Inhibition of Histamine Release from RBL-2H3 Cells by Zoledronate Did Not Affect Rab27a/Doc2a Interaction

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Mast cell (MC) exocytosis is organized by prenylated protein, including Rab families. Among Rab proteins, Rab3a, Rab27a, and Rab11 are responsible for exocytosis arrangement. Rab3a and Rab27a are contributed to exocytosis by interacting with other exocytosis proteins. Zoledronate administration disrupted the Rab prenylation process that affected its interaction with other proteins, and finally, its function. The present study has investigated the effect of zoledronate on the histamine release (HR) from RBL-2H3 cells. The main focus is to answer the question of whether zoledronate affects Rab27a/Doc2a interaction. Histamine release on RBL-2H3 cells after zoledronate or clodronate administration was measured using HPLC-fluorometry. Dinitrophenylated bovine serum albumin (DNP-BSA) (20 ng/mL) or ionomycin (1 µM) are used as secretagogues. Calcium (Ca2+) influx observation was performed using Fura-2A/M. In situ proximity ligation assay (PLA) is used to investigate Rab27a/Doc2a interaction after bisphosphonates (BPs) treatment. Histamine concentration measurement with HPLC-fluorometry showed that zoledronate (30, 100 µM) inhibited HR from antigen-activated RBL-2H3 cells. Zoledronate showed less inhibition in cells activated with ionomycin. Intracellular Ca2+ concentration and Ca2+ flux rate from the extracellular compartment was not changed by zoledronate administration. No changes in Rab27a/Doc2a interaction after zoledronate treatment. Histamine release inhibition by zoledronate in DNP-BSA-activated RBL-2H3 cells is not related to the disruption of Rab27a/Doc2a interaction and is not involve the change in Ca2+ influx.

Key words zoledronate; histamine release; mast cell; Rab27a; Doc2a; protein interaction

INTRODUCTION

Nitrogen-containing bisphosphonates (including zoledronate) are acted by inhibiting farnesyl pyrophosphate synthase3 and geranylgeranyl transferase that important for proteins prenylation.2 This action caused the impairment of localization, and finally, the function of prenylated-proteins. Zoledronate was reported to decrease membrane-to-cytoplasmic translocation, and finally, the function of prenylated-proteins. Zoledronate administration disrupted the Rab prenylation process that affected its interaction with other proteins, and finally, its function. The present study has investigated the effect of zoledronate on the histamine release (HR) from RBL-2H3 cells. The main focus is to answer the question of whether zoledronate affects Rab27a/Doc2a interaction. Histamine release on RBL-2H3 cells after zoledronate or clodronate administration was measured using HPLC-fluorometry. Dinitrophenylated bovine serum albumin (DNP-BSA) (20 ng/mL) or ionomycin (1 µM) are used as secretagogues. Calcium (Ca2+) influx observation was performed using Fura-2A/M. In situ proximity ligation assay (PLA) is used to investigate Rab27a/Doc2a interaction after bisphosphonates (BPs) treatment. Histamine concentration measurement with HPLC-fluorometry showed that zoledronate (30, 100 µM) inhibited HR from antigen-activated RBL-2H3 cells. Zoledronate showed less inhibition in cells activated with ionomycin. Intracellular Ca2+ concentration and Ca2+ flux rate from the extracellular compartment was not changed by zoledronate administration. No changes in Rab27a/Doc2a interaction after zoledronate treatment. Histamine release inhibition by zoledronate in DNP-BSA-activated RBL-2H3 cells is not related to the disruption of Rab27a/Doc2a interaction and is not involve the change in Ca2+ influx.

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MATERIALS AND METHODS

All the experimental procedures performed in this study are available in Sahid et al.6 and Liu et al.4 Brief explanations are described below.

Histamine Release Measurement Incubate 2 × 10⁵ RBL-2H3 cells/well in Eagle’s minimum essential medium (MEM) containing monoclonal immunoglobulin E (IgE) against dinitrophenylated bovine serum albumin (DNP-BSA) at 37°C with 5% CO2. Continue the incubation for 30 min or 24 h after the addition of zoledronate, clodronate (10–100 µM), or culture medium. Then, replace the medium with 200 µL PIPES buffer containing 20 ng/mL DNP-BSA and incubate for 30 min at 37°C. When ionomycin (1 µM) was used, the incubation was done for 15 min. Histamine concentration was determined by the HPLC-fluorometry.

Calcium Influx Measurement Incubate 1 × 10⁵ RBL-2H3 cells/well for 24 h in the presence of zoledronate (100 µM), clodronate (100 µM), or MEM medium. Replace the medium with Ca2+-free PIPES buffer containing Fura-2A/M (2 µM), continue the incubation for one hour. Read sample for 1100 s with an excitation wavelength of 340/380 nm and emission of 510 nm. Period 1–100 s is to measure the fluorescence intensity without secretagogues; 100–700 s is to measure the fluorescence after secretagogues addition in a Ca2+-free condition; 700–1100 s is to measure fluorescence intensity after the addition of 2 mM Ca2+. Relative fluorescence units (R.F.U.) ratio (340/380 nm) was calculated as the Ca2+ signal.

In Situ Proximity Ligation Assay (PLA) Antibodies

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against Doc2a and Rab27a (Santa Cruz Biotechnology, CA, U.S.A.) were used at 100 times diluted concentration from the manufacturer stock original concentration. Rab27a/Doc2a interaction is detected by the in situ PLA (Merck, Darmstadt, Germany). Cellular actin structures were stained with Acti-Stain 488 Phalloidin (Cytoskeleton, CO, U.S.A.) with the final concentration of 100 nM. Cells were mounted with a medium containing 4''-6-diamidino-2-phenylindole (DAPI; ThermoFisher Scientific, MA, U.S.A.). The quantification of protein interaction (represented by red dot) was analyzed using the Duolink Image Tools (Sigma-Aldrich, MO, U.S.A.).

**Statistic Analysis**

Results were depicted as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed with two-tailed assuming unequal variances t-test using Microsoft Excel data analyzer. Comparison analysis between groups was done using one-way or two-way ANOVA with Tukey’s multiple comparisons tests. 

**RESULTS**

**Zoledronate Inhibited HR**

Clodronate inhibited 23.37 ± 2% and 39.25 ± 5.45% of HR when 30 and 100 µM concentration were used, respectively (Fig. 1A). Zoledronate induced a higher magnitude of HR inhibition compared to clodronate. At 30 µM, it has 44.52 ± 2.47% of HR suppression. In ionomycin-activated cells, zoledronate HR inhibition was reduced to near 10 and 20% at 30 and 100 µM, respectively (Fig. 1B). This phenomenon was not observed with clodronate treatment. The effect of clodronate and zoledronate only appeared after a long incubation time (24h), but not a short incubation time (30 min) (Fig. 1C).

**Zoledronate Did Not Affect Ca^{2+} Mobilization**

DNP-BSA addition at 100 s did not increase the Ca^{2+}-Fura-2 fluorescence intensity (Fig. 2A). In contrast, the administration of 1 µM of ionomycin at 100 s increased the fluorescence intensity (Fig. 2C). Since the buffer used did not contain ethylenediaminetetraacetic acid (EDTA) or other chelating agents, traces of extracellular Ca^{2+} could probably enter the cells by binding to ionomycin but not with DNP-BSA. Ionomycin was reported to induce Ca^{2+} uptake from the extracellular medium into the intracellular compartment. The addition of extracellular Ca^{2+} (2 mM) at 700 s rapidly increased the fluorescence to reach its maximum intensity (Figs. 2A, C), with no remarkable difference in the ratio of fluorescence unit (R.F.U. 340/380) between each group in antigen- and ionomycin-activated cells. Because the fluorescence intensity of Ca^{2+}-Fura-2 complex was affected by the Ca^{2+} that entering the cells and the Ca^{2+} released from the intracellular store, we also calculated the influx rate between secretagogue-control, clodronate- or zoledronate-treated group was at a comparable level both in cells activated with DNP-BSA or ionomycin (Figs. 2B, D).
Zoledronate Did Not Affect Rab27a/Doc2a Interaction

Low Rab27a/Doc2a interaction was observed in the group without DNP-stimulation (Fig. 3A). Rab27a/Doc2a interaction was significantly increased in the group with DNP stimulation (without bisphosphonate administration) as marked by the increase of red spot number per cell. The number of red spots in DNP-activated cells treated with zoledronate is at a comparable level with those in the DNP-activated group without zoledronate administration. Quantification of red spot number per cell in antigen-activated cells showed that zoledronate and clodronate administration did not significantly change the number of red spots compare to the group without secretagogue-stimulation (Fig. 3B).

DISCUSSION

In the design of the experiment, clodronate was applied as a negative control for zoledronate. Clodronate is metabolized into non-hydrolyzable ATP analog and does not interrupt protein prenylation. Thus, we postulated that clodronate would not inhibit HR and have no effect on Rab27a/Doc2a interaction. Unexpectedly, clodronate was shown to inhibit HR. Even though both HR inhibition by zoledronate and clodronate is independent of Ca\(^{2+}\) mobilization, the HR inhibition by zoledronate and clodronate might involve different machinery. These unexpected results are not the focus of our investigation in this study; hence no further investigation is made.

Simvastatin was previously shown to interfere Rab27a/Doc2a interaction. Zoledronate and simvastatin inhibition of HR are not affecting Ca\(^{2+}\) mobilization and could be recovered by geranylgeraniol administration. Zoledronate and simvastatin only inhibited histamine release after prolonged incubation (24 h). This could be understood as both drugs interrupt the biochemical process that produces protein prenylation precursors. Thus, the effect of both drugs on HR will obviously appear when there is an accumulation of unprenylated protein (defect in normal function). The more incubation time, the more accumulation of unprenylated protein could be expected. Most of Rab prenylation are mediated by the addition of geranylgeranyl moiety to the protein structure by geranylgeranyltransferase II, which is the target enzyme of zoledronate. These facts lead us to postulate that zoledronate might inhibit HR from MCs by disturbing Rab27a/Doc2a interaction. Clodronate is expected to have no interference in Rab27a/Doc2a interaction. In situ PLA results showed that both of zoledronate and clodronate are failed to disturb the Rab27a/Doc2a interaction in DNP-BSA-activated cells. Clodronate failure to disturb Rab27a/Doc2a interaction is appropriate with its biochemical mechanism of action. The failure of zoledronate was unexpected, and we were not anticipating this. Zoledronate treatment is expected to affect global protein prenylation, and thus, disturbing Rab27a/Doc2a interaction. The rationalization of this finding might be related to the variation in the expression level, prenylation rate, and function of Rab protein. In general, Rab proteins have three main functions, i.e., controlling endocytosis, controlling secretion/exocytosis, and manage traffic to, in, and from the Golgi complex. Rabs protein can be divided into three groups.
based on their involvement in exocytosis mediated by different secretagogues, *i.e.*, Rabs that mediated antigen stimulation; Rabs that mediated Ca\(^{2+}\)-ionophore and phorbol ester stimulation; Rabs that mediated both stimulations.\(^5\) Rab proteins have different expression levels\(^6\) and prenylation rates.\(^9\) Rab27a is among Rab proteins that responsible for MC exocytosis. This protein has a low expression level\(^8\) and prenylation rate.\(^9\) We speculate that low prenylation rate protein, *i.e.*, Rab27a, is less affected by zoledronate administration. This speculation appeared contradictory to our previous study using simvastatin.\(^6\) Rab27a function should also be less affected by simvastatin.\(^6\)

Rab27a is important in the fusion of granules with the plasma membrane. Granules should be transported to the near plasma membrane area from the inner cytosol. Many other Rab proteins are involved in the regulation of granule trafficking. As the simvastatin inhibition site is more upstream in the mevalonate pathway than that of zoledronate (see graphical abstract), simvastatin is expected to interrupt the function of more Rab proteins.

Simvastatin- or zoledronate-treated MC co-administered with mevalonolactone, geranylgeraniol, or farnesol shows different HR recovery patterns.\(^4,6\) The broader effects of simvastatin (HR can only be recovered by the co-administration of mevalonolactone) highlight the possibility of wide interruption on granule traffic and fusion. In contrast, the more specific effect of zoledronate highlights that lesser proteins are affected. Broader effect of statin on protein prenylation is supported by the study using zoledronate and fluvasatin. In this study, the ratio of unprenylated-to-prenylated Ras protein is stable after zoledronate treatment, but increased after fluvasatin administration.\(^20\) The disturbance of Rab27a/Doc2a interaction could result from the interruption of Rab27a prenylation or failure of granule trafficking to the plasma membrane area. Thus, the less effect of zoledronate on low prenylation rate proteins is needed for further detailed investigation.

The non-global effect of zoledronate on protein prenylation was also reported previously.\(^21\) Zoledronate decreased membrane-to-cyttoplasmic fractions ratio of RhoA, Cdc42, and Rab6, but less significant in Rac1.\(^2\) RhoA, Cdc42, and Rac1 belong to the Rho protein family, which is prenylated by the addition of farnesyl pyrophosphate or geranylgeranyl pyrophosphate by farnesyltransferase and geranylgeranyltransferase I, respectively.\(^23\) These Rho proteins are also important components of MC exocytosis. We could not link the effect of

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Fig. 3. Detection of Rab27a/Doc2a Interaction in DNP-BSA (20 ng/mL)-Activated RBL-2H3 Cells with in Situ Proximity Ligation Assay (PLA)

Red spots indicated the interaction between Rab27a and Doc2a (A). Quantification of PLA positive spots in cells (B). Error bars in each group represent S.E.M. of number of red spot/cell, *n* = 1. *p* < 0.05 significant t-test results versus control medium (without DNP-BSA administration) group. White lines showed the representative red spots in each cell (red spots are above the white lines). (Color figure can be accessed in the online version.)
zoledronate to RhoA, Rac1, and Cdc42 to Rab27a or other Rab proteins, as there is no information available about the expression level and prenylation rate of these proteins.

Even though the hierarchy of protein prenylation was reported, there are no/limited studies available that explain the effect of zoledronate to the degree of particular protein prenylation, especially for Rab proteins. Thus, we only focus on Rab27a/Doc2a interaction that previously reported. Future study about Rab protein prenylation hierarchy is crucial to elaborate the mechanism/machinery involved and its correlation with zoledronate effect on exocytosis process.

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Conflict of Interest The authors declare no conflict of interest.

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