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Targeting cytosolic phospholipase A_2 by arachidonyl trifluoromethyl ketone prevents chronic inflammation in mice

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Abstract

Cytosolic phospholipase A_2 ($cPLA_2$) plays a pivotal role in inflammation by catalyzing the release of arachidonic acid, a substrate for lipoxygenase and cyclooxygenase enzymes, from membrane phospholipids. In the present study we examined the role of $cPLA_2$ in inflammatory responses through the use of a specific inhibitor of the enzyme, $cPLA_2$, arachidonyl trifluoromethyl ketone (AACOCF3). Interestingly, we observed that AACOCF3 is an inhibitor of chronic but not acute inflammatory responses. Specifically, AACOCF3 inhibited phorbol 12-myristate 13-acetate (PMA)-induced chronic ear edema in mice. Additionally, oral treatment of ovalbumin-sensitized/ovalbumin-challenged BALB/c mice with 20 mg/kg AACOCF3 prevented the development of airway hyper-responsiveness in a model of asthma. Furthermore, AACOCF3 decreased cellular recruitment in the airway lumen and airway inflammation after the ovalbumin challenge. Taken together, these results suggest that a potent and specific chemical inhibitor of $cPLA_2$ may be useful for the treatment of chronic inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and asthma.

Keywords: Inflammation; Acute; Chronic; Inhibitor; Phospholipase A_2

1. Introduction

The metabolism of arachidonic acid via the 5-lipoxygenase and cyclooxygenase pathways leads to the formation of pro-inflammatory leukotrienes and prostaglandins (Fitzpatrick and Soberman, 2001). These arachidonic acid metabolites, collectively known as eicosanoids, play a key role in the pathogenesis of a number of inflammatory conditions, including allergic rhinitis, rheumatoid arthritis, inflammatory bowel disease, psoriasis, cancer, and asthma (Henderson, 1994; Gijon and Leslie, 1997; Bonventre, 1999; Gijon and Leslie, 1999; Henderson et al., 2002; Hegen et al., 2003).

The first step in the generation of eicosanoids is the release of arachidonic acid from membrane phospholipids (Bonventre and Sapirstein, 2002). Arachidonic acid is released by the hydrolysis of membrane glycerophospholipids at the sn-2 position by the action of phospholipase A_2 (PLA_2) enzymes (Borsch-Haubold, 1998; Balsinde et al., 1999). The PLA_2s encompass a large family of enzymes (Kramer and Sharp, 1997; Bonventre, 1999). At least 19 different types of PLA_2s have been reported in the literature that include several forms of secreted PLA_2 ($sPLA_2$), group IV Ca^{2+}-independent PLA_2 ($iPLA_2$), and Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) (Kramer and Sharp, 1997; Bonventre, 1999). Although the physiological roles of $sPLA_2$ and $iPLA_2$ are not completely understood, involvement of $cPLA_2$ in the generation of eicosanoids is well documented (Kramer and Sharp, 1997). Most tissues and inflammatory cells, including platelets, neutrophils, monocytes, macrophages, eosinophils, basophils and mast cells, with the exception of lymphocytes, express $cPLA_2$ (Kramer and Sharp, 1997; Bonventre, 1999). Stimulation of $cPLA_2$ in platelets can be induced by thrombin or a calcium ionophore (Bartoli et al., 1994; Riendeau et al., 1994); in neutrophils and macrophages via PMA or zymosan (Qiu and Leslie, 1994; Garcia Pastor et al., 1999); and in mast cells with

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IgE/antigen (Malaviya et al., 1993; Nakatani et al., 1994; Fujishima et al., 1999; Nakatani et al., 2000; Burke et al., 2001) or bacteria (Malaviya et al., 1996; Malaviya and Abraham, 2000). In unstimulated cells, $cPLA_2$ is found in the cytosol (Kramer and Sharp, 1997). Following cell stimulation, $cPLA_2$ is phosphorylated and translocates to the nuclear membrane where it interacts with membrane phospholipids to release arachidonic acid (Kramer et al., 1996).

Studies using $cPLA_2$-knockout mice and specific inhibitors support the notion that $cPLA_2$ plays an essential role in health and disease (Bonventre, 1999; Gijon and Leslie, 1999; Sapirstein and Bonventre, 2000). Although $cPLA_2$-deficient mice that were generated by targeted disruption of the *$cPLA_2$ gene* in embryonic stem cells did not show gross developmental or behavioral abnormalities, they did show significant defects in inflammatory processes (Sapirstein and Bonventre, 2000). Because eicosanoids (especially COX products) play a key role in the pathogenesis of rheumatoid arthritis (Sundy, 2001), Hegen et al. (2003) examined the role of $cPLA_2$ in a mouse model of collagen-induced arthritis. The authors showed that $cPLA_2$-null mice on the DBA/1LacJ background are resistant to collagen-induced arthritis (Hegen et al., 2003). In another study, Uozumi et al. (1997) examined the role of $cPLA_2$ in asthma by using $cPLA_2$-knockout (KO) mice on the C57BL/6 background. Marginal differences between wild-type and $cPLA_2$-KO mice were observed in the development of the acute anaphylactic response, implying that eicosanoids may not be required for the full development of that response (Uozumi et al., 1997). However, a marked reduction in alveolar lumen thickening and complete attenuation of airway hyper-responsiveness to methacholine was observed in the $cPLA_2$-KO mice, suggesting that $cPLA_2$ is important for the late phase asthmatic response (Uozumi et al., 1997). Because full expression of the asthmatic response in mice depends on the genetic background of the strain (Lloyd et al., 2001), we investigated the role of $cPLA_2$ in allergic inflammation using the BALB/c strain. This strain is highly responsive to allergic challenge (Leong and Huston, 2001). C57BL/6 mice, on the other hand, have several genetic defects that might have affected the outcome of the inflammatory process (Leong and Huston, 2001) in the study by Uozumi et al. (1997). Thus, the purpose of the study was to evaluate the effect of AACOCF3 in allergic inflammation in BALB/c strain of mice.

One of the key features of human asthma is the structural changes in the airways commonly known as tissue remodeling (Vignola et al., 2003). This remodeling is characterized by the thickening of the bronchial wall and fibrosis (Vignola et al., 2003). Using a mouse model of bleomycin-induced pulmonary fibrosis, Nagase et al. (2002) showed that $cPLA_2$-KO mice have significantly attenuated pulmonary inflammation and fibrosis. Taken together, these reports suggest that $cPLA_2$ is a key element of inflammatory reactions and that targeting $cPLA_2$ may offer an attractive approach for the treatment of chronic inflammatory diseases such as asthma and rheumatoid arthritis.

In the last decade, considerable effort has been made to develop specific and potent inhibitors of $cPLA_2$ for the treatment of chronic inflammatory diseases (Sapirstein and Bonventre, 2000). An arachidonic acid analog in which the –COOH group of arachidonic acid is replaced by COCF3, i.e., arachidonyl trifluoromethyl ketone (AACOCF3), has been reported by Street et al. (1993). It is a selective, and slow, but tight binding, inhibitor of $cPLA_2$, and has been widely used to examine the role of $cPLA_2$ in biological processes including several animal models of inflammation (Ackermann et al., 1995; Myou et al., 2001; Nagase et al., 2003; Kalyvas and David, 2004). When given intraperitoneally (i.p.), AACOCF3 inhibited allergen induced eosinophilic infiltration in the airway lumen and airway hyper-responsiveness in guinea pigs (Myou et al., 2001). AACOCF3 also prevented hypoxic pulmonary vasoconstriction in mice when given i.p. (Ichinose et al., 2002). Recently, $cPLA_2$ has been implicated in the development of experimental autoimmune encephalitis, a rodent model of multiple sclerosis using AACOCF3 in mice to reverse experimental autoimmune encephalitis (Kalyvas and David, 2004). These results demonstrate that AACOCF3 is an inhibitor of $cPLA_2$-dependent biological responses in vivo.

In the present study, we further investigated the effect of AACOCF3 in models of both acute and chronic inflammation in BALB/c strain of mice. Our findings show that AACOCF3 is an inhibitor of chronic but not acute inflammatory responses in mice. Most interestingly, when given orally prior to antigen challenge in a BALB/c mouse model of allergic asthma, AACOCF3 prevents allergen-induced airway hyper-responsiveness and airway inflammation. While $cPLA_2\alpha$ is a likely mechanism for the observed effects it cannot be verified in these experiments.

2. Materials and methods

2.1. Materials

2.1.1. Mice

Male and female BALB/c mice, between 5 and 7 weeks old, were obtained from ACE Laboratories (PA). Mice were housed in a controlled environment (12-h light/12-h dark photoperiod, 22 ± 1 °C, 60 ± 10% relative humidity), which is fully accredited by the United States Department of Agriculture. Mice were provided free access to autoclaved pellet food and tap water. The Institutional Animal Care and Use Committee (IACUC) approved the studies.

2.1.2. Reagents

Grade V chicken egg ovalbumin (ovalbumin), methacholine, PMA, eosin, hematoxylin, Giemsa stain, formalin, ethanol, bovine serum albumin (BSA) and betamethasone were purchased from Sigma/Aldrich Chemical Co. (St. Louis, MO). AACOCF3 was purchased from Cayman Chemicals (Ann Arbor, MI). Imject Alum was purchased from Pierce (Rockford, IL).

2.2. Mouse model of acute inflammation

Our mouse model of acute inflammation employed was a slight modification of the procedure of Stanley et al. (1991). Briefly, the left ear of a mouse was painted topically with 10 µl of test compound in acetone/water (99:1), 10 min prior to PMA

challenge. The right ear was painted with an equal volume of the vehicle alone. To examine the effect of AACOCF3 following intraperitoneal challenge on ear edema, groups of mice were injected with 10 or 30 mg/kg AACOCF3, 30 min prior to PMA challenge. Control mice were injected with vehicle (0.5% methylcellulose). A transient inflammatory condition was induced by a topical application of 10 µl of PMA (400 ng/ml) painted on the left ear, with an equal volume of vehicle applied to the right ear. Six hours later, mice were sacrificed and 6 mm^2 diameter ear punch biopsies were collected and weighed.

2.3. Mouse model of chronic inflammation

The chronic skin inflammation model had a duration of 1 week, and has also been described previously (Burke et al., 2001). Briefly, 10 µl of a solution of PMA (400 ng/ml) in acetone/water (99:1) was applied to the left ear on days 0, 2, 5, and 7. On days 5 and 6, when ear edema and redness were clearly evident, AACOCF3 was given twice a day (i.p.), with a 6-h interval between doses. Only one drug treatment was given on day 7. Control mice were treated with an equal volume of vehicle using the identical dose schedule. Six hours after the drug and PMA treatments on day 7, mice were sacrificed and 6 mm^2 diameter ear punch biopsies were collected and weighed. Weight differential comparisons between the control group and drug-treated group were interpreted as percent inhibition of inflammation by the test compound. Typically, 1% betamethasone was used as a positive control. The results of ear edema were expressed as net increase in ear weight after subtraction of the weight of 6 mm^2 diameter ear punch biopsies from vehicle-treated mice.

2.4. Mouse model of allergic asthma

Female BALB/c mice were injected i.p. with 20 µg of ovalbumin adsorbed on 2.25 mg Imject alum on days 0 and 14 as has been described previously (Takeda et al., 1997). On days 28, 29, and 30, mice were challenged for 20 min with 5% ovalbumin via their airways using an ultrasonic nebulizer (Malaviya et al., 2000). To examine the effect of the $cPLA_2$ inhibitor AACOCF3 on allergic asthma, mice were treated orally (in 0.5% methylcellulose) or intraperitoneally (in 2% dimethyl sulfoxide) with AACOCF3 30 min before and 6 h after the ovalbumin challenge on days 28, 29, and 30. On day 31 mice were treated twice orally or intraperitoneally with AACOCF3, with a 6-h interval between doses. Mice were assessed for airway responsiveness on day 32.

2.5. Determination of airway responsiveness

Airway responsiveness was measured in unrestrained mice by noninvasive whole body plethysmography using a BioSystem plethysmographic instrument (BUXCO, Troy, NY) as described earlier (Malaviya et al., 2000). To measure the responsiveness to methacholine, mice were placed in the chamber and baseline readings were taken and averaged for 5 min. Mice were nebulized with saline or methacholine at increasing doses (1–10 mg/ml) for 2 min, and the Penh (enhanced Pause) readings were taken and averaged for 5 min after each nebulization.

2.6. Assessment of eosinophil infiltration

After airway responsiveness measurements on day 32, lungs were thoroughly lavaged with 1 ml of saline and total leukocytes were counted. (Malaviya et al., 2000). The lavage fluid was centrifuged, and the supernatant was removed. The cell pellet was resuspended in saline containing 0.1% BSA to yield a final cell concentration of 0.1×10^6 cells/ml. Cytospin smears made from the cell suspension were stained with Giemsa, and the number of eosinophils was determined.

2.7. Histological analysis of lungs

Separate groups of mice were used for these analyses. Mice were sacrificed and their lungs were fixed in 10% buffered formalin under constant pressure. After routine paraffin embedding, 5-µm sections were cut and stained with H&E. The sections were examined via light microscopy.

2.8. Data analysis

Comparisons of data among each experimental group were carried out using the Student's *t*-test. Data are presented as mean ± S.E.M. p values <0.05 were taken as significant.

3. Results

3.1. Effect of the $cPLA_2$ inhibitor AACOCF3 on ear edema in mice

Topical application of PMA to mouse ears results in an acute inflammatory response, characterized by erythema and edema; this response reaches a maximum by 6 h and subsides by 24 h (Stanley et al., 1991). Multiple applications of PMA have been shown to produce a more prolonged inflammatory response characterized by ear edema, inflammatory cell influx and epidermal cell hyperplasia (Stanley et al., 1991; Burke et al., 2001).

We examined the contribution of $cPLA_2$ in both acute and chronic inflammation by using the $cPLA_2$ inhibitor AACOCF3. We first tested the effect of AACOCF3 on PMA induced-acute ear edema. Mice were treated with AACOCF3 topically (Fig. 1A) 10 min prior to PMA challenge, or intraperitoneally (Fig. 1B) 30 min prior to PMA challenge. Six hours after the PMA challenge, animals were sacrificed and 6 mm^2 biopsy punches of the ear specimens were weighed. Neither topical, as shown in Fig. 1A (8.9 ± 0.6 mg versus 9.5 ± 0.6 mg; $p = 0.3$) nor intraperitoneal AACOCF3 administration as shown in Fig. 1B (8.6 ± 1.0 mg versus 8.5 ± 0.9 mg and 7.3 ± 0.8 mg; $p = 0.4$) had any significant effect on net ear weight. Thus, $cPLA_2$ derived inflammatory mediators (eisosanoids) may not be important in the development of PMA induced acute ear edema in mice.

We next tested AACOCF3 in a mouse model of chronic inflammation. To this end, we used a well-characterized mouse

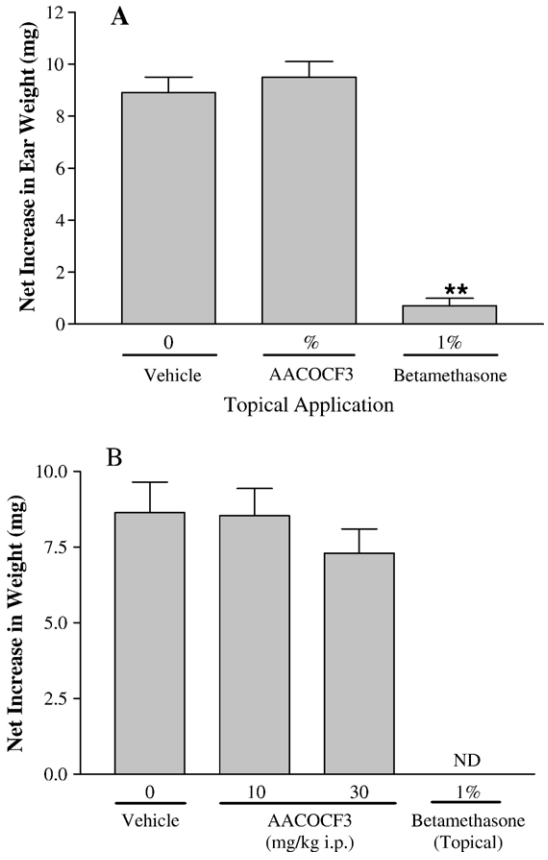

Fig. 1. Inhibition of PMA induced acute ear edema by AACOCF3. Mice were treated with a topical application of PMA as has been described in Materials and Methods. To study the effect of a $cPLA_2$ inhibitor on PMA-induced ear edema, mice were treated with AACOCF3 topically (A) or intraperitoneally (B) before the PMA application. After 6 h ears were excised and weighed to measure edema. 1% betamethasone (topical) was used as a control. Mean ear weight of vehicle treated mice was 6.88 ± 0.15 mg. Results are expressed as net increase in ear weight (PMA treated − vehicle treated). The data points represent the mean ± S.E.M. values obtained in 5 mice. ND = not detectable. $^{**}p < 0.001$ compared with the vehicle-treated group as determined by the Student's *t*-test.

model of chronic ear edema (Stanley et al., 1991; Burke et al., 2001) in which six topical applications of PMA are made over a 10-day period. Although the exact mechanism of this chronic inflammation is not fully elucidated, $cPLA_2$ products seem to play a key role (Stanley et al., 1991; Burke et al., 2001). Using this model, Burke et al. (2001) have shown that topical application of BMS-229724, a $cPLA_2$ inhibitor, results in significant inhibition of both ear edema and neutrophil influx. As shown in Fig. 2, i.p. treatment of AACOCF3 inhibited PMA-induced ear edema by 72% (8.2 ± 0.92 mg versus 2.3 ± 0.91 mg; $p < 0.001$). Taken together, these results suggest that $cPLA_2$ regulates PMA-induced chronic but not the acute inflammatory response in mice.

3.2. Effect of the $cPLA_2$ inhibitor AACOCF3 on allergen induced leukocyte infiltration in airway lumen in a mouse model of allergic asthma

Allergic asthma is characterized by inflammation of the airway caused by the recruitment of inflammatory cells including T cells, eosinophils and mast cells to the airway parenchyma (Arm and Lee, 1992; Wasserman, 1994). $cPLA_2$ seems to play a critical role in the development of the inflammatory response in asthma. In a previous study, Uozumi and Shimizu examined the role of $cPLA_2$ using $cPLA_2$-KO mice on the C57BL/6 background (Uozumi et al., 1997).

Because the full asthmatic response depends on the genetic background of the mouse strain used (Leong and Huston, 2001), we sought to replicate the above results using the BALB/c mouse strain. We utilized a well-characterized mouse model of allergic asthma to study the effect of a $cPLA_2$ inhibitor AACOCF3 on airway inflammation. In this model, sensitization of mice with ovalbumin followed by airway challenge with the same antigen triggers the production of high levels of ovalbumin-specific IgE and IgG1 antibodies (Hamelmann et al., 1997b). In our experiments mice were sensitized by repeated intraperitoneal injections of ovalbumin and then challenged via the airway with ovalbumin, mimicking a natural model of allergic sensitization. Forty-eight hours after the last ovalbumin challenge we assessed airway inflammation in the ovalbumin-sensitized and phosphate buffered saline (PBS)-challenged mice; ovalbumin-sensitized and ovalbumin-challenged (ovalbumin) mice; and AACOCF3-treated ovalbumin-sensitized and challenged (ovalbumin + AACOCF3) mice.

Eosinophils are the most prominent cells involved in the airway inflammation of asthmatics and are found in large numbers in both sputum and bronchoalveolar lavage fluids (Hamelmann et al., 1997a; Malaviya et al., 2000; Malaviya and Uckun, 2001). Because $cPLA_2$-derived inflammatory mediators, especially leukotrienes, play a key role in the recruitment of leukocytes in the airway lumen in asthma, we compared the total leukocyte count and eosinophils in bronchoalveolar lavage samples of PBS-challenged and ovalbumin-challenged mice,

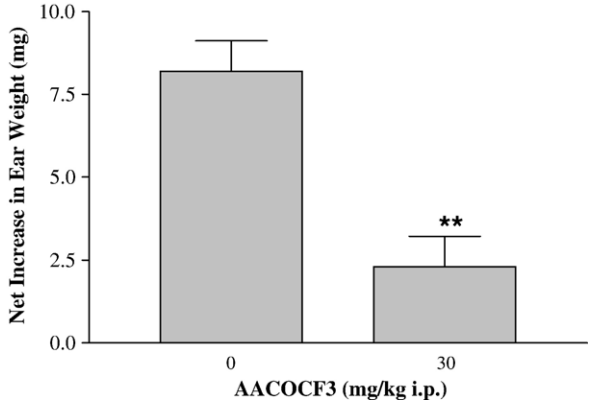

Fig. 2. Inhibition of PMA induced chronic ear edema by AACOCF3. Mice were treated with a topical application of PMA as has been described in Materials and Methods. To study the effect of a $cPLA_2$ inhibitor on PMA-induced ear edema, mice were treated with 30 mg/kg AACOCF3 i.p. 30 min before the PMA application. After 6 h since the last application of PMA, mice ears were excised and weighed to measure edema. Mean ear weight of vehicle-treated mice was 10.1 ± 0.74 mg. Results are expressed as "net increase in ear weight" (PMA treated − vehicle treated). The data points represent the mean ± S.E.M. values obtained in 5 mice. $^{**}p < 0.001$ compared with vehicle-treated group as determined by the Student's *t*-test.

with or without AACOCF3 treatment. We first examined the dose response effect of AACOCF3 (10 and 30 mg/kg intraperitoneally) on cellular infiltration in the bronchoalveolar lavage fluid in a mouse model of allergic asthma (Fig. 3). Significantly higher numbers of total leukocytes ($0.36 \pm 0.14 \times 10^5$ cells/ml versus $1.25 \pm 0.32 \times 10^5$ cells/ml; $p < 0.05$) were present in the bronchoalveolar lavage fluids of mice challenged with ovalbumin compared to mice challenged with PBS (Fig. 3). Treatment of ovalbumin-challenged mice with AACOCF3 intraperitoneally resulted in the inhibition of cellular infiltration in the bronchoalveolar lavage fluids of mice in a dose dependent fashion (Fig. 3). At 10 and 30 mg/kg AACOCF3 inhibited accumulation of leukocytes in the BAL by 80% and 100% respectively (Fig. 3). Dexamethasone, which was used as a control, completely attenuated cellular response in the bronchoalveolar lavage of the ovalbumin-challenged mice. We next compared the total leukocyte counts and eosinophils in bronchoalveolar lavage samples of PBS-challenged and ovalbumin-challenged mice with and without oral administration of AACOCF3. As shown in Fig. 4, higher numbers of total leukocytes ($0.54 \pm 0.08 \times 10^5$ cells/ml versus $1.5 \pm 0.46 \times 10^5$ cells/ml; $p < 0.05$; Fig. 4A), and increased numbers of eosinophils ($0 \pm 0 \times 10^5$ eosinophils/ml versus $0.46 \pm 0.1 \times 10^5$ eosinophils/ml; $p < 0.05$; Fig. 4B) were present in the bronchoalveolar lavage fluids of mice challenged with ovalbumin compared to mice challenged with PBS. Pretreatment of mice with AACOCF3 orally resulted in a decrease of total leukocytes by 90% ($1.5 \pm 0.46 \times 10^5$ cells/ml versus $0.6 \pm 0.1 \times 10^5$ cells/ml; $p < 0.05$; Fig. 4A), and a decrease of total eosinophils by 80% ($0.46 \pm 0.1 \times 10^5$ eosinophils/ml versus $0.1 \pm 0.1 \times 10^5$ eosinophils/ml; $p < 0.05$; Fig. 4B).

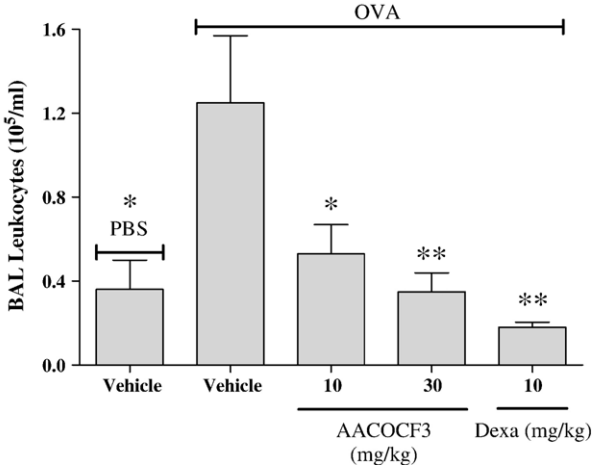

Fig. 3. Inhibition of leukocyte accumulation in bronchoalveolar lavage of ovalbumin-challenged mice by AACOCF3 following intraperitoneal administration. The lungs of the mice were lavaged and total leukocytes counts taken. The results are expressed as 10^5 cells/ml in bronchoalveolar lavage specimens. The data points represent the mean ± S.E.M. values obtained in 5 mice. *$p < 0.05$, comparison of OVA challenged group with PBS challenged and OVA + 10 mg/kg AACOCF3 treated groups; **$p < 0.001$, comparison of OVA challenged group with OVA + 30 mg/kg AACOCF3 treated and OVA + Dexamethasone (Dexa) treated groups as determined by the Student's *t*-test.

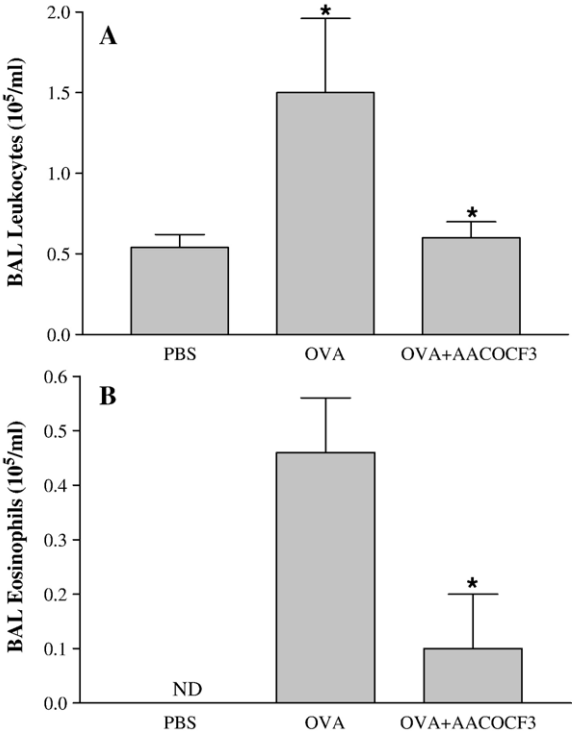

Fig. 4. Inhibition of total leukocyte and eosinophil infiltration in bronchoalveolar lavage of ovalbumin-challenged mice by AACOCF3. The lungs of the mice were lavaged and total leukocytes (A) and eosinophil (B) counts were determined. The results are expressed as 10^5 cells/ml in bronchoalveolar lavage specimens. The data points represent the mean ± S.E.M. values obtained in 6 to 11 mice. ND = not detectable; *$p < 0.05$, comparison of OVA challenged group with PBS challenged and OVA + AACOCF3 treated groups, as determined by the Student's *t*-test.

3.3. Effect of the cPLA$_2$ inhibitor AACOCF3 on allergen-induced airway inflammation in mice

We next examined the effect of AACOCF3 on airway inflammation. Histological changes in the lungs of PBS-challenged mice were barely detectable (Fig. 5A). However, striking histological changes, including marked infiltration of eosinophils and mononuclear cells in the peribronchial and perivascular areas of the lung interstitium of ovalbumin-treated mice, were observed (Fig. 5B). Examination of bronchial mucosa revealed hypertrophy and hyperplasia of goblet cells with hypersecretion of mucus in the ovalbumin-challenged (Fig. 5E) but not in PBS-challenged (Fig. 5D) mice. These results are consistent with previous reports (Henderson et al., 1996; Takeda et al., 1997; Malaviya et al., 2000). Using a similar sensitization and challenge protocol Takeda et al. (1997) have shown marked increase in eosinophilic inflammation in the lungs of ovalbumin-challenged mice. Histological analysis of the lungs of 20 mg/kg AACOCF3-treated mice revealed that the influx of both eosinophils and mononuclear cells were markedly reduced (Fig. 5C) compared with the control group (Fig. 5B). Notably, treatment of mice with AACOCF3 also inhibited goblet cell hypertrophy and hyperplasia in mice (Fig. 5F).

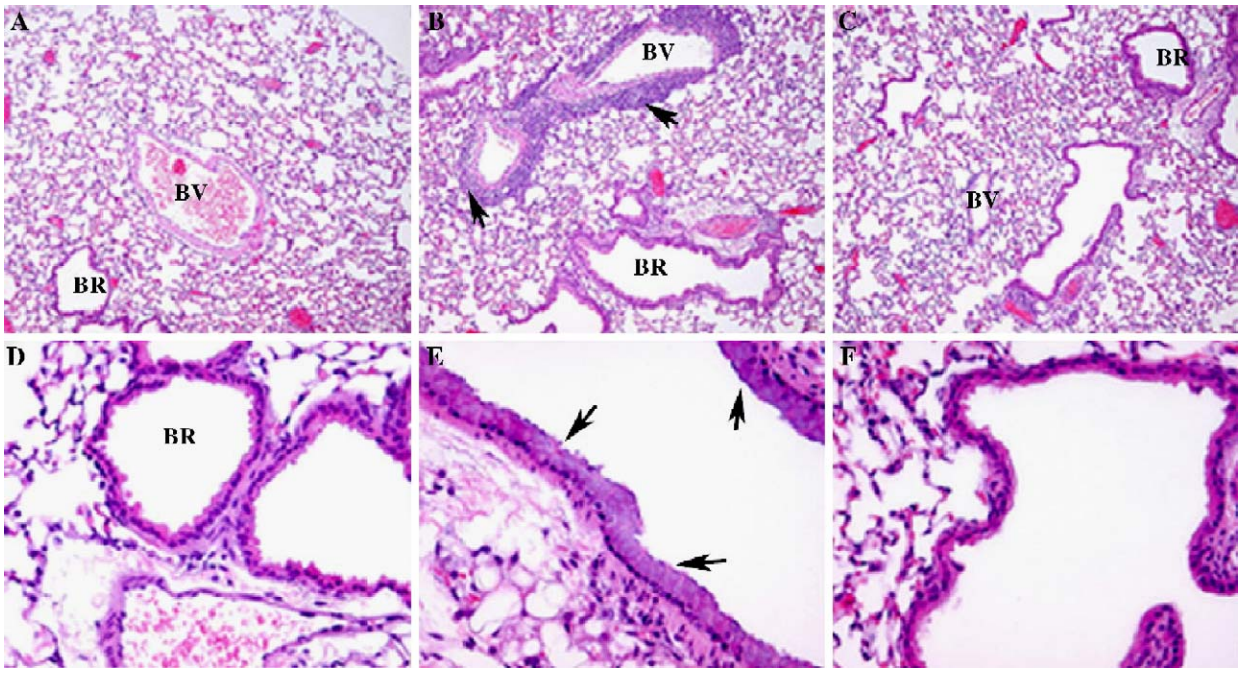

Fig. 5. Inhibition of pulmonary inflammation by AACOCF3. Lung tissues, obtained from (A and D) PBS-sensitized/PBS challenged (PBS), (B and E) OVA-sensitized/OVA-challenged (OVA) mice and (C and F) OVA-sensitized/OVA-challenged/AACOCF3 treated (OVA + AACOCF3) mice, were stained with hematoxylin and eosin. (A) Note that no cell infiltrates are present around blood vessel (BV) or bronchiole (BR). (D) No hyperplasia or hypertrophy of bronchial epithelium is observed. (B) Note thick perivascular cuffs composed of macrophages, lymphocytes, plasma cells, and eosinophils (arrow). (E) Also note hypertrophy and hyperplasia of goblet cells (arrow). (C) Note that no infiltrates are present around the BV. (F) Also note that bronchial epithelium is comparable to control (D) and that goblets cells are absent. Similar results were obtained in an independent experiment. (A–C) 10× magnification. (D–F) 40× magnification.

3.4. Effect of the cPLA$_2$ inhibitor AACOCF3 on allergen-induced airway hyper-responsiveness in mice

Airway hyper-responsiveness of asthmatic patients is maintained as a result of persistent airway inflammation. We next examined the effect of the cPLA$_2$ inhibitor AACOCF3 on airway hyper-responsiveness using whole body plethysmography in non-restrained conscious mice (Hamelmann et al., 1997a). Bronchial hyper-responsiveness of PBS-challenged mice, ovalbumin-challenged mice, and AACOCF3 treated ovalbumin challenged mice to inhaled methacholine was examined. The results are shown in Fig. 6. Mice treated with ovalbumin showed higher Penh in response to methacholine compared with control (PBS) mice. Treatment of ovalbumin-sensitized mice with 20 mg/kg AACOCF3 prevented development of airway hyper-responsiveness by 56%. Thus, AACOCF3 is also an inhibitor of airway hyper-responsiveness in BALB/c mice.

4. Discussion

cPLA$_2$ plays a key role in inflammation by its ability to release arachidonic acid from cell membrane glycerophospholipids (Kramer and Sharp, 1997). The oxidative metabolism of released arachidonic acid by 5-lipoxygenase and cyclooxygenase (COX) results in the generation of pro-inflammatory leukotrienes and prostaglandins (Kramer and Sharp, 1997). These pro-inflammatory mediators possess a wide spectrum of biological activities and alone, or in combination, are responsible for the clinical signs and symptoms of a variety of clinical conditions (Kramer and Sharp, 1997).

Arachidonyl trifluoromethyl ketone (AACOCF3) is an inhibitor of cPLA$_2$ (Street et al., 1993). It is a slow but tight-

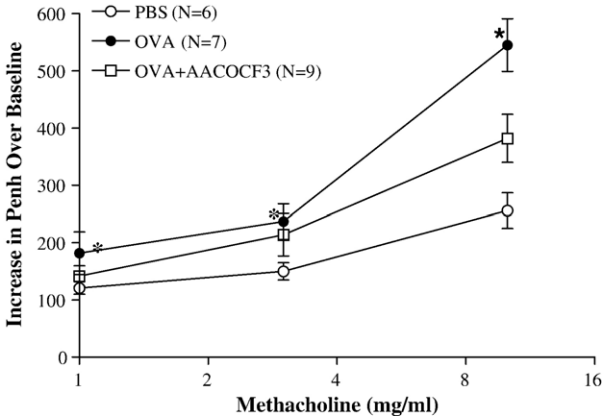

Fig. 6. Inhibition of airway responsiveness by AACOCF3. Mice were sensitized with ovalbumin on days 0 and 14 i.p. as detailed under Materials and Methods. On days 28, 29, and 30, mice were challenged for 20 min with 5% ovalbumin using an ultrasonic nebulizer. After 48 h, airway responsiveness to increasing doses of methacholine was assessed by measurement of Penh. To study the effect of the cPLA$_2$ inhibitor on methacholine-induced airway hyper-responsiveness, mice were treated orally with 20 mg/kg of AACOCF3 or vehicle 30 min before OVA challenge on days 28–30 and once on day 31. The data points represent the mean ± S.E.M. values obtained in 6 to 9 mice. *$p < 0.05$, comparison of OVA challenged group with PBS challenged and OVA + AACOCF3 treated groups at the respective time points, as determined by the Student's *t*-test.

binding inhibitor of Group IV cPLA$_2$ as shown by a nonlinear progress curve and a very slow dissociation rate (Street et al., 1993). It binds to the active site of cPLA$_2$ (Street et al., 1993) and is widely used to examine the role of cPLA$_2$ in biological responses in vitro and in vivo (Ackermann et al., 1995; Myou et al., 2001; Nagase et al., 2003; Kalyvas and David, 2004). Although AACOCF3 is considered to be a potent and relatively specific inhibitor of cPLA$_2$ in vitro, there is direct evidence that in platelets it also inhibits cyclooxygenase activity under the condition when exogenous arachidonic acid is added to the cells (Riendeau et al., 1994). In addition, AACOCF3 has also been shown to inhibit sPLA$_2$ and iPLA$_2$ activities (Ackermann et al., 1995; Balsinde et al., 1999). However, AACOCF3 was considered to be more selective for cPLA$_2$ than sPLA$_2$ or iPLA$_2$ (Ackermann et al., 1995). Therefore, we utilized AACOCF3 in the present investigation to examine the role of cPLA$_2$ in acute and chronic inflammation using well-characterized mouse models of inflammation. Our results show that cPLA$_2$ is required for the full expression of chronic inflammation in mice. Our conclusion is based on the observations that AACOCF3 inhibited PMA-induced chronic ear edema and airway hyper-responsiveness in a mouse model of allergic asthma.

AACOCF3 inhibited PMA-induced chronic, but not acute, ear edema in BALB/c mice, implying that cPLA$_2$ seems to be involved in chronic but not acute inflammation. Although the mechanism of PMA-induced acute edema in mice is not fully understood, mast cell-derived inflammatory mediators, such as histamine, tumor necrosis factor-α (TNFα) and proteases seem to play a role; thus, anti-histamines and mast cell inhibitors significantly block PMA-induced acute edema (Carlson et al., 1985; Wershil et al., 1988; Tomimori et al., 2002). However, such inhibitors have very little effect on the PMA-induced chronic response (Stanley et al., 1991), implying that other inflammatory mediators such as cytokines, chemokines or arachidonic acid metabolites may be involved.

Although a role for arachidonic acid metabolites in the development of acute inflammatory responses has also been documented, our results are in accordance with the study of Myou et al. (2001). Those authors showed that the treatment of guinea pigs with 20 mg/kg i.p. AACOCF3 did not inhibit immediate bronchoconstriction after ovalbumin challenge, but significantly attenuated the late phase response. On the other hand, Uozumi et al. (1997), using a mouse model of allergic asthma have shown that a deficiency of cPLA$_2$ results in a 25% reduction in lung resistance in a mouse model of ovalbumin-induced acute anaphylaxis. This effect may not be due to the defective eisosanoid response per se, however, it could be due to the other impairments owing to the cPLA$_2$ deficiency in the cPLA$_2$-null mice. Nakatani et al. (2000) have shown that mast cells cultured from the bone marrow cells of cPLA$_2$-null mice release significantly reduced amounts of histamine compared to mast cells cultured from bone marrow cells of wild-type mice in response to allergic challenge (Nakatani et al., 2000). One possibility is that some of these responses may be due to known genetic defects in the mouse strain (C57BL/6) that was used to create cPLA$_2$-null mice. It has been shown that C57BL/6 mice have a point mutation for the *mast cell protease 7 gene* (mMCP-7) (Hunt et al., 1996). MCP-7 is a tryptase expressed by connective tissue mast cells and it is selectively released into the plasma of V3 mastocytosis mice undergoing passive systemic anaphylaxis (Huber et al., 1992). Because fibrinogen is a physiologic substrate of mMCP-7, it can regulate mast cell-mediated acute inflammatory reactions by controlling clot formation and fibrinogen/integrin-dependent cellular responses (Huber et al., 1992; Ramos et al., 1992). Thus, the reduced anaphylactic response as shown by Uozumi et al. (1997) may have been caused by the mutation of mMCP-7 that occurs in C57BL/6 mice used in the study.

Because the full asthmatic response depends on the genetic background of the mouse strain used (Leong and Huston, 2001), we sought to replicate the above results in the BALB/c mouse strain by using AACOCF3. The role of cPLA$_2$ in allergic asthma has previously been shown by Uozumi et al. (1997) using cPLA$_2$-null mice on the C57BL/6 background, and by Myou et al. (2001) in guinea pigs, by the use of AACOCF3. However, different strains of mice produce distinct pathogenic phenotypes even if sensitization and challenge conditions are kept constant. It is well documented in the literature that BALB/c mice have greater AHR response to ovalbumin than C57BL/6 mice (Leong and Huston, 2001). Even isolated tracheal smooth muscles from BALB/c mice are more contractile than those from C57BL/6 mice (Leong and Huston, 2001). In addition to the mMCP-7 defect discussed earlier, C57BL/6 mice also have impairment in the translation of interleukin (IL)-9 (Leong and Huston, 2001). Furthermore, BALB/c mice develop higher levels of IgE and cellular response than C57BL/6 mice (Leong and Huston, 2001) and tend to exhibit a more Th$_2$-skewed response than C57BL/6 mice (Leong and Huston, 2001).

Since the BALB/c mouse model is widely used to study the pathogenic mechanisms involved in disease processes, and also as a pre-clinical animal model for testing drug candidates by the pharmaceutical industry, it was of interest to examine the role of cPLA$_2$ in the BALB/c mouse model of allergic asthma. Notably, our data shows that when AACOCF3 is given orally or intraperitoneally for 4 consecutive days, twice a day, it prevents airway inflammation. cPLA$_2$ derived cysteinyl leukotrienes (such as leukotriene C$_4$, D$_4$ and E$_4$), and prostaglandins such as prostaglandin D$_2$ and thromboxane A$_2$, have been shown to contribute significantly to the pathogenesis of asthma (Narita et al., 1996; Spahr and Krawiec, 2004). Specifically, LTC$_4$ acts as a powerful secretagogue by causing goblet cell proliferation, whereas LTD$_4$ and PGD$_2$ cause bronchoconstriction and airway hyper-responsiveness (Spahr and Krawiec, 2004). Mice with a deficiency in the PGD$_2$ receptor also have attenuated eosinophil infiltration in their bronchoalveolar lavage upon allergen challenge and in the airway hyper-reactivity to methacholine (Spahr and Krawiec, 2004). In our experiments, AACOCF3 when given intraperitoneally inhibited cellular infiltration in bronchoalveolar lavage fluids of ovalbumin-challenged mice in a dose-dependent fashion. At 30 mg/kg intraperitoneally AACOCF3 completely attenuated the cellular response in the

airway lumen of ovalbumin-challenged mice. Following oral administration at 20 mg/kg AACOCF3 inhibited cellular infiltration in bronchoalveolar lavage fluids of ovalbumin-challenged mice by 90%, however, at the same dose methacholine-induced airway hyper-reactivity was inhibited by only 56%, implying that non-cPLA$_2$ dependent mechanisms might be involved in the induction of the AHR response.

cPLA$_2$ may also affect the inflammatory response by its involvement in the regulation of secretion of TNFα, IL-6, IL-1 and IL-8 (Camandola et al., 1996). These cytokines play an important role in the pathogenesis of allergic asthma (Drazen et al., 1996). Thus, inhibition of cPLA$_2$ by AACOCF3 may result in the inhibition of these inflammatory mediators in vivo, which in turn reduced the airway hyper-responsiveness and airway inflammation in mice.

Clearly, not all the cPLA$_2$-dependent mechanisms are detrimental (pro-inflammatory). Beneficial effects of cPLA$_2$-derived anti-inflammatory mediators have also been documented (Clarkson et al., 1998; Laye and Gill, 2003; Linton and Fazio, 2004; Spahr and Krawiec, 2004). Therefore, use of cPLA$_2$ inhibitors for the treatment of clinical conditions should be used with caution. PGE$_2$, and PGI$_2$ are known vasodilators and thus may have protective roles in the pathophysiology of conditions associated with angiotensin II and norepinephrine (Bonventre, 1999). PGE$_2$ has also been reported to protect the kidney against toxic injury (Paller and Manivel, 1992) and regulate the secretion of Th$_1$ cytokines from monocytes by regulating intracellular cAMP (Reder et al., 1994). In addition, cPLA$_2^{-/-}$ mice have been shown to exhibit fertility defects similar to cyclooxygenase-1- and cyclooxygenase-2-deficient mice (Bonventre, 1999; Sapirstein and Bonventre, 2000). Although, absence of cPLA$_2$ in the homozygous cPLA$_2^{-/-}$ mice yielded litters of normal size with a predicted Mendelian genetic distribution, female cPLA$_2^{-/-}$ mice produced small litters and the pups usually did not survive (Bonventre, 1999; Sapirstein and Bonventre, 2000). This indicates the pivotal role of cPLA$_2$ in the maintenance of the normal reproductive process. Female cPLA$_2^{-/-}$ often failed implantation during pregnancy (Sapirstein and Bonventre, 2000). Since prostaglandins are involved in the vascular permeability of the endometrium in normal murine pregnancy, anti-inflammatory eicosanoids such as prostaglandin F$_2$α, and prostaglandin E$_2$ produced by the maternal-fetal-placental compartments are also important for uterine contractions and cervical dilation during labor (Rajabi and Cybulsky, 1995; van der Weiden et al., 1996). Thus, deactivation of cPLA$_2$ may contribute to the severe reproductive abnormalities.

The most compelling example is the association of cyclooxygenase-2 enzyme inhibition with higher risks of myocardial infarction and stroke (Clarkson et al., 1998; Fosslien, 2005; Garcia Rodriguez, 2001; Krotz et al., 2005). This adverse effect of a cyclooxygenase-2 inhibitor has been shown to be due to the suppressed formation of prostaglandin I$_2$, a cyclooxygenase-2 derived anti-inflammatory mediator known to inhibit platelet aggregation, cause vasodilatation, and prevent the proliferation of vascular smooth muscle cells in vitro. Also the individual cardiovascular effects of prostaglandin I$_2$ in vitro contrast with those of thromboxane A$_2$, the major cyclooxygenase-1 product of platelets (not inhibited by a cyclooxygenase-2 inhibitor), which causes platelet aggregation, vasoconstriction, and vascular proliferation. Thus inhibition of pro-inflammatory mediators with cPLA$_2$ inhibitors, such as AACOCF3, may have similar detrimental outcomes.

In conclusion, our preliminary data presented here provides experimental evidence that cPLA$_2$ is an important regulator of chronic but not acute inflammation in mice. Specifically, we have shown that mice treated with a cPLA$_2$ inhibitor, AACOCF3, showed decreased ear edema in the PMA-induced mouse model of chronic inflammation. Furthermore, AACOCF3 showed significant biologic activity in a mouse model of allergic asthma. Treatment of ovalbumin-sensitized female BALB/c mice with AACOCF3 prevented the development of airway hyper-responsiveness and decreased cellular recruitment in the airway lumen after the ovalbumin challenge. Because AACOCF3 also exerts its effect on cyclooxygenases (Reddy and Herschman, 1997), iPLA$_2$ and sPLA$_2$ (expressed in chronic inflammatory conditions) (Ackermann et al., 1995; Balsinde et al., 1999), although with a lesser degree, the inhibition of these enzymes by AACOCF3 cannot be ignored in our experimental models. Additional studies are required to further investigate the role of cPLA$_2$ in chronic inflammatory conditions possibly by using more specific cPLA$_2$α inhibitors.

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