

Peroxynitrite Modulates Release of Inflammatory Mediators from Guinea Pig Lung Mast Cells Activated by Antigen-Antibody Reaction

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Key Words

Mast cells · Peroxynitrite · Reactive oxygen species · Antioxidants · Intracellular Ca^{2+} level · Phospholipase A_2

Abstract

Background: Peroxynitrite (ONOO^-), the product of the reaction between the superoxide anion (O_2^-) and nitric oxide (NO), is produced during inflammatory disease and may be a major cytotoxic agent. No reports are available as to whether ONOO^- generates or modulates inflammatory mediator release from activated guinea pig lung mast cells. In this study, we explored the modulatory role of intracellular ONOO^- on inflammatory mediator release (histamine and leukotrienes) from activated mast cells. **Methods:** Guinea pig lung mast cells were purified by the enzyme digestion, and by using the rough and discontinuous Percoll density gradients. Mast cells were sensitized with IgG1 (anti-ovalbumin) antibody and challenged with ovalbumin (OVA). The intracellular ROS formation was determined by following the oxidative production of 2', 7'-dichlorofluorescein diacetate (DCFH-DA), dihydrorhodamine 123 (DHR), and anti-nitrotyrosine antibody immunofluorescence. Histamine was assayed using a fluorometric analyzer, leukotrienes by radioimmunoassay, intracellular Ca^{2+} levels by confocal

scanning microscopy, and PLA_2 activity using prelabeling of [^3H]arachidonic acid. **Results:** ROS detected by DCFH-DA weakly increased in mast cells activated with OVA (1.0 g/ml), and the ROS so generated was inhibited by ebselen (50 μM). However, the ROS detected by DHR increased 3-fold under the same conditions. Peroxynitrite scavengers sL-MT, DMTU, and inhibitor FeTPPS inhibited ROS formation but the NADPH oxidase inhibitor diphenyleneiodonium (DPI) only partially inhibited this formation. Dimethyl thiourea (DMTU) and seleno-L-methionine (sL-MT) inhibited the tyrosine nitration of cytosolic proteins, the release of histamine and leukotrienes, Ca^{2+} influx, and the PLA_2 activity evoked by mast cell activation. **Conclusion:** The data obtained suggests that the ROS generated by the antigen/antibody reaction activated mast cells is ONOO^- , and that this modulates the release of inflammatory mediators via Ca^{2+} -dependent PLA_2 activity.

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Introduction

Reactive oxygen species (ROS) such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\text{OH}^\cdot$), nitric oxide (NO), and peroxynitrite (ONOO^-) are ubiquitous molecules produced by various

cell types as the results of diverse enzyme systems [1, 2]. ROS are known to modulate the activities of many kinases, phosphatases, and transcription factors [3]. Moreover, ROS has been reported to participate in the pathogenesis of various diseases, and in particular in the pathogenesis of asthma [4], ischemia/reperfusion injury [5], bronchoconstriction [6], postburn inflammatory response [7], and cardiopulmonary bypass [2].

ONOO⁻ is a potent oxidant and is formed by rapid reaction between free radical NO and O₂ [2, 8, 9], and it is more reactive than either of these precursors. This generation of ONOO⁻ results in a reduced bioavailability of NO, which diminishes its physiological functions and its strong antioxidant action on free radicals and metal-mediated processes [10]. Moreover, ONOO⁻ damages pulmonary surfactants [11] and pulmonary epithelial cells [12], and induces airway hyperresponsiveness in guinea pigs [13, 14]. It also nitrates amino acids such as cysteine, methionine, tryptophan, selenium, and hemoglobin tyrosine [8, 15–22], and the extent of this nitration is a stable biomarker of ONOO⁻. ONOO⁻ acts as a signaling molecule by triggering the release of arachidonic acid in PC12 cells [23, 24]. Moreover, endogenous and exogenous ONOO⁻ is causally linked to DNA damage, the peroxidation of lipids, antioxidant depletion [25], and calpain-like apoptosis [26]. In addition, ONOO⁻ generation has been demonstrated in many pathologic conditions including asthma [27], inflammation [28], sepsis and autoimmune disease [16], and in nasal polyps. ONOO⁻ is scavenged by dimethyl thiourea (DMTU) [29], *L*-methionine [21], or selenoproteins [30, 31].

Mast cells have been reported to be implicated in many biological responses, for example, allergy, host responses to parasites and neoplasms, acute and chronic inflammation, tissue remodeling, and wound healing [32]. The aggregation of high-affinity immunoglobulin E receptors (FcεRI) or FcγRI on mast cells and basophils activates these cells to release inflammatory mediators, cytokines, and this is followed by allergic reactions [33].

Many studies have found that various mast cells also generate intracellular ROS including H₂O₂, •O₂, OH⁻ and NO following incubation with stimuli or antigens. Moreover, the ROS so generated have been associated with the release of mediators via mast cell activation [34–37].

In contrast, the effects of NO on mast cells are complex, multifarious, and diverse in terms of adhesion, reactivity, and phenotypic heterogeneity. NO suppresses antigen-induced degeneration, mediator release, and cytokine expression [38, 39]. However, the degranulation of C1.MC/C57.1 (mouse bone marrow-derived mast

cells) and RBL-2H3 cell (rat basophilic leukemia cell) lines by NO is variable, selective and depends on phenotype [40]. NO may also contribute to the induction and the progress of the allergic reactions on the ocular surface [41].

As the above reports illustrate, many studies have focused on the effects of ROS on mast cells in association with allergic diseases in various cells and tissues. However, the generation of ROS in guinea pig lung mast cells activated by antigen-antibody reaction via FcγRI cross-linking, and the relationship between this generated ROS and inflammatory mediator release have not been reported. Therefore, in this study we investigate whether various types of ROS, including ONOO⁻, generated in the guinea pig lung mast cells are involved in the processes of mast cell degranulation and leukotriene synthesis.

Materials and Methods

Animals

Hartley albino female guinea pigs, with body weights of 200–250 g, were used in this study. The animals were maintained in accordance with the ‘Principles of Laboratory Animal Care’ issued by Association for Assessment and Laboratory Animal Care (AAALAC).

Drugs and Solutions

The following substances were used: ovalbumin (OVA), collagenase (type I), elastase (type IV, porcine pancreatic), complete Freund’s adjuvant, Fluo-3 AM, glucose, dimethyl thiourea (DMTU), seleno-*L*-methionine (sL-MT), cytochrome c, Percoll, diphenyleneiodonium, ebselen, *N*-monomethyl-*L*-arginine (NMMA), *o*-phenanthroline (Sigma Chemical, St. Louis, Mo., USA); 5,10,15,20-tetrakis-(4-sulfonatophenyl)-propylrinate iron (III) chloride (FeTPPs) (Calbiochem, La Jolla, Calif., USA); anti-nitrotyrosine (rabbit immunoaffinity-purified IgG, Upstate, Lake Placid, N.Y., USA); rabbit IgG-FITC (Molecular Probes Alexa Fluor Dyes); gelatin (Yakuri Pure Chemicals, Osaka, Japan); LTD₄ assay kit, [³H]arachidonic acid (60–100 Ci/mmol; specific activity, 2.22–3.7 Tbq/mmol) (NEN, Korea); 2′-7′-dichlorofluorescein diacetate (DCFH-DA), dihydrorhodamine (DHR) 123 (Molecular Probe, Eugene, Oreg., USA); NO kit (R & D, Minneapolis, Minn., USA). All chemicals used were of the best quality commercially available.

Active Sensitization Protocol (Anti-OVA Antibody Production)

Ten outbred female guinea pigs were first immunized by foot pad injections of a mixture of 50 µg OVA and complete Freund’s adjuvant. One week after immunization, the animals received intradermal injections of 100 µg OVA in one side of the back and 200 µg of OVA in the other side of the back. The animals were sacrificed 1 week later, and serum samples were stored in aliquots at –70°C until required. Serum antibody titers were determined by passive cutaneous anaphylaxis (PCA) as described previously [42]. Serum IgG1 antibody was separated by affinity column chromatog-

raphy. Guinea pig blood serum was applied to an anti-IgG2 affinity column, and 0.1 M citric acid (pH 2.1) was used and eluted. IgG1 was passed through and absorbed IgG2 antibody was rinsed with 0.2 M sodium carbonate (pH 11.3). The separated IgG1 was concentrated under pressure for experiments. The titers of anti-OVA were 1,600–3,200, and sera were used to prepare passively sensitized mast cells.

Guinea Pig Lung Mast Cell Preparations

Guinea pig lung mast cells were isolated and purified using a modification of a previously reported method [43]. Briefly, lungs obtained from 16 unsensitized guinea pigs (3 g/each lung) were perfused with 50 ml of modified Tyrode's buffer consisting of (millimolar): NaCl, 137; NaH₂PO₄, 0.36; KCl, 2.6; CaCl₂, 1; MgCl₂, 1.5; NaHCO₃, 25; glucose, 1 g/l; gelatin, 1 g/l, pH 7.4. After removing large airways and blood vessels, the lungs were minced with a McIlwain tissue chopper (Mickle Laboratory Engineering, Goms-hall, England). Pooled lung tissues were treated for 15, 15 and 25 min with 125 U/g tissue and 5 U/g tissue with collagenase and elastase, respectively. Freed cells were separated from the residual tissue by filtration through mesh and a Nynetex mesh (100 µm). The cells were washed with Tyrode buffer (without CaCl₂ and MgCl₂) containing gelatin (TG buffer), layered over gradients consisting of 10 ml of Percoll (density, 1.045 g/ml), and centrifuged at 1,400 rpm for 25 min. Pelleted cells (containing mast cells) were resuspended in TG buffer, and further purified using a discontinuous Percoll density gradient (consisting of densities of 1.06, 1.07, 1.08, 1.09, and 1.10 g/ml). This gradient was then centrifuged at 1,400 rpm for 25 min (~3.5 × 10⁷ cells/gradient). The cell band between densities 1.09 and 1.10 g/ml, which contained mast cells in highest purity (1–2 × 10⁷) was removed, washed with TGCM buffer, and designated the partially purified mast cell preparation. Mast cells counts were made by alcian blue staining, and cell viabilities were determined by trypan blue exclusion. Cell viabilities were consistently above 98%, and the purity range of the purified mast cells was 80–90%.

Determination of Intracellular ROS by Flow Cytometry

Purified mast cells (4 × 10⁵) were incubated with anti-OVA antibody (1 ml/10⁶ cells) at 37°C for 45 min, washed, and incubated with 2'-7'-dichlorofluorescein diacetate (DCFH-DA, 5 µM) or DHR 123 (DHF, 5 µM) for 5 min [44]. The cells were challenged with 1.0 µg/ml OVA for the indicated period of time, and then subjected to FACS. In all experiments, antioxidants (50 µM eb-selen, 50 µM diphenyleneiodonium (DPI), 10 µM FeTPPS, 10 mM DMTU, 30 µM sL-MT, or 100 µM o-phenanthroline) or NMMA (100 µM) were added 30 min before OVA challenge. The concentrations of all antioxidants were optimized by preliminary study. All experiments in this study were performed in blocks of five or more.

Mediator Release due to Mast Cell Activation

Purified mast cells (4 × 10⁵) were passively sensitized with anti-OVA antibody (1 ml/10⁶ cells) for 45 min at 37°C in a shaking water bath. After this incubation period, the cells were washed, resuspended in TGCM buffer, and challenged with 1.0 µg/ml of OVA for 10 min or 20 min. The reaction was terminated by placing the tubes in an ice bath. Supernatants obtained after centrifugation were analyzed for histamine and leukotrienes. For leukotriene determinations, 0.1% gelatin (final concentration) was added to su-

pernatants because leukotrienes decompose in air. Antioxidants were added to mast cells 30 min before OVA challenge (1.0 µg/ml) [43].

Histamine Assay

Histamine was analyzed using an automated fluorometric method with a dialyzer, as previously described [43]. The sensitivity of the assay was approximately 1–20 ng/ml of histamine, and the amount of histamine released was expressed as a percentage of the total histamine present in unstimulated cells.

Leukotriene Radioimmunoassay (RIA)

The leukotriene contents of cell supernatants were determined by RIA as described previously [45]. Leukotriene antibody was diluted in buffered saline (5 mM MES, HEPES adjusted to pH 7.4 with 1 N NaOH) containing 0.1% gelatin. Each assay tube contained 100 µl of sample supernatant, antibody (50 µl of a 1:1,000 dilution), and 50 µl of [³H]leukotriene D₄ (LTD₄, 2,500–3,000 cpm) in buffered saline. Incubation was conducted for 2 h at 4°C, and the reaction was terminated by adding 0.5 ml of dextran-coated charcoal (200 mg charcoal and 20 mg dextran-mixed with 100 ml buffered saline). Five min later, the mixture was centrifuged at 800 g at 4°C and 0.4 ml of this supernatant was added to Aquasol (NEN Research Products) for liquid scintillation spectrometry counting (Packard, Model 3225). Standard curves were constructed in the presence of antigen using LTD₄. The detection limit of the assay was 0.045 pmol of LTD₄ and results are expressed as pmol/4 × 10⁵ cells.

Determination of the Superoxide Anion (O₂⁻)

Superoxide anion (O₂⁻) production was measured as described previously [46]. Briefly, 50 µl of sensitized mast cells (4 × 10⁵), 172.5 µl PBSG (PBS containing 0.9 mM CaCl₂, 0.5 mM MgCl₂, and 7.5 mM glucose), 5.0 µl SOD (3 mg/ml) or 5.0 µl H₂O, 12.5 µl cytochrome c (75 µM) and OVA (1.0 µg/ml) were added to each well of a 96-well plate and then incubated at room temperature for 20 min. The O₂⁻ generated by the reduction of cytochrome c was read using a microplate reader (Spectra Max, Molecular Devices, Sunnyvale, Calif., USA) at 550 nm for 20 min in kinetic mode.

Determination of Total Nitric Oxide (NO) Production

Purified mast cells (4 × 10⁵) were sensitized with anti-OVA antibody (1 ml/10⁶ cells) and challenged with OVA (1.0 µg/ml) for 20 min. Total nitric oxide released into the supernatant was measured using an ELISA Kit (R & D) [47]. Nitrate reductase was added to the samples before nitrite determination.

Estimation of Tyrosine Nitration by Immunofluorescence

Peroxy-nitrite-mediated tyrosine nitration was estimated using a modification of the method of Thiyagarajan and Shaarma [48], using the anti-nitrotyrosine antibody/immunofluorescence technique. Briefly, mast cells (4 × 10⁵) passively sensitized with anti-OVA antibody (1 ml/10⁶ cells) were challenged with 1.0 µg/ml of OVA for 30 min, and then 1 × 10⁵ cells were spun onto glass slides using a cytospin. The cells were then fixed with 0.1% glutaraldehyde in PBS for 15 min on ice, washed with PBS, and permeabilized with 0.1% Triton X-100 for 15 min. After blocking with 2% BSA for 1 h at room temperature, they were incubated with 10 µg/ml anti-nitrotyrosine overnight at room temperature. They were then washed with PBS, incubated with a 1:100 dilution of anti-rabbit IgG-fluo-

rescence-conjugated secondary antibody for 1 h in the dark. After mounting, the cells were examined under a fluorescent microscope. DMTU (10 mM), sL-MT (30 μ M) or NMMA (100 μ M) were added to the mast cells 30 min before OVA challenge.

Determination of Intracellular Ca^{2+} Level ($[Ca^{2+}]_i$)

Sensitized mast cells (4×10^5) were incubated for 30 min after adding Fluo-3 AM (5 μ M) and fixed on a slide glass treated with poly-L-lysine. OVA antigen (1.0 μ g/ml) was then flowed over the slide glass to stimulate the cells. $[Ca^{2+}]_i$ values of single mast cells were determined by measuring optimal density (ODs) using an LSM 510 laser scanning microscope (Carl Zeiss, Germany) [43]. Antioxidants (10 mM DMTU, 30 μ M sL-MT, or 100 μ M NMMA) were added to the mast cells 30 min before the OVA challenge (1.0 μ g/ml).

Determination of Phospholipase A_2 (PLA₂) Activity during Mast Cell Activation

The activity of PLA₂ was determined using the method described by Shin et al. [49]. Sensitized mast cells (1×10^6) were preincubated with [³H]arachidonic acid (1.0 μ Ci, 0.01 mmol) at 37°C for 1 h. These prelabeled mast cells were then washed twice and resuspended in TGCM buffer, sensitized anti-OVA antibody (1 ml/ 10^6 cells) for 45 min, and stimulated with 1.0 μ g/ml OVA for 30 min. This reaction was stopped on ice, and the activities of supernatants obtained by centrifugation were determined using a liquid-scintillation counter. DMTU (10 mM) or sL-MT (30 μ M) was added to the mast cells 30 min before the OVA challenge.

Statistical Analysis

Experimental data are presented as means $\pm$ SEM. Differences between control and experimental groups were analyzed using the Student's *t* test for unpaired data. *p* values below 0.05, 0.01 or 0.001 were considered significant.

Data obtained from FACS analysis for ROS generation were tested for significance using the Kolmogorov-Smirnov test (for the analysis of histograms from flow systems) using CellQuest FACS analysis software (Becton and Dickinson, UK).

Results

Intracellular ROS Generation by Activated Guinea Pig Lung Mast Cells

When normal mast cells were treated with hydrogen peroxide (H₂O₂, 100 μ M) for 20 min, the FACS profile immediately shifted to the right (fig. 1a). However, the treatment of unsensitized mast cells for 5, 10, 15, 20, 30, or 60 min with OVA at 0.1, 1.0, or 10.0 μ g did not induce a FACS profile shift (data not shown).

When sensitized guinea pig lung mast cells (4×10^5 cells/400 μ l anti-OVA antibody) were activated with OVA (0.1, 1.0, or 10.0 μ g) for 10 s to ~20 min, ROS was slightly generated intracellularly after a 1.0 μ g/ml OVA challenge for 30 s to ~1 min using DCFH-DA fluorescent probes (fig. 1a). Moreover, the H₂O₂ scavenger, ebselen

(50 μ M), completely scavenged the ROS generated (fig. 1a), and the $\cdot$ OH⁻ and ONOO⁻ scavenger DMTU (10 mM) completely scavenged the generated ROS (data not shown). However, the peroxynitrite (ONOO⁻) scavenger sL-MT (30 μ M) and the specific NO synthase inhibitor NMMA (100 μ M) both failed to inhibit the ROS detected by DCFH-DA (data not shown). These data indicate that the small amounts of ROS species generated by mast cell activation may have been H₂O₂ or $\cdot$ OH⁻.

We examined whether this ROS was detectable using fluorescent probes DHR because the ROS generated were barely detectable by DCFH-DA. Sensitized mast cells (4×10^5 cells/400 μ l anti-OVA antibody) were challenged with OVA (0.1, 1.0 and 10.0 μ g/ml) for 20 min and ROS generation was followed using DHR (fig. 1b). ROS levels peaked when the cells were stimulated with 1.0 μ g/ml OVA for 30 s to ~1 min by 3-fold. The amount of ROS generated by 10.0 μ g/ml OVA was similar to that by 1.0 μ g/ml OVA (fig. 1b). DHR is known to detect mainly H₂O₂, hypochlorous acid (HOCl), and ONOO⁻, but not to detect $\cdot$ OH⁻ [10]. Thus, this result indicates that intracellular ROS such as H₂O₂, HOCl, or ONOO⁻ are produced in guinea pig lung mast cells activated by OVA/anti-OVA antibody.

Effects of Antioxidants on Inflammatory Mediator Release by Activated Mast Cells

Based on the data above, we first examined whether the ROS generated is related to mediator release. We first examined whether H₂O₂ itself affects mediator release in unstimulated mast cells. However, H₂O₂ (0.1, 10, 30, 50, 100, 500 μ M and 1 mM) alone failed to induce histamine release and leukotriene synthesis.

When mast cells (4×10^5 cells) sensitized with anti-OVA antibody were challenged by 0.1 μ g/ml or 1.0 μ g/ml OVA, the histamine release evoked by both concentrations of OVA was similar (i.e., $33.5 \pm 2.59\%$ and $31.4 \pm 1.72\%$, respectively); the histamine level by 10.0 μ g/ml of OVA was $25.7 \pm 3.79\%$. This is why 1.0 μ g/ml OVA (high concentration) of the same concentration that was used for ROS generation in the mast cell activation was chosen. Pretreatment of mast cells with DMTU (10 mM), sL-MT (30 μ M), or FeTPPS (10 μ M) inhibited histamine release by 86.6, 84.8, and 40.7%, respectively, compared to the OVA alone (table 1). NMMA (100 μ M) showed an inhibitory effect of up to 21.3%.

DMTU (10 mM) completely inhibited leukotrienes synthesis when sensitized mast cells were challenged with 1.0 μ g/ml OVA (658.7 ± 9.32 pmol/ 4×10^5 cells). sL-MT (30 μ M) or FeTPPS (10 μ M) also inhibited leukotriene

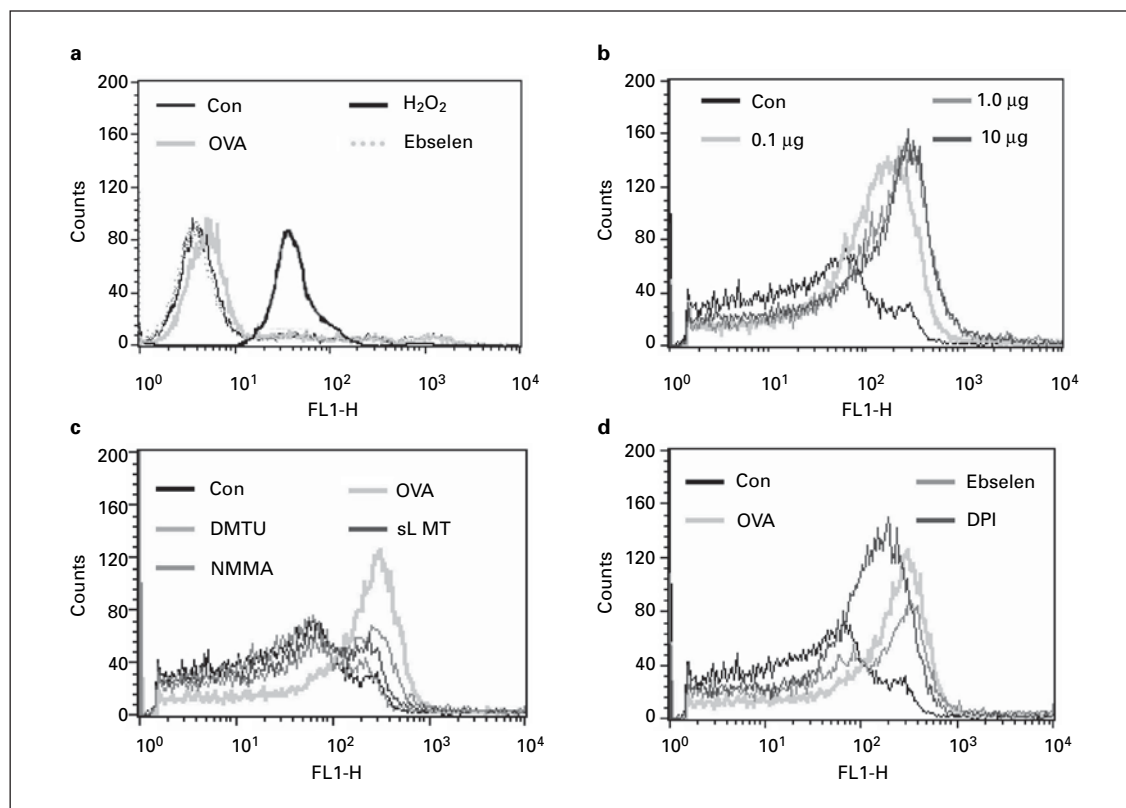


Fig. 1. Effects of antioxidants on the ROS or peroxynitrite generated from guinea pig lung mast cells activated with OVA/anti-OVA antibody reaction. **a** DCFH-DA (5 μ M final concentration). **b** Dihydrorhodamine 123 (10 μ M final concentration) as used for ROS quantitation. Positive control ROS generated from mast cells treated with H_2O_2 (100 μ M) alone (a). The generated ROS was determined by counting of 10,000 cells using the Kolmogorov-Smirnov test for analysis of histograms. **c**, **d** Each antioxidant effect by DHR dye. Experiments were performed in blocks of six. Con = Non-stimulation; H_2O_2 = hydrogen peroxide; OVA = ovalbumin; DMTU = dimethyl thiourea; sL-MT = seleno-*L*-methionine; NMMA = N-monomethyl-*L*-arginine.

release by 78.0 and 21%, respectively, but NMMA showed only weak inhibition (approximately 14.9%) (table 1).

The $\cdot\text{O}_2^-$ scavenger DPI also inhibited histamine and leukotriene release by mast cell activation by 61.4 and 16.0%, respectively. However, the selective H_2O_2 scavenger ebselen (50 μ M) increased histamine release by 78.8%, but did not affect leukotriene release.

Characterization of ROS Species Generated by Activated Guinea Pig Lung Mast Cells

As inflammatory mediator release from activated mast cells was inhibited by antioxidants, we examined the ROS types produced. It has been reported that two pools of NADPH oxidase exist in the plasma and granular membranes of mast cells [50], and DPI is known to scavenge NADPH oxidase, and to potently inhibit $\cdot\text{O}_2^-$ production. Thus, we examined whether DPI inhibited ROS produc-

tion. DPI (100 μ M) partially inhibited ROS production by up to 25–30% by DHR. However, DPI also inhibits NO synthase (NOS) and H_2O_2 [50]. To examine this further, we examined the effects of the NOS inhibitor (NMMA) and of the H_2O_2 inhibitor (ebselen) on the ROS detected by DHR (fig. 1d). NMMA was found to inhibit up to 90% of the ROS generated (fig. 1c), but ebselen inhibited the ROS produced by ≤ 10 –15% (fig. 1d). These data infer that the ROS generated by mast cell activation is $\cdot\text{O}_2^-$ and NO, and small amounts of H_2O_2 .

If $\cdot\text{O}_2^-$ and NO are generated during mast cell activation, then they would be expected to be rapidly converted into ONOO $^-$. Therefore, we examined whether the ROS detected by DHR is in fact ONOO $^-$ by using different antioxidants. The ONOO $^-$ scavenger sL-MT (30 μ M) [24, 31] or the nonspecific ONOO $^-$ scavenger, DMTU (10 mM) [29], inhibited ROS production (fig. 1c). Spe-

Table 1. Effects of antioxidants on histamine release and on the production of leukotrienes in mast cells activated by OVA/anti-OVA antibody

	Histamine %	Leukotrienes pmol/4 × 10 ⁵ cells
OVA (1.0 µg/ml)	31.4 ± 1.71	658.7 ± 9.32
DMTU (10 mM)	4.5 ± 0.47 (86.6)**	0.0 (100)***
sL-MT (30 µM)	4.8 ± 0.09 (84.8)**	144.8 ± 3.00 (78.0)**
FeTPPS (10 µM)	18.6 ± 1.13 (40.7)*	520.4 ± 28.2 (21.0)*
DPI (100 µM)	12.1 ± 0.92 (61.4)**	553.3 ± 13.5 (16.0)
NMMA (100 µM)	24.7 ± 1.46 (21.3)*	560.3 ± 15.0 (14.9)
Ebselen (50 µM)	55.9 ± 2.14 (78.8)**	635.2 ± 18.9 (3.6)

Purified mast cells (4 × 10⁵ cells) were passively sensitized with anti-OVA antibody (1 ml/10⁶ cells) and then challenged with 1.0 µg/ml OVA. Antioxidants were pretreated 30 min before OVA challenge. Figures in parentheses are the percentage reductions evoked by the antioxidants. Experiments were performed in blocks of eight. OVA = Ovalbumin; DMTU = dimethyl thiourea; sL-MT = seleno-*L*-methionine; DPI = diphenyleneiodonium; NMMA = N-monomethyl *L*-arginine. * *p* < 0.05; ** *p* < 0.01 means a reduction vs. OVA alone. ** *p* < 0.01 means an increase vs. OVA alone.

cifically the ONOO⁻ inhibitor FeTPPS (10 µM) [51] inhibited ROS production by 80–85% (data not shown). FeTPPS catalyzes the isomerization of ONOO⁻ to nitrate anion, and sL-MT specifically protects the ONOO⁻ against oxidation and nitration more than that of methionine or FeTPPS. Therefore, we chose to use sL-MT rather than FeTPPS or methionine.

Generation of the Superoxide Anion (O₂⁻) and Nitric Oxide (NO) by Activated Mast Cells

As ROS generated was completely inhibited by ONOO⁻ scavengers, but was not completely inhibited by DPI treatment, we examined whether O₂⁻ and NO are generated during mast cell activation. Mast cells (4 × 10⁵ cells) activated with 1.0 µg/ml OVA/anti-OVA antibody showed remarkably increased O₂⁻ generation for ≤ 30 s. Moreover, this elevated O₂⁻ was inhibited by DMTU or sL-MT, but not by NMMA (table 2). DPI also inhibited O₂⁻ elevation by 27%.

Mast cells (4 × 10⁵ cells) activated by OVA/anti-OVA antibody showed NO upregulation from 3.2 ± 0.91 µmol/l to 11.1 ± 2.65 µmol/l for 30 s (table 2). Moreover, this NO was significantly scavenged by DMTU or sL-MT (table 2), and was completely inhibited by NMMA-specific nitric oxide synthase (NOS) inhibitor. Thus, the above suggest that the NO and O₂⁻

Table 2. Effects of antioxidants on superoxide anion and nitric oxide levels generated by guinea pig lung mast cells activated with OVA/anti-OVA antibody reaction in the absence or presence of antioxidants

	O ₂ ⁻ , nmol/min/ 4 × 10 ⁵ cells	Total NO µmol/l
Control	6.9 ± 0.77	3.2 ± 0.91
OVA	42.2 ± 3.84*	11.1 ± 2.65*
DMTU (10 mM)	16.9 ± 4.61 (60.0) ⁺	2.4 ± 0.80 (78.4) ⁺
sL-MT (30 µM)	16.6 ± 1.49 (60.7) ⁺	2.5 ± 0.90 (77.5) ⁺
DPI (100 µM)	30.8 ± 3.11 (27.0)	6.3 ± 0.47 (43.2)
NMMA (100 µM)	38.5 ± 3.03 (8.8)	3.1 ± 0.33 (72.1) ⁺

Purified mast cells (4 × 10⁵ cells) were sensitized with anti-OVA antibody (1 ml/10⁶ cells) and then challenged with 1.0 µg/ml OVA. Reduced superoxide anion levels were assessed by following cytochrome c reduction using a spectrofluorometer, and nitric oxide was followed using ELISA. Figures in parentheses are the percentage reduction evoked by the antioxidants. Experiments were performed in blocks of eight. Control = Non-stimulation; DMTU = dimethyl thiourea; sL-MT = seleno-*L*-methionine; DPI = diphenyleneiodonium; NMMA = N-monomethyl-*L*-arginine. * *p* < 0.05 compared with control. ⁺ *p* < 0.05 compared with OVA alone.

generated by mast cell activation are converted rapidly into ONOO⁻.

Estimation of Tyrosine Nitration Generated by Activated Guinea Pig Lung Mast Cells

To confirm that the ROS species generated by mast cell activation is ONOO⁻, the tyrosine nitration of amino acid (protein) (a stable biomarker of ONOO⁻ [18–22]), was estimated in mast cells activated with OVA/anti-OVA antibody by immunofluorescence assay. The immunoreactivity of nitrotyrosine residues increased by ~40% in the cytosol of activated mast cells versus the control (fig. 2). Moreover, DMTU (10 mM) or sL-MT (30 µM) considerably reduced this signal intensity in activated mast cells by ~100 and ~62%, respectively, and NMMA (100 µM) also inhibited it by ~31% (fig. 2). Moreover, the degrees of inhibition of nitrotyrosine residue formation by antioxidants were similar to that of the fluorescence profiles of ROS by FACS (fig. 1c, d).

Effects of Antioxidants on Intracellular Ca²⁺ ([Ca²⁺]_i) Levels in Activated Mast Cells

An increase in [Ca²⁺]_i is necessary for the degranulation of inflammatory mediators during the mast cell activation. Thus, the effects of antioxidants on [Ca²⁺]_i were determined at the single mast cell level by measuring

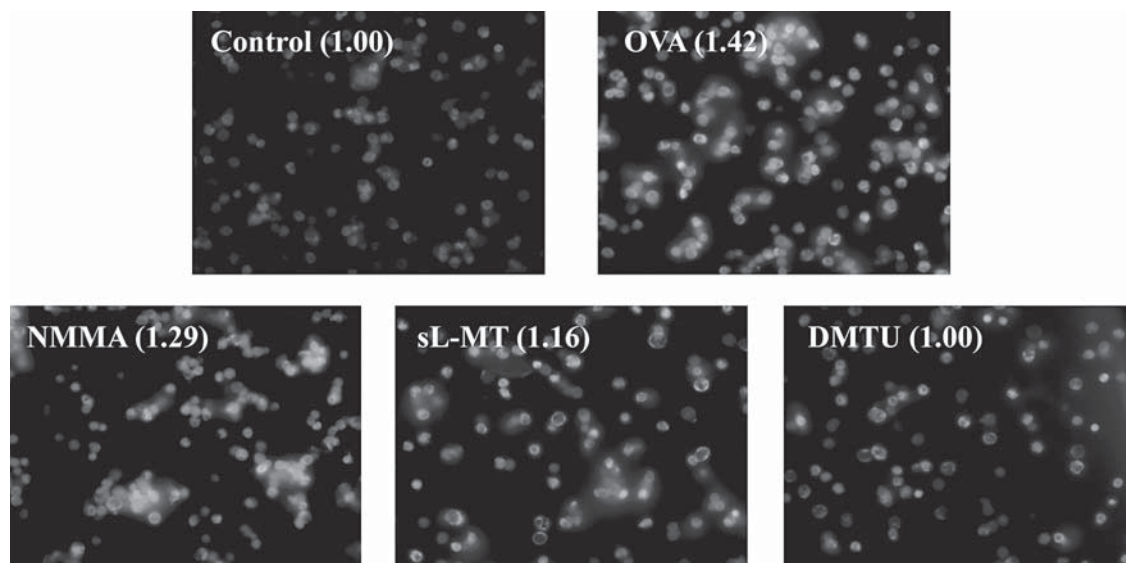


Fig. 2. Immunofluorescence photomicrographs of the nitrated tyrosine residues of cytosolic proteins in guinea pig lung mast cells activated with OVA/anti-OVA antibody. Cells were incubated on slides with 10 $\mu\text{g/ml}$ anti-nitrotyrosine, as described in 'Materials and Methods'. Parentheses represent relative intensity versus the control. Experiments were performed in blocks of six. For abbreviations, see figure 1.

fluorescence intensities. As shown in figure 3, the exposure of sensitized mast cells (4×10^5 cells) to 1.0 $\mu\text{g/ml}$ OVA rapidly increased the $[\text{Ca}^{2+}]_i$ level (from 1.00 ± 0.012 to 1.94 ± 0.055 OD). This increase in $[\text{Ca}^{2+}]_i$ level was reduced by DMTU (10 mM) or sL-MT (30 μM) (fig. 3). NMMA (100 μM) also inhibited this increase in $[\text{Ca}^{2+}]_i$ by 20–30%. However, NMMA inhibited this increase to a lesser extent. This inhibitory effect of NMMA on $[\text{Ca}^{2+}]_i$ was similar to the ROS FACS profiles and mediator releases.

Effects of Antioxidants on Phospholipase A_2 (PLA_2) Activity in Activated Mast Cells

$[\text{Ca}^{2+}]_i$ levels in mast cells are associated with membranous PLA_2 in the synthesis of lipid mediators such as leukotrienes and prostaglandins. And, ONOO^- has recently been reported to stimulate the release of arachidonic acid from PC12 cells, an effect that is mediated through the peroxynitrite-dependent activation of PLA_2 [24, 25]. Therefore, we investigated whether the ONOO^- scavengers, DMTU (40 mM) or sL-MT (30 μM), inhibit PLA_2 activity. PLA_2 activity increased by 53.2% in mast cells activated with 1.0 $\mu\text{g/ml}$ OVA (from $18,058 \pm 649$ to $27,660 \pm 3,145$ cpm). This increased PLA_2 activity decreased by 35.0% after DMTU treatment (from $27,660 \pm 3,145$ to $17,978 \pm 2,197$ cpm), and by 54.9% after sL-

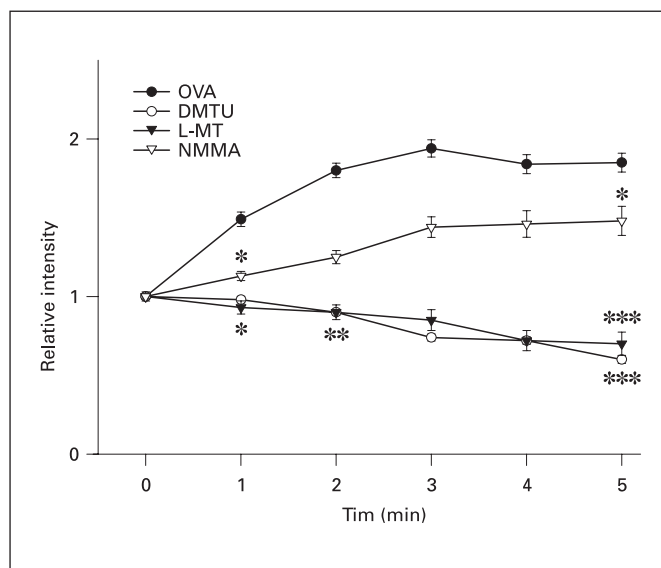


Fig. 3. The effects of antioxidants on Ca^{2+} influx in activated single mast cells. Fluo-3 AM (5.0 μM) was added to mast cells (4×10^5 cells) and incubated for 30 min, and the cells were fixed and activated on glass slides. The data shown are optical fluorescence densities versus the control. Experiments were performed in blocks of eight. For abbreviations, see figure 1. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with OVA challenge alone.

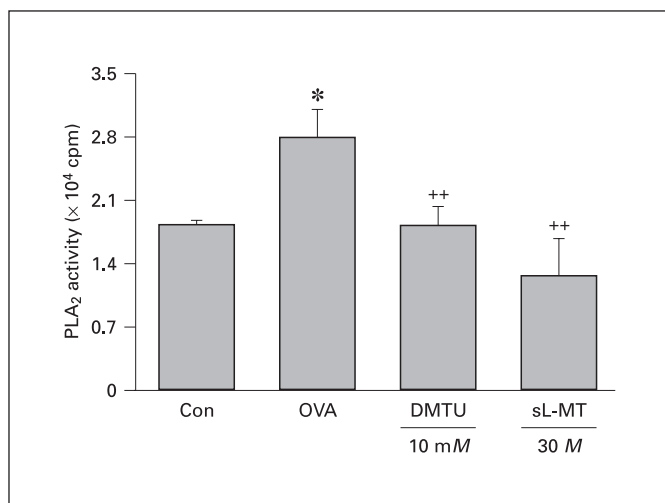


Fig. 4. Effects of antioxidants on PLA₂ activity in activated guinea pig lung mast cells. Labeled phospholipid levels were determined as described above. NMMA inhibited PLA₂ activity by 17%. Experiments were performed in blocks of eight. For abbreviations, see figure 1. ** $p < 0.05$ vs. control; ++ $p < 0.01$ vs. OVA challenge alone.

MT treatment (from $27,660 \pm 3,145$ to $12,480 \pm 4,164$ cpm) versus OVA challenge alone ($27,660 \pm 3,145$ cpm) (fig. 4). NMMA treatment had an inhibitory effect on PLA₂ activity by 17% (from $27,660 \pm 3,145$ to $22,958 \pm 2,530$ cpm).

Discussion

Oxidative stress is known to arise in many pathophysiologic conditions due to an imbalance between oxidants and antioxidants. Such conditions include allergic diseases, rheumatoid arthritis, and atherosclerosis [16]. Changes in ROS generation and redox potential have also been reported to modulate the function of various mast cells. Moreover, several studies have been performed on the role of ROS in the cells, e.g., the RBL 2H3 cell line [15, 35], rat peritoneal mast cells [34], and human basophils [36]. However, the role of ROS, particularly of ONOO⁻, in guinea pig lung mast cells, which are known to produce human mast-cell-like responses with respect to mast cell activation, has not been investigated. We undertook this study to investigate the hypothesis that antigen-IgG1-activated mast cells may generate specific ROS forms, and that these may modulate inflammatory mediator release. The study results show that peroxynitrite

(ONOO⁻), a product of ROS, is mainly generated by guinea pig lung mast cells activated with antigen/antibody reaction via FcγRI cross-linking, and that this generated ONOO⁻ is related to the release of inflammatory mediators via a Ca²⁺/PLA₂ pathway. Our results also show that DMTU scavenges ONOO⁻ more than sL-MT.

When sensitized mast cells challenged with OVA are followed by DCFH-DA, the oxidized DCF measures endogenous levels of H₂O₂ and hydroxyl radical ([•]OH⁻), but DCFH-DA does not directly measure the levels of superoxide anion ([•]O₂⁻). As shown in figure 1a, the amounts of ROS detected by DCFH-DA were low, but DHR showed much higher levels of ROS generation (fig. 1b). This generated ROS was also found to cause inflammatory mediator release (table 1). Moreover, various antioxidants (except the H₂O₂ scavenger ebselen) inhibited histamine release. Leukotriene synthesis was strongly inhibited by DMTU or sL-MT, but other scavengers showed only weak inhibition. The organoseleno compound ebselen acts catalytically by reducing ONOO⁻ to nitrite as a first step, and it also has the ability to mimic glutathione peroxidase in cerebral ischemia [30], and to act as a H₂O₂ scavenger [50]. We found that ebselen remarkably increased histamine release and weakly scavenged ONOO⁻, and that sL-MT (another seleno compound) strongly scavenged histamine and ONOO⁻ (fig. 1c, d). This increased effect of ebselen may have been due to the enhancement of oxidative modifications by ebselen under conditions of cellular glutathione depletion [10]. sL-MT is incorporated into protein at random in place of methionine and has been proposed to act as an ONOO⁻ reductase [16, 30]. Therefore, mechanisms of ebselen and sL-MT may differ, and sL-MT probably acts as an ONOO⁻ scavenger and not an ONOO⁻ decomposition catalyst. FeTPPS catalyses the isomerization of ONOO⁻ to nitrite anion, and thereby reduces nitrogen dioxide and hydroxyl radical levels [51]. However, FeTPPS also inhibited ONOO⁻ and histamine release, but only weakly inhibited leukotriene release (table 1); we are unable to explain this result. As a result of the above, we chose to examine the effect of two antioxidants (sL-MT and DMTU), which remarkably inhibited histamine and leukotrienes. sL-MT is a relatively specific ONOO⁻ scavenger (positive control), whereas DMTU is a nonspecific scavenger, but a strong scavenger for ONOO⁻. Recently, sL-MT was reported to strongly scavenge ONOO⁻ [30, 31], but it also scavenges NO [52]. Thus, it is difficult to identify specific ROS scavengers.

Our results imply that the ROS generated by mast cell activation is ONOO⁻, which was reduced by either DMTU

[24, 25, 29] or sL-MT [10, 16] (fig. 1c). DMTU or sL-MT strongly inhibited histamine release and leukotriene synthesis (table 1), and O_2^- and NO were generated during mast cell activation and also scavenged by DMTU or sL-MT (table 2). Thus, the ratio of $^{\bullet}O_2^-$ and NO plays an important role in the production of ROS. Equal fluxes of NO and $^{\bullet}O_2^-$ can stimulate lipid peroxidation via ONOO $^-$ generation, whereas a high NO to $^{\bullet}O_2^-$ ratio can reduce peroxidation because of the ability of NO to scavenge ROS radicals, and a low ratio might favor pro-oxidant via ONOO $^-$ generation [10, 53]. In the present study, a low NO to $^{\bullet}O_2^-$ ratio was observed after mast cell activation, which concurs with reports that ONOO $^-$ induces mediators (histamine and leukotrienes) [23–25] and then induces airway hyperresponsiveness in guinea pigs [13, 14].

The antioxidants used in the present study lacked ROS type selectivity and specificity. Therefore, we separately confirmed peroxynitrite-induced tyrosine nitration (a known stable biomarker of ONOO $^-$) in the cytosol of mast cells (fig. 2). However, the selective targeting of ONOO $^-$ to specific tyrosines has not been previously reported in guinea pig lung mast cells. Tyrosine nitration is not specific of ONOO $^-$, although tyrosine nitration is recognized as a footprint of ONOO $^-$. Other nitrating species may be released from cells, as recently indicated by the promotion of nitration by myeloperoxidase (MPO) [54], and by eosinophil peroxidase (EPO) [19, 55]. However, these alternative mechanisms of nitration appear to be more restricted than the nitration mediated by ONOO $^-$. This peroxidase mechanism requires the presence of a specific enzyme (e.g., myeloperoxidase or eosinophil peroxidase). Moreover, the nitration by myeloperoxidase may be mediated by H_2O_2 or nitrite; however, only small amounts of H_2O_2 were generated under our experimental conditions. Therefore, we suggest that in the present study nitrotyrosine was generated by ONOO $^-$ rather than that by MPO.

Peroxynitrite formation in most cases requires both NO and O_2^- , and NO is generated by the activation of endogenous NOS in various cells including mast cells [38–40]. O_2^- formation is more complex than that of NO because O_2^- sources are dependent on cell types, and include activated macrophages, neutrophils, and other cell types in the immune system. Moreover, these entities generate $^{\bullet}O_2^-$ via NADPH oxidase (flavoprotein) and NOS. However, the NADPH oxidase inhibitor DPI did not completely inhibit the ROS generation detected by DHR or O_2^- -generated by bioassay (fig. 1d; table 2). Therefore, it can be inferred that O_2^- formation is the result of something other than a NADPH oxidase mechanism.

Arachidonic acid is released from membrane phospholipids via the activation of PLA $_2$, which triggers histamine release by mast cell activation [43]. Moreover, the metabolism of arachidonic acid may be regarded as a source of free radicals, such as, O_2^- and H_2O_2 [56]. The enzyme responsible for the metabolism of arachidonic acid is known as prostaglandin endoperoxide synthase (PGHS). Therefore, it can be inferred that O_2^- is generated by the metabolism of arachidonic acid via PGHS in guinea pig lung mast cells. However, this does not rule out the involvement of NADPH oxidase in guinea pig lung mast cell activation.

In addition, if $^{\bullet}O_2^-$ is generated by mast cell activation, it can react with either NO or H_2O_2 . The reaction between $^{\bullet}O_2^-$ and H_2O_2 rapidly produces $^{\bullet}OH^-$, which is not detected by DHR. H_2O_2 generation was observed at only barely detectable levels ($\leq 10\%$) in our system. Nevertheless, to examine the possibility of $^{\bullet}OH^-$ generation we approached the issue indirectly using a cell permeable iron chelator, o-phenanthroline (100 μM), which inhibits the $^{\bullet}OH^-$ generated via the reaction between $^{\bullet}O_2^-$ and H_2O_2 . The ESR spectroscopy spin trap probe TEMPOL-H readily penetrates plasma membranes and allows the detection of free radicals, particularly of $^{\bullet}OH^-$ and $^{\bullet}O_2^-$, with high sensitivity and specificity [57]. Unfortunately, we do not have this facility, and thus we assessed indirectly whether $^{\bullet}OH^-$ is generated during mast cell activation, using o-phenanthroline. Because under our experimental conditions the hydroxyl radical is generated, ONOO $^-$ is likely to be generated more in the presence of o-phenanthroline than in the absence of o-phenanthroline [58]. However, in the presence of o-phenanthroline, ONOO $^-$ generation was slightly reduced (data not shown), suggesting that $^{\bullet}OH^-$ is not produced during mast cell activation. Moreover, HOCl may also be generated by activated myeloperoxidase after mast cell activation, and this can be detected by DHR. However, HOCl is only detectable using specific and selective antibody against chlorinated proteins, but no suitable guinea pig antibody is available.

Based on these results, we suggest that the main ROS generated by mast cell activation is ONOO $^-$; in addition, it appears that small amounts of H_2O_2 are generated after mast cell activation. However, this issue will only be resolved by further study of HOCl and $^{\bullet}OH^-$.

The causal involvement of NO in the pathogenesis of asthma remains controversial [15, 34, 40]. In our experiment, a specific inhibitor of NO synthase, NMMA, weakly inhibited the release of inflammatory mediators (table 1). Matsui et al. [15] found that NMMA failed to ex-

hibit any inhibitory effect at concentrations ranging from 8 μM to 1 mM, whereas Brooks et al. [34] found that NMMA enhances histamine release [40]. This difference may be associated with the variability and selectivity of the phenotypes of mast cells. Moreover, H_2O_2 concentration per se itself did not induce mediator release. Our data are not in agreement with a report which found that the incubation of rat peritoneal mast cells with H_2O_2 caused significant histamine release, we can only attribute this to the variable phenotypes of mast cells.

$[\text{Ca}^{2+}]_i$ is closely related to inflammatory mediator release from mast cells activated by antigen/antibody reactions [43]. ROS was found to increase $[\text{Ca}^{2+}]_i$ after mast cell activation [50, 59], and ONOO^- was found to markedly elevate $[\text{Ca}^{2+}]_i$ in human articular chondrocytes [26]. $[\text{Ca}^{2+}]_i$ elevation has a number of effects on mast cells, e.g., on membranous PLA_2 activity which facilitates the synthesis of lipid mediators like leukotrienes and prostaglandins [43]. Moreover, ONOO^- was previously found to prompt the release of arachidonic acid via PLA_2 in PC12 cells [15, 23, 24]. Our data (fig. 3, 4) support these findings. Therefore, ONOO^- may play an important role in the release of mediators via Ca^{2+} -dependent PLA_2 ac-

tivity in mast cells activated by OVA/anti-OVA reaction via $\text{Fc}\gamma\text{RI}$ cross-linking, and DMTU and sL-MT may have a strong scavenging effect on ONOO^- .

However, NMMA inhibits Ca^{2+} -dependent PLA_2 activity and inflammatory mediator releases less than those of sL-MT and DMTU (table 1; fig. 3, 4). Thus, it could be inferred that NO weakly promotes inflammatory mediator release, but other mechanisms may be involved, and further study is warranted.

The present study suggests that the ONOO^- generated by $\text{Fc}\gamma\text{RI}$ -dependent mast cell activation may contribute to modulating the release of inflammatory mediators via Ca^{2+} -dependent PLA_2 activity. Our results also provide some insight of the clinical applications of ONOO^- in allergic disorders such as asthma.

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