EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 32, No. 2, 67-71, June 2000

Major house dust mite allergen, *Der p I*, activates phospholipase D in human peripheral blood mononuclear cells from allergic patients: Involvement of protein kinase C

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Accepted 19 May 2000

Abbreviations: PC, phosphatidylcholine; PEt, phosphatidylethanol; PLD, phospholipase D; HDM, house-dust-mite; PKC, protein kinase C; PLC, phospholipase C; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; PA, phosphatidic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; $InsP_3$, inositol 1,4,5-triphosphate

Abstract

The major house-dust-mite allergen, *Der p I*, stimulates the phospholipase D (PLD) in peripheral blood mononuclear cells (PBMC) from allergic patients with maximal responses after 30 min exposure. At 30 min, *Der p I* stimulated PLD activity by 1.4-fold in mild, 1.6-fold in moderate and 2-fold in severe allergic patients over control values ($p < 0.05$). When the cells were pretreated for 24 h with phorbol myristate acetate to down-regulate protein kinase C (PKC), PLD stimulation by *Der p I* was largely abolished. These results indicate that in PBMC from allergic patients, *Der p I* can stimulate PLD activity, and that PKC activation is involved in this stimulation.

Keywords: *Der p I*, phospholipase D, protein kinase C, allergy, PBMC

Introduction

House dust mites (HDM) are the most common source of aeroallergens, which can cause clinical symptoms ranging from extrinsic asthma to atopic dermatitis (Hoyne *et al*., 1996). One of the major mite allergens, designated *Der p I*, has been purified from *D. pteronyssinus* and shown to react with anti-mite IgE antibodies in up to 80% of allergic sera (Lind and Lowenstein, 1983; Krillis *et al*., 1984). High levels of anti-*Der p I* IgE, IgA and IgG are present in 90% of patients who have positive skin tests to *Der p I* extracts, whereas nonsensitized subjects have only low titers of anti *Der p I* IgG (Platts and Chapman, 1987; Roche *et al*., 1997).

Der p I is the first HDM allergen cloned by recombinant DNA technology (Chua *et al*., 1988; Dilworth *et al*., 1991). The cDNA analysis showed that they are cysteine proteases in the same family as papain and actinidin and have 222 residues with a calculated MW of 25 kD (Thomas and Smith, 1998). The cysteine protease activity of *Der p I* increases airway mucosal permeability, and may thereby contribute to the pathogenesis of airway inflammation and hyperresponsiveness by a nonimmunological mechanism (Roche *et al*., 1997).

It has been reported that *Der p I* induces proliferative T cell responses in the majority of patients with asthma and atopic dermatitis, who are sensitized to *Der p I* (Rawle *et al*., 1984; OBrien *et al*., 1992). Recently, several investigators have suggested that the mite-specific proliferating T cells are mainly type 2 T-helper (Th2) cells secreting interleukin-4 and -5 (Looney *et al*., 1994).

Despite these increasing data, very little attention has been given to the potential intracellular signaling mechanisms of the pathogenesis induced by HDM allergens. It is well known that ligation of the antigen receptors on T cells (TCR) results in the activation of key signal transducing systems such as the phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C-γ (PLC-γ) and increase of intracellular free Ca^{2+} concentration (Howe and Weiss, 1995). However, T cell activation, via the TCR, has been shown to occur in the absence of activation of PLC (ORourke and Mescher, 1988). Moreover, the growing body of evidence has indicated that PLC-mediate hydrolysis of PIP_2 is not sufficient to stimulate cell proliferation (Exton, 1994).

In recent years, phosphatidylcholine (PC)-hydrolysis by phospholipase D (PLD) has been suggested to play an important role in proliferative responses of a wide range of mitogens and growth factors (Exton, 1997). PLD catalyzes the hydrolysis of phospholipids, mainly PC, resulting in the formation of phosphatidic acid (PA), which acts by itself as a cellular messenger (Exton, 1990) or

can be transformed by PA phosphohydrolase into diacylglycerol (DAG), which is essential for the activation of protein kinase C (PKC) (Nishizuka, 1995). More recently, PLD has been shown to be coupled to TCR in the leukemic T cell line, Jurkat, resulting in the induction of proliferation of T cells (Reid *et al*., 1997).

In this study, we report that the HDM allergen, *Der p I* induces PLD activation in PBMC isolated from allergic patients. In addition, we also demonstrate that the activation of PLD is partially regulated by PKC. These results suggest a potentially important role of PLD in *Der p I*-induced allergenic pathogenesis.

Materials and Methods

Subjects

Seven patients who referred with symptoms of allergic diseases and had positive skin prick tests to house dust mite extracts (*Dermatophagoides farinae* and *Dermatophagoides pterynyssinus*) were studied. The mean age of the patients was 27 years old (range: 22 to 34 years). Among the healthy people who do not have any clinical and family history of allergy, three healthy volunteers with serum IgE values less than 150 IU/mL were included.

Skin prick tests and Serum IgE levels

Skin prick tests were performed with allergen extracts of house dust mite (ALK, Denmark & Greer Lab, USA). Histamine was used as a positive control (10 mg/ml), and a diluent of an unbuffered saline containing 0.03% human serum albumin (ALK, Copenhagen, Denmark), was used as a negative control. Reactions to each skin test solution were measured 15 min after the pricks. The contours of each wheal reaction were outlined with a fine filter tip pen. The contours were then transferred to the record sheet by means of translucent tapes. The size of each wheal is documented as the mean of the longest diameter (a) and the diameter perpendicular to it at its mid-point (b): (a + b)/2. Wheals with more than half of the histamine reaction after subtracting the response to the diluent control were considered as expressions of clear-cut positivity. Total serum IgE was measured by the paper radioimmunosorbent test (Kabi-Pharmacia, Uppsala, Sweden). The lower detection limit of IgE was < 1.5 IU/ml.

PBMC culture

Heparinized blood (100~150 ml) was collected from donors sensitized or nonsensitized to *Der p I* and peripheral blood mononuclear cells (PBMC) were purified from heparinized blood by density gradient centrifugation as previously described (Nasert *et al*., 1996). The cells were washed three times in Hank's balanced salt solution and cultured at 37°C in RPMI-1640 medium (Gibco BRL, USA) supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells grown at 37°C in a humidified CO_2 controlled (5%) incubator were then washed with RPMI-1640 medium containing 0.1% (w/v) bovine serum albumin, 100 units/ml penicillin and 100 μg/ml streptomycin (serum-free medium) and incubated in a serum-free medium at 37°C for 1 day before the treatment with *Der p I* (No. 70720, Allergopharma Joachim Ganzer, Hamburg, Germany).

Determination of PLD activity

PLD activity was determined by the formation of PEt as described elsewhere (Kim *et al*., 1999). Briefly, PBMC cultured on 6-well plates were metabolically labeled with 1 μCi/ml of [^{3}H]palmitate in serum free medium for 18 h. The cells were then pretreated with 1% ethanol for 15 min before the treatment with *Der p I*. In some experiments the cells were treated with phorbol myristate acetate (PMA) (100 nM) for 24 h to down-regulate PKC before labeling with [^{3}H]palmitate. After treatment of *Der p I* for the designated time, the cells were quickly washed with ice-cold PBS and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer (Bligh and Dyer, 1959), and PEt was separated by a thin layer chromatography using a solvent system of ethyl acetate/iso-octane/acetic acid/water (110:50:20:100, v/v) (Park *et al*., 1999). The regions corresponding to the authentic PEt bands were identified with 0.002% (w/v) primulin in 80% (v/v) acetone, scraped and counted using a liquid scintillation counter.

Results

The patients were divided into three categories (severe,

Table 1. The Characteristics of Subjects

	Pt No.	Skin prick test	sIgE (IU/mL)	Clinical History
Severe	2	D.f (++++), D.p (++++)	3,227 ± 179.5	AR, AS, AD, AA
Moderate	2	D.f (+++), D.p (+++)	2,378 ± 238.3	AD, AR
Mild	3	D.f (+), D.p (++)	1,354 ± 155.7	AD, AR
Normal	3	Normal	43 ± 5.5	None

D.f: Dermatophagoides farinae, D.p: Dermatophagoides pterynyssinus, AA: allergic asthma, AR: allergic rhinitis, AS: allergic sinusitis, AD: atopic dermatitis, sIgE: serum total IgE level.

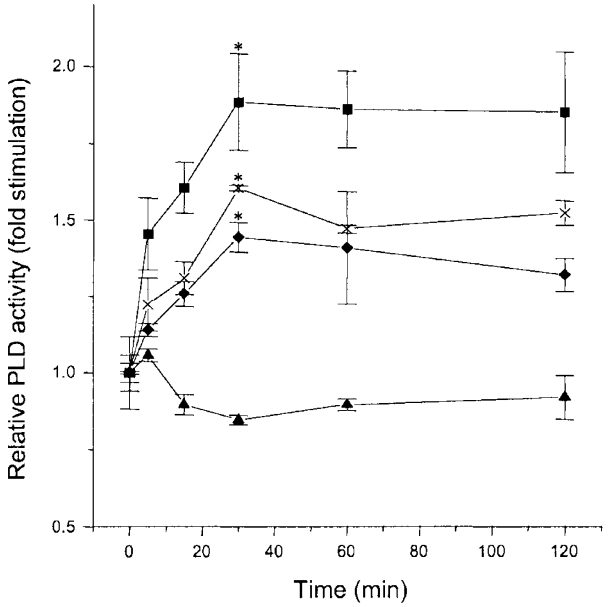

Figure 1. The effects of *Der p* 1 on PLD activity in PBMC from normal (▲) and allergic patients (severe, ■; moderate, ✕ ; mild, ◆). PBMC were labeled with [^{3}H]palmitate and then treated with *Der p* 1 for the time indicated. Relative PLD activity was determined by the measurement of [^{3}H]phosphatidylethanol formation in the presence of 1% ethanol and expressed as a percentage of [^{3}H]phospholipids. Results are the mean ± SD from the experiment performed in triplicate. Statistics were determined using one-way ANOVA. *, $p < 0.05$ (*vs* normal).

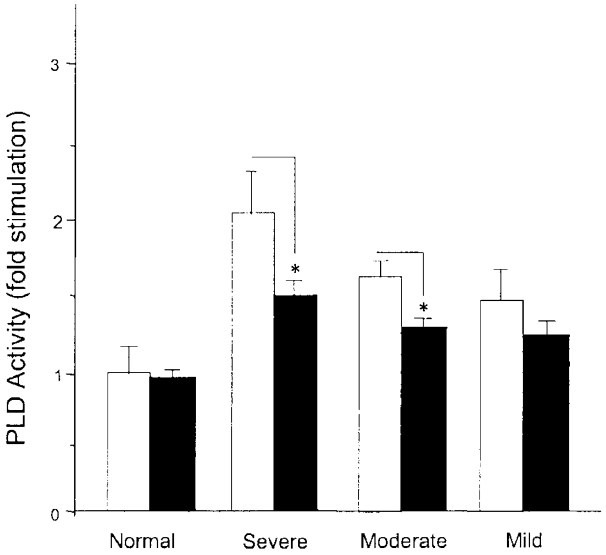

Figure 2. Effects of PKC down-regulation on *Der p* 1-mediated PLD activity. PBMC were treated in the absence (□) or presence (■) of 100 nM PMA for 24 h. The cells were then labeled with [^{3}H]palmitate before treatment with *Der p* 1 (10 μg/ml) for 30 min, followed by the measurement of relative PLD activity. Results are the mean ± SD from the experiment performed in triplicate. Statistics were determined using one-way ANOVA. *, $p < 0.05$ (*vs* PMA none treatment).

moderate and mild) on the basis of clinical severity. The total IgE level in serum of severe allergic patients (3,227 ± 179.5 IU/ml) was significantly higher than in those of moderate (2,378 ± 238.3 IU/ml) and mild (1,354 ± 155.7 IU/ml) allergic patients and normal control (43 ± 5.5 IU/ml) as shown Table 1.

The effects of *Der p I* (10 mg/ml) on PLD activity in PBMC from normal individuals (as a control) and allergic patients after various times of exposure are illustrated in Figure 1. A significant effect on PLD activity was seen in PBMC from allergic patients after 20 min of treatment with *Der p I*. The PLD activity was increased above normal values after *Der p I* treatment at each time point examined, with maximal responses after 30 min exposure. At 30 min, *Der p I* stimulated PLD activity by 1.4-fold in mild, 1.6-fold in moderate and 2-fold in severe allergic patients over control values ($p < 0.05$).

To determine whether *Der p I*-induced PLD stimulation occurred as a consequence of PKC activation, PBMC were exposed to PMA (100 nM) for 24 h before the treatment of *Der p I*. The results are illustrated in Figure 2. PKC down-regulation by the long-term treatment of PMA had no intrinsic effect on basal PLD activity. PLD activity in allergic PBMC that were previously down-regulated was significantly diminished compared to that in *Der p I*-treated non-PKC down-regulated cells ($p < 0.05$). Although PLD activity in PKC down-regulated cells of allergic patients was still significantly higher than that in the equivalent normal PBMC ($p < 0.05$), PKC down-regulation decreased the extent of activation of PLD by *Der p I* by about 50%.

Discussion

The present study demonstrates that the house-dust-mite allergen, *Der p I*, stimulates PLD activity in PBMC from allergic patients. PLD activity was measured through the formation of PEt from labeled phospholipids, predominantly PC, which is considered the definitive assay for PLD activity (Gomez-Munoz *et al.*, 1994; Liscovitch and Chalifa, 1994).

Although our results were obtained from PBMC instead of enriched specific cell types, according to previous results (Stanford *et al.*, 1991; Bacon *et al.*, 1998), *Der p I*-induced PLD activation was probably involved in T cell activation. For several years, it appeared that the early events of T cell activation were involved with PLC that catalyze the hydrolysis of PIP_2 into the second messenger, inositol 1,4,5-triphosphate (IP_3) and DAG (Berridge and Irvine, 1984). However, several subsequent studies have emphasized the role of tyrosine phosphorylation events in T cell activation, and two recent studies have cast doubt on the necessity of phosphoinositide hydrolysis for T cell activation (June *et al.*, 1990; Mustelin *et al.*, 1990). Several investigators have suggested that activation of PLD resulting in PA production may be an important intracellular signaling pathway and that the PA

has mitogenic properties for various cell types (Exton, 1994). Furthermore, more recent reports demonstrate that the antigen receptors on mature and immature T cells were coupled to PLD activation and that this activation is dependent upon PKC (Reid *et al*., 1997).

In the present experiments, *Der p I*-induced PLD activation partially occurred as a consequence of PKC activation. This conclusion is based on the following results. *Der p I*-induced PLD activation was significantly abrogated by prior long-term exposure to PMA, which has been demonstrated to lead to PKC down-regulation in various cell types (Kraft *et al*., 1994). The expression of mRNA for PKC-α, -β, -ε, and -ζ, but not-γ or -δ isozymes, has been detected in T cells (Freire-Moar *et al*., 1991). Among these isotypes, the most striking changes were observed in PKC-ε isozyme in T cells activation (Harris *et al*., 1995). As PKC-ζ is phorbol independent and not responsive to PKC down-regulation (Wang *et al*., 1995), we could hypothesize that PKC-α or PKC-β alone or in conjunction with PKC-ε mediates *Der p I*-induced PLD activity. The relationship between PKC isoforms and PLD activity is the subject of ongoing study in our laboratory.

In our present study, even if the PKC was down-regulated, *Der p I*-induced PLD activation was not completely inhibited, implying that another activation mechanism was needed for *Der p I*-induced PLD activation. PLD activity can be regulated by multiple types of signals (Liscovitch and Chalifa, 1994). Both heterotrimeric and low molecular weight G proteins have been implicated in PLD activation. Many investigators suggested a key role for the involvement of the small molecular weight G proteins ADP-ribosylation factor (ARF) and RhoA in agonist-induced PLD activation in certain cell types (Gomez-Cambronero and Keire, 1998). A recent report demonstrated that the potent agonist of T cell activation, RANTES, induced potent PLD activation by the activation of ARF and RhoA (Bacon *et al*., 1998). However, it is not yet clear whether these small molecular weight G proteins may be the regulator for *Der p I*-induced PLD activation.

In conclusion, the data presented in this report demonstrates that the HDM allergen, *Der p I*, induces activation of PLD in PBMC from allergic patients. The present study also suggests that *Der p I*-induced stimulation of PLD activity is partially dependent upon PKC. The exact mechanism regulating PLD in *Der p I*-stimulated PBMC from allergic patients remains to be dissected.

Acknowledgements

This study was supported financially by the Korea Science and Engineering Foundation (KOSEF) & Ministry of Science and Techonology (MOST) through the Research Center for Bio-Medicinal Resources (Bio-MedRRC), Pai-Chai University, Taejon, Korea

References

Bacon, K. B., Schall, T. J. and Dairaghi, D. J. (1998) RANTES activation of phospholipase D in Jurkat T cells: requirement of GTP-binding proteins ARF and RhoA. *J. Immunol.* 160: 1894-1900

Berridge, M. J. and Irvine, R. F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312: 315-321

Bligh, K. M. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. *J. Biochem. Physiol.* 37: 911-917

Chua, K. Y., Stewart, G. A., Thomas, W. R., Simpson, R. J., Dilworth, R. J., Plozza, T. M. and Turner, K. J. (1988) Sequence of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J. Exp. Med.* 167: 175-182

Dilworth, R. J., Chua, K. Y. and Thomas, W. R. (1991) Sequence analysis of cDNA coding for a major house dust mite allergen, Der f 1. *Clin. Exp. Allergy* 21: 25-32

Exton, J. H. (1990) Signaling through phosphatidylchone breakdown. *J. Biol. Chem.* 265: 1-4

Exton, J. H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochem. Biophys. Acta* 1212: 26-34

Exton, J. H. (1997) Phospholipase D: enzymology, mechanism of regulation, and function. *Physiol. Rev.* 77: 303-320

Freire-Moar, J., Cherwinski, H., Hwang, F., Ransom, J. and Webb, D. (1991) Expression of protein kinase C isozymes in thymocyte subpopulations and their differential regulation. *J. Immunol.* 147: 2405-2409

Gomez-Cambronero, J. and Keire, P. (1998) Phospholipase D: A novel major player in signal transduction. *Cell. Signal.* 10: 387-397

Gomez-Munoz, A., Martin, A., OBrien, L. and Brindley, D. N. (1994) Cell-permeable ceramides inhibit the stimulation of DNA synthesis and phospholipase D activity by phosphatidate and lysophosphatidate in rat fibroblasts. *J. Biol. Chem.* 269: 2937-2943

Harris, W., Gollapudi, S. and Gupta, S. (1995) Anti-CD3-induced changes in protein kinase C isozymes expression in human CD4+ and CD8+ T lymphocytes. *J. Clin. Immunol.* 15: 232-241

Howe, L. R. and Weiss, A. (1995) Multiple kinases mediate T cell receptor signaling. *Trends Biochem. Sci.* 20: 59-67

Hoyne, G., Brourne, T., Kristensen, N., Hetzel, C. and Lamb, J. (1996) From epitope to peptides to immunotherapy. *Clin. Immunol. Immunopathol.* 80: S23-S30

June, C. H., Fletcher, M. F., Ledbetter, J. A. and Samelson, L. E. (1990) Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimu-

lation. *J. Immunol.* 144: 1591-1599

Kim, J. -H., Yoon, Y. -D., Shin, I. and Han, J. -S. (1999) Effects of ceramide, the Fas signal intermediate, on apoptosis and phospholipase D activity in mouse ovarian granulosa cells in vitro. *IUBMB Life* 48: 445-452

Kraft, A. S., Anderson, W. B., Cooper, H. L. and Sando, J. J. (1982) Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. *J. Biol. Chem.* 257: 13193-13196

Krillis, S., Baldo, B. A. and Basten, A. (1984) Antigen and allergens from the common house dust mite *Dermatophagoides pteronyssinus*. Part II. Identification of the major IgE binding antigen by crossed radioimmuno-electrophoresis. *J. Allergy Clin. Immunol.* 74: 142-150

Lind, P. and Lowenstein, H. (1983) Identification of allergens in *Dermatophagoides pteronyssinus* mite body extract by crossing radioimmuno-electrophoresis with two different rabbit antibody pools. *Scand. J. Immunol.* 17: 263-273

Liscovitch, M. and Chalifa, V. (1994) Signal-activated phospholipase D. In *Signal-Activated Phospholipases* (Liscovitch, M. eds) pp. 31-63, Landes, Austin

Looney, R. J., Pudiak, D. and Rosenfeld, S. I. (1994) Cytokine production by mite-specific T-cells from donors with mild atopic disease. *J. Allergy Clin. Immunol.* 93: 476-483

Mustelin, T., Coggeshall, K. M., Isakov, N. and Altman, A. (1990) T cell antigen receptor-mediated activation of phospholipase C require tyrosine phosphorylation. *Science* 247: 1584-1587

Nasert, S., Burtchen, N. and Kussebe, F. (1996) Stimulation of IgE and IgA production by CD45RA T helper cells in atopic patients. *J. Immunol.* 157: 441-448

Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular response. *FASEB J.* 9: 484-496

OBrien, R. M., Thomas, W. R. and Wooton, A. M. (1992) T-cell responses to the purified major allergens from the house dust mite *Dermatophagoides pteronyssinus*. *J. Allergy Clin. Immunol.* 89: 1021-1031

ORourke, A. M. and Mescher, M. F. (1988) T cell receptor-mediated signaling occurs in the absence of inositol phosphate production. *J. Biol. Chem.* 263: 18594-18600

Park, B.-J., Kim, J.-H., Han, J.-S. and Jung, P.-M. (1999) Effect of ceramide on apoptosis and phospholipase D activity in FRTL-5 thyroid cells. *Exp. Mol. Med.* 31(3): 142-150

Platts-Mills, T. A. E. and Chapman, M. D. (1987) Dust mites: immunology, allergic disease, and environmental control. *J. Allergy Clin. Immunol.* 80: 755-775

Rawle, F. C., Mitchell, E. B. and Platts-Mills, T. A. E. (1984) T-cell responses to the major allergen from the house dust mite *Dermatophagoides pteronyssinus*, antigen P 1: comparison of patients with asthma, atopic dermatitis, and perennial rhinitis. *J. Immunol.* 133: 195-201

Reid, P. A., Gardner, S. D., Williams, D. M. and Harnett, M. M. (1997) The antigen receptors on mature and immature T lymphocytes are coupled to phosphatidylcholine-specific phospholipase D activation. *Immunology* 90: 250-256

Roche, N., Chinet, T. C. and Huchon, G. J. (1997) Allergic and nonallergic interaction between house dust mite allergens and airway mucosa. *Eur. Respir. J.* 10: 719-726

Stanford, J., Cunningham, G. R., Strupp, J. A., Henderson, G. S., Exton, J. H. and Bocckino, S. B. (1991) Activation of phospholipase D: a signaling system set in motion by perturbation of the T lymphocyte antigen receptor/CD3 complex. *Cell Regul.* 2: 841-850

Thomas, W. R. and Smith, W. (1998) An update on allergens: Hous-dust-mite allergens. *Allergy* 53: 821-832

Wang, X. D., Kiang, J. G. and Smallridge, R. C. (1995) Identification of multiple protein kinase C isoforms in FRTL-5 thyroid cells. *Thyroid* 5: 137-140