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Nerve Growth Factor Activates Mast Cells Through the Collaborative Interaction with Lysophosphatidylserine Expressed on the Membrane Surface of Activated Platelets

Keiko Kawamoto,* Junken Aoki,† Akane Tanaka,* Atsuko Itakura,* Hiroyuki Hosono,† Hiroyuki Arai,† Yasuo Kiso,‡ and Hiroshi Matsuda2*

Effect of nerve growth factor (NGF) on platelet-associated mast cell activation was investigated. Although neither NGF alone nor platelets alone induced significant 5-hydroxytryptamine (5-HT) release from rat peritoneal mast cells, marked 5-HT release was detected when costimulated with NGF and calcium ionophore-activated platelets. This response reached maximal levels as early as 5 min after the initiation of the coinubcation and was completely blocked by anti-NGF Ab or by an inhibitor for a tyrosine kinase of the trkA NGF receptor. Paraformaldehyde-fixed platelets activated with either calcium ionophore or thrombin exhibited the collaborative ability, suggesting the possible involvement of some membrane molecules expressed on activated platelets in mast cell activation. Because activation of platelets induced expression of phosphatidylserine (PS) and/or lysoPS on membrane surface, and since lysoPS, unlike PS, initiated the NGF-induced 5-HT release, lysoPS expressed on activated platelets may be involved in the mast cell activation. Moreover, intradermal injection of NGF and activated platelets into the rat skin increased local vascular permeability. These findings suggested that NGF collaboratively worked with membrane lysoPS of activated platelets to induce mast cell activation. Thus, NGF released in response to inflammatory stimuli may contribute to mast cell activation in collaboration with locally activated platelets in the process of inflammations and tissue repair. The Journal of Immunology, 2002, 168: 6412–6419.

Nerve growth factor (NGF) is a well-characterized neurotrophic factor that is commonly believed to play a crucial role in the development and maintenance of the central and peripheral nervous systems including sympathetic and sensory neurons (1–3). Two classes of NGF receptors have been identified by their relative affinities; the low-affinity NGF receptor is a 75-kDa glycoprotein, and the high-affinity receptor is a 140-kDa molecule containing a tyrosine kinase domain that is encoded by the trkA proto-oncogene (TrkA) (4, 5). In addition to neurotrophic activity, increasing evidences give rise to possible multifunctional properties of NGF on immunocompetent cells including lymphocytes, monocytes/macrophages, and mast cells through functional NGF receptors (4–11). In prior studies, we have demonstrated that NGF promotes not only granulocyte differentiation from human PBMC and murine bone marrow cells (12, 13), but also differentiation of connective tissue-type mast cells from murine bone marrow cells and bone marrow-derived culture mast cells (14). NGF is capable of supporting survival of neutrophils (15), eosinophils (16), and mast cells (9) by preventing apoptosis, and enhancing functional properties of neutrophils, eosinophils, macrophages, and mast cells: phagocytosis, superoxide production, matrix metalloproteinase-9 production, and chemotaxis (11, 12, 16–20). These experimental findings strongly support a possibility that NGF acts as a cytokine that is capable of modulating inflammatory responses and tissue repair. In fact, the topical application of NGF to cutaneous wounds accelerates the rate of wound healing in normal and diabetic mice (21).

Mast cells are often abundant along blood vessels, and generate and release a number of vasoactive mediators including histamine, serotonin (5-hydroxytryptamine; 5-HT), and leukotrienes by cross-linking of FcεRI-IgE with its specific Ag (22). In addition to the immunological stimulation, intradermal or s.c. administration of NGF to rats causes immediate vasodilatory responses characterized by the degranulation of local mast cells (23, 24). In contrast with the in vivo reports, NGF alone is insufficient to induce chemical mediator release from rat peritoneal mast cells (PMC) and the addition of exogenous phosphatidylserine (PS) or lysoPS, a deacylated PS derivative, to NGF is necessary for the mast cell activation (8, 25, 26). However, the mechanisms by which NGF and serinephospholipids induce mast cell activation and their pathophysiological roles have been poorly understood.

PS is a membrane phospholipid component normally distributed at the internal side of the plasma membrane. Activation of platelets by thrombin or calcium ionophore results in loss of membrane asymmetry and expression of PS and/or lysoPS on their cell surface, which is provided to catalyze hemostatic plug formation and blood coagulation (27, 28). In the tissue repair process, circulating

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1 Abbreviations used in this paper: NGF, nerve growth factor; TrkA, high affinity receptor for NGF; 5-HT, 5-hydroxytryptamine; PMC, peritoneal mast cell; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PS-PLA2, PS-specific phospholipase A2; MAPK, mitogen-activated protein kinase; P38K, phosphatidylinositol 3-kinase; PLCγ, phospholipase C-γ; RBL-2H3 cell, rat basophilic leukemia cell.

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platelets rapidly adhere to the subendothelial connective tissue exposed by vascular injury. Following this event, attached platelets are activated by a perivascular matrix (27, 28). Activated platelets most likely provide the phospholipid molecules at the affected sites. Therefore, we speculated that NGF may interact with platelets accumulating at the site of injured blood vessels, where mast cells locate abundantly, resulting in mast cell activation in vivo. To examine this hypothesis, we investigated whether activation of mast cells was modulated by NGF and platelets in vitro, by measuring 5-HT release from rat PMC. We report herein a novel collaborative interaction between NGF and surface lysoPS on activated platelets, thereby leading to mediator release from mast cells in vitro and in vivo.

Materials and Methods

NGF and other reagents

2.5S NGF purified from murine submaxillary glands was a gift from A. M. Stanisz and J. Bienenstock (McMaster University, Hamilton, Canada). Neurotrophic activity of the NGF preparations was determined as described previously (12). Cytochrome c and busulfan were purchased from Sigma-Aldrich (St. Louis, MO). PS, lysoPS, lysophosphatidic acid (ly-soPA), lysophosphatidylcholine (lysoPC), lysophosphatidylethanolamine, and lysophosphatidylinositol were purchased from Avanti Polar Lipids (Alabaster, AL). We also used K252a (Calbiochem, La Jolla, CA), PD98059 (New England Biolabs, Beverly, MA), and LY294002 (Calbiochem), PS-specific phospholipase A1 (PS-PLA1), was purified as described previously (29). Unless otherwise indicated, all chemicals and Abs were purchased from Sigma-Aldrich.

Isolation of rat PMC

Outbred male Wistar rats (8–16 wk of age) (Clea Japan, Tokyo, Japan) were kept in our laboratory provided with food and water ad libitum >1 wk before they were sacrificed. PMCs were purified by Percoll solution (Pharmacia Biotech, Uppsala, Sweden) as described previously (18). Sedimented PMC (>96% purity and 98% viability) were washed twice and resuspended at a concentration of 105 cells/ml in DMEM supplemented with 10% FCS.

Preparation of platelets

Whole-blood anticoagulated with 0.38% of trisodium citrate was collected from the exposed external jugular vein of normal rats under ether anesthesia. Platelet-rich plasma was prepared by centrifugation of blood samples at 120 × g for 13 min. To activate platelets, platelet-rich plasma was incubated with 1μM calcium ionophore A23187 at 37°C for 30 min without agitation, and washed twice with PBS containing 0.1% BSA and 300 ng/ml PGI2 (Ono Pharmaceutical, Tokyo, Japan). Washed platelets were resuspended at a concentration of 2 × 109 cells/ml in prewarmed DMEM supplemented with 10% FCS. In some experiments, we used platelets fixed with 1% paraformaldehyde in PBS at 4°C for 20 min following the stimulation with 1μM calcium ionophore A23187 or 0.2 U/ml thrombin. After the fixation, platelets were washed three times with PBS. Platelets showed no aggregation in the process of the treatment indicated above.

[3H]-HT release from PMC

5-HT release was determined as described previously (9). Briefly, purified PMC (105 cells/ml) resuspended in DMEM supplemented with 10% FCS were incubated at 37°C for 1 h with 1μCi/ml of [3H]-HT (specific activity: 26.3 Ci/mM; New England Nuclear, Boston, MA). The cells were washed five times with ice-cold PBS containing 0.1% BSA to remove unincorporated [3H]-HT. A total of 105 radioabeled PMC were incubated in 1 ml of DMEM with or without various concentrations of NGF, various numbers of platelets, and/or various aminophospholipids at 37°C for 30 min, unless especially indicated. After centrifugation for 5 min at 800 × g, aliquots (200 μl) of the supernatants were dissolved in 3 ml of a scintillation mixture (Ready Protein; Beckman Coulter, Fullerton, CA) and counted for β emissions in a scintillation counter (LS500; Beckman Coulter). Total incorporated cpm of PMC were obtained from cell pellets lysed with 1 ml of 1% Triton X-100 for 30 min on ice. The percentage of [3H]-HT release was calculated as: supernatant cpm/total cpm × 100. The percentage of release of unstimulated PMC was <10%.

Treatment of RBC with calcium ionophore

Venous blood obtained from healthy volunteers was diluted with an equal volume of PBS, and centrifuged in Percoll solution as described above. Sedimented RBC were resuspended at a 50% packed-cell volume in buffer containing 70 mM NaCl, 80 mM KCl, and 10 mM HEPES, and 1 mM CaCl2 (pH 7.4), and then stimulated with 10μM A23187 at 37°C for 2–3 h (30). After stimulation, PS expressed on the surface of RBC was assessed with FITC-conjugated annexin V (BD Pharmingen, San Diego, CA) according to the manufacturer’s instruction. By this procedure, >65% cells expressed PS on their surface. To prepare surface lysoPS “positive” RBC, PS-expressing RBC were treated at 37°C for 10 min with PS-PLA1, which is capable of allowing to accumulate lysoPS on the RBC surface (29). The RBC preparations were washed and fixed with 1% paraformaldehyde for 10 min.

Intracellular calcium mobilization

Freshly isolated PMC were incubated with 2 μM fura 2 acetoxyethyl ester (Dojin, Tokyo, Japan) in Tyrode’s buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 0.1% BSA, and 10 mM HEPES (pH 7.4)) at 37°C for 1 h, and then resuspended in 500 μl (106 cells) of the same buffer in a stirring cuvette. Following stimulation with 50 ng/ml NGF in the presence of 5 μM lysoPS or 106 activated platelets, cytosolic calcium of PMC was measured by monitoring fluorescence intensity at an emission wavelength of 510 nm, and excitation wavelengths of 340 and 380 nm using a CAF-110 (JACS, Tokyo, Japan) with a V3.0 software program.

Pretreatment of PMC with signal transduction inhibitors

Before a 5-HT release assay, PMC were preincubated for 1 h with the following inhibitors: 50 ng/ml K252a, a TrkA inhibitor (31); 100 μM PD98059, a mitogen-activated protein kinase (MAPK) kinase inhibitor (32); or 5 μM LY294002, a phosphatidylinositol 3-kinase inhibitor (33). The PMC were pretreated with inhibitors or a diluent solution, resuspended in DMEM, and allowed to demonstrate activating effect of NGF and platelets.

Western blot analysis of phosphorylated proteins

Autophosphorylation of TrkA, MAPK, PI3K, and phospholipase C-γ (PLCγ) was examined by using a modification of the method described previously (18). Briefly, PMC were suspended in serum-free medium at a density of 2 × 106 cells/ml and followed by stimulation with NGF (50 ng/ml), lysoPS (5 μM), and/or activated platelets (108 cells/ml) at 37°C for 5 or 30 min. Cells were lysed with lysis buffer (1.0% Nonidet P-40, 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 1 μg/ml leupeptin) for 5 min, and then frozen and thawed three times. Lysates were centrifuged at 15,000 rpm, 10 min, and supernatants were incubated with anti-PI3K Ab (Upstate Biotechnology, Lake Placid, NY) or anti-PLCγ 1 (Santa Cruz Bio- technology, Santa Cruz, CA) conjugated with protein-A beads at 4°C overnight with gentle rotation. The boiled immunocomplex samples were subjected to 10% SDS-PAGE and electrically transferred to a membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was incubated at 4°C overnight with the anti-phosphotyrosine mouse Ab and followed to reincubate with peroxidase-conjugated second Ab. For detection of phosphorylated TrkA and MAPK, equal amounts of whole-cell lysates were resolved by SDS-PAGE. The phosphorylation of TrkA or MAPK in each sample was assessed using rabbit polyclonal Ab (Cell Signaling Technology, Beverly, MA) that recognize the two different phosphorylated forms of each protein. The immunoreactive bands were visualized with an enhanced chemiluminescent detection reagent (Amersham, Arlington Heights, IL).

Plasma extravasation assay

Rats anesthetized with pentobarbital sodium were intradermally injected with 50 μl of 100 ng/ml NGF with or without 5 × 105 activated or resting platelets fixed with paraformaldehyde into the shaved dorsal skin, followed by i.v. injection of 1.0% Evans blue dye. The same concentration of cytchrome c was served as a same m.w. control. Injection sites were marked on the skin for orientation. Thirty minutes later, the dorsal skin was removed, and OD of dye infiltration were digitalized by using Gel Print 200/VGA and Basic Quantifier (Genomic Solutions, Ann Arbor, MI).

In vivo pretreatment with busulfan

To induce thrombocytopenia, rats were pretreated with busulfan according to the method reported previously (34). Briefly, rats were injected i.p. with busulfan (20 mg/kg body weight) twice 10 and 13 days before the plasma.
extravasation assay. The number of platelets in blood collected from the retro-orbital plexus were counted.

Real-time PCR quantification of rat cytokines

A quantitative RT-PCR was used to determine mRNA levels of cytokines in rat basophilic leukemia cells (RBL-2H3 cells; Health Science Research Resources Bank, Osaka, Japan). Total RNA was isolated from 5 × 10⁵ cells stimulated with 50 ng/ml NGF, 5 × 10⁶ activated platelets, and/or 5 μM lysOPs using a TRizol reagent (Life Technologies, Rockville, MD), according to the manufacturer’s instructions. A total of 1 μg of total RNA was reverse-transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies) and an oligo(dT) primer. Specific primers for amplification were based on published sequences for IL-3, IL-4, IL-10, TNF-α, IFN-γ, GM-CSF, and β-actin (35, 36). A quantitative PCR was performed using a SYBR Green PCR core reagent kit (PE Applied Biosystems, Tokyo, Japan) following the thermal cycling programs: stage 1, 50°C for 10 min; stage 2, 95°C for 3 min; stage 3, 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Fluorescence intensity was measured in real-time during extension steps for a SYBR Green assay by using a Nippon Bio-Rad thermal cycler (iCycler iQ detection system; Nippon Bio-Rad Laboratories, Tokyo, Japan). The no-template control was not amplified in the 40-cycle PCR.

Statistical analysis

Two-tailed Student’s t test was done for statistical analysis of the data, and p < 0.05 was taken as the level of significance.

Results

5-HT release from PMC induced by NGF and activated platelets

We examined 5-HT release from PMC (10⁵) cultured with a suboptimal dose of NGF (50 ng/ml) and platelets (10⁹) for 30 min at 37°C. As shown in Fig. 1A, neither NGF nor platelets alone could induce mediator release from mast cells. However, striking augmentation of 5-HT release (63.8%) was observed when PMC were cultured with 10⁹ A23187-activated platelets and 50 ng/ml NGF simultaneously. These data indicated that NGF and platelets had synergistic effect on in vitro mast cell activation without requiring exogenous serinephospholipids. Whereas coincubation of PMC with unstimulated resting platelets showed little enhancement of NGF-induced 5-HT release, the effect of activated platelets was a nearly 3-fold increase than that of resting platelets. Calcium ionophore is well-known to act as a strong secretagogue for mast cells. To eliminate the possibility that the small amount of A23187 was contaminated in the platelet suspension, we examined the releaseability of the second washing from the activated platelets. The supernatants had no effect on 5-HT release from PMC in the presence or absence of 50 ng/ml NGF (3.5 and 4.3%, respectively). Hence, we used activated platelets for following experiments.

When various concentrations of NGF (0.5, 5, 50, and 500 ng/ml) were added to 10⁹ PMC and 10⁹ A23187-activated platelets, significant release of 5-HT was observed in a dose-dependent manner; this effect was observed at concentrations as low as 0.5 ng/ml (Fig. 1B). We next examined the influence of numbers of platelets on NGF-induced mast cell activation. 5-HT release was also dependent on the number of activated platelets together with a fixed dose (50 ng/ml) of NGF; the minimal effect of the 5-HT release was detected when coincubated with 10⁷ activated platelets (Fig. 1C). Neutralizing Abs against NGF were provided to verify the specificity of the NGF effect on PMC in the presence of activated platelets. Addition of anti-NGF Ab completely abrogated the 5-HT release induced by NGF and activated platelets (Fig. 1C). Control Ab showed no positive effect on 5-HT release. As shown in Fig. 1D, this 5-HT release dependent on both NGF and activated platelets reached roughly maximal levels as early as 5 min after the initiation of the incubation with 50 ng/ml NGF and 10⁹ platelets.

Effect of fixation of activated platelets on NGF-induced 5-HT release

Activated platelets secrete chemical mediators which are capable of stimulating mast cells. Therefore, we conducted experiments with platelets fixed with paraformaldehyde to clarify whether some soluble factors released from platelets might be involved in mast cell activation in our in vitro system. Platelets were fixed with 1% paraformaldehyde after stimulation with calcium ionophore

FIGURE 1. 5-HT release from PMC stimulated with NGF and activated platelets. Released [³H]5-HT was measured by a scintillation counter as described in Materials and Methods. Each value represents the mean ± SE of four to six separate experiments. A, A total of 10⁵ PMC were incubated with 10⁹ resting platelets or with 10⁹ platelets activated with 1 μM A23187 in the presence or absence of 50 ng/ml NGF at 37°C for 30 min. *, p < 0.001; when compared with PMC alone. B, A total of 10⁵ PMC were stimulated with various concentrations of NGF in the presence of 10⁹ activated platelets for 30 min. *, p < 0.01; when compared with PMC plus activated platelets. C, A total of 10⁵ PMC were incubated with increasing numbers of activated platelets in the presence of 50 ng/ml NGF containing control Ab or anti-NGF Ab (1/1000 dilution). *, p < 0.001; when compared with PMC plus NGF. †, p < 0.001; when compared with control Ab. D, A total of 10⁵ PMC were stimulated simultaneously with 50 ng/ml NGF and 10⁹ activated platelets for 0, 5, 15, 30, and 60 min. *, p < 0.001; when compared with PMC alone.
A23187 or thrombin. The fixed activated platelets to 50 ng/ml NGF led to significant 5-HT release and marked degranulation of mast cells (Fig. 2, A and B). Thus, we concluded that the fixation of activated platelets did not substantially alter their ability to modulate the NGF-dependent mast cell activation.

**Effect of aminophospholipids on NGF-induced 5-HT release**

Previous studies show that NGF-mediated mediator release from mast cells requires the presence of exogenous serinephospholipids such as PS or lysoPS (8, 25, 26). PS is a membrane component that is normally distributed in an inner leaflet of a phospholipid bilayer. Activation of platelets by calcium ionophore leads to not only surface expression of membrane PS, but also accumulation of lysoPS by the subsequent degradation (28, 37, 38). Therefore, we examined the effect of either PS, lysoPA, lysoPC, lysolysophosphatidylethanolamine, lyso phosphatidylglycerol, or lysoPS on NGF-induced mast cell activation. As shown in Fig. 3, lysoPS induced significant 5-HT release from mast cells in the presence of NGF, but the other aminophospholipids did not.

To further clarify the possible involvement of serinephospholipids expressed on membrane surface in the mast cell activation, we examined whether PS- or lysoPS-expressing RBC mediated NGF-induced 5-HT release from PMC. Treatment of RBC with calcium ionophore has been reported to cause loss of phospholipid asymmetry resulting in PS expression on their surface (30). Furthermore, Sato et al. (29) have recently identified a new enzyme of the lipase family secreted from rat-activated platelets, termed PS-PLA1, that specifically acts on PS and produce lysoPS. By using this enzyme, we prepared two RBC samples that expressed PS or lysoPS on their surface. When RBC were treated with A23187, >65% of them were positive for binding of annexin V, which was slightly higher than activated platelets (Fig. 4). However, the addition of 10^6 PS-expressing RBC to PMC showed no significant release of 5-HT despite the presence of 50 ng/ml NGF (Table I). In contrast, the treatment of PS-expressing RBC with PS-PLA1 led to significant NGF-induced 5-HT release from PMC (Table I). Thus, we concluded that NGF was capable of inducing 5-HT release from PMC in the presence of lysoPS but not PS.

**Effect of NGF and platelets on calcium mobilization of PMC**

Because NGF-dependent histamine release of mast cells is inhibited by EDTA (25), we measured cytosolic calcium mobilization of mast cells loaded with fura 2 acetoxy methyl ester in response to addition of NGF and activated platelets. A very slight increase of intracellular calcium levels was observed in single addition of 50 ng/ml NGF, 5 μM lysoPS, or 10^6 activated platelets, respectively (Fig. 5). In contrast, simultaneous addition of NGF and A23187-activated platelets led to a marked increase of intracellular calcium levels, which reached the maximum levels within 200 msec; and the combination of NGF and lysoPS also significantly increased calcium influx (Fig. 5).

**Signal transduction pathways triggered by NGF and activated platelets**

The biological effects of NGF on target cells are mediated by specific cell surface receptors with different affinities: p75 and TrkA.
We next attempted to determine a dye extravasation after intradermal injection of NGF and activated platelets. Increased vascular permeability by intradermal injection of NGF-MEDIATED MAST CELL ACTIVATION

We next attempted to determine a dye extravasation after intradermal injection of NGF

activating platelets

Table 1. Effect of serinephospholipids-expressing RBC on NGF-induced 5-HT release from PMC

<table>
<thead>
<tr>
<th>Group</th>
<th>NGF (50 ng/ml)</th>
<th>Activated Cells (10⁶)</th>
<th>PS-PLA₂ Treatment*</th>
<th>[³H]5-HT Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>None</td>
<td>–</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>B</td>
<td>–</td>
<td>Platelets</td>
<td>–</td>
<td>4.4 ± 1.3</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>Platelets</td>
<td>–</td>
<td>38.6 ± 0.4</td>
</tr>
<tr>
<td>D</td>
<td>–</td>
<td>RBC</td>
<td>–</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>RBC</td>
<td>–</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>F</td>
<td>–</td>
<td>RBC</td>
<td>+</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>G</td>
<td>+</td>
<td>RBC</td>
<td>+</td>
<td>16.1 ± 0.4*</td>
</tr>
</tbody>
</table>

* Platelets and RBC were pretreated with calcium ionophore A23187 and in two groups, PS-expressed RBC were treated with PS-PLA₂ at 37°C for 10 min. Each value represents the mean ± SE of four separate experiments.

**p < 0.01; when compared to groups D, E, and F.

Intracellular calcium mobilization of PMC stimulated with NGF in the presence of lysoPS or activated platelets. After preincubation with 2 μM fura-2 acetoxymethyl ester, 10⁶ PMC were treated with 50 ng/ml NGF in the presence or absence of 5 μM lysoPS or 10⁶ A23187-activated platelets. Arrows indicate the time point when reagents were applied.

FIGURE 5. Intracellular calcium mobilization of PMC stimulated with NGF in the presence of lysoPS or activated platelets. After preincubation with 2 μM fura-2 acetoxymethyl ester, 10⁶ PMC were treated with 50 ng/ml NGF in the presence or absence of 5 μM lysoPS or 10⁶ A23187-activated platelets. Arrows indicate the time point when reagents were applied.

FIGURE 6. Suppressive effects of signal transduction inhibitors on NGF-induced 5-HT release from PMC. PMC were preincubated with either 50 ng/ml K252a, 100 μM PD98059, 10 μM LY294002, or assay medium for 1 h before costimulation with 50 ng/ml NGF and 10⁶ A23187-activated platelets for 30 min. Each value represents the mean ± SE of four separate experiments.* p < 0.001; when compared with NGF alone.

activated platelets on mast cell activation in vivo. As shown in Fig. 6A, markedly increased extravasation was observed at the injection site of the skin when a mixture of 5 ng NGF and 5 × 10⁶ platelets activated with A23187 or thrombin was administered, whereas injection of NGF alone induced a mild vasodilative response. In controls with PBS vehicle, 5 ng cytochrome c, or activated platelets alone, no or slight response was noted. To evaluate a role of circulating platelets in NGF-induced mast cell activation in vivo, busulfan was injected into rats before intradermal injection with NGF and activated platelets. The injection was induced marked reduction in the number of circulating platelets (<2%) 13 days later. Pretreatment with busulfan suppressed the NGF-induced vasodilative response by about half level as compared with that in control rats (Fig. 6B).

mRNA levels of cytokines

Because NGF increases mRNA levels of several cytokines in mast cells (35), we next examined an effect of activated platelets or lysoPS on NGF-mediated cytokine production by real-time PCR quantification. We used rat basophilic leukemia cell line RBL-2H3 instead of rat PMC. As detected in rat PMC, simultaneous stimulation by NGF and activated platelets caused significant degranulation response in RBL-2H3 cells (data not shown). Treatment with NGF alone induced a marked increase in mRNA expression of IL-3, IL-4, TNF-α, IFN-γ, and GM-CSF, whereas a slight, but not significant, increase in mRNA expression of IL-10 was detected (Fig. 9). However, neither lysoPS nor activated platelets
substantially modulated the NGF effect on cytokine gene expression.

Discussion

Mast cells bear specific receptors for IgE and the reaction of cell-bound IgE molecules with multivalent Ag induces release of a variety of chemical mediators and cytokines from mast cells, which greatly contribute to acute and chronic allergic in

placements. In addition to this immunological stimulation, cytokines and growth factors directly modulate mast cell functions (22). Although intradermal injection of NGF has been reported to induce plasma extravasation, how NGF caused the activation of mast cells at the injection site has been conclusively unclear (23, 24). In this study, we clearly demonstrated that NGF led to mediator release from mast cells through the collaborative interaction with activated platelets in vitro. This effect was not substantially influenced by a fixation of activated platelets with paraformaldehyde, suggesting that certain membrane molecules, such as PS or lysoPS (8, 25, 26) expressed on the platelet surface after stimulation with calcium ionophore or thrombin may be involved in NGF-mediated mast cell activation. Because NGF-induced 5-HT release was detected in the presence of lysoPS or lysoPS-expressing RBC, but not PS or PS-expressing RBC, lysoPS rather than PS may act as an alternative potential molecule for NGF-induced mast cell activation. The effect of lysoPS on in vivo histamine release from rat PMC is 1000-fold greater than that of PS (39) and a kind of stimulation leads to PS-PLA, release from platelets which specifically acts on PS to produce lysoPS, but not on any other lipids (29). These findings support our experimental results that degradation of membrane PS by PS-PLA, produced lysoPS in the process of their activation and the membrane lysoPS was strongly involved in the NGF-induced mediator release from mast cells. Although some previous reports suggested that the potentiating effect of lysoPS on mediator release of mast cells was required conversion to PS after incorporation into mast cell membrane (40, 41), we ruled out this possibility because the fixation of activated platelets with paraformaldehyde had no influence on 5-HT release from PMC.

Recently, lysophospholipids such as lysoPS, lysoPA, and lysoPC have been reported to induce a transient increase of cytосolic calcium levels in a human T cell line (42), suggesting the presence of the same receptor for the lysophospholipids on the target cell surface. In PMC, lysoPS, unlike the other lysophospholipids, triggered NGF-induced 5-HT release, whereas it led little calcium influx, suggesting that mast cells might express receptors which are capable of recognizing lysoPS. However, because isotope-labeled lysoPS failed to bind to CD36 expressed on rat macrophages
(data not shown), a signal of lysoPS might be mediated through some receptors different from CD36.

Binding of NGF to TrkA on PMC rapidly induces autophosphorylation of tyrosine residue of the receptor, which leads to activation of downstream signal cascades including MAPK and PI3K, thereby resulting in chemotactic movement (9, 18). Simultaneous addition of MAPK kinase and PI3K inhibitors completely blocked NGF-induced 5-HT release from PMC even in the presence of activated platelets, suggesting that 5-HT release may be mediated through both the signal transduction cascades. However, because activated platelets did not influence tyrosine phosphorylation of the individual signal molecules even in the presence of NGF, and because NGF alone was not capable of releasing 5-HT despite leading to phosphorylation of both MAPK and PI3K molecules, the signaling mechanisms by which lysoPS-expressing platelets exert its effect have been unclear.

NGF stimulation in the presence of lysoPS not only induces degranulation of mast cells but also increases the production of several cytokotkines including TNF-α (35). Therefore, we examined the effect of activated platelets and lysoPS on NGF-inducible inflammatory cytokine mRNA expression by quantitative real-time PCR. The mRNA levels of IL-3, IL-4, IL-10, TNF-α, IFN-γ, and GM-CSF were increased in response to NGF, but those were not modulated by addition of lysoPS or activated platelets. Thus, NGF and activated platelets may act collaboratively on 5-HT release, but not on cytokine gene expression. However, as NGF does not significantly increase the production of TNF-α protein even in the presence of lysoPS (43), the NGF-mediated effect on cytokine production might be limited to the gene expression level.

We confirmed the collaborative action of NGF and activated platelets on mediator release from resident tissue mast cells in rat skin by in vivo extravasation assay. This result implies that the novel activation pathway to mast cells presented here may occur in the pathophysiological condition. NGF alone showed slight effect of vascular permeability. It may be caused by the interaction between circulating platelets probably activated in the injection site and injected NGF because rats with thrombocytopenia manifested significant reduction in NGF-induced vascular permeability. NGF is rapidly released from salivary glands into blood stream in response to fighting stress in rats and mice and serum levels of NGF were increased up to 300 ng/ml (44). If circulating naïve platelets could induce mast cell activation together with NGF, an increase in serum levels of NGF in such conditions would cause fatal systemic shock by massive mast cell degranulation. We consider that the collaborative interaction between NGF and activated platelets demonstrated in this study may occur in the presence of endothelial cell denudation, particularly in the microvasculature at local damaged and inflamed tissues. We found that skin wound led to rapid increase in NGF levels in peripheral blood and affected sites of mice, and that local application of NGF accelerated the wound healing process (21). Correspondingly, increased levels of NGF in local inflammatory tissues or peripheral blood have been found in patients with systemic sclerosis, multiple sclerosis, chronic arthritis, and vernal keratoconjunctivitis (46–50). Platelets circulating in blood immediately accumulate at the site of injury or hemorrhage leading to their morphological change and biochemical activation. In contrast, mast cells are residential cells adjacent to the endothelium in the normal connective and mucosal tissues. Therefore, NGF locally and/or systemically produced in response to inflammatory stimuli may act with activated platelets recruited to injured sites to modulate the functions of mast cells in vivo. Our results strongly support this hypothesis, and suggest a possible role for NGF in many pathophysiological conditions that lead to mast cell activation. Activation of mast cells induces the release of histamine, leukotrienes, and produces inflammatory cytokotkines, resulting in the recruitment and activation of circulating leukocytes to the area of allergic and nonallergic inflammation. Mast cells also contribute to innate immunity to bacterial infection (51–53). The striking cooperative effect of NGF with platelets on mast cell activation may contribute to development of acute and chronic inflammations and wound healing process at damaged tissues.

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**References**


