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SHORT COMMUNICATION

Promotion of microtubule acetylation plays an important role in degranulation of antigen-activated mast cells

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Abstract

Objective The aim of this study was to investigate whether microtubule acetylation is triggered by antigen stimulation and how it affects mast cell degranulation.

Methods The RBL-2H3 cell line was used as a model for mast cells. Acetylation of α-tubulin was analyzed by Western blotting. Intracellular distribution of α-tubulin and acetylated α-tubulin was observed by immunostaining. Degranulation was monitored by measuring the activity of β-hexosaminidase secreted into cell supernatants. Tukey–Kramer test was used to compare differences between groups.

Results Microtubule acetylation proceeds globally in mast cell cytoplasm after antigen stimulation in addition to accelerated formation of microtubule-organizing centers. Pretreatment with 5Z-7-oxozeaenol (5 μmol/l), an inhibitor of TGF-β-activated kinase 1, which is a key activator of α-tubulin acetyltransferase 1, did not affect the distribution and acetylation of microtubules in resting cells; however, it significantly suppressed antigen-evoked microtubule acetylation and their reorganization, and subsequent degranulation ($95.0 \pm 1.2\%$ inhibition, $n = 3$, $P < 0.01$).

Conclusions These results provided new insight into the post-translational modifications of microtubule to regulate mast cell degranulation.

Keywords Mast cell · Degranulation · Microtubule acetylation · Microtubule-organizing center

Introduction

Within minutes of the crosslinking of IgE bound to high-affinity IgE receptors (FcεRI) by multivalent antigen, allergic mediators such as histamine and serotonin pre-stored in intracellular granules are secreted from mast cells [1]. The movement of secretory granules toward the plasma membrane where they fuse to release allergic mediators is dependent on the microtubule tract [2]. Microtubules are key cytoskeletal elements that dynamically assemble from heterodimers of α- and β-tubulin. Tubulins are subject to various post-translational modifications (PTMs) such as acetylation, which affect microtubule dynamics, their organization, and interaction with motor proteins [3]. For instance, Lys40 of α-tubulin is a predominant acetylation site. The enzymes involved in this acetylation is α-tubulin acetyltransferase 1 (αTAT1), which is activated by TGF-β-activated kinase 1 (TAK1) [4]. Though acetylation at Lys40 of α-tubulin does not affect microtubule structure, it protects long-lived microtubules and promotes kinesin-1 binding and its transport [5]. However, it is unclear whether microtubule acetylation is triggered by antigen stimulation. We have studied the role of microtubule acetylation in mast cell degranulation.

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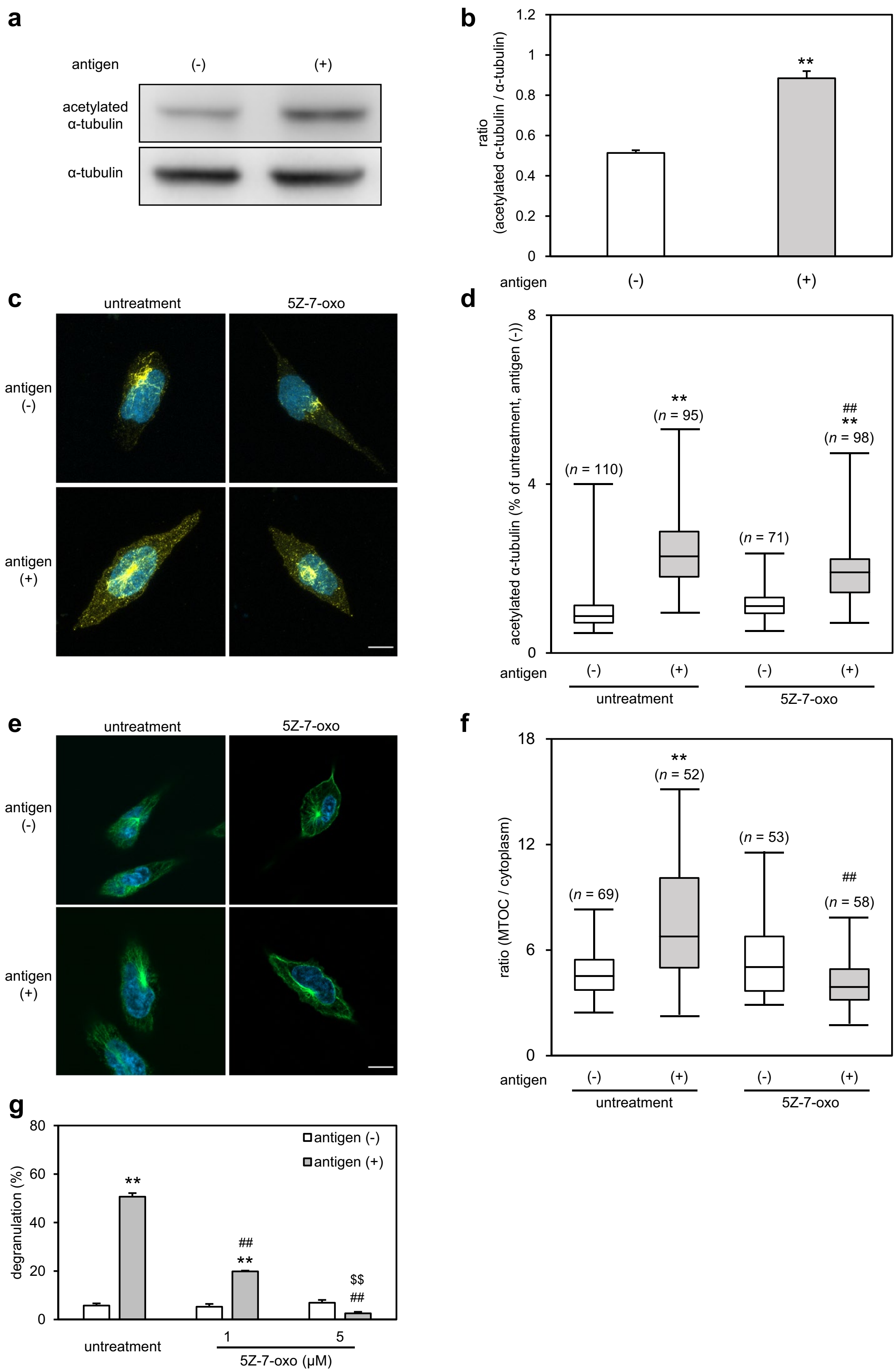
Materials and methods

Western blot analysis

RBL-2H3 cells sensitized with anti-dinitrophenyl (DNP) IgE were stimulated with DNP-conjugated bovine serum albumin (DNP-BSA; 50 ng/ml) for 10 min. The cells were fixed in 2% paraformaldehyde, harvested with a rubber policeman in cold lysis buffer [6]. Then, the lysates were maintained at room temperature for 30 min, followed by


a
antigen
(-)
(+)
acetylated α-tubulin
α-tubulin
b
ratio (acetylated α-tubulin / α-tubulin)
1.2
1
0.8
0.6
0.4
0.2
0
**
antigen
(-)
(+)
c
untreatment
5Z-7-oxo
antigen (-)
antigen (+)
d
acetylated α-tubulin (% of untreatment, antigen (-))
8
4
0
(n = 110)
**
(n = 95)
(n = 71)
##
**
(n = 98)
antigen
(-)
(+)
(-)
(+)
untreatment
5Z-7-oxo
e
untreatment
5Z-7-oxo
antigen (-)
antigen (+)
f
ratio (MTOC / cytoplasm)
18
12
6
0
(n = 69)
**
(n = 52)
(n = 53)
##
(n = 58)
antigen
(-)
(+)
(-)
(+)
untreatment
5Z-7-oxo
g
degranulation (%)
80
60
40
20
0
antigen (-)
antigen (+)
**
##
**
$$
##
untreatment
1
5
5Z-7-oxo (μM)

◂**Fig. 1** **a**–**f** Promotion of the acetylation and reorganization of microtubules following antigen stimulation in RBL-2H3 cells. **a** Western blot analysis of α-tubulin acetylation. **b** Relative optical density of acetylated α-tubulin normalized to total α-tubulin. Results are expressed as mean ± SEM from three independent experiments. **c** Z-stack images of confocal fluorescence microscopy with double staining of nuclei (blue) and acetylated α-tubulin (yellow). **d** Normalized fluorescence intensity of acetylated α-tubulin to cells without 5Z-7-oxo pretreatment nor antigen stimulation. **e** Confocal fluorescence images with double staining of nuclei (blue) and α-tubulin (green). **f** Ratio of fluorescence intensity of α-tubulin in MTOCs to that in cytoplasm. Bars = 10 μm. $^{**,\#\#}P<0.01$ vs. resting cells and 5Z-7-oxo-untreated cells, respectively. **g** The percentage of released β-hexosaminidase to the total content in cells stimulated with antigen for 30 min. Results are expressed as mean ± SEM from three independent experiments. $^{**,\#\#,\$\$}P<0.01$ vs. resting cells, 5Z-7-oxo-untreated cells, and 5Z-7-oxo-treated cells (1 μmol/l), respectively

centrifugation at 15,000×*g* for 10 min at 0 °C. The resulting supernatants were solubilized by treatment with NuPAGE LDS sample buffer (Thermo Fischer Scientific) for 3 min at 100 °C. Subsequently, proteins were separated using 10% SDS–PAGE. Further, the separated proteins were transferred to a PVDF membrane with an electroblotter. After blocking with 5% BSA in PBS with 0.1% Tween® 20, membranes were probed with mouse anti-acetylated-α-tubulin antibody (1:500) (Santa Cruz Biotechnology) or mouse anti-α-tubulin antibody (1:1000) (Sigma–Aldrich) and treated with HRP-labeled goat anti-mouse IgG antibody (1:2000) (Abcam). Lastly, immunoreactivity was detected by ECL (GE Healthcare) with a LAS-4000 (Fuji Film) and analyzed by ImageJ (NIH).

Immunostaining analysis

Immunostaining analysis was performed using previously described procedures [6]. Briefly, RBL-2H3 cells sensitized with anti-DNP IgE were stimulated with DNP-BSA (50 ng/ml) for 10 min. The cells were fixed in 2% paraformaldehyde, permeabilized in 0.3% Triton X-100, and blocked in 1% BSA. They were probed with mouse anti-acetylated-α-tubulin antibody or mouse anti-α-tubulin antibody. The cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Thermo Fischer Scientifi) and 0.1 μg/ml 4,6-diamidino 2-phenylindole (DAPI). DAPI and Alexa Fluor 488 signals were observed under a confocal laser scanning microscope (LSM-800; Carl Zeiss). The fluorescence image of α-tubulin was presented as a single optical section, whereas that of acetylated α-tubulin was presented as a z-stack image that merged optical sections of 0.4 μm intervals. Quantification of acetylated microtubule was performed by measuring the averaged fluorescence intensity inside cell periphery. The accumulation of α-tubulin in microtubule-organizing centers (MTOCs) was analyzed by modifying the procedure previously reported [7]. After the α-tubulin fluorescent signal was measured in circles with radii of 1 and 5 μm in MTOCs and cytoplasm, respectively, in a single cell, ratio of fluorescence intensity in MTOCs to that in cytoplasm was calculated.

β-Hexosaminidase assay

β-Hexosaminidase assay was performed using previously described procedures [6].

Statistical analysis

Tukey–Kramer test was used to compare differences between groups. Results were considered statistically significant at $P<0.05$.

Results

Western blotting analysis showed that the acetylated α-tubulin was significantly increased in RBL-2H3 cells stimulated with antigen (Fig. 1a, b). Although the acetylated microtubule resided only in the central regions of resting cells, acetylation was observed to be significantly promoted and extended from the central region to the periphery of cells according to antigen stimulation (Fig. 1c, d). In particular, acetylated tubulins were observed in a dot-like distribution in the cytoplasm of activated cells. The accumulation of α-tubulin in MTOCs, which was used as an index of microtubule reorganization, significantly increased after antigen stimulation (Fig. 1e, f). Whereas pretreatment with 5Z-7-oxozeaenol (5Z-7-oxo; 5 μmol/l) (Sigma–Aldrich) for 30 min [4], an inhibitor of TAK1 that is a key activator of αTAT1, did not affect intracellular distribution and acetylation levels of microtubules in resting cells, it significantly and expectedly suppressed acetylation and reorganization of microtubules triggered at 10 min after antigen addition (Fig. 1c–f). Further, pretreatment with 5Z-7-oxo significantly inhibited degranulation after antigen stimulation in a dose-dependent manner (60.8 ± 0.7 and 95.0 ± 1.2% inhibition, 1 and 5 μmol/l pretreatment, respectively, $n=3$, $P<0.01$) (Fig. 1g).

Discussion

These data are the first demonstration that microtubule acetylation occurred in mast cell activation. The inhibition of microtubule acetylation by 5Z-7-oxo induced the suppression of microtubule reorganization and degranulation following antigen stimulation, suggesting that αTAT1-mediated microtubule acetylation promoted mast cell degranulation through microtubule stabilization. The promoted acetylation probably affects the mobility of granules along the microtubule tract after antigen stimulation [5]. We here demonstrated that microtubule acetylation was promoted after antigen stimulation and played an important role in degranulation in mast cells. Because the relationship between microtubule PTMs and mast cell activation is unknown, our findings can provide novel information surrounding the molecular mechanisms regulating mast cell degranulation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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