

RESEARCH ARTICLE

# rhPLD2 suppresses chronic inflammation reactions in a guinea pig asthma model\*

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## Abstract

**Background:** Asthma is a complex inflammatory disorder of the airways, and research on alternative therapeutic strategies has attracted attention. This study aimed at hypersusceptibility and toxicity of recombinant human phospholipase D2 (rhPLD2) in guinea pigs. We determined the behavioral responses in the model of immediate hypersensitivity animals and changes of eosinophil levels following use of the drugs. Special attention was given to the effects of rhPLD2 *in vivo* on the guinea pig with chronic persistent asthma and the mechanism involved.

**Methods:** To investigate the effect of rhPLD2 on the expression of protein kinase C (PKC), and to examine the activity of signal transducer and activator of transcription 1 and 5a in the lung of the guinea pig with chronic asthma. Guinea pigs with chronic asthma were divided into five groups: a saline group, a dexamethasone 5.0 mg group, and rhPLD2 (1.5, 2, or 3 mg) groups. Non-sensitized animals were as normal control group. PKC expression was measured by immunohistochemistry, alterations of STAT1 and STAT5a were detected by TransAM transcription factor assay kits.

**Results:** rhPLD2 (3.0 mg) decreased PKC expression to baseline and inhibited STAT1 activity compared with that of the saline group ( $p < 0.01$ ).

**Conclusion:** The rhPLD2 may suppress the chronic inflammatory reaction through down-regulating PKC expression and STAT1/STAT5a activity in the lung. The rhPLD2 may be a suitable therapeutic target for asthma.

**Keywords:** Recombinant human phospholipase D2 asthma, protein kinase C. STAT Family. inflammation

## Introduction

Asthma is increasing in prevalence all over the world. This disease is involved the development of airway inflammation, which is associated with eosinophils, neutrophils, and lymphocytes, as well as the presence of airway hyperresponsiveness. T lymphocytes are critical components in asthma pathogenesis.<sup>(1)</sup>

During lymphocyte activation, phospholipase stimulation occurs downstream of T-cell antigen receptor activation, hydrolyzing phosphatidylinositol 4,5-diphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG), both of which lead ultimately to the activation of protein kinase C (PKC). Therefore, PKC activity may be changed in lymphocytes in bronchial

asthma.<sup>(2,3)</sup> JAK-STAT signaling also regulate T-cell growth and differentiation.<sup>(4,5)</sup> Knockouts of STAT proteins are embryonic lethal or show defects in proliferation and signal transduction.

PLD is found in bacteria, fungi, plants, and animals, and is controlled by multiple hormones, neurotransmitters, growth factors, and cytokines.<sup>(6)</sup> It transforms phosphatidylcholine (PC) into phosphatidic acid (PA) to affect inflammation.<sup>(7)</sup> PLD2 essentially requires phosphatidylinositol bisphosphate for its enzymatic activity. Through the hydrolyzing PC creating choline and PA, this bio-active protein also functions in signaling transduction, and exocytosis in mast cell, which may involved in the release of inflammatory factors. The

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wild-type PLD2 has four conserved functional regions, which were critical for catalysis *in vitro* and for PLD function *in vivo*. PLD affects PKC and JAK-STAT signaling pathways. PLD2 contains PH domain, PX domain, CRI, CRII, CRIII, CRIV and CT domain. Previous studies showed although CRI and CRIV of PLD2 contains a copy of the HKD motif, they can not function alone.<sup>(8–10)</sup> The rhPLD2 is our self-made engineering protein product modified from the wild-type PLD2<sup>(11,12)</sup> □ which contains the main functional domains of PLD2. In the present study, we used guinea pig as a chronic asthmatic model in determining the possible role of rhPLD2 in the treatment with asthma. The results showed that rhPLD2 can significantly repress the activity of p65 when the guinea pigs with chronic asthma.<sup>(13,14)</sup> Therefore, it is important to examine the relationship among rhPLD2, PKC, STAT1, STAT5a, and the role of rhPLD2 in asthma inflammation.

## Materials and methods

### Guinea pig model with chronic asthma

Specific pathogen-free guinea pigs, 200 ± 20 g, were purchased from SLAC (Shanghai, China). Guinea pigs were sensitized by intraperitoneal injection of 250 mg/kg of ovalbumin (OVA) on day 0. After 2 weeks, they were challenged with 1% OVA in double-distilled water as antigen exposures. Filtered air was passed through a Pari BOY nebulizer (Bairui corp. German) to generate an aerosol. The size distribution of the aerosol was determined using a particle counter (Aerodynamic Particle Sizer, TSI). The aerosol sizes were distributed log-normally with a count median aerodynamic diameter of 0.82 µm and geometric SD of 1.46 µm. A mean OVA concentration of 3.8 ng/mL was measured in the chamber during the exposures. It took another 20 days till chronic asthma developed in the guinea pig.

### Experimental protocols

Thirty-five guinea pigs with chronic asthma were stochastically divided into 5 groups, with treatments of intraperitoneal injection of NS, DXM and a series of concentration of rhPLD2 respectively, 45 min before the onset of asthma for three times. Non-sensitized animals were used as the control group (Table 1).

### Collection of specimen

The guinea pigs were killed by cervical dislocation 24 h after the last aeroallergen challenge. The lungs were removed from both non-sensitized and sensitized groups for histological analysis, immunohistochemistry of PKC, and detection of the nuclear proteins, STAT1 and STAT5a. Samples were then centrifuged at 4000 rpm for 10 min at 4°C followed by collection of supernatant into 0.5 mL EP at –80°C.

### Hematoxylin and eosin staining

Lung tissue was fixed in 10% formaldehyde (pH 7.2) and embedded in paraffin blocks. Five-micrometer tissue

sections were mounted on slides, then deparaffinated and rehydrated for histology and immunohistochemistry. Hematoxylin and eosin staining (HE) was then performed.

### Immunohistochemistry

A monoclonal antibody PKC (mouse IgG) specific for a common epitope of PKCα and PKCβ was used. Lung specimens the control and asthmatic guinea pigs were treated in the same way. All samples were coded and sections were analyzed in a blinded manner. Sections were immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Then sections were incubated with 5% bovine serum albumin to reduce background, followed by incubation with the primary antiserum overnight at 4°C. Sections were incubated with biotin-conjugated goat anti-mouse IgG for 45 min. The avidin-biotin-peroxidase complex was added to the sections for 45 min. Immunostaining was visualized by immersion in diaminobenzidine and counterstained with the nuclear stain, hematoxylin. Negative control used PBS instead of primary antibodies. The percentage of immunostained spots was assessed using a computerized image-analysis system (Image Pro Plus, Media Cybernetics, Shanghai, China).

### TransAM STAT family kits for STAT1-DNA, STAT5a-DNA binding

We followed the instructions of the Nuclear and Cytoplasmic Extraction Kit from KANGCHEN. Lung tissue of 100 mg was cut into small pieces, PBS (1–2 mL) was added, and then the tissue was homogenized in a tissue homogenizer. Pellet the cells by centrifugation at 500g for 5 min and the supernatant was removed. The packed cell volume was estimated and 0.2 mL of the CER-A mix was added. Vortex the tube vigorously for 15 seconds to fully resuspend the cell pellet. Incubate the tube on ice for 10 min. Add 11 µL of ice-cold CER-B to the tube. Centrifuge the tube for 5 min at 13,000g in a microcentrifuge. Transfer the supernatant (cytoplasmic extract) fraction to a clean pre-chilled tube. Place this tube on ice until use or storage. Resuspend the insoluble (pellet) fraction, which contains nuclei, in 100 µL of ice-cold NER. Vortex the fraction for 15 s. Return the sample to ice and continue vortexing for 15 s every 10 min, for a total of 40 min. Centrifuge the tube at 13000g in a microcentrifuge for 10 min. Transfer the supernatant (nuclear extract) fraction to a clean pre-chilled tube. Store all extracts at –80°C until use.

Table 1. Treatment and group of animals.

| Group          | Number of animals | Dosage of treatment |
|----------------|-------------------|---------------------|
| Saline group   | 7                 | 1 mL                |
| DXM            | 7                 | 5 mg/kg             |
| rhPLD2         | 7                 | 3.0 mg/kg           |
| rhPLD2         | 7                 | 2.0 mg/kg           |
| rhPLD2         | 7                 | 1.5 mg/kg           |
| Normal control | 7                 | None                |

TransAM STAT family kits (active motif) were used to determine the binding activity of STAT1 and STAT5a. The nuclear extracts were prepared by a nuclear extract Kit (KANGCHEN) and added to multi-well plates with an immobilized oligonucleotide that contained the consensus binding site for the given transcription factor. The quantity of bound transcription factor was then determined with a primary antibody and a secondary antibody conjugated to horseradish peroxidase in ELISA-type assay.

### Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical analysis was performed with SPSS 11.0 (SPSS Inc, Chicago, IL).

## Results

### Histology

Aerosolized OVA induced widespread inflammatory infiltration, primarily around airway walls (bronchi and bronchioli) and blood vessels. Lung tissue in OVA-challenged guinea pigs was also characterized by gross alterations in the structural integrity of the airway walls, epithelial cell shedding, and extensive mucosal edema (Figure 1).

### PKC expression in lung tissues

Immunohistochemistry showed intensive expression of PKC, especially inflammatory cells in the experimental group (Figure 2A). DMX treatment reduced PKC immunoreactivity (Figure 2B). In the rhPLD2 (3.0mg) treated group, an intermediate level of PKC immunoreactivity, less than that of the control but higher than that of DXM (Figure 2C), was observed. The immunostaining in the rhPLD2 (2.0 and 1.5mg) was slightly higher than that of the 3.0mg rhPLD2 (Figure 2D and 2E). The control lung tissue showed the lowest immunoreactivity for PKC (Figure 2F). The negative control showed no PKC staining

(Figure 2G). DXM and rhPLD2 (3.0mg) decreased PKC expression with significant difference compared with the saline group ( $p < 0.01$ ).

### Binding activity of STAT1 and STAT5a

DXM and rhPLD2 (3.0mg) inhibited STAT1 activity for ( $0.2648 \pm 0.0102$ ) and DXM ( $0.2973 \pm 0.0325$ ) (Figure 3A). Lower doses of rhPLD2 (2.0 and 1.5mg) also inhibited STAT1 activity (Figure 3A;  $p < 0.01$ ).

rhPLD2 and DXM also inhibited STAT5a activity significantly at different statistical levels,  $p < 0.05$  for DXM and  $p < 0.01$  for rhPLD2, respectively (Figure 3B).

## Discussion

### rhPLD2 and PKC

PKC is a signal transduction molecule that can influence cell proliferation,<sup>(15)</sup> such as passively affecting HASMC proliferation.<sup>(16)</sup> Asthmatic serum induces HASMCs to release autocrine mitogenic factors that activate phospholipase C (PLC) to hydrolyze PC to DAG and activate PKC persistently. The present study sought to determine the relationship between rhPLD2 and PKC.

OVA increased PKC expression, which could be blocked by DXM, as shown previously.<sup>(17)</sup> rhPLD2 dose-dependently also blocked PKC activation, with similar efficiency to DXM. Overexpression of a catalytically inactive variant of PLD2, but not PLD1, blocked insulin-dependent activation of PLD and phosphorylation of MAPK.<sup>(18)</sup> PLD2 may enhance PKC expression by producing DAG, and rhPLD2 may block this pathway to reduce PKC expression.

### rhPLD2 and STAT

Cytokines affect inflammation via the STATs. OVA induces lymphocyte proliferation via STAT1 and STAT5a activation. Allergens can induce STAT1 and STAT5a phosphorylation on tyrosine residues, which may regulate cell

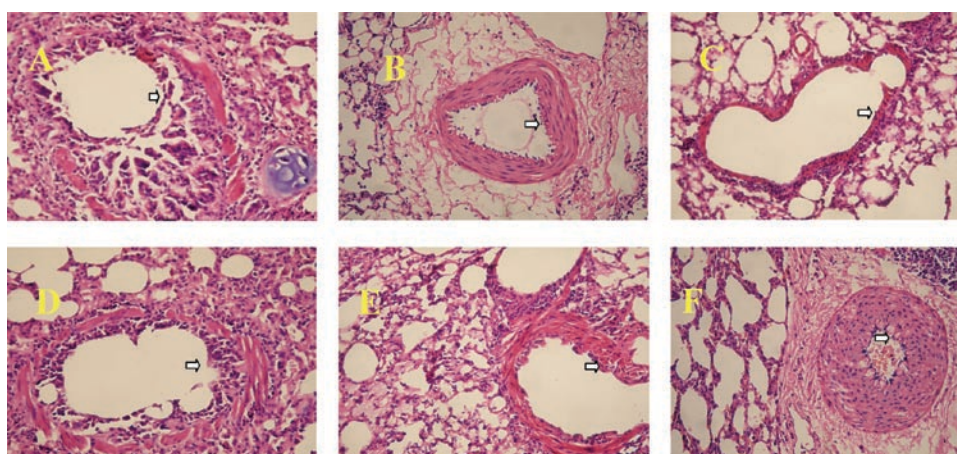


Figure 1. HE staining of lung tissue ( $\times 400$ ). (A) (saline group): Gross alterations in the structural integrity of the airway walls, epithelial cell shedding and extensive mucosal edema. (B) (DXM 5.0mg): Reduction in infiltrating inflammatory cells, epithelial cell shedding, and mucosal edema. (C) (rhPLD2 3.0mg): Similar reductions in infiltrating inflammatory cells, epithelial cell shedding, and mucosal edema, (D) (rhPLD2 2.0mg); (E) (rhPLD2 1.5mg); (F) (normal control): Integrity of airway walls with no epithelial cell shedding or mucosal edema.

proliferation by allergens in asthma.<sup>(19)</sup> In the present study, OVA induced activation of STAT1 and STAT5a in the lung tissue, while DXM could block that induction. rhPLD2 also showed higher ability in blocking that induction.

The role of wild-type PLD2 in signal transduction has been universally accepted. PKC can be activated by PLD2, and the activation of PKC trigger cascade of signal transduction, which results in an activation of some transcript factor.<sup>(20)</sup> The wild-type PLD2 could directly phosphorylate STAT, and this phosphorylation may be blocked by rhPLD2. Alternatively, rhPLD2 may inhibit NF- $\kappa$ B activity to block STAT. We then postulate that

rhPLD2, through gene recombination, has become signal transduction anergy, but hold the receptor site for Rho to compete with the wild-type PLD2 so that the activation of PKC was blocked. In the biological world, there often exists such a phenomenon: the homologue proteins may compete with the same wild-type proteins or the proteins shared with the consensus conformation.<sup>(21)</sup>

Therefore, after extrinsic rhPLD2 went into cytoplasm, owing to its homologue conformation with native PLD2, it may block the transport of native PLD2 so as to reduce the expression of native PLD2 by puzzling the cell recognizing system. Besides the homologue theory, our previous data indicate that rhPLD2 can inhibit the secretion

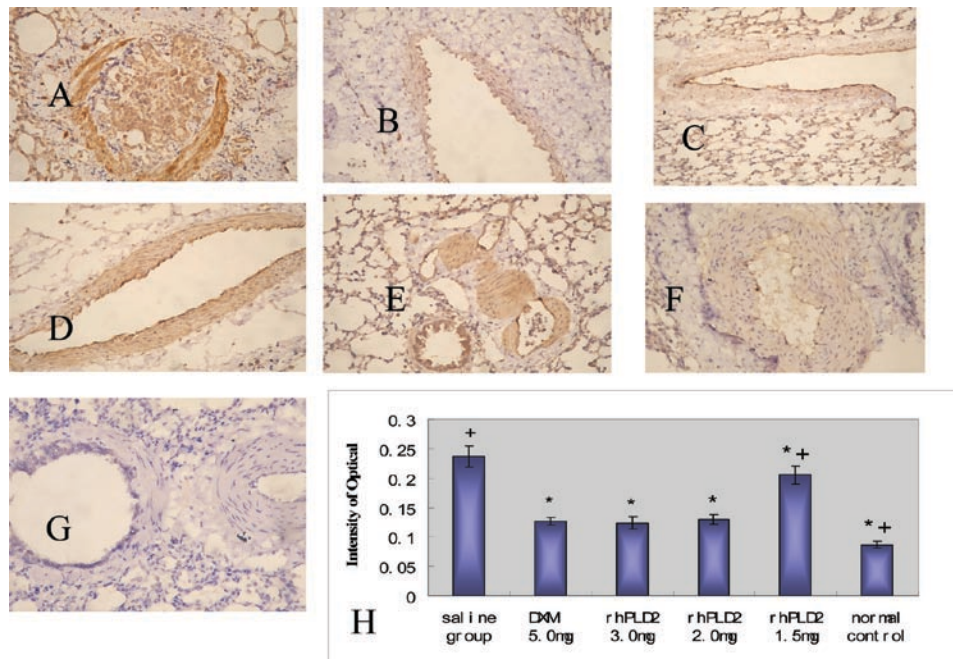


Figure 2. Immunostaining of PKC in lung tissues ( $\times 400$ ). (A) Saline group (asthma); (B) DXM 5.0 mg; (C) rhPLD2 3.0 mg; (D) rhPLD2 2.0 mg; (E) rhPLD2 1.5 mg; (F) normal control; (G) Negative control. (H) Immunohistochemical average optical densities of PKC expression (\*compared with saline group  $p < 0.01$ , +compared with DXM,  $p < 0.01$ ).

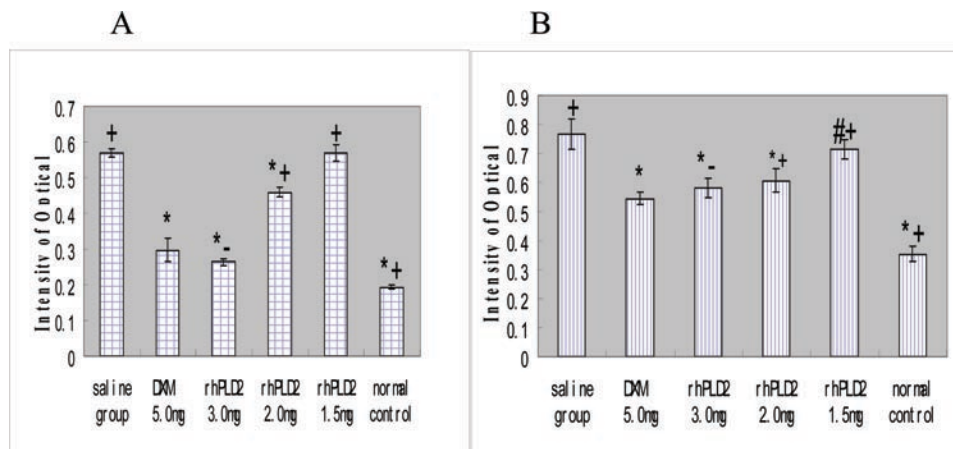


Figure 3. Binding activity of STAT1 and STAT5a. (A) Average optical densities of STAT1 (\*compared with saline group  $p < 0.01$ , +compared with DXM 5.0 mg group,  $p < 0.01$ , —compared with DXM 5.0 mg group,  $p < 0.05$ ). (B) Average optical densities of STAT5a (\*compared with saline group  $p < 0.01$ , #compared with saline group  $p < 0.05$ , +compared with DXM 5.0 mg group,  $p < 0.01$ , —compared with DXM 5.0 mg group,  $p < 0.05$ ).

of PAF, which is a potentially inflammatory mediator. After binding its receptor, PAF can promote to express and exocytose a series of inflammatory proteins. Then the deduction of PAF perhaps contributed to the depression of PKC. Finally, PKC signaling may interact with the STAT signaling pathway,<sup>(22)</sup> and rhPLD2 may inhibit STAT signaling by inhibiting PKC signaling.

In the present study, we found that inflammation increased PKC in airway smooth muscle, increased STAT1 and STAT5a activation in the lung tissue. rhPLD2 could block those changes, indicating a relationship among PKC, STAT1, STAT5a, and rhPLD2 in airway inflammation in asthma. The results also suggested that rhPLD2 may be a suitable therapeutic target for asthma.

## Declaration of interest

The authors report no conflicts of interest.

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