Inhibitory action of roxithromycin on histamine release and prostaglandin D_2 production from ß-defensin 2-stimulated mast cells

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Abstract. The long-term, low-dose therapy with the 14-membered macrolides is well known to be effective for treatment of chronic airway inflammation. Although the mode of macrolides on neutrophils, monocytes, and epithelial cells has been investigated, the effect of macrolides on mast cell function is sparsely reported on. We first examined the effect of roxithromycin (RXM) on mast cell functions activated by human ß-defensin-2 (hBD-2). In this study, histamine release, prostaglandin D_2 (PGD_2) production, and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured in the absence and presence of RXM, using rat peritoneal mast cells stimulated with hBD-2. RXM, at doses of 12.5 and 25 μg/ml, significantly inhibited the histamine release from mast cells ($p<0.05$). In addition, PGD_2 production induced by hBD-2 was significantly reduced by RXM at 6.25 ($p<0.05$) and 12.5 μg/ml ($p<0.01$). Furthermore, the hBD-2-induced increase of $[Ca^{2+}]_i$ in mast cells was inhibited by 6.25 and 12.5 μg/ml of RXM ($p<0.05$). The present findings suggest that RXM modulates mast cell activation induced by hBD-2 via a Ca^{2+} signal pathway, thereby possibly alleviating chronic airway inflammation.

Introduction

Long-term low-dose macrolide therapy was first introduced for the treatment of diffuse panbronchiolitis in the 1980s (1). In the 1990s, it was also an effective treatment for chronic rhinosinusitis (2-4). The efficacy of this macrolide therapy on chronic rhinosinusitis is believed to be due to their anti-inflammatory effects (5), but the precise mechanisms have not been clarified yet.

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Chronic rhinosinusitis is a common upper respiratory tract disease (6). Patients complain of continuous rhinorrhea, nasal obstruction, postnasal drainage, hyposmia, and sometimes facial pain and headache. The underlying pathophysiology of chronic rhinosinusitis is postulated to be controlled by a Th1- and/or 2-mediated cascades (7,8). The number of mast cells, eosinophils and IgE-positive cells were significantly increased in a variety of chronic rhinosinusitis (9,10). This inflammatory cascade is initiated by bioactive mediators such as histamines that are secreted mainly from mast cells. A variety of modulatory effects of macrolides on inflammatory cells are shown in *in vitro* studies (5), but the action of macrolides on mast cell functions is not well understood.

Human ß-defensin (hBD), an antimicrobial peptide, is predominately expressed at epithelial tissues, where it participates in the host defense by killing invading microorganisms, as well as promoting both innate and adaptive immune responses (11). Among four hBDs identified so far, hBD-2 is mainly expressed in skin as well as in the respiratory and gastrointestinal tracts (12). hBD-2 mRNA was detected in the turbinate mucosa and nasal polyps of patients with chronic sinusitis, but not in normal nasal mucosa (13,14). In addition, hBD-2 peptide was identified in both normal and inflamed maxillary sinuses where it was secreted from the maxillary sinus epithelia (15). A line of studies published by our research groups propose that hBDs modulate the inflammatory responses by recruiting mast cells to inflammation foci and inducing the degranulation as well as prostaglandin production from this cell population (16-19). However, there is no known report on the effect of macrolides on mast cell functions mediated by epithelial cell-derived hBD-2. A novel mechanism of macrolide revealed by the present study may contribute to a better understanding of the clinical effectiveness of long-term low-dose macrolide therapy on inflammatory airway disorders.

Materials and methods

Reagents. Roxithromycin (erythromycin 9-(-O-[2-methoxy-ethoxy] methyloxime; RXM) was purchased from Sigma Chemical Co. (St. Louis, MO). The agent was dissolved in dimethylsulfoxide (DMSO) at a concentration of 200 mg/ml, and then diluted with Tyrode's buffer (26 mM NaCl, 1 mM

KCl, 2 mM HEPES, 5.6 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% bovine serum albumin, pH 7.4). Final concentration of DMSO was less than 0.3% (20). hBD-2 was purchased from Peptide Institute, Inc. (Osaka, Japan). Histamine enzyme immunoassay (EIA) kit was purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Prostaglandin (PG) D_2-MOX EIA kit was obtained from Cayman Chemical Company (Ann Arbor, MI).

Preparation of mast cells. Mast cells were obtained from male Sprague-Dawley rats weighing 400-500 g by lavage of peritoneal cavity with 50 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 0.1% bovine serum albumin. After centrifugation, the cells were suspended in 10 ml of modified Eagle's minimal essential medium (MEM; Sigma) with 10% fetal calf serum (FCS), and layered on 15 ml of 75% Percoll solution (Amersham, Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 600 g for 25 min at room temperature, mast cells were resuspended in MEM with 1% bovine serum albumin, and washed once with Tyrode's buffer without Ca^{2+} and Mg^{2+}. Toluidine blue staining was used as purity criterion (90-99%) (16-19).

Measurement of histamine release. Mast cells at $2x10^5$ cells/ml were preincubated at 37˚C for 5 min in the absence or presence of RXM (6.25-25 μg/ml), and then stimulated with hBD-2 at 37˚C for 5 min in a volume of 100 μl Tyrode's buffer. The reaction was terminated by placing test tubes in an ice-cold water bath, followed by centrifugation at 200 g for 10 min at 4˚C. Histamine content in the supernatants was measured by histamine EIA kit according to the manufacturer's instruction. Total cellular histamine was determined using the cell samples that were incubated without hBD-2 at 37˚C for 5 min and then sonicated for 30 sec. Histamine release was expressed as the percentage of total cellular histamine.

Measurement of PGD_2. Mast cells at a concentration of $5x10^5$ cell/ml were preincubated at 37˚C for 5 min in the absence or presence of RXM (6.25-12.5 μg/ml), and then stimulated with hBD-2 at 37˚C for 2 h in a volume of 100 μl Tyrode's buffer. The reaction was terminated by placing test tubes in an ice-cold water bath, followed by centrifugation at 200 g for 10 min at 4˚C. The supernatants were used for PGD_2 quantification by an EIA kit, according to the manufacturer's instructions.

Measurement of intracellular Ca^{2+} mobilization. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by using a fluorometric imaging plate reader (FLIPR) and a Calcium 3 assay kit according to the manufacturer's instructions (Molecular Devices, Sunnyvale, CA). Briefly, 100 μl of mast cell ($2x10^6$ cells/ml) suspensions were applied to each well of Biocoat poly-D-lysine 96-well black/clear plate (Becton Dickinson, Meylan Cedex, France), and preincubated at 37˚C for 2 h in the absence or presence of RXM (6.25-12.5 μg/ml). Dye solution (100 μl) containing a chelating agent for external Ca^{2+} was added into each well and incubated at 37˚C for 1 h. Subsequently, the plates were inserted into a FLIPR, and the fluorescence (relative fluorescence unit: RFU) was determined before and after the addition of 50 μl of hBD-2 at the indicated concentrations.

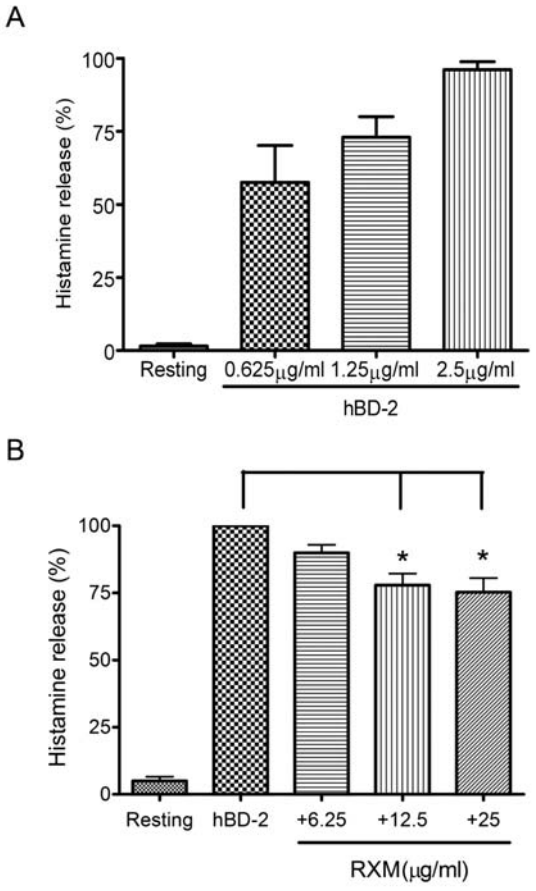

Figure 1. Effect of RXM on histamine release from hBD-2-stimulated mast cells. (A) Mast cells were incubated for at 37˚C for 5 min without (Resting) or with hBD-2 (0.625-2.5 μg/ml). Histamine release is expressed as a percentage of total cellular histamine. Each bar represents the mean ± SE of 4 separate experiments. (B) Mast cells were preincubated at 37˚C for 5 min in the absence or presence of RXM (6.25-25 μg/ml), and then stimulated by 1.25 μg/ml of hBD-2 at 37˚C for 5 min. Mast cells were also preincubated in the absence of RXM and further incubated without hBD-2 (Resting). Histamine release is expressed as a percentage of mast cells stimulated in the absence of RXM. Each bar represents the mean ± SE of 3 separate experiments. *$p<0.05$.

Statistical analysis. Data are expressed as mean ± SE, and analyzed for significant difference by a one-way ANOVA with multiple comparison test (StatView®, Abacus concept, Berkely, CA). Differences were considered statistically significant at $p<0.05$.

Results

Evaluation of histamine-releasing activities of hBD-2. To evaluate the histamine-releasing activities of hBD-2, mast cells were incubated with various concentrations of hBD-2. At resting conditions without hBD-2, little response of histamine release was observed (Fig. 1A). hBD-2 induced the histamine release from cultured mast cells in a dose-dependent manner. Since hBD-2 at 1.25 μg/ml increased histamine release ~70%, we performed the following studies using 1.25 μg/ml hBD-2,

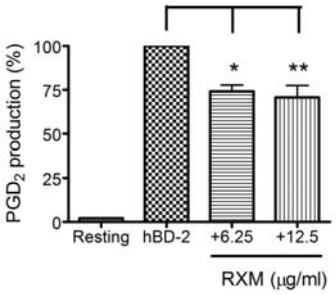

Figure 2. Effect of RXM on PGD_2 production from hBD-2-stimulated mast cells. Mast cells were preincubated at 37˚C for 5 min in the absence or presence of RXM (6.25-12.5 μg/ml), and then stimulated by 1.25 μg/ml of hBD-2 at 37˚C for 2 h. Mast cells were also preincubated in the absence of RXM and further incubated without hBD-2 (Resting). PGD_2 production is expressed as a percentage of mast cells stimulated in the absence of RXM. Each bar represents the mean ± SE of 3 separate experiments. $^{*}p<0.05$; $^{**}p<0.01$.

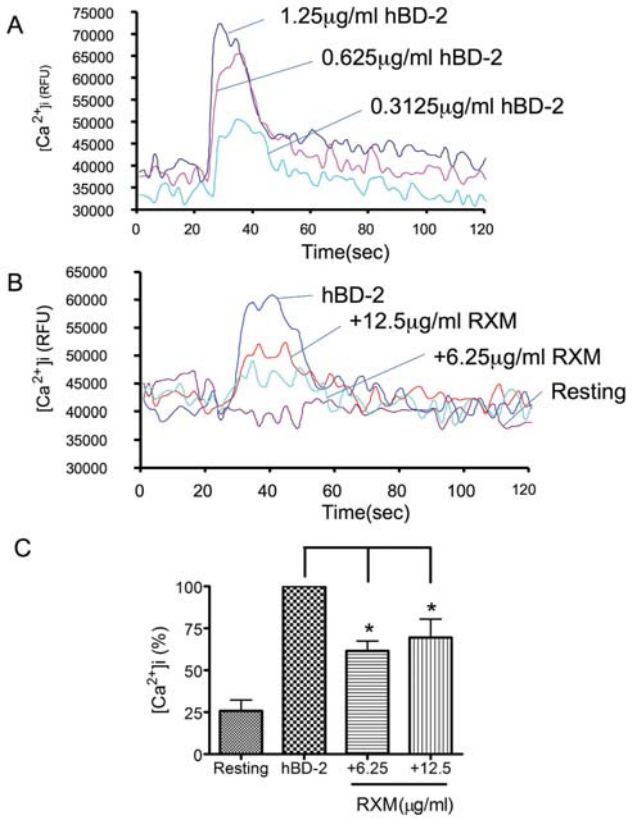

Figure 3. Effect of RXM on hBD-2-induced $[Ca^{2+}]_i$. (A) Mast cells were preincubated at 37˚C for 2 h without RXM and then incubated with Ca^{2+} fluorescence dye at 37˚C for 1 h. The cells were stimulated with various concentrations of hBD-2 (0.3125-1.25 μg/ml) in the external medium chelating Ca^{2+}. (B) Mast cells were preincubated at 37˚C for 2 h in the absence or presence of RXM (6.25-12.5 μg/ml), and then incubated with Ca^{2+} fluorescence dye at 37˚C for 1 h. The cells were stimulated without (Resting) or with 0.3125 μg/ml of hBD-2. (C) The changes in $[Ca^{2+}]_i$ are expressed as a percentage of that in mast cells stimulated in the absence of RXM. Each bar represents the mean ± SE of 4 separate experiments. $^{*}p<0.05$.

which is considered the most effective dose to evaluate the effect of macrolides.

Effect of RXM on histamine release from hBD-2-stimulated mast cells. We next examined the effect of RXM on histamine release from hBD-2-stimulated mast cells. Mast cells were stimulated by hBD-2 in the presence of various concentrations of RXM. The histamine release from mast cells induced by 1.25 μg/ml hBD-2 was significantly inhibited by the addition of 12.5 and 25 μg/ml RXM ($p<0.05$), but not by 6.25 μg/ml (Fig. 1B).

Effect of RXM on PGD_2 production in hBD-2-stimulated mast cells. PGD_2 is known as one of the major biological mediators released from mast cells. We examined the actions of RXM on PGD_2 production of mast cells stimulated by 1.25 μg/ml of hBD-2. hBD-2-induced PGD_2 production from mast cells was suppressed by RXM at 6.25 μg/ml ($p<0.05$) and 12.5 μg/ml ($p<0.01$)(Fig. 2).

Effect of RXM on hBD-2-induced $[Ca^{2+}]_i$. The final series of experiments were conducted to clarify the underlying mechanism of inhibitory effects of RXM on the histamine release and PGD_2 production from hBD-2-stimulated mast cells. Since it is well known that the intracellular Ca^{2+} signal plays a key role in the process of histamine release and PG biosynthesis of mast cells (21,22), we examined the effect of RXM on $[Ca^{2+}]_i$ in mast cells. Stimulation with hBD-2 resulted in the increase of $[Ca^{2+}]_i$ in mast cells dose-dependently from 0.3125 to 1.25 μg/ml (Fig. 3A). The addition of RXM at 6.25 and 12.5 μg/ml suppressed the elevation of $[Ca^{2+}]_i$ induced by 0.3125 μg/ml hBD-2 (Fig. 3B). Fig. 3C exhibited the average of relative changes in $[Ca^{2+}]_i$ in which RXM at 6.25 and 12.5 μg/ml significantly inhibited the hBD-2-induced increase in $[Ca^{2+}]_i$ ($p<0.05$)(Fig. 3C).

Discussion

We previously reported that hBD-2 stimulates rat peritoneal mast cells to mobilize intracellular Ca^{2+} in a G-protein-phospholipase C-dependent manner, resulting in histamine release or PGD_2 production (16,17). We first evaluated the effects of RXM on the biological activities of mast cells stimulated by hBD-2. All of the responses such as histamine release, PGD_2 production, and increased $[Ca^{2+}]_i$ by hBD-2-stimulated mast cells were inhibited by RXM. A few experiments reported on the effect of macrolides on mast cell function. Clarithromycin was demonstrated to induce cell death in mast cells (23). In contrast to the present study, Shimane *et al* (24) reported that RXM (~100 μg/ml) did not inhibit histamine release from mouse mast cells stimulated by chemical substances (compound 48/80 and calcium ionophore A23187) and immunologic stimulants using mouse IgE and anti-mouse IgE. The discrepancy between these two experiments may be due to a difference in the source of mast cells, stimulants, administered dose of RXM, experimental conditions, etc.

The present study indicates that the mode of inhibitory effect of RXM on mast cell functions could be explained by the inhibition of the $[Ca^{2+}]_i$ increase. The $[Ca^{2+}]_i$ increase resulting from stimulus-response coupling of cell surface receptors is composed of two sources, an initial transient derived from

the mobilization from intracellular pool and a subsequent phase of sustained entry from extracellular milieu (25). In the present study, only Ca^{2+} release from intracellular pool was investigated because the assay medium contained a chelating agent for external Ca^{2+}. Therefore, the present study indicates that RXM can inhibit the Ca^{2+} mobilization from internal store.

The pathophysiology of chronic rhinosinusitis is likely multifactorial, namely upper respiratory tract infection, allergy, anatomical anomalies, and immunological deficiencies. Since various pathogens are highly cultured from chronic rhinosinusitis (26), it is easy to suppose that hBD-2 is secreted from sinus mucosa as evidenced by the previous reports (13-15). Mast cells are suggested to play an important role in the formation of nasal polyps and various types of chronic rhinosinusitis not only through IgE-mediated process but also non-IgE-mediated inflammatory events (27,28). hBD-2 derived from the inflamed sinus epithelia is assumed to participate in inflammation by activating mast cells to release histamine and PGD_2 (16-19). Thus, we advocate that an inhibitory effect of RXM on mast cell activation induced by hBD-2 is a novel mechanism of macrolides. In conclusion, the present findings suggest that macrolides alleviate inflammatory reactions by modulating mast cell functions.

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