



Trimellitic anhydride induces low-grade mast cell degranulation without specific IgE



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ABSTRACT

Objectives: Low-molecular-weight (LMW) substances are known to be causative agents of occupational asthma (OA) and occupational rhinitis (OR). Although most LMW substances are irritants or allergens, some can cause immediate type immunoglobulin E (IgE)-mediated allergic reactions. Trimellitic anhydride (TMA) is one such LMW substance, which is known as an immunological sensitizer. However, the exact molecular biological details of the effects of TMA remain unclear.

Methods: We measured the β -hexosaminidase release from mast cells after directly exposing the cells to various LMW substances. The tyrosine phosphorylation of whole cellular molecules and the phosphorylation of extracellular signal-regulated kinase (ERK) were assessed by immunoblot assay.

Results: Among the LMW substances tested, only TMA induced β -hexosaminidase release. However, the mast cell degranulation induced by TMA was lower than that induced by an antigen or a calcium ionophore. Moreover, the pattern of tyrosine phosphorylation of whole cellular molecules was quite different between IgE-mediated antigen stimulation and TMA exposure. The TMA effect on mast cells was independent of not only IgE but also Ca^{2+} influx. ERK phosphorylation was not detected in mast cells exposed to TMA.

Conclusions: TMA induced mild degranulation of mast cells without IgE, even though the phosphorylation of ERK was not detected. This reaction suggests that TMA affects humans even upon first exposure. Therefore, it is imperative to avoid human exposure to high concentrations of TMA. In order to stop the development of severe asthma in individuals with OR, we need to be able to identify cases of OR caused by TMA as soon as possible.

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1. Introduction

Occupational exposure is regarded as one of the environmental factors responsible for allergic rhinitis. Occupational rhinitis (OR), defined as an inflammatory disease of the nasal mucous membrane without infection, develops in the work place. However, the disease is distinguished from work-exacerbated rhinitis, which is natural

rhinitis aggravated by occupational exposure. Despite that OR is not a rare disease, its accurate diagnosis is difficult in some cases. It is very important to identify OR patients and to treat them appropriately in daily clinical cases. The major symptoms of OR are sneezing, rhinorrhea, and persistent or intermittent nasal obstruction. The cause includes substances that are significantly associated with the work place or occupational exposure, resulting in the decrease of nasal breath flow and excessive nasal secretions or rhinorrhea. There are two general classifications of OR [1,2], with the classification by Castano (i.e., immunological type and irritant-induced) currently being the main one. The immunological type is dependent on immunoglobulin E (IgE), and thus the mechanism of immune reaction is similar to that of normal allergic rhinitis. The causative agents of OR are divided into low-molecular-weight (LMW) and high-molecular-weight (HMW) substances [3]. Most HMW substances are glycoproteins of animal or plant origins. When humans are sensitized by HMW substances, an immediate IgE-mediated

Abbreviations: LMW, low molecular weight; OA, occupational asthma; OR, occupational rhinitis; IgE, immediate immunoglobulin E; TMA, trimellitic anhydride; HMW, high molecular weight; IgE, immunoglobulin E; IL, interleukin; DNP, dinitrophenylated; BSA, bovine serum albumin; PD, piecemeal degranulation.

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allergic reaction ensues, resulting in OR. Similarly, some LMW substances can cause immediate IgE-mediated allergic reactions, but most work as irritants. It is postulated that irritants have either an irritation effect to cause neurogenic inflammation or a corrosive effect to inflict direct damage to airway epithelial cells; however, details of the exact mechanism remain unclear.

Mast cells play important roles in the development of allergic disease and the inflammatory process and in protection against gram-negative bacteria. A previous study using an animal model revealed that interleukin (IL)-33 and FcεRI play essential roles in the development of rhinitis [4]. The RBL-2H3 cell line is a mast cell line that was cloned, by the limited dilution technique, from leukemia cells isolated from rats that had been treated with a chemical carcinogen. RBL-2H3 cells have been used for studying IgE–FcεRI interactions [5] and signaling pathways for degranulation [6], as well as to test novel mast cell stabilizers [7].

The activation of mast cells is known to induce eosinophilic inflammation in the tissue. In this study, we sought to investigate the mechanism by which mast cells are affected after direct exposure to LMW substances. To this end, the β-hexosaminidase release assay was used to test several LMW substances. Upon the finding that trimellitic anhydride (TMA) was the only LMW substance that could cause β-hexosaminidase release, we studied this substance in further detail. By excluding Ca²⁺ from the buffer, we determined whether Ca²⁺ mobilization was essential for the TMA-induced phenomenon. In addition, we investigated the effect of TMA on mast cell signal transduction by immunoblot analysis.

2. Material and methods

Cell culture and low-molecular-weight substances. RBL-2H3 cells were maintained in Dulbecco's modified Eagle's medium containing 100 U/mL of penicillin and 10% heat-inactivated fetal calf serum. Diphenylmethane diisocyanate, toluene diisocyanate, hexamethylene diisocyanate, pyromellitic dianhydride, and TMA (Tokyo Chemical Industry, Tokyo, Japan) were used as commercial preparations dissolved in ethanol. Lithium chloride, cadmium chloride, nickel chloride hexahydrate, chromic chloride hexahydrate, cobalt chloride hexahydrate (Nacalai, Kyoto, Japan), ammonium persulfate, and ammonium thioglycolate (Sigma, St. Louis, MO, USA) were dissolved in water. These were each adjusted to 5 or 10 mM before adding to the medium with cells.

Analysis of β-hexosaminidase release. Antigen-induced degranulation was determined by the measurement of β-hexosaminidase release. RBL-2H3 cells (10⁵) were seeded in 24-well plates and cultured overnight with anti-2,4-dinitrophenylated IgE mAb (anti-DNP IgE, clone SPE-7; Sigma). The cells were washed once with Tyrode-Hepes buffer (10 mM Hepes, pH 7.4, 127 mM NaCl, 4 mM KCl, 0.5 mM KH₂PO₄, 1 mM CaCl₂, 0.6 mM MgCl₂, 10 mM LiCl₂, 5.6 mM glucose, and 0.1% bovine serum albumin (BSA)) and then stimulated with 1–1000 ng/mL of the antigen DNP-BSA (LSL, Tokyo, Japan). The cells were also stimulated with 0.25–2 μM of the Ca²⁺ ionophore A23187 (Sigma) or 5–10 mM of LMW substances in the absence of anti-DNP IgE in the same buffer. After incubation for 1 h at 37 °C, the medium was recovered and reacted with the substrate p-nitrophenyl-N-acetyl-β-D-glucopyranoside (Nacalai) in 0.1 M sodium citrate buffer (pH 4.5) for 30 min at 37 °C. The reaction was terminated by the addition of 0.2 M glycine buffer (pH 10.7). The release of the product 4-P-nitrophenol was monitored by measuring its absorbance at 405 nm, using a microplate reader (Spectra Max 250; Molecular Devices, Sunnyvale, CA, USA). The released β-hexosaminidase activities were expressed as a percentage of maximal release induced by 1% NP-40 in Tyrode-HEPES buffer.

Table 1

Known causative low-molecular-weight substances of occupational rhinitis used in this study. A), B), C) are used in polyurethane resin factories. D), E) are used in epoxy resin factories. F), G), H), I), J) are metals used in electrical device factories. K), L) are used as hair dye.

A) Diphenylmethane diisocyanate	G) Cadmium chloride (II)
B) Toluene diisocyanate	H) Nickel chloride hexahydrate (II)
C) Hexamethylene diisocyanate	I) Chromic chloride hexahydrate (III)
D) Pyromellitic dianhydride	J) Cobalt chloride hexahydrate (II)
E) Trimellitic anhydride	K) Ammonium persulfate
F) Lithium chloride (II)	L) Ammonium thioglycolate

Immunoblotting. RBL-2H3 cells were seeded into a dish and cultured overnight with anti-DNP IgE mAb. The cells were washed twice with Tyrode-Hepes buffer and activated by 30 ng/mL of the DNP-BSA antigen or 10 mM of TMA. Cells were washed twice with ice-cold PBS and then solubilized in lysis buffer containing 1% NP40 (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 100 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin) on ice. Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electronically transferred to a polyvinylidene difluoride transfer membrane (Merck Millipore, Billerica, MA, USA). After blocking with 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20), the blots on the membrane were reacted with the indicated primary antibodies, followed by horseradish peroxidase-conjugated second antibodies (Bio-Rad, Hercules, CA, USA) in TBST. Proteins were visualized by the enhanced chemiluminescence reagent (Western Lightning, PerkinElmer, Winter Street Waltham, MA, USA). Anti-phospho-extracellular signal-regulated kinase (ERK) (Thr202/Tyr402) and anti-ERK antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell viability assays. RBL-2H3 cells were trypsinized after 1 h exposure to TMA or vehicle (ethanol), counted by trypan blue dye exclusion assay. The percentage of dead cells in living cells was shown as a result. All experiments were conducted independently in triplicate.

3. Results

Exposure to TMA induced mast cell degranulation. At first, we examined the effects of several LMW substances on mast cells. The substances we used in this study, all known causative agents of OR, are shown in Table 1. The β-hexosaminidase release was not observed with the same volume of vehicle (ethanol) that was used as controls (Fig. 1). Although the degranulation was induced in DNP-sensitized RBL-2H3 cells by DNP-BSA stimulation, we didn't find the effect of DNP-BSA in the absence of anti-DNP IgE on mast cells degranulation. Moreover, we also didn't find the effect of anti-DNP IgE in the absence of DNP-BSA on degranulation. The suspension of hexamethylene diisocyanate (C) was too turbid to allow measurement of β-hexosaminidase release. We found that among the LMW substances tested, only TMA induced β-hexosaminidase release. The β-hexosaminidase release was more obvious with 10 mM TMA than with the 5 mM concentration. This effect became smaller when 20 mM was used, and the phenomenon was not evident at 100 mM. We concluded that the effect of TMA was not dose dependent and used 10 mM as the optimal concentration in the subsequent experiments. On the other hand, pyromellitic dianhydride could not induce degranulation of mast cells despite that it is also an anhydride like TMA. However, the cells stimulated by TMA could release β-hexosaminidase without IgE. This suggested that the TMA-induced degranulation of mast cells is independent of IgE crosslinking. However, the degranulation induced by TMA was lower than that induced by the antigen or calcium ionophore.

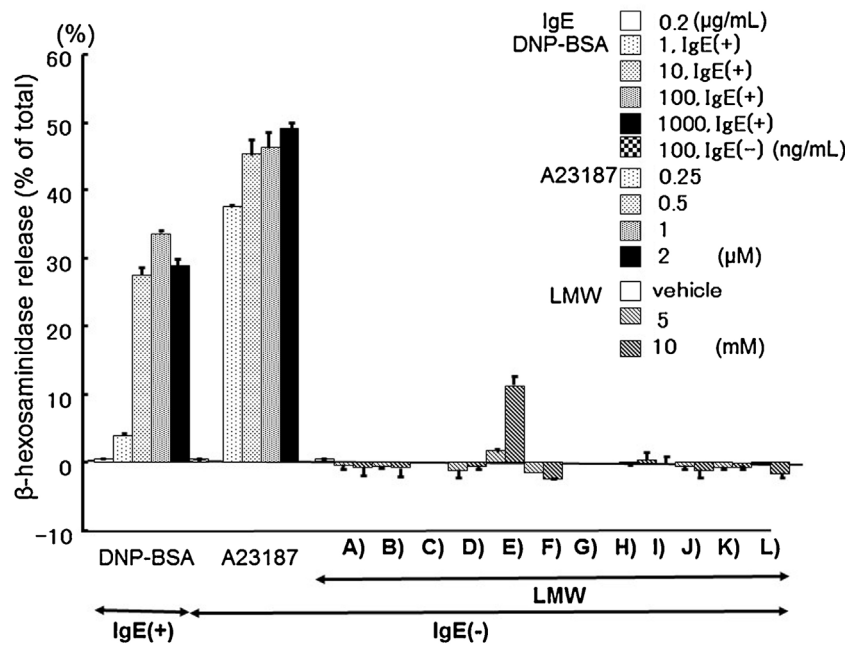


Fig. 1. Analysis of mast cell degranulation induced by low-molecular-weight substances. Results are the mean values \pm SD from 3 independent experiments.

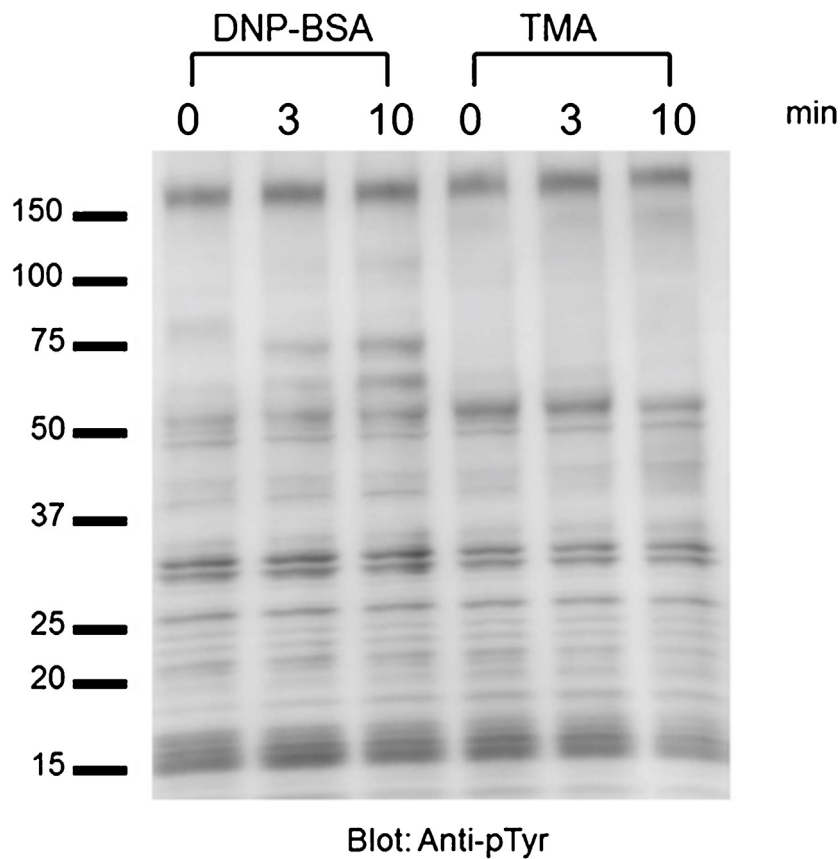


Fig. 2. Immunoblotting analysis of the effect of trimellitic anhydride (TMA) on mast cells. The phosphorylation of whole cellular molecules by antigen or TMA. Results are representative of 3 independent experiments.

TMA did not cause tyrosine phosphorylation of mast cell proteins. Tyrosine phosphorylation of various adaptor proteins induces degranulation after aggregation of Fc ϵ R1. Therefore, we tested the tyrosine phosphorylation of whole cellular molecules in RBL-2H3 cells. After 3 or 10 min of 10 mM TMA exposure, RBL-2H3 cells

were solubilized in the lysis buffer. Antigen stimulation resulted in the phosphorylation of some molecules. However, unlike antigen stimulation, TMA did not induce the tyrosine phosphorylation of whole cellular molecules (Fig. 2). This result confirmed that

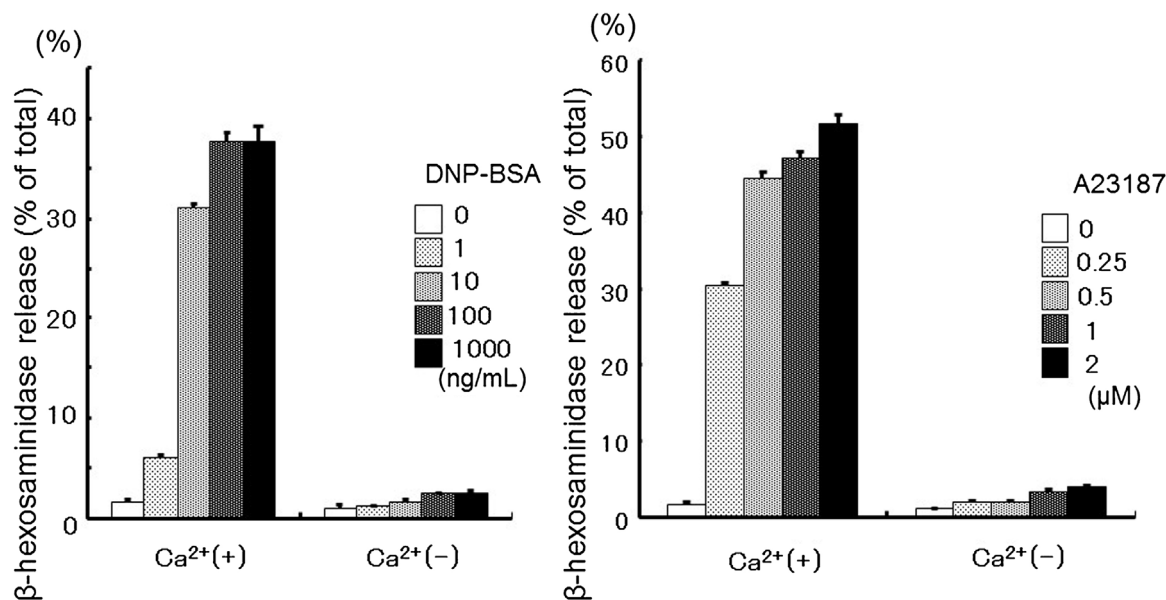


Fig. 3. Suppression of RBL-2H3 cell degranulation in the presence or absence of Ca^{2+} . Degranulation by antigen or calcium ionophore in the presence or absence of Ca^{2+} . These experiments were conducted in the absence of anti-DNP IgE. Results are the mean values \pm SD from 3 independent experiments.

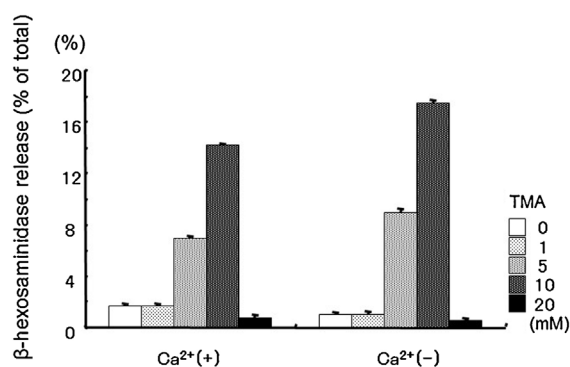


Fig. 4. Degranulation of mast cells by trimellitic anhydride in the presence or absence of Ca^{2+} . Results are the mean values \pm SD from 3 independent experiments.

the TMA stimulation induce degranulation by different mechanism from IgE-mediated degranulation.

TMA worked independently of calcium mobilization. The influx of Ca^{2+} is critical for IgE-mediated mast cell degranulation. However, because TMA induced degranulation without IgE, this suggested that TMA could induce degranulation without Ca^{2+} influx. Thus, we investigated whether or not this TMA-induced phenomenon required Ca^{2+} . To this end, we tested the effect of the absence or presence of Ca^{2+} in Tyrode-Hepes buffer on RBL-2H3 cells degranulation. Antigen-induced or A23187-induced degranulation was abrogated in the absence of Ca^{2+} (Fig. 3). In contrast, TMA elicited degranulation regardless of the absence of Ca^{2+} (Fig. 4).

The phosphorylation of extracellular signal-regulated kinases was not detected in mast cells exposed to TMA. Additionally, we tested other effects of TMA on the RBL-2H3 cells. We could not find the phosphorylation of ERK of RBL-2H3 cells exposed to TMA (Fig. 5), whereas antigen stimulation resulted in ERK phosphorylation. The effect of TMA was expressed even after 3 min of its exposure to the cells.

TMA exposure didn't induce significant cell death compared to vehicle. Finally, we tested whether TMA can elicit cell death by trypan blue exclusion assay. We didn't find significant difference on the cell viability between vehicle and TMA exposure (Fig. 6). The

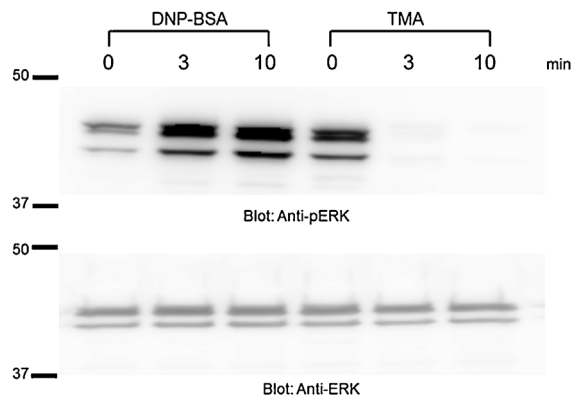


Fig. 5. Immunoblotting analysis of the effect of trimellitic anhydride (TMA) on mast cells. The phosphorylation of ERK mediated by antigen or TMA. Results are representative of 3 independent experiments.

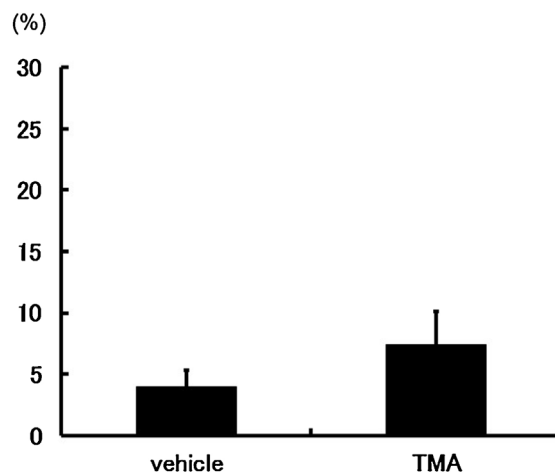


Fig. 6. Trypan blue exclusion assay. The percentage of dead cells among total cell number are shown after vehicle or 10 mM TMA exposure for 1 h. Results are the mean values \pm SD from 3 independent experiments.

impact of TMA on cell viability was same whether in the presence or absence of anti-DNP IgE.

4. Discussion

In the present study, among several LMW substances known to cause OR, we found that only TMA induced degranulation of mast cells (Fig. 1). The pattern of tyrosine phosphorylation after TMA exposure was different from that of Fc ϵ RI-mediated stimulation. The TMA-induced degranulation was independent of IgE and Ca²⁺ influx. Moreover, we could not find the immediate phosphorylation of ERK in TMA-exposed mast cells.

Some of the LMW substances cause immediate IgE-mediated allergic reactions, but most work as irritants. However, details of the exact mechanism of which remain unclear. It is important to investigate how LMW substances work as inflammation inducer for the developing of appropriate treatment. RBL-2H3 cells are useful as a model for mast cell degranulation [8]. Therefore, we investigated the impact of LMW substances on mast cell degranulation as screening.

TMA is a LMW reactive compound used in paints, in printing inks, and for the hardening of epoxy and alkyd resins and vinyl plasticizers. It is also known as a causative agent of occupational asthma (OA) and OR [9,12]. Moreover, TMA induces the early and late phase reactions in CHS, and mast cells may be required for TMA-induced CHS [10]. These symptoms are immunological in nature, as serum antibodies against TMA can be demonstrated [11]. In this way, it is regarded that TMA behaves as an allergen after sensitization and is a typical respiratory allergen. In general, HMW substances induce IgE-dependent allergic reactions after sensitization. On the other hand, LMW substances are too small to be recognized as an antigen. Therefore, TMA might work as a hapten, which is recognized as an antigen only after conjugation with albumin or other endogenous host proteins. According to the previous report, only BSA-conjugated TMA could induce the production of cytokine, whereas TMA alone could not [13]. A previous study reported that OR and conjunctivitis are developed before OA [14]. Another study revealed that although TMA was a highly reactive compound, conjugation to a suitable protein was necessary to induce chemical mediator production by alveolar macrophages [13]. This suggests that TMA works as a hapten. TMA induces the release of histamine in the nasal mucous membrane and conjunctiva mucous membrane without sensitization. Because nasal discharge includes albumin in abundance, TMA can easily conjugate with albumin and be recognized as an antigen, which then induces sensitization. We speculate that this sensitization develops OA. Adverse health effects of TMA are due to its effects as an irritant, an immunological sensitizer, or a combination of both [15]. It is considered that TMA has an immediate toxic effect on mast cells before sensitization, and behaves as an allergic antigen after sensitization.

A previous study revealed that TMA elicits significant airway obstruction and eosinophilia in a non-sensitized animal, and with even greater airway obstruction occurring in a sensitized animal [16]. Using animal model, it is shown that TMA induced typical morphological changes of the inflamed skin, strong infiltration with T cells, major histocompatibility complex positive cells, eosinophils, and mast cells, a T-helper cell-type 2 cytokine profile and strong increase of serum IgE levels [17]. The exact mechanism by which eosinophilic inflammation is induced by TMA remains unclear. Mast cells are known to have an important role in inducing eosinophilic inflammation, and they constitute a positive feedback loop. Our data revealed that TMA induced the degranulation of mast cells directly without TMA specific IgE. The pattern of tyrosine phosphorylation stimulated by TMA was different from that by Fc ϵ RI, and there was no change of tyrosine phosphorylation of

whole cellular protein in cells stimulated by TMA (Fig. 2). Another study also revealed no change in activity of the protein tyrosine kinase Lyn in guinea pig airway tissue after TMA exposure [18]. Mercuric chloride also has effects similar to TMA, in that it has been shown to induce mast cell degranulation independently of Fc ϵ RI aggregation and failed to induce any detectable changes in protein phosphorylation Dasty et al., 1999. It suggests that this reaction could be responsible, at least in part, for the formation of eosinophilic inflammation.

Classically, antigen-specific IgE is essential for anaphylactic degranulation. The antibodies against TMA will then bind to the high-affinity IgE receptors (Fc ϵ RI) on mast cells [19–21] and exposure of TMA causes cross-linking of the IgE receptors with subsequent release of chemical mediators, which results in bronchoconstriction and attraction of inflammatory cells [22–24]. It is increasingly recognized that besides IgE, mast cells can also be activated by innate receptors such as Fc-receptors, Toll-like receptors, tumor necrosis factor receptors, NOD-like receptors, and even physical stimuli [25,30]. The mast cell degranulation by these signaling pathways usually requires Ca²⁺ influx. However, our study revealed that Ca²⁺ influx was not essential for the degranulation induced by TMA (Fig. 4). Whereas anaphylactic degranulation is characterized by the formation of large degranulation sacs within the cell as granules fuse to each other, piecemeal degranulation (PD) involves the formation of small vesicles that appear to shuttle granule contents to the plasma membrane. PD has been interpreted as a slow secretory process that affects the release of granule-stored materials in a period of hours or days [26]. Ca²⁺ influx is not necessary for PD [27]. It has been reported that phorbol 12-myristate 13-acetate and formyl-met-leu-phe can induce PD. Previous studies have reported that the bacterial toxins streptolysin O and α -toxins form pores in the plasma membrane and allow efflux of cytosol protein and ions [28,29,31]. Using the trypan blue exclusion assay, we investigated whether TMA can elicit cell death. There was no significant difference between TMA exposure and solvent on cell viability (Fig. 6). It suggests that degranulation by TMA is not induced by simple cell lysis or death. The impact of TMA on mast cells was saturated and occurred only over limited range of concentration *in vitro*. Therefore, we speculated that TMA affects RBL-2H3 cells through a receptor mediated mechanism similar to IgE crosslinking. A previous cohort study stated allowable concentration of TMA [32,33]. According to the ACGIH, threshold limited values time weighted average (TLV-TWA) is 0.0005 mg/m³ and threshold limited values short term exposure limit (TLV-STEL) is 0.002 mg/m³ [34]. Compared to these values, 10 mM is very high concentration. The impact of TMA is dose-dependent *in vivo*, although our data showed the effect of TMA on RBL-2H3 cells was not dose-dependent *in vitro*. However, TMA stimulation might have a role in mast cell degranulation, further study is needed to reveal the mechanism involved. TMA is a well known respiratory sensitizer, although the irritant mechanism remains unclear. Our findings suggest that TMA induces degranulation without TMA specific IgE. Therefore, TMA will affect human health, even if it is first exposure.

There are several studies on the effect of TMA on cells. It was reported that the complement system played an important role in TMA-induced inflammation [35]. Moreover, both sensitization and the complement system are required for TMA-induced eosinophilia [36]. The macrophage scavenger receptor is considered to be a likely cause of this TMA-induced phenomenon [13]. Direct effects of TMA on mast cell activation *in vivo* were found in previous study [10]. According to the study, mast cell-deficient mice developed only late phase reaction, while congenic normal mice showed both the early and the late phase reactions. In a previous review, it was indicated that this direct toxicity might also be important in the etiology of TMA hypersensitivity [37].

We conducted further studies to reveal other TMA effects on the mast cells. The activated mast cells not only released pre-made histamine but also synthesized inflammatory cytokines and chemokines. MAP kinase has an important role in synthesizing these chemical mediators. Intracellular signal transduction, including the phosphorylation of ERK, is subsequently followed by NF- κ B translocation, leading to the production of cytokines and chemokines. Western blot analysis revealed the absence of ERK phosphorylation after TMA exposure (Fig. 5). The phosphorylation of p38 and of c-Jun N-terminal kinase was also not evident (data not shown).

We investigated the β -hexosaminidase release as degranulation indicator in this study and our previous studies. Previous study suggests that mast cell granule β -hexosaminidase is responsible for protection against bacterial invasion, but not involved in the allergic response [38]. We found that only TMA could induce degranulation among some LMW substances, so that this phenomenon is not a result from simple error including bacterial contamination. In the current study, we could not observe any response in RBL-2H3 cells toward the other LMW substances tested, despite that they are known causative agents of OR. RBL-2H3 cells showed high sensitivity to cadmium (Cd) transport via Zrt/Irt-related protein 8 [39]. Therefore, the cells incubated with Cd had poor cell survival due to the cytotoxicity of Cd. A previous study revealed that LMW ammonium persulfate induced degranulation without inducing cytokine secretion but was PI3 kinase independent [40]. The report concluded that the oxidizing activity induced by ammonium persulfate might be crucial in the development of OR. However, we could not detect degranulation of RBL-2H3 cells treated with ammonium persulfate. It has been reported that mechanistic pathways are unique to individual allergens, among ovalbumin and TMA and *Aspergillus* [41]. Similarly, there might be differences in effect on mast cells among the same type of LMW substances. Further study is needed to elucidate these differences.

We concluded that TMA exposure results in the immediate mild degranulation of mast cells via an IgE-independent pathway. The effect of TMA exposure was lower than Fc ϵ RI-mediated degranulation. However, TMA is also known for its airway-irritating effects in humans and, according to the modified Royal College of General Practitioners 1995 three-star system, is classified as moderate evidence for its association with OA [42]. TMA induced respiratory irritant reactions at high concentrations in rats, and these reactions could easily be distinguished from IgE-mediated allergic reactions [43]. Moreover, persistent chronic inflammation induces asthma, and persistent exposure of TMA causes sensitization and results in allergic inflammation. Symptoms of asthma and increased bronchial responsiveness may persist in spite of the avoidance of further TMA exposure [44]. Therefore, we need to find ways to identify OR patients more quickly in order to treat them and stop the development of severe asthma.

Transparency document

The <http://dx.doi.org/10.1016/j.toxrep.2016.09.001> associated with this article can be found in the online version.

Conflict of interest

Authors declares that there is no conflict of interest.

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