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Secretion of MCP-1, IL-8 and IL-6 Induced by House Dust Mite, *Dermatophagoides pteronissinus* in Human Eosinophilic EoL-1 Cells

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Abstract: The house dust mite (*Dermatophagoides pteronissinus*) is an important factor in triggering allergic diseases. The function of eosinophils, particularly in the production of cytokine or chemokine, is critical in understanding the pathogenesis of inflammatory diseases. In this study, we examined whether *D. pteronissinus* extract (DpE) induces the expression of monocyte chemotactic protein 1 (MCP-1)/CCL2, IL-8/CXCL8, and IL-6 that mediate in the infiltration and activation of immune cells and in its signaling mechanism in the human eosinophilic cell line, EoL-1. DpE increased the mRNA and protein expression of MCP-1, IL-8, and IL-6 in a time- and dose-dependent course in EoL-1 cells. In our experiments using signal-specific inhibitors, we found that the increased expression of MCP-1, IL-8, and IL-6 due to DpE is associated with Src family tyrosine kinase and protein kinase C δ (PKC δ). In addition, the activation of extracellular signal-regulated kinase (ERK) is required for MCP-1 and IL-8 expression while p38 mitogen-activated protein kinase (MAPK) is involved in IL-6 expression. DpE induced the phosphorylation of ERK and p38 MAPK. PP2, an inhibitor of Src family tyrosine kinase, and rottlerin, an inhibitor of PKC δ, blocked the activation of ERK and p38 MAPK. DpE induces the activation of ERK and p38 MAPK via Src family tyrosine kinase and PKC δ for MCP-1, IL-8, or IL-6 production. Increased cytokine release due to the house dust mite and the characterization of its signal transduction may be valuable in understanding the eosinophil-related pathogenic mechanism of inflammatory diseases.

Key words: *Dermatophagoides pteronissinus*, eosinophils, MCP-1, IL-6, IL-8

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INTRODUCTION

Eosinophils are major inflammatory effector cells. Eosinophilia in blood and tissue is a feature of allergic disorders such as atopic dermatitis and bronchial asthma (Homey et al., 2006; Foster et al., 2008). The presence of eosinophils during the occurrence of chronic inflammatory diseases has been associated with tissue pathology. In addition, eosinophils are potential sources of cytokines that may influence chronic inflammatory reactions and other biological responses (Costa et al.,1993). A recent study reported that eosinophils release inflammatory cytokines and adhesion molecules due to the house dust mite after coculturing with bronchial epithelial cells (Wong et al., 2006). MCP-1/CCL2 is a potent chemokine attracting monocytes, and IL-8/CXCL8 functions as a chemoattractant and a regulator of neutrophils. IL-6 increases acute phase proteins in acute inflammation and exerts lymphocyte activation in chronic inflammation (Rossi and Zlotnik A., 2000; Shakoory et al., 2004; Gabay 2006). The establishment of the human eosinophilic leukemia cell line, EoL-1, has been proved to be useful as an in vitro model for studying the properties of eosinophils, as well the mechanism of their inflammatory responses and released products (Mayumi., 1992; Goldstein et al., 1996). The house dust mite, *Dermatophagoides pteronissinus*, is a major allergen that causes the incidence and chronic inflammation of allergic diseases(Arlian and Platts-Mills., 2001; Friedmann., 1999). *D. pteronissinus* may induce a direct or an indirect inflammatory response as well as a dysregulation of IgE production (Thomas et al., 2002). The extract of house dust mite increases IL-8, TNF-α, and GM-CSF via NF-κB and the proteases derived from *D. pteronissinus* activate eosinophils that cause cytokine production (Wong et al., 2006; Coward et al., 2004). Specific immunotherapy such as sublingual therapy has been tested for the treatment

of atopic dermatitis and asthma using the house dust mite's extract (Wong et al., 2006; Bousquet et al., 2007). In a previous report, we elucidated that *D. pteronissinus* extract (DpE) increases the production of MCP-1, IL-8, and IL-6 in monocytes (Lee et al., 2008). Thus, to further clarify the function of DpE in eosinophils as initiators of allergic diseases, we investigated cytokine production from eosinophils and the involved mechanism.

MATERIALS AND METHODS

DpE preparation

The *D. pteronissinus* extract (DpE) was supplied from Arthropods of Medical Importance Resource Bank (Yonsei University College of Medicine, Seoul, Korea). DpE extract was dissolved in PBS (1 mg/mL). EndotrapTrap Red (Lonza, MD) was used for the removal of endotoxin in DpE according to the manufacturer's instructions. Endotoxin level was measured by Limulus amebocyte lysate QCL-1000 test (Cambrex, MD) and the level was 0.005 EU/mg of DpE.

Materials and cell culture

Both RPMI-1640 and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Gaithersburg, MD). Signal protein inhibitors such as PP2, rottlerin, Ro-31-8425, PD98059 and SB202190 were obtained from Calbiochem (San Diego, CA). LPS (Escherichia coli 055:B5) was purchased from Sigma-Aldrich (St.Louis, MO). Antibodies against ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-ERK, p38 MAPK, and phospho-p38 were obatained from Cell Signaling Technology (Beverly, MA). Human EoL-1 cells were obtained from the Ricken Cell Bank (Tsukuba, Japan) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-6 and IL-8 in the supernatant were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using OptEIA Set IL-6 and IL-8 (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. The concentration of MCP-1 in the supernatant was measured with a DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions using an ELISA reader, BIO-TEK ELx808. All assays were performed in three independent experiments. The concentration was calculated using a linear-regression equation obtained from the standard absorbance values.

Semi-quantitative RT-PCR

RT-PCR was performed to measure the relative quantities of mRNA for MCP-1, IL-6, and IL-8 in either control or mite-stimulated cells. The total RNA was extracted from the cells using Trizol reagent as described in the manufacturer's instructions. To prepare the cDNA, the total RNA (2 μg) was incubated at 37°C for 90 min using the first-strand cDNA synthesis kit (Promega, Madison, WI). Primer sequences and product lengths in this study are used as described in a previous study (Lee et al., 2008). PCR was carried out with an initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 59 or 72°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. GAPDH was used as an internal control for each PCR reaction. The final PCR products were separated on 1% agarose gels and then visualized by ethidium bromide staining.

Western blotting

Cells were lysed in cytosolic lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na3VO4, and protease inhibitors). Protein samples (30 μg/well) were separated by 10% SDS-PAGE and transferred to nitrocellulose filters. The blots were incubated with anti-phospho-ERK or anti-phospho-p38 antibody and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech.). The same membranes were stripped and reprobed with anti-ERK2 or anti-p38 antibody as an internal control.

Statistical analysis data were expressed as the means± SD. The statistical differences were analyzed using the Student's t test in the SPSS statistical software package (Version 10.0, Chicago, IL). A significant value was defined as $P<0.01$.

RESULTS

The mRNA and protein production of MCP-1, IL-8, and IL-6 as induced by DpE in EoL-1 cells

We first investigated the levels of cytokine proteins after treatment with DpE in EoL-1 cells. The protein secretion and mRNA production of MCP-1, IL-8, and IL-6 was evaluated by ELISA and RT-PCR, respectively. Results show that DpE increased the protein release of MCP-1, IL-8, and IL-6 in a time-dependent manner (Fig. 1A). The cytokine expression gradually elevated from the 3 h point, and reached a maximum level at 6 or 12 h. Thereafter, the production decreased. Furthermore, although LPS increased the cytokine level, DpE is more effective than LPS in elevating cytokine at the same concentration. As shown in Fig. 1B, the alteration of cytokine mRNA was similar to that of the protein. We next examined whether the expression of MCP-1, IL-8, and IL-6 was affected after a dose-dependent treatment of DpE. The result shows that DpE induces both the cytokines and their mRNA (Fig. 2).

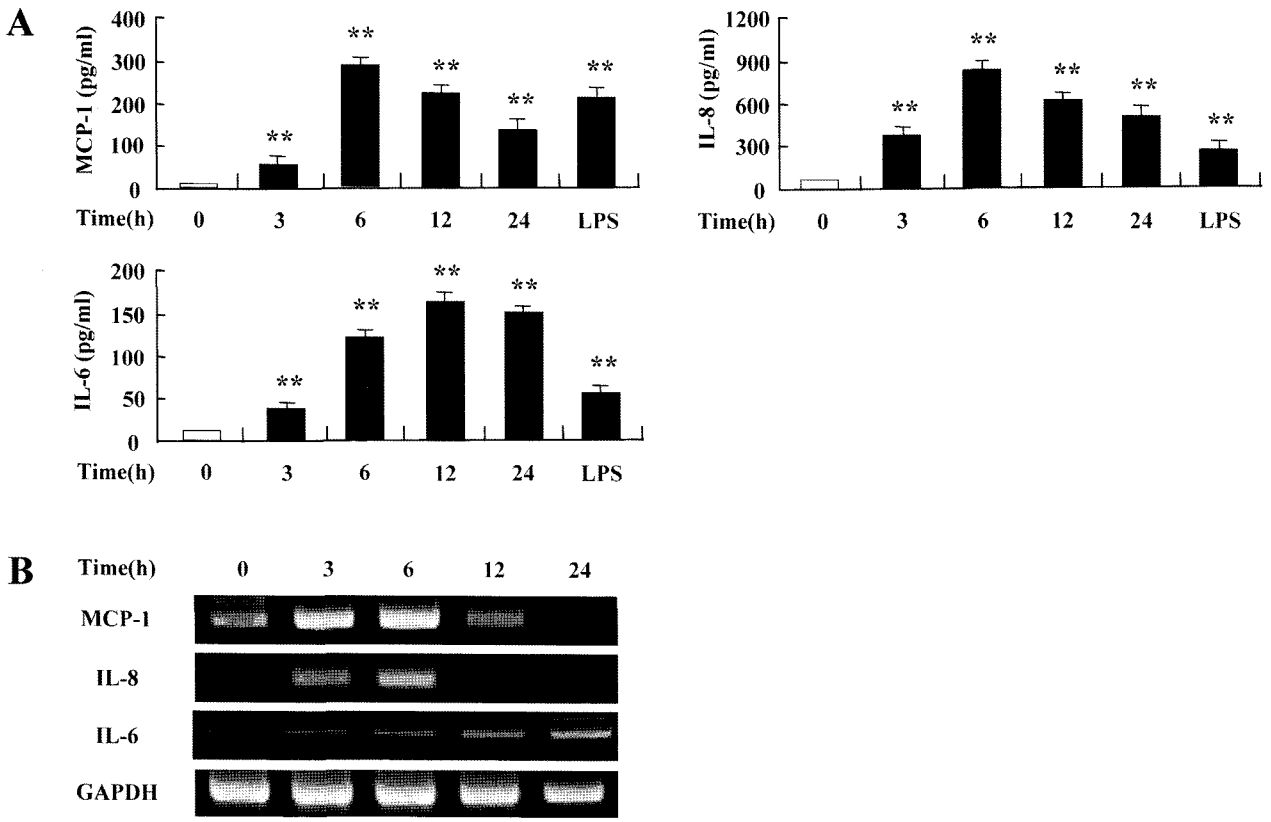

Fig. 1. DpE elevates the expression of MCP-1, IL-8 and IL-6 in a time-dependent manner. A, Serum starved EoL-1 cells were stimulated with 1 μg/mL DpE or LPS for the indicated time. The supernatnat was collected and analyzed by ELISA as described in the Methods section. $^{**}P<0.01$ was considered a significant difference between the untreated group and DpE-treated group or between the untreated group and LPS-treated group. B, Total RNA was extracted from the harvested cells. RNA levels of MCP-1, IL-8 and IL-6 were analyzed by RT-PCR as described in the Methods section. The bands were normalized with GAPDH. Data are expressed as representative of three individual experiments.

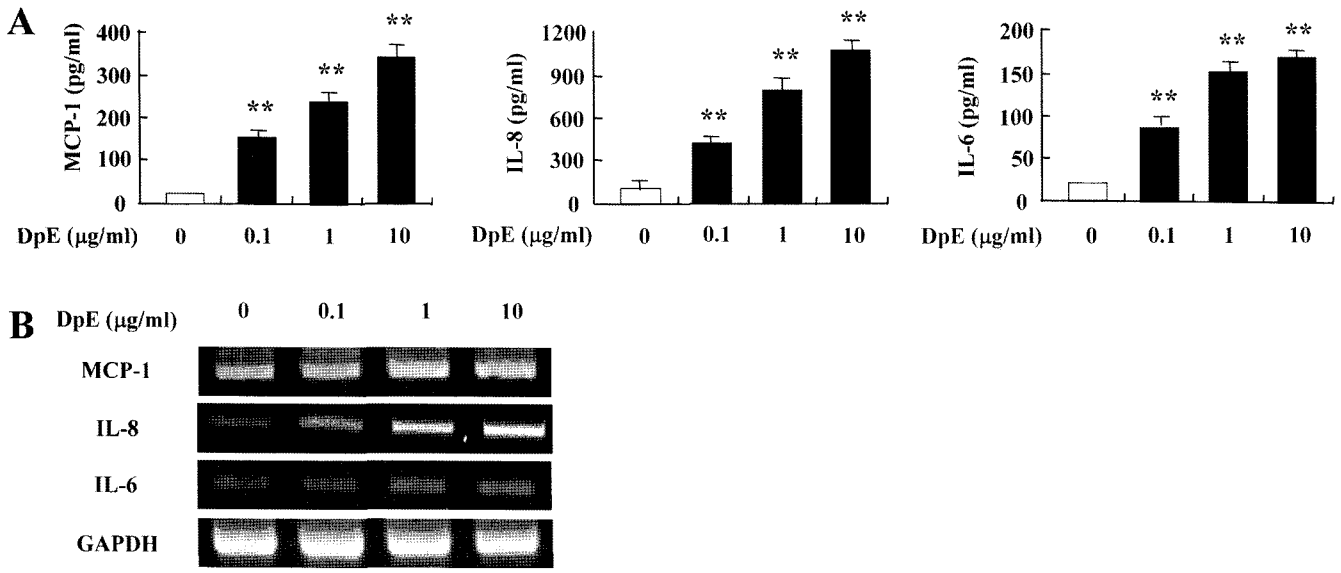

Fig. 2. DpE increases the production of MCP-1, IL-8 and IL-6 in a dose-dependent manner. A, Serum starved EoL-1 cells were stimulated with the indicated dose of DpE for 6 h, for detection of MCP-1 and IL-8, or 12h, for IL-6 detection. The supernatnat was collected and analyzed by ELISA as described in the Methods section. $^{**}P<0.01$ was considered a significant difference between the untreated group and DpE-treated group. B, Total RNA was extracted from the harvested cells. RNA levels of MCP-1, IL-8 and IL-6 were analyzed by RT-PCR as described in the Methods section. The bands were normalized with GAPDH. Data are expressed as representative of three individual experiments.

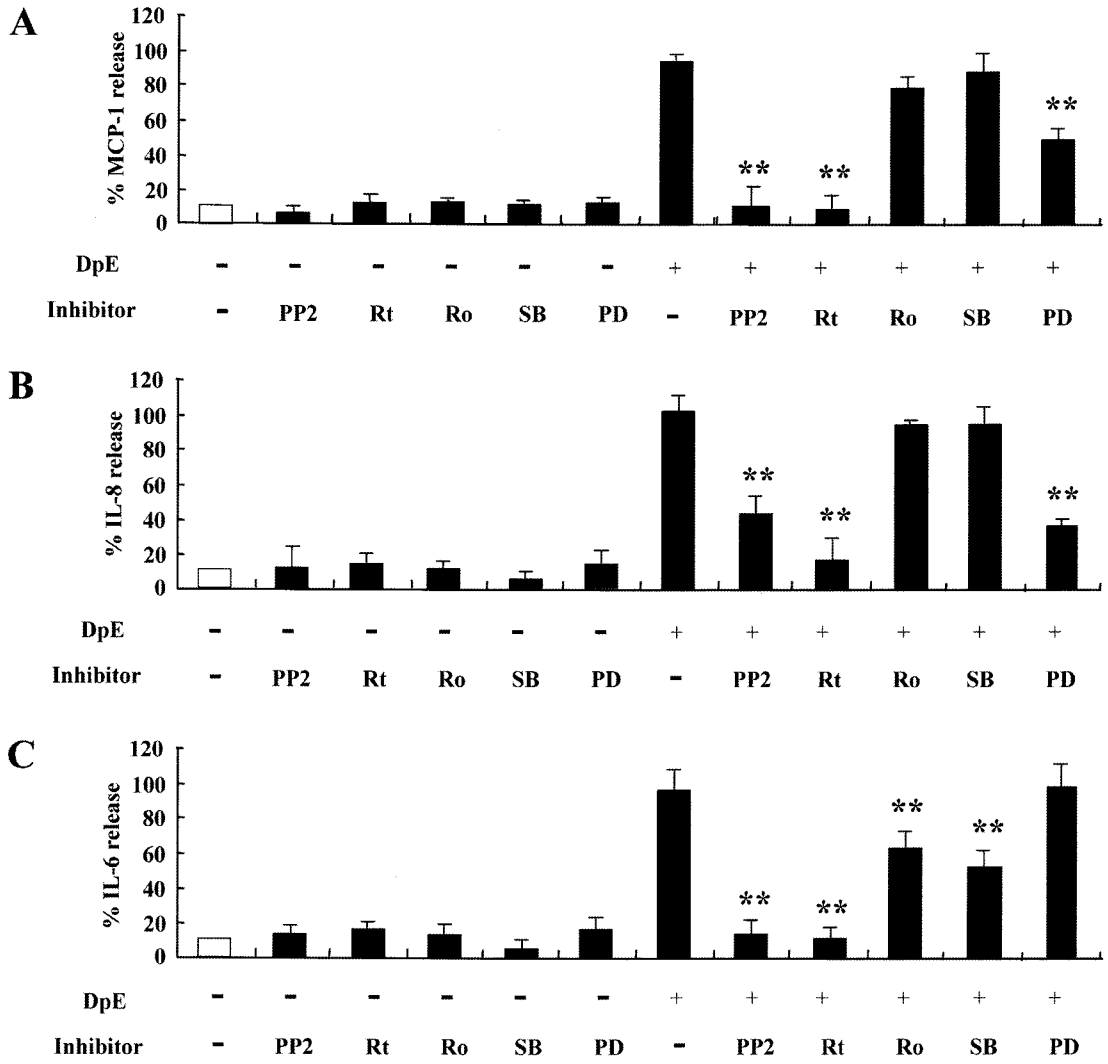

Fig. 3. DpE transduces its effect on MCP-1, IL-8 and IL-6 expression via Src family tyrosine kinase, PKC δ and MAPK. Serum starved EoL-1 cells were pre-incubated in the absence or presence of 5 iM PP2, 5 μM rottlerin (Rt), 100 nM Ro-31-8425 (Ro), 5 μM SB202190 (SB) or 5 μM PD98059 (PD) for 30 min. After treatment with 1 μg/mL DpE for for 6 h, for detection of MCP-1 (A) and IL-8 (B), or 12 h, for IL-6 detection (C). The supernatant was collected and analyzed by ELISA as described in the Methods section. Data are expressed as the means±SD of three independent experiments and are presented as the percentage of cytokine concentration (the cytokine level in the DpE-treated cells is set at 100%). $^{**}P<0.01$ was considered a significant difference between DpE-treated group and inhibitor-treated group.

Involvement of Src family tyrosine kinase, PKC δ, and MAPK in cytokine release of MCP-1, IL-8, and IL-6 as induced by DpE

To further characterize how DpE induces the cytokine release in EoL-1 cells, we examined the signaling proteins linked to this mechanism. EoL-1 cells were pre-treated with PP2, an inhibitor of Src family tyrosine kinase, and with rottlerin, a PKC δ blocker before the stimulation of DpE. Both inhibitors significantly decreased MCP-1, IL-8, and IL-6 ($P<0.01$) (Fig. 3). PD98059, an upstream inhibitor of ERK, inhibited both MCP-1 and IL-8 secretion. DpE-induced IL-6 expression was blocked by SB202190, an inhibitor of p38 MAPK. The specific inhibitors have no effect on cytokine production in the absence of DpE. These results indicate that Src family tyrosine kinase and PKC δ are associated with the signal transduction triggered by DpE, which leads to the up-regulation of MCP-1, IL-8, and IL-6. The involvement of p38 MAPK and ERK depends on one kind of increased cytokine. In a dose-dependent experiment using the aforementioned inhibitors, we were able to confirm that the signal proteins are involved in cytokine expression (Fig. 4). The data from Fig. 4 are consistent with the data shown in Fig. 3.

Activation of ERK and p38 MAPK via Src family tyrosine kinase and PKC δ in response to DpE

We examined the phosphorylation of ERK and p38 MAPK by performing Western blotting because PD98059 and SB202190 blocked the increased expression of cytokine. The phosphorylation of ERK and p38 MAPK reached their

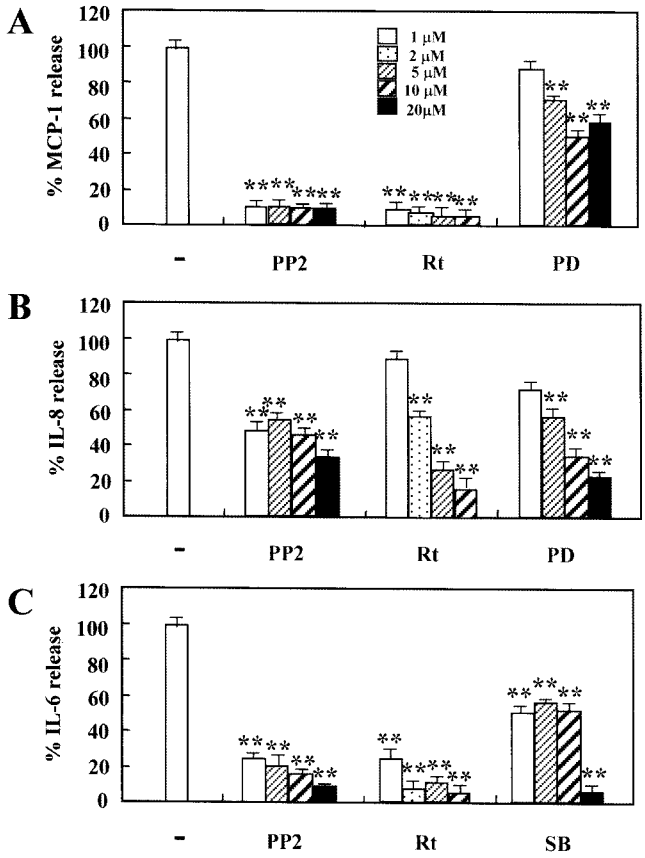

Fig. 4. The increased expression of MCP-1, IL-8 and IL-6 is inhibited by PP2, rotterin, PD98059 or SB202190 in a dose-dependent course. Serum starved EoL-1 cells were pre-incubated in the absence or presence of PP2, rottlerin (Rt), PD98059 (PD) or SB202190 (SB) in the indicated concentration for 30 min. After treatment with 1 μg/mL DpE for for 6 h, for detection of MCP-1 (A) and IL-8 (B), or 12h, for IL-6 detection (C). The supernatant was collected and analyzed by ELISA as described in the Methods section. Data are expressed as the means±SD of three independent experiments and are presented as the percentage of cytokine concentration (the cytokine level in the DpE-treated cells is set at 100%). $^{**}P<0.01$ was considered a significant difference between DpE-treated group and inhibitor-treated group.

maximum level 1 h after stimulation with DpE (Fig. 5). The activation of ERK and p38 MAPK was blocked by both PP2 and rottlerin. These results indicate that Src family tyrosine kinase and PKC are upstream molecules of activation of ERK and p38 MAPK due to DpE.

DISCUSSION

Recent studies about eosinophils and house dust mite show that they play important roles in the pathogenesis of allergic diseases. In this study, we investigated whether DpE is effective in the up-regulation of cytokine expression in EoL-1 cells as an in vitro model of human eosinophils. We demonstrated that DpE increases both mRNA expression and protein secretion of MCP-1, IL-8, and IL-6 (Figs. 1 and 2) and unveiled the associated mechanism where Src family tyrosine kinase and PKC δ are commonly involved in the increased expression of MCP-1, IL-8, and IL-6. ERK activation is required for both MCP-1 and IL-8 expression, while p38 MAPK activation is needed for IL-6 expression. The chemokines MCP-1 and IL-8 produced by the eosinophils have been associated with a number of inflammatory diseases (Mukaida et al.,1998; Terakawa et al., 2006; Steube et al., 2000). MCP-1 is a powerful monocyte chemoattractant. IL-8 production has been observed in eosinophilic diseases such as atopic dermatitis and asthma. IL-6 is known as a pleiotropic cytokine and is associated with cell proliferation, movement, and anti-apoptosis depending on the cell type (Hirano, 1998). In our experiments, we found that DpE increases the protein secretion of MCP-1, IL-8, and IL-6 more effectively than does LPS. The increase of the cytokines is caused by mRNA expression, not by the secretion of protein-containing granules. Our previous study reported that DpE-induced cytokine release in EoL-1 cells is less than in THP-1 cells, although the results have proven that DpE is involved in the regulation of cytokine release by eosinophils (Lee et al., 2008). For the detailed mechanism on cytokine expression due to DpE, we investigated the alternation of cytokine expression using signal protein-specific inhibitors. We found that DpE increases the expression of MCP-1, IL-8, and IL-6 via Src family tyrosine kinase and PKC δ (Figs. 3 and 4). Cationic stimulation activates Src family protein kinase including Src, Lyn, and Hck. This signal leads to the induction of the release of eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (Adamko et al., 2008). The Src family protein kinase is also involved in the granule release induced by IL-8 (Barlic et al., 2000). PKC δ is a novel PKC isoform of serine/threonine kinase that acts as an important regulator in human eosinophils. PKC δ in mature eosinophils increases NADPH oxidase activity and superoxide production after PMA, IL-5, or LTB4 treatment (Bankers-Fulbright et al., 2001; Sepulveda et al., 2005; Sano et al., 2005). For the first time, we found that Src family kinase and PKC δ in human eosinophils are associated with cytokine expression, an expression which is induced by DpE. After Src family protein kinase and PKC δ activation, DpE triggers different signals for the expression of MCP-1, IL-8, and IL-6. While ERK plays a key role in MCP-1 and IL-8 induction, p38 MAPK functions as an important molecule in IL-6 expression. Although DpE activates common upstream molecules such as Src family protein kinase and PKC δ for cytokine up-regulation, the process of the different downstream signals by DpE needs to be elucidated. As shown in Fig. 3C, Ro-31-8425 weakly inhibits IL-6 secretion. Further experiments will be needed to better understand the underlying mechanisms.

House dust mite has been known as one of the major factors in inducing allergic diseases. A variety of allergens

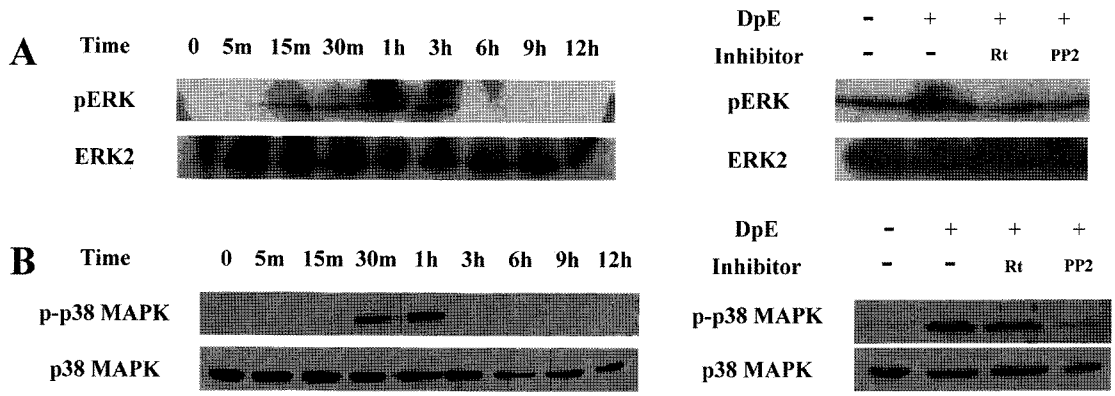

Fig. 5. DpE induces both ERK and p38 MAPK activation through Src family tyrosine kinase and PKC δ. Serum starved EoL-1 cells were stimulated with 1 μg/mL DpE for the indicated time (left panel) or EoL-1 cells were pre-incubated in the absence or presence of 10 μM PP2 or 5 μM rottlerin (Rt) before treatment with 1 μg/mL DpE for 30 min (right panel). Proteins were extracted, separated on 10% SDS–polyacrylamide gels (50 μg/lane) and transferred to nitrocellulose membrane. The degree of activation was detected by Western blotting with anti-phopho-ERK (pERK) (A) or anti-phospho-p38 (p-p38 MAPK) antibody (B). The membrane was stripped and reprobed with anti-ERK2 or anti-p38 antibody as an internal control.

such as serine and cysteine proteases is included in mite extracts and different groups of mite are classified on the basis of their protease activity (Thomas et al., 2002). However, we used the total extract of mite in this study because mite extract increases cytokine production through a protease-independent mechanism as shown in recent papers (Lee et al., 2008; Kauffman et al., 2006). In addition, both serine and cysteine inhibitors have no effect on the increased cytokine expression (data not shown).

In conclusion, the study has shown that DpE induces MCP-1, IL-8, and IL-6 secretion. DpE transduces the signal of MCP-1 and IL-8 production via Src family tyrosine kinase/PKC δ/ERK pathway and IL-6 expression because DpE requires an Src family tyrosine kinase/PKC δ/p38 MAPK cascade. This study has helped to reveal the pathogenic mechanism of eosinophil-associated diseases caused by *D. pteronissinus*, which includes deviations of cytokine expression. The elucidated mechanism may contribute to the understanding of the process of and therapy for eosinophilic diseases such as allergic diseases.

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