount of TNF-α and IL-4 in the cultured media were determined using rat OptEIA ELISA kits, according to the manufacturer's protocol (BD Biosciences, San Jose, CA).

2.8. Western blot analysis

RBL-2H3 cells (1×10^6 cells/well) were cultured in 6 well-plates and followed by sensitization with 20 ng/ml DNP-specific IgE for 12 h. The cells were washed twice with fresh culture medium, and pretreated with furaltadone for 30 min. After stimulation with 25 ng/ml DNP-HSA for 7 min, the reaction was terminated on ice and followed by washing with cold phosphate-buffered saline (PBS) twice. Cells were lysed by adding 100 μl lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P–40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 mg/ml pepstatin, and a protease-inhibitor cocktail tablet) per well. Following centrifugation of the cell lysate at $15{,}000 \times g$ for 5 min, the supernatant was mixed with 3 × Laemmli buffer and followed by protein denaturation at 95 °C for 5 min. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. Following incubation with each specific antibody in a TBS-T (Tris-buffered saline containing 0.1% Tween 20) buffer containing 5% BSA, the membrane was incubated with horseradish peroxidase-labeled secondary antibody. Protein bands were detected with an enhanced chemiluminescence detection kit (ThermoFisher Scientific, Waltham, MA).