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Roles of Mitogen-Activated Protein Kinase Pathways for Mediator Release from Human Cultured Mast Cells

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ABSTRACT. Human cultured mast cells (HCMC) secrete histamine, sulfidoleukotrienes (LTs), and prostaglandin D_2 (PGD_2), and produce a variety of cytokines after aggregation of high-affinity receptors for IgE (FcεRI). With respect to the mitogen-activated protein kinase (MAPK) family, extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 mitogen-activated protein kinase (p38 MAPK) are known. To investigate the roles of these kinase pathways for mediator release from human mast cells, we examined the participation of the activation of these kinases in mediator release, using 1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene (U0126), an ERK pathway inhibitor, and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), a p38 MAPK pathway inhibitor. U0126 inhibited ERK activation, LT and PGD_2 release, and granulocyte macrophage-colony stimulating factor (GM-CSF) production after stimulation of HCMC. SB203580, on the other hand, potentiated JNK activation and GM-CSF production. The findings of the present study demonstrated that: (i) the release of arachidonic acid metabolites is mediated by the ERK pathway; (ii) GM-CSF production may be driven by both the ERK and JNK pathways; and (iii) the p38 MAPK pathway negatively regulates the JNK pathway. This suggests that MAPK pathways play important roles in mediator release from human mast cells. BIOCHEM PHARMACOL **60**;4:589–594, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. human cultured mast cells (HCMC); mediator release; signal transduction; extracellular signal-regulated kinases (ERKs); c-Jun NH_2-terminal kinases (JNKs); p38 mitogen-activated protein kinase (p38 MAPK)

Mast cells appear to play a central role not only in causing immediate allergic reaction but also in developing late-phase reaction, and have been recognized as one of the most important cell types in the pathophysiology of allergic diseases [1, 2]. Cross-linking of the high-affinity receptors for IgE (FcεRI) induces the sequential aggregation of FcεRI and the release of inflammatory mediators. Mast cells are known to release histamine, arachidonic acid metabolites (LTs§ and PGD_2) [3], and many cytokines such as TNF-α [4], IL-8 [5], MIP-1α [6], and GM-CSF [3–6]. Since both human and rodent mast cells are reported to be highly heterogeneous [7, 8], mast cells from different species or different tissues may generate different mediator arrays. Therefore, the pathways that control mast cell mediator release may not be identical, depending on species or tissues.

In rodent systems, signal transduction events following aggregation of FcεRI have been analyzed vigorously. Generally, aggregation of FcεRI by antigen results in a cascade of intracellular signals [8–10]. The activation of PKC and the elevation of intracellular Ca^{2+} concentration are believed to be the primary synergistic signals required for secretion. Additionally, activation of three major classes of MAPKs has been reported [11, 12]. Although degranulation was assumed to be a MAPK-independent response, release of arachidonic acid is thought to be dependent on phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) by ERK in RBL-2H3 cells [13]. TNF-α production is regulated by an MEK kinase-regulated JNK pathway [11]. Additionally, p38 MAPK may negatively regulate the activation of ERK and the responses mediated by this kinase [14].

HCMC, which resemble lung-type human mast cells, are good tools for studying human mast cells. We previously reported that Ca^{2+} and PKC signaling in HCMC showed similarities to those in mouse and rat mast cells [15], whereas analyses of the activation of MAPKs in human mast cells have been limited. Therefore, we examined the

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§ *Abbreviations*: LT, leukotriene; PGD_2, prostaglandin D_2; TNF-α, tumor necrosis factor-α; IL, interleukin; MIP, macrophage inflammatory protein; GM-CSF, granulocyte macrophage-colony stimulating factor; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase; JNK, c-Jun NH_2 terminal kinase; and HCMC, human cultured mast cells.

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effects of 1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene (U0126) [16], an MEK inhibitor, and of 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1*H*-imidazole (SB203580) [17], a p38 MAPK inhibitor, on the activation of HCMC to elucidate the roles of MAPKs in the production of a cytokine, GM-CSF. At the same time, we also examined the release of arachidonic acid metabolites (LTs and PGD_2), and degranulation (histamine release) for comparison. In the present study, we demonstrated the activation of MAPKs and their roles in mediator release following FcεRI aggregation.

MATERIALS AND METHODS

Agents

U0126 and SB203580 were obtained from Promega and Calbiochem, respectively.

Human Mast Cell Culture

HCMC were obtained from human umbilical cord blood cells using a method described previously with some modifications [18]. Briefly, mononuclear cells (MNC) were separated from heparinized umbilical cord blood by Lymphocyte Separation Medium (ICN Biomedicals Inc.) gradient. $CD34^+$ cells were purified from MNC using the $CD34^+$ progenitor cell isolation kit (Miltenyi Biotec) and the Magnetic Cell Sorting System (Miltenyi Biotec). Purified $CD34^+$ cells were cultured in α-minimum essential medium (α-MEM; GIBCO-BRL Laboratories) containing 15% fetal bovine serum (Filtron Pty Ltd.) in the presence of 80 ng/mL of human recombinant stem cell factor (Kirin Brewery) and 50 ng/mL of human recombinant IL-6 (Kirin). The immunoperoxidase staining for tryptase and chymase was performed by a method previously established [19]. HCMC employed in the present study were from a more than 15-week-old culture with > 99% purity. Cells were almost 100% positive for tryptase and 14% positive for chymase in the immunophenotypic analysis.

Chemical Mediator Release and Cytokine Production

HCMC were sensitized with human myeloma IgE (Chemicon Int. Inc.) at 37° overnight. In the experiment on IgE-mediated chemical mediator (histamine, LTs, and PGD_2) release, cells were resuspended at 1×10^5 cells/mL in Tyrode's solution containing HEPES and BSA (Seikagaku Kogyo Co.) (126 mM NaCl, 4 mM KCl, 0.64 mM NaH_2PO_4, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.1% glucose, 10 mM HEPES, 0.03% BSA, pH 7.4) and treated with agents for 10 min. Then the cells were challenged with anti-human IgE antibody (anti-IgE; 1 μg/mL, DAKO) at 4 μg/mL (optimal concentration [18]) for 30 min. To examine the effect of agents on cytokine (GM-CSF) production, cells were resuspended at 1×10^5 cells/mL in fresh culture medium, treated with test agents for 10 min, and then challenged with anti-IgE at 2 μg/mL for 6 hr. Histamine was quantified by a method of post-column derivatization as previously reported [20]. For the analysis of LTs, PGD_2, and GM-CSF, we used commercial ELISA kits (LTs, Bühlmann Laboratory; PGD_2, Cayman Chemical Co.; GM-CSF, R&D Systems, Inc.).

Detection of MAPK Activation

ERKs, JNKs, and p38 MAPK are known as MAPKs. Inhibitors and anti-IgE-treated mast cells were lysed with ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5; 0.15 M NaCl; 1% IGEPAL CA-630; 10 μg/mL of aprotinin; 10 μg/mL of leupeptin; 1 mM EDTA; and 1 mM Na_3VO_4). Cell lysates were prepared separately, using 1×10^5 cells/lane (10 μg protein/lane) for ERK and p38 MAPK, and 3×10^5 cells/lane (30 μg protein/lane) for JNK. Analyses of proteins by SDS–PAGE and transfer to PVDF membrane (PVDF-Plus, pore size = 0.45 μm; Micron Separations, Inc.) were performed as described previously [21]. Proteins were detected using the immunoblotting technique with antibodies against phosphorylated forms of ERK (Phospho-p44/42 MAP Kinase antibody; New England Biolabs, Inc.), JNK (Phospho-SAPK/JNK antibody; New England Biolabs, Inc.), and p38 MAPK (Phospho-p38 MAPK antibody; New England Biolabs, Inc.). Horseradish peroxidase-conjugated antibody against rabbit IgG (Promega) was used as a secondary antibody. Finally, proteins were visualized using the ECL-plus system (Amersham). Visualized bands were analyzed using National Institutes of Health Image software (Wayne Rasband, National Institutes of Health).

Statistical Analysis

Values in the figures represent the means ± SEM of three or four independent experiments. One-way ANOVA/Dunnett's post-hoc procedure was used to assess the difference between control and treatment groups. $P < 0.05$ was considered to be significant.

RESULTS

IgE-Mediated Activation of ERKs, JNKs, and p38 MAPK in HCMC

Since it has been shown previously that ERKs, JNKs, and p38 MAPK can be activated through IgE-mediated stimulation in murine mast cell lines [11, 12], we determined the kinetics of activation of these MAPKs in anti-IgE-stimulated HCMC. The maximal activation of ERKs and p38 MAPK was observed at 5 min, and the maximal activation of JNKs was observed at 15 min after anti-IgE stimulation (Fig. 1). The activation of MAPKs returned to their basal levels at 60–120 min. ERK2 appeared to be preferentially phosphorylated in the anti-IgE-stimulated HCMC, compared with ERK1. Although we could detect JNK2 activation, the signal was very weak in comparison with JNK1 activation. Thus, we only evaluated JNK1 activation, and

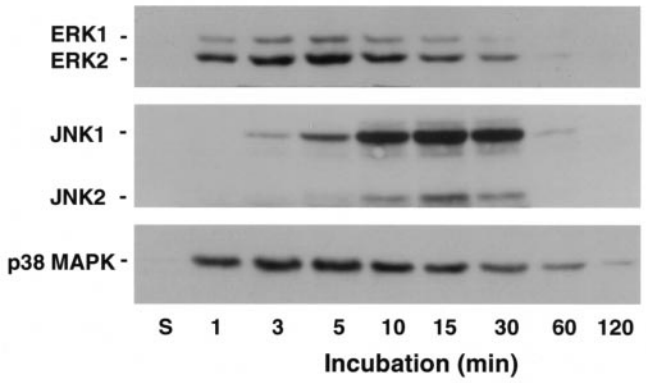

FIG. 1. IgE-mediated ERK, JNK, and p38 MAPK activation in HCMC. Sensitized HCMC were challenged with anti-IgE (4 μg/mL) for the indicated periods, and total cellular proteins were analyzed by SDS–PAGE and western blotting. Phosphorylation of ERK, JNK, and p38 MAPK was detected with phospho-specific antibodies. HRP-conjugated antibody was used as a secondary antibody. Proteins were visualized using the ECL-plus system. The result presented is representative of three different experiments. S = spontaneous.

analyzed it as JNK activation in the following experiments. Since we attempted to determine the activation of these three MAPKs simultaneously in inhibitor experiments, we detected the activation at 10 min after anti-IgE stimulation.

Selectivity of U0126 upon Activation of MAPKs in HCMC

U0126 was identified initially as an inhibitor of AP-1-driven gene transcription and later was shown to directly block ERK phosphorylation by its upstream kinases, MEK1 and MEK2, but the concentration used did not significantly inhibit the related MAPK family members MAPK kinase 3 (MKK3), MKK4, MKK6, ERK2, JNK1, or p38 MAPK [16]. HCMC were stimulated with anti-IgE in the presence or absence of various concentrations of U0126 for 10 min. As shown in Fig. 2, U0126 blocked ERK1/2 activation concentration dependently, and significant inhibition was observed from 0.1 μM, although it scarcely inhibited JNK1 and p38 MAPK activation at concentrations up to 3 μM.

These findings demonstrated that U0126 is a selective inhibitor of the ERK pathway in HCMC.

Selectivity of SB203580 on MAPK Activation in HCMC

The pyrimidyl imidazole compounds as exemplified by SB203580 have been demonstrated previously to inhibit p38 MAPK specifically [17]. SB203580 is known to inhibit the kinase activity of p38 MAPK but not p38 MAPK activation. As shown in Fig. 3, SB203580 did not affect the activation of ERKs or p38 MAPK at the concentrations examined (0.1 to10 μM). In contrast, SB203580 potentiated JNK activation 1.5- to 2-fold compared with the SB203580 non-treated group at 1–10 μM.

Effects of U0126 on IgE-Mediated Release of Mediators from HCMC

Since we found that U0126 inhibited ERK activation selectively for up to 3 μM, we examined the effects of U0126 (0.01 to 3 μM) on the release of histamine, LTs, PGD_2, and GM-CSF. First, we examined IgE-mediated histamine release (degranulation), which is thought not to be regulated by MAPKs. U0126 did not affect histamine release (Fig. 4). The release of LTs and PGD_2 and GM-CSF production, on the other hand, were inhibited concentra-

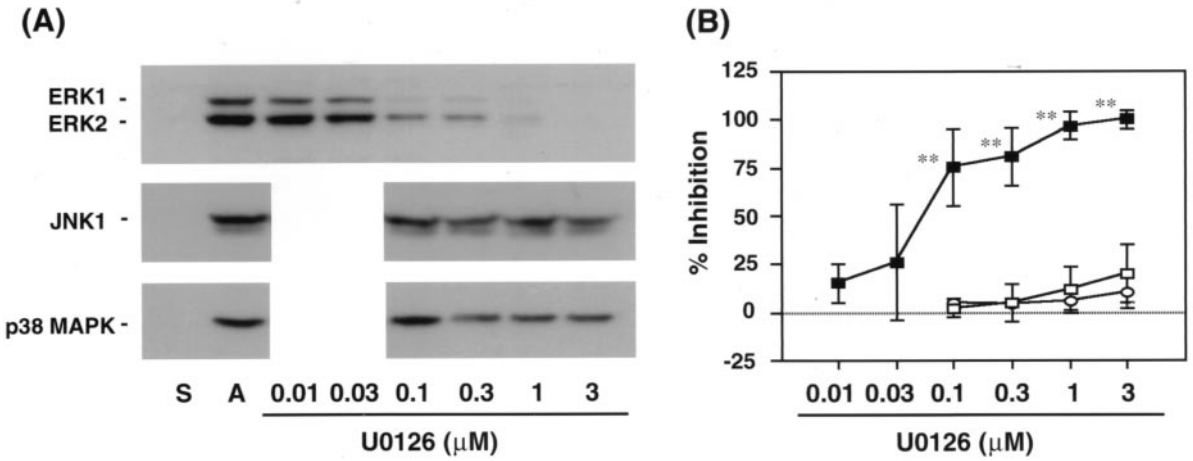

FIG. 2. Effects of U0126 on IgE-mediated ERK, JNK, and p38 MAPK activation in HCMC. (A) Sensitized HCMC were treated with various concentrations of U0126 for 10 min, and then challenged with anti-IgE (4 μg/mL) for 10 min. Total cellular proteins were analyzed by SDS–PAGE and western blotting. Horseradish peroxidase-conjugated antibody was used as the secondary antibody. Proteins were visualized using the ECL-plus system. The result presented is representative of three different experiments. S = spontaneous; and A = anti-IgE stimulation. (B) Visualized bands were analyzed using the National Institutes of Health Image software. Phosphorylation of ERK (■), JNK (□), and p38 MAPK (○) were detected with phospho-specific antibodies. Each value represents the means ± SEM (N = 3 or 4) of the analyzed data. Key: (**) $P < 0.01$ (Dunnett's post-hoc procedure).

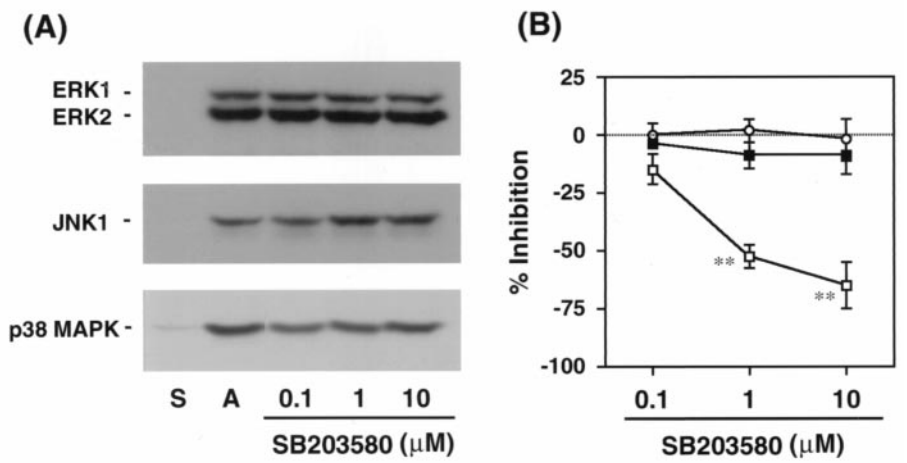

FIG. 3. Effects of SB203580 on IgE-mediated ERK, JNK, and p38 MAPK activation in HCMC. (A) Sensitized HCMC were treated with various concentrations of SB203580 for 10 min, and then challenged with anti-IgE (4 μg/mL) for 10 min. Total cellular proteins were analyzed by SDS–PAGE and western blotting. Horseradish peroxidase-conjugated antibody was used as the secondary antibody. Proteins were visualized using the ECL-plus system. The result presented is representative of three different experiments. S = spontaneous; A = anti-IgE stimulation. (B) Visualized bands were analyzed using the National Institutes of Health Image software. Phosphorylation of ERK (■), JNK (□), and p38 MAPK (○) were detected with phospho-specific antibodies. Each value represents the mean ± SEM (N = 3 or 4) of the analyzed data. Key: (**) $P < 0.01$ (Dunnett's post-hoc procedure).

tion dependently (Fig. 4), similar to the suppression of ERK activation by U0126 (Fig. 2). GM-CSF production was strongly inhibited when compared with the release of LTs and PGD_2. Additionally, 2′-amino-3′-methoxyflavone (PD 98059), an MEK inhibitor that binds directly to the nonphosphorylated form of MEK and blocks its activation by Raf-mediated phosphorylation [22], also suppressed LT and PGD_2 release and GM-CSF production (data not shown).

Effects of SB203580 on IgE-Mediated Release of Mediators from HCMC

Next, we examined the effects of SB203580. SB203580 did not affect histamine or PGD_2 release, but slightly inhibited the release of LTs at 10 μM (Fig. 5). In contrast, SB203580 potentiated GM-CSF production 2- to 3-fold against controls (SB203580 non-treated group) at 3–10 μM (Fig. 5). At low concentrations (0.1 to1 μM), GM-CSF production was slightly but not significantly inhibited by SB203580.

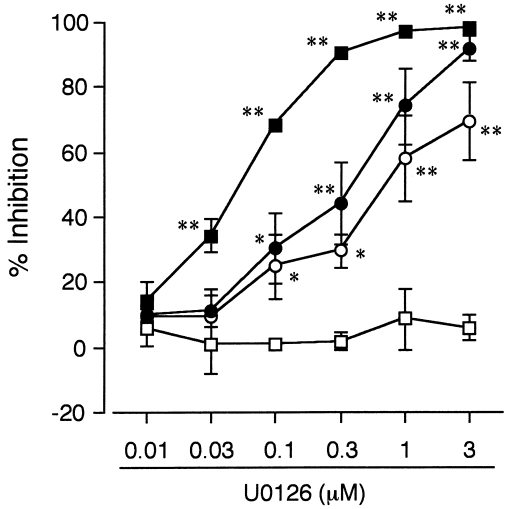

FIG. 4. Effects of U0126 on IgE-mediated release of histamine (□), LTs (●), and PGD_2 (○) and GM-CSF (■) production from HCMC. Sensitized cells were challenged with anti-IgE for 30 min (histamine, LTs, and PGD_2) or 6 hr (GM-CSF). U0126 was added 10 min before the anti-IgE challenge. Values are expressed as the percent inhibition of the control mediators release. Each value represents the mean ± SEM of four different experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ (Dunnett's post-hoc procedure).

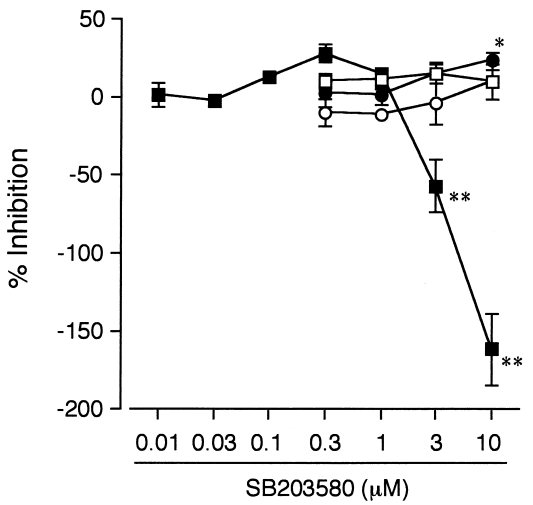

FIG. 5. Effects of SB203580 on IgE-mediated release of histamine (□), LTs (●), and PGD_2 (○) and GM-CSF (■) production from HCMC. Sensitized cells were challenged with anti-IgE for 30 min (histamine, LTs, and PGD_2) or 6 hr (GM-CSF). SB203580 was added 10 min before the anti-IgE challenge. Values are expressed as the percent inhibition of the control mediators release. Each value represents the mean ± SEM of four different experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ (Dunnett's post-hoc procedure).

DISCUSSION

Mast cells are especially notable cells because they can degranulate to release stored histamine, and can synthesize and release arachidonic acid metabolites and cytokines [3, 6, 23]. These mediators contribute to the variety of symptoms in allergic diseases, such as asthma [24]. Released histamine participates in vasodilation or bronchoconstriction, and LTs or PGD_2 induce airway smooth muscle constriction [25]. Variously produced cytokines contribute to the production of IgE, the expression of adhesion molecules, and the accumulation and activation of inflammatory cells [26–28]. For the allopathic treatment of asthma, mediator antagonists may be useful, although for the treatment of ongoing pathology of asthma, especially the regulation of the shift to chronic airway inflammation or the status of remodeling, the inhibition of mediator release may be crucial. To regulate mast cell mediator release in human allergic diseases, the signaling pathways involved in these processes must be analyzed. Similar intracellular events have been reported to be involved in FcεRI-mediated signaling pathways in rodent and human mast cells [29, 30]. We have also shown that Ca^{2+} and PKC signals are important for the release of histamine and LTs in human mast cells similar to rodent mast cells [15]. The participation of MAPKs in the release of arachidonic acid metabolites and cytokine production has been reported in rodent mast cells [11–14], whereas their roles in human mast cells have been investigated insufficiently. Suzuki *et al.* [29] reported that ERK1 and ERK2 are activated after cross-linking of FcεRI, and commented that these kinases play important roles in the signal transduction pathways in HCMC. We, therefore, designed the present study for the analysis of MAPK activation, and determined the participation of these kinases in mediator release from HCMC using various inhibitors.

At first, we confirmed the kinetics of MAPK activation. Although the activation of ERKs and p38 MAPK reached a maximum within 5 min and then declined gradually, JNK activation reached a maximum at 15 min after anti-IgE stimulation. Since the release of histamine and LTs from HCMC is almost complete after 15 min [18], JNK activation is unlikely to be involved in these signaling pathways.

From the findings of the U0126 experiments, activation of ERKs appears to be closely related to the release of arachidonic acid metabolites and the production of GM-CSF. Since specific inhibition of the ERK pathway suppressed arachidonic acid metabolite release and GM-CSF production, we concluded that the ERK pathway contributes to the release of these mediators after cross-linking of FcεRI in HCMC. The different inhibitory potencies of U0126 for arachidonic acid metabolite release and GM-CSF production may depend on the different sensitivities to ERKs of PLA_2 activation and GM-CSF-related transcription factor activation. The importance of the ERK pathway for arachidonic acid release and TNF-α production has been demonstrated using PD 98059 [14] or quercetin [13], a kinase inhibitor, in RBL-2H3 cells. However, Ishizuka *et al.* [11] reported that an MEK kinase-regulated JNK pathway but not the ERK2 pathway regulates TNF-α production in MC/9 murine mast cells. Miura *et al.* [31] reported that ERKs might selectively regulate the pathway leading to arachidonic acid metabolite (LTC_4) generation, but not cytokine (IL-4) production in human basophils. Although basophils share several features similar to human mast cells, the lineage of basophils has been reported to be closer to that of eosinophils than to the mast cell lineage [32]. Therefore, it is possible to consider that basophils, HCMC, and some rodent mast cells may use the ERK pathway differently.

In epithelial cells or monocytes, p38 MAPK is known to regulate a transcriptional factor, thereby controlling mediating cytokine synthesis such as TNF-α or IL-1β. SB203580 is well characterized as a p38 MAPK inhibitor [17], and was used in a signal transduction study on rodent mast cells [14]. However, in RBL-2H3 cells, p38 MAPK negatively regulates the activation of ERK, arachidonic acid release, and TNF-α production [14]. Thus, we investigated the role of the p38 MAPK pathway in mediator release using SB203580. SB203580 is known to inhibit the kinase activity of p38 MAPK, but not p38 MAPK activation. In the present study, SB203580 did not affect the ERKs, p38 MAPK activation, or arachidonic acid metabolite release. However, SB203580 enhanced FcεRI-mediated JNK activation at 10 min and GM-CSF production at 6 hr after cross-linking in HCMC. The correlation between JNK activation and GM-CSF production may confirm that the JNK pathway drives GM-CSF-regulating transcriptional factor and then regulates GM-CSF production. Although SB203580 potentiated JNK activation at 1 μM, it failed to potentiate GM-CSF production at the same concentration. SB203580 has also been reported recently to act on the serine/threonine kinase Raf-1 in quiescent smooth muscle cells [33]. Thus, it is suggested that SB203580 acts on Raf-1 activation also in HCMC. Nevertheless, Raf-1-mediated ERK activation was not affected by SB203580; hence, we can eliminate this possibility, at least in HCMC. Additionally, it will be necessary in future studies to detect the molecules responsible for the slight inhibition of release of LTs at 10 μM and GM-CSF production at 0.3 μM.

Finally, histamine release was not regulated by these MAPK pathways. This reaction, as reported previously, was driven by Ca^{2+} and PKC signaling [15].

In conclusion, the above findings suggest that the release of arachidonic acid metabolites is mediated by the ERK pathway, and that GM-CSF production may be driven by both the ERK and JNK pathways. Furthermore, the p38 MAPK pathway negatively regulated the JNK pathway, at least in HCMC. We demonstrated the roles of activation of MAPKs in mediator release in human mast cells, and presented some differences between human mast cells and previously reported rodent mast cells.

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