Article

Human Recombinant PLD2 Can Repress p65 Activity of Guinea Pigs of Chronic Asthma *in vivo*

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This article is to investigate the effect of human recombinant phospholipase D2 (rhPLD2) *in vivo* on the expression of nuclear transcription factor p65 in chronic asthma of guinea pigs. After treating the guinea pigs with chronic asthma by rhPLD2, the crude nuclear extraction was assayed with TransAM Transcription Factor Assay Kit for the activity of pulmo tissue nuclear transcription factor p65. Compared with the healthy guinea pigs, the activity of nuclear transcription factor p65 in guinea pigs of chronic asthma is much higher than that of control groups. Our results showed that rhPLD2 markedly depressed the activity of p65 when the guinea pigs were attacked by chronic asthma. *Cellular & Molecular Immunology*. 2006;3(4):307-310.

Key Words: rhPLD2, nuclear transcription factor p65, asthma, guinea pig

Introduction

As a pulmonary disorder, asthma was characterized by the generalized reversible obstruction of airflow and airway hyperresponsiveness, an exaggerated bronchospastic response to nonspecific agents such as methacholine and histamine or specific antigens. More recently, greater concerns have been raised due to the ever-increasing incidence of asthma. The number of the patients has hit 160,000,000 all over the world. In China, the incidence is up to 1%. The juveniles less than 10 year old account for more than half of the total patients. Now asthma has become the biggest contributer to the hospitalization of juveniles (1).

In contrast to the vast majority of injury and repair responses in the lung and other organs, asthmatic inflammation frequently starts in childhood and persists throughout the life of the afflicted individual. The asthma-like inflammation and physiologic dysregulation seen in these models is an end-result of the cellular and molecular events involved in sensitization, Th2 cell development, Th2 cytokine elaboration, and the activation of Th2 cytokine effector pathways. Interventions that inhibit any of these steps can appear to have a beneficial effect on the asthma-relevant readouts measured (2). However, since it is likely that antigen sensitization, Th2 cell development, and Th2 cytokine elaboration have already occurred in patients with established disease and/or a disease exacerbation, interventions at these sites will likely be unuseful therapeutically. Therefore, interventions that regulate Th2 cytokine effector pathways are attractive as therapies.

More recently, phospholipase D2 has been implicated in a wide range of physiological processes and diseases including inflammation, secrection, mitogenesis, neuronal and cardiac stimulation, and the respiratory burst in neutrophils. PLD2 catalyzes the hydrolysis of phospholipids, usually phosphatidylcholine, to generate phosphatidic acid. Phosphatidic acid may act directly as a signaling molecule or can be further metabolized to form diacylglycerol by phophatidic acid phosphohydrolase, the latter may function as an activator of protein kinase C isoforms and other diacylglycerol-dependent enzymes (3). PLD2 can be activated in cells by a variety of extracellular agonists including those that bind to G protein-coupled receptors and those that stimulate receptor tyrosine kinase. Therefore, PLD2 may act as key enzyme involved in cytokine effector pathways. rhPLD2 has been made by ourselves by engineering protein production modified from the wild-type PLD2. The wild-type PLD2 as one of the distinct PLD isoforms, has been cloned since 1997. The open reading frame is predicted to encode a 933-amino acid protein. It has four conserved functional regions, which were critical for catalysis *in vitro* and for PLD function *in vivo*. In order to verify whether rhPLD2, as therapy intervention, could block the signal transduction, we set up guinea pig model of persistent chronic asthma, treated with rhPLD2, and then detected NF-κB, which as a transcription

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factor, regulates a wide range of target inflammation cytokine, enzyme, receptor, and so on. Our results showed that rhPLD2 can significantly repress the activity of p65 when the guinea pigs were attacked by chronic asthma.

Materials and Methods

Animals

Male guinea pigs with weight about 250 ± 50 g were purchased from Shanghai Shenwang Animal Breed Corporation. The animals were housed in specific pathogen-free environments and were allowed access to food and water ad libitum.

OVA exposure

A 1% solution of OVA in double-distilled water was used for antigen exposures. Filtered air was passed through an 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.

Guinea pig model of chronic asthma

All guinea pigs were initially sensitized to and then challenged with OVA before the onset of rhPLD2 treatment: guinea pigs were given an intraperitoneal injection of OVA (250 mg/kg, suspended in 1 ml NS) at day 1, followed by inhalation of OVA (1% solution) at day 14. After that, every animal was performed by an administration of nebulized 1% OVA for about 5 min every other day. It will take another 20 days till the guinea pig model of chronic asthma was built up. Nonsensitized control animals received only the PBS.

Treatment of animals

Fifty-two guinea pigs attacked by chronic asthma are stochsticly divided into 5 groups, followed by consecutive treatment with an intraperitoneal injection of NS (1 ml), DXM (5 mg/kg) and a series of concentration of rhPLD2 (1.5 mg/kg, 3 mg/kg, 6 mg/kg) respectively before earlier 45 min of the onset of asthma 3 times. Each group included 10 guinea pigs at least.

Collection of specimen

Lung tissue of different groups of guinea pig was prepared as follows: after the guinea pig was killed, lung tissue was immediately incubated in NS to remove blood, and then chopped with scissors and fibrous tissue was discarded. The pellets were stored at -70^{o}C until used.

Extraction of nuclear proteins from tissue samples

Tissue nuclear proteins were extracted from whole tissue samples by the method of M. Audrey Koay. Briefly, lung tissue stored at -70^{o}C was thawed. Tissue of 100 mg was mechanically homogenized for 10 - 15 times in liquid nitrogen, to which 4 ml of buffer E [150 mM NaCl, 10 mM Hepes, 0.6% (v/v) NP-40, 0.2 mM EDTA, 0.5 mM PMSF, pH 7.9] was added. The homogenate was transferred to a 15 ml Falcon tube and centrifuged at 850× g in a tabletop centrifuge for 30 s to remove cellular debris. The supernatant was then transferred to a 50 ml falcon tube and incubated on ice for 5 min prior to being centrifuged for 10 min at 3,500× g. The supernatant was collected as cytoplasmic extract. The pellet was resuspended in 300 μl of buffer F [25% (v/v) glycerol, 20 mM Hepes, 0.5 M NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, pH 7.9] and incubated on ice for 30 min. Following centrifugation at 14,000 rpm in an Eppendorf microcentrifuge for 2 min, the supernatant was collected as the nuclear extract and frozen at -70^{o}C. Protein concentration in nuclear extracts was determined by using the Bradford assay. Briefly, 0.5 mg of protein standards (BSA) was aliquoted to the wells corresponding to the following vulumes: 0, 1, 2, 4, 8, 12, 16, 20 μl/well and then standard buffer was added to each well to 20 μl/well. The sample well was also added 20 μl nuclear extract diluted properly in standard buffer following by aliquoted 200 μl G250 to each well. Incubate for 5 min at room temperature, then read absorbance on a spectrophotometer at 450 nm. Our results showed that protein concentration was about 0.5 mg/ml - 0.8 mg/ml.

Detection of transcript factor p65

The performance was followed as TransAM NF-κB kit. Recombinant NF-κB p65 was used as protein standards. The OD of each well was read, the standard curve was drawn and the concentration of NF-κB p65 was calculated according to the standard curve.

Data analysis

Statistical significance was evaluated with the program SPSS 11.5 for Windows. Student's *t*-test and ANOVA were employed for comparing groups of samples, as appropriate. The p value < 0.05 was considered significant.

Results

Preparation of standard curve

The standard curve was obtained as described in the method section. Our results evidently showed that the OD value was positively related to the amount of transcript factor p65. The equation of standard curve is shown in Figure 1.

Effect of intervening factor on recombinant of guinea pig attacked by chronical asthma

Detection of p65 activity was performed by using Active Motif Kit. The following equation was used to determine the activity of p65:

$$Oda = Odt - Odc$$

(Oda indicates actual value，Odt indicates the total value unsubtracted background value, Odc indicates the value that the wild-type competing oligonucleotide was added to the

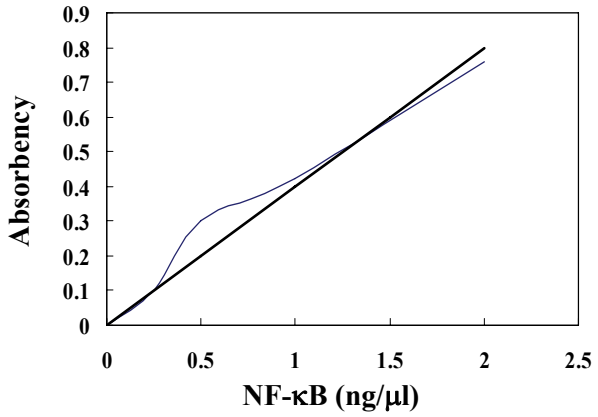

Figure 1. Detection of the concentration of recombinant NF-κB p65. (y = 0.3985x, R^2 = 0.965).

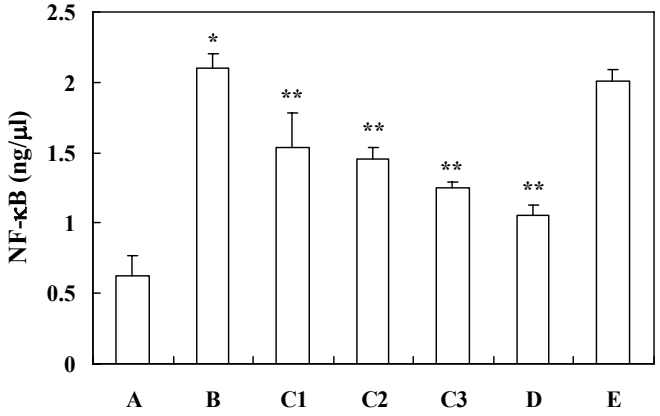

Figure 2. Comparation of p65 activity for different groups. (A) healthy guinea pigs, n = 8; (B) guinea pigs of chronic asthma, n = 8; (C1) treated with rhPLD2 (1.5 mg/kg), n = 11; (C2) treated with rhPLD2 (3.0 mg/kg), n = 11; (C3) treated with rhPLD2 (6.0 mg/kg), n = 10; (D) treated with DXM, n = 10; (E) treated with NS, n = 10. *p < 0.05, **p < 0.01.

well, which was used to determine the non-specific binding between p65 and oligonucleotide.)

As shown in Figure 2, when guinea pigs were attacked by chronic asthma, the activity of p65 was significantly increased (p < 0.001). Our results showed that both rhPLD2 and DXM can reduce the transcription activity of p65, but there was no difference of the curative effect between rhPLD2 and DXM (p = 0.736). In the experiments, although guinea pigs treated with NS also showed some trend of repression of p65 activity, but the extent of its repression of transcript factor p65 was very limited. In the group of asthmatic condition, the OD values (mean ± SD) was 0.87500 ± 0.096670, the guinea pig treated with NS had OD value about 0.72863 ± 0.090955, while the healthy guinea pigs comparatively had low OD (0.72863 ± 0.090955). Furthermore, when compared with the guinea pig treated with rhPLD or DXM, we found the marked difference in repression of p65 between rhPLD2 and NS, or DXM and NS, which indicated that the decrease of p65 activity was because of spontaneous catabation of asthmatic syndrome.

Discussion

Our studies clearly demonstrate that rhPLD2 and DXM can repress the activity of p65 when guinea pig model of chronic asthma was induced by OVA, also did our previous studies showed that rhPLD2 plays a vital role in reduction of IL-5 and IL-13 in bronchial lavage buffer and the expression of MMP-9 in lung tissue. Together, it indicates that rhPLD2 seems to hold promise as an effective alternative to the traditional asthmatic therapy. Unlike the mechanism of DXM inhibition of the expression of a wide range of inflammatory factors, which involves that DXM binding its receptor, then as whole complex, translocates into nuclear and acts as transcript factor to repress a variety of cytokine expression, rhPLD2 may possess other mechanisms to inhibit asthma attack. However the details of the mechanism involved are not clear.

PLD2 has been widely implicated in the transduction of intracellular signals in higher eukaryotic organism. PLD2, as one of isoforms of PLD, performs the same function as PLD1 does, catalyzing the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. A role for PLD2 has been implicated in membrane trafficking, cytoskeletal reorgnization, receptor endocytosis, exocytosis. Although until recently there are few reports about the role of PLD2 in the activation of Th2 cytokine effector pathways, our previous experimental results evidently showed that rhPLD2 can repress the expression of IL-5 and IL-13 (data not shown). This discovery does surprise us. We were bewildered why rhPLD2 had the reversed function of wild-type PLD2 in signal transduction. Because transcript factor p65 has a potential role in regulation of a series of inflammation factors (such as cytokines, immunoreceptors, and enzymes), and IL-13, IL-5 just belong to the target genes of p65, we then further investigated the correlation between rhPLD2 and p65. We set up guinea pig model of chronic asthma challenged with OVA, and intervened these asthma animals with NS, DXM, and rhPLD2 respectively. Our results firstly showed that rhPLD2 had an effect on repression of the expression of p65 that enhanced its expression when guinea pigs were attacked by chronic asthma, and rhPLD2 had a paralleled efficacy as DXM, while had a rather predominant capability in reduction of p65 than NS

It is indeed an attraction for us that rhPLD2 has a role in repression of p65 as much as DXM does. It's a provocative discovery. DXM, as we all know, which has been used as traditional means to abate symptom of chronic asthma, has inevitable side-effect on the patient with long-term treatment. It indicates that rhPLD2 may act as an effective alternative to the traditional asthma treatment.

It is really interesting that rhPLD2 as well as DXM have effect of reduction of p65. Some investigators had probed the

mechanism of the effect of DXM in repression of the expression of p65. In 1995, two experimental groups had independently demonstrated that glucocorticoids can block the nucleocytoplasmic shuttling of NF-κB to reduce its transcript activity *via* enhancing the expression of IκBα (4, 5). Our results are consistent to their viewpoint. However, other people hold different points. The experiment of mutation of glucocorticoids receptor showed that the improvement of the expression of IκBα and the change of NF-κB activity possessed two independently bio-chemical processes. Their results indicated that the mutation of glucocorticoids receptor simultaneously repressed the expression of IκBα and the activity of p65, there must be another way access to regulation of p65 activity (6-8).

The role of PLD2 in signal transduction has been universally accepted. PKC can be activated by PLD2, and the activation of PKC triggers cascade of signal transduction, which results in the activation of some transcript factor (9). In order to determine the correlation PLD2 and NF-κB as well as the role of PLD2 in asthma, we used our recombinant human PLD2 (rhPLD2) to intervene the guinea pig model of chronic asthma. As shown in the result section, we discovered that rhPLD2 could markedly repress of p65 activity. It is a pity that we didn't make a detection of IκBα so that we couldn't verify the relationship of rhPLD2, IκBα and the activity of p65. However, the previous studies provided us some clues for the regulation of NF-κB by PLD2. There was a report showing that a guanine nucleotide exchange factor named Lbc could activate NF-κB. Another report also showed that a kind of bacterial tripeptide fMet-Leu-Phe (fMLP) can stimulate the activation of NF-κB, and this function of fMLP required the small GTPase RhoA in human peripheral blood monocytes, while RhoA is just the regulator of PLD2 (10). We then postulate that rhPLD2, through gene recombination, has become signal transduction anergy, but holds the rather receptor site for Rho to compete with the wild-type PLD2 so that the activation of p65 was blocked.

In conclusion, our studies suggested that intraperitoneal administration of rhPLD2 can repress the p65 activity, and this treatment also provides protection against guinea pig chronic asthma challenged by OVA. Further studies of its mechanism involved are currently undergoing.

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