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Platelet-Activating Factor Receptor Develops Airway Hyperresponsiveness Independently of Airway Inflammation in a Murine Asthma Model

Satoshi Ishii, Takahide Nagase, Hideo Shindou, Hajime Takizawa, Yasuyoshi Ouchi, and Takao Shimizu

Lipid mediators play an important role in modulating inflammatory responses. Platelet-activating factor (PAF) is a potent proinflammatory phospholipid with eosinophil chemotactic activity in vitro and in vivo. We show in this study that mice deficient in PAF receptor exhibited significantly reduced airway hyperresponsiveness to muscarinic cholinergic stimulation in an asthma model. However, PAF receptor-deficient mice developed an eosinophilic airway inflammatory response at a comparable level to that of wild-type mice. These results indicate an important role for PAF receptor, downstream of the eosinophilic inflammatory cascade, in regulating airway responsiveness after sensitization and allergen challenge. The Journal of Immunology, 2004, 172: 7095–7102.

Bronchial asthma is a complex disease of the lung characterized by reversible airway obstruction, chronic airway inflammation, and airway hyperresponsiveness (AHR) to nonspecific stimuli. The progression of airway inflammation involves several cell types, including CD4+ Th2 cells, eosinophils, and mast cells (1). The immunopathogenic role of Th2 cells is suggested by the roles of their products, such as IL-4, IL-5, and IL-13 in the recruitment and activation of the primary effector cells of the allergic response, eosinophils and mast cells. Activation of these cells results in the release of many inflammatory mediators that seem to induce AHR individually or coordinately (2, 3), although the precise molecular mechanisms predisposing to the development of AHR in asthmatics are largely unknown. The hypothesis that airway inflammation is responsible for AHR is based on the finding of a significant relationship between the parameters of airway inflammation and AHR (4, 5) and on the observation that inhaled steroids reduce both airway inflammation and AHR (6, 7). However, a number of studies in asthmatic patients have cast doubt on the requirement of airway inflammation for AHR (see review in Ref. 8). In addition, dissociation of AHR from airway inflammation has also been reported in some mouse models of asthma, because IL-5-deficient BALB/c mice partially developed AHR by OVA sensitization/challenge in the absence of airway inflammation (9). Conversely, IL-10-deficient C57BL/6 mice failed to develop AHR even in the presence of robust airway inflammation (10).

Platelet-activating factor (PAF; 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent phospholipid mediator with various biological activities besides platelet activation (11). PAF acts by binding to a G protein-coupled seven-transmembrane receptor (12–16). PAF has long been implicated in the pathophysiological mechanisms of asthma (17), because exogenous PAF closely mimics many of the clinical features of asthma, including AHR (18, 19), bronchoconstriction (18), tracheal fluid secretion (20), and airway microvascular leakage (21) in animals and humans. PAF is detected in bronchoalveolar lavage (BAL) fluid from asthmatic patients but not from nonallergic subjects (22). Eosinophils and mast cells activated in asthmatic airways may be the cellular origins of PAF, because these cells are known to produce PAF in response to various stimuli in vitro (23, 24). Furthermore, PAF was reported to be a potent chemotactic factor for eosinophils (25) and to induce eosinophil degranulation in vitro (26). By using PAF receptor-deficient (pafr−/−) and PAF receptor-overexpressing mice, we have previously demonstrated that PAF plays a critical role in anaphylaxis and acute injury in the lung (27, 28), suggesting that PAF mediates early-phase responses of allergy and inflammation in the tissue. However, the importance of PAF in the development of the allergen-induced AHR and chronic inflammation associated with asthma has not yet been investigated in pafr−/− mice. To define the role of PAF in the late-phase responses of allergy, we used an established murine asthma model, where mice were immunized with aluminum hydroxide adjuvant-adsorbed OVA and challenged with aerosolized OVA. In this study, we describe that pafr−/− mice, sensitized and challenged with OVA, displayed reduced AHR despite a significant eosinophilic airway inflammatory response. PAF may contribute to AHR in asthmatics independently of the eosinophilic airway inflammation.

Materials and Methods

Mice

pafr−/− mice were produced on a mixed C57BL/6 × 129/Ola genetic background as described previously (27). In the present study, pafr−/− mice and

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3 Abbreviations used in this paper: AHR, airway hyperresponsiveness; PAF, platelet-activating factor; BAL, bronchoalveolar lavage; LT, leukotriene; PAS, periodic acid-Schiff; Rl, total lung resistance; EC200 Rl, effective concentration of methacholine required to double the basal Rl.
the corresponding wild-type (paf-4+) control mice have been backcrossed for 6–10 generations onto a BALB/c genetic background. The animals were maintained in a light-dark cycle with light from 7:00 a.m. to 8:00 p.m. at 22°C. Mice were fed with a standard laboratory diet and water ad libitum. All of the mice in this study were used under a protocol approved by the Animal Care and Use Committee of Tokyo University.

During the course of the backcrossing, we selected mice homozygous for the intact allele of the group IIA phospholipase A2 gene that is linked to the PAF receptor gene on murine chromosome 4 (29, 30). The genetic deficiency, inbred mice are deficient in group IIA phospholipase A2 due to a congenital dissection of the gene, whereas BALB/c mice have an intact allele for group IIA phospholipase A2 (29, 31).

Thus, our selection was able to exclude the possible effects of group IIA phospholipase A2 deficiency, which may cause an abnormal metabolism of PAF, on the phenotypes of paf-4+ mice. For genotyping by PCR, genomic DNAs were isolated from tail biopsies. The PCR for PAF receptor alleles was performed with 10 pmol of each primer and 2.5 U of KOD Dash DNA polymerase (Toyobo, Osaka, Japan) in a 50-μl final volume. The PCR profile involved a 2-min denaturation step at 94°C, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 2 s, and extension at 74°C for 30 s. The primers were as follows: forward, 5′-TATGCTGACCTGTCCTCTCCTGAG-3′, and reverse, 5′-TTATGGCGACTTGTTGGTGAGG-3′; for detecting the intact PAF receptor allele; and forward, 5′-GGCTGCTGGCCGCGATATCATGCTGGGAAAATGATGAC-3′, and reverse, 5′-AGGATGCGATAGCCACAATGATGAC-3′, for detecting the disrupted PAF receptor allele. The former set of primers amplified a 287-bp DNA fragment, and the latter PCR product consisted of ~900 bp. The PCR for group IIA phospholipase A2 alleles was performed with 10 pmol of each primer and 1.0 U of Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan) in a 40-μl final volume. The PCR profile involved a 2-min denaturation step at 94°C, followed by 35 cycles of denaturation at 95°C for 30 s, and annealing and extension at 65°C for 3 min. The primers were as follows: forward, 5′-TGTACCTGTCCCTCAAGAGCTGAC-3′, and reverse, 5′-TCACTCTTCTCCAGGCGTCTGAGC-3′, producing 673- and 674-bp DNA fragments from genomic DNA with the intact or mutant allele, respectively. HidII digestion of the PCR products produced polymorphic fragments of 291, 267, and 115 bp with the intact allele or 368, 191, and 115 bp with the mutant allele. The DNA fragments were detected by 2% agarose gel electrophoresis, and ethidium bromide staining.

**Experimental design**

Male and female mice at the age of 8–15 wk were used. Within each experimental group, the sex ratio and the backcross generation were equal, and the age did not differ significantly.

**Sensitization and challenge protocol**

Eosinophilic pulmonary inflammation was induced according to the method of Foster et al. (32) with slight modification. Briefly, mice were sensitized on days 0 and 14 by i.p. injection of 50 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO)/1 mg of aluminum hydroxide (Imject Alum; Pierce, Rockland, IL) in 200 μl of 0.9% sterile saline (Otsuka, Tokyo, Japan). Nonsensitized mice received only 1 mg of aluminum hydroxide [Alum; Pierce, Rockland, IL] in 200 μl of 0.9% pyrogen-free saline. On day 23, the sensitized mice were exposed three times at 1-h intervals to an aerosol of OVA (10 mg/ml) for 30 min. The aerosol challenge protocol was then repeated every second day thereafter for 8 days. Mice were studied 16–22 h after the last aerosol challenge.

**Serum and BAL fluid samples**

For the collection of blood and BAL fluid, mice were anesthetized with 1.5 g of urethane per kilogram of body weight by an i.p. injection at a volume of 10 ml/kg, and placed in the supine position. The blood was taken by cutting the femoral vein and artery. The blood sample was collected in 0.9% saline for 30 min. The nonsensitized mice received saline only. The exposed three times at 1-h intervals to an aerosol of OVA (10 mg/ml) in phosphate-buffered saline at 37°C until use. The lower limits of detection for BAL fluid were 5.0, 5.0, and 1.5 pg/ml, respectively. The total level of cysteinyl leukotrienes (LTC4, LTD4, and LTE4) in the BAL fluid was evaluated by an enzyme immunoassay kit from Amersham (Piscataway, NJ). The lower limit of detection was 15 pg/ml.

**Lung histology**

After the blood collection, the lungs were removed and fixed in 10% phosphate-buffered formalin. From the paraffin-embedded right and left lobes of lung, three sections of 3-μm thickness were prepared at the upper, middle, and lower positions of each lobe, and stained with either H&E or periodic acid-Schiff (PAS). A semiquantitative scoring system was used to grade the size of lung infiltrates in the H&E-stained sections, where +5 signifies a widespread infiltrate around the majority of vessels and bronchioles, and =1 signifies a small number of infiltrates. The total score represents the sum of the scores of both lobes. The goblet cell hyperplasia in the PAS-stained sections was graded by a semiquantitative scoring system (0 = <5% goblet cells in airway epithelium; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = >75%) as performed by McMullen et al. (33). The sum of the airway scores from right lobe was divided by the number of airways examined (16–29 per mouse), and expressed as PAS score in arbitrary units. For both semiquantitative scoring, randomized and blinded slides were graded by SI.

**Measurement of airway responsiveness**

A separate group of mice was anesthetized with a mixture of ketamine and pentobarbital (35 mg/kg each) by i.p. injection. A metal cannula was inserted into the trachea of a tracheostomized mouse. The total lung resistance (Rl) of a mechanically ventilated mouse was measured as previously described (34). Saline and methacholine (acetyl)-b-methylcholine chloride; Wako, Osaka, Japan) were inhaled at a positive end-expiratory pressure of 3 cmH2O. At the start of the protocol, two deep inhalations (3-fold the tidal volume) were delivered to standardize the volume history. All animals were then challenged with the saline aerosol for 2 min. The aerosol was generated with an ultrasonic nebulizer (Ultra-Neb100; DeVilbiss, Somerset, PA) connected to a breathing system. The aerosol was delivered through the inspiratory line into the trachea. Measurements of 10-s duration were made during the tidal ventilation beginning 1 min after the administration of the saline aerosol. This represented the baseline measurement. Subsequently, each dose of the methacholine aerosol was administered for 2 min in a dose-response manner (0.3125, 0.625, 1.25, 2.5, 5.0, 10, and 20 mg/ml saline). During the experiments, oxygen gas was continuously supplied to the ventilatory system. Airway responsiveness was assessed as the effective concentration of methacholine.

The concentrations of cytokines in the BAL fluid were determined using murine ELISA kits obtained from Endogen (Woburn, MA) for IL-4 and IL-5, and R&D Systems (Minneapolis, MN) for IL-13. Whole-lung samples were homogenized on ice using a rotor/stator type tissue homogenizer (Physoctron; Microtec, Chiba, Japan) for 40 s in 8 ml of PBS containing the protease inhibitor mixture per gram of lung tissue. After centrifugation at 18,000 × g for 10 min, the resulting supernatants were stored at −80°C until use. The total amount of PAF receptor inhibitor mixture was per gram of lung tissue. After centrifugation at 18,000 × g for 10 min, the resulting supernatants were stored at −80°C until use. The lower limits of detection for IL-4, IL-5, and IL-13 were 5.0, 5.0, and 1.5 pg/ml, respectively. The total level of cysteinyl leukotrienes (LTC4, LTD4, and LTE4) in the BAL fluid was evaluated by an enzyme immunoassay kit from Amersham (Piscataway, NJ). The lower limit of detection was 15 pg/ml.

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the ANOVA showed significance. For four groups, the difference was evaluated by ANOVA. When parametric analysis (t test) was used to determine the levels of difference between controls (data not shown). However, there were no significant differences between pafrr++/+ mice given OVA and pafrr−/− OVA mice (t test).

### Results

#### Serum Ig levels

In both pafrr++/+ and pafrr−/− mice, aeroallergen challenge was associated with a significant increase in the serum levels of total and OVA-specific IgE, compared with their respective saline-treated controls (data not shown). However, there were no significant differences between pafrr++/+ and pafrr−/− mice, when either the total or OVA-specific IgE level was compared. Similarly to the IgE levels, we found no difference in the OVA-specific IgG1 levels between pafrr++/+ and pafrr−/− mice (data not shown).

#### Inflammatory cell recruitment in BAL fluid

The recovery of cells from the BAL fluid of saline-aerosolized pafrr++/+ and pafrr−/− mice revealed a predominance of alveolar macrophages in both groups, without any significant difference between the numbers (Table I). Aerosol challenge of mice with OVA induced a drastic increase in the total cell number compared with mice given aerosolized saline (Table I). Differential cell counts revealed that the infiltrates in both genotypes were mainly composed of eosinophils. However, the total numbers of cells and the proportions of eosinophils, lymphocytes, macrophages/monocytes, and neutrophils did not differ between pafrr++/+ and pafrr−/− mice given OVA (Table I). These data imply that pafrr−/− mice were capable of recruiting significant numbers of inflammatory cells into the airway lumen after OVA challenge in a manner similar to pafrr++/+ mice.

#### Th2 cytokine and cysteinyl leukotriene levels in BAL fluid

We assessed the levels of the Th2 cytokines IL-4, IL-5, and IL-13 in the BAL fluid (Table II). In saline-treated mice of either genotype, the levels of the Th2 cytokines were near or below the limit of detection. Aeroallergen-challenged pafrr++/+ and pafrr−/− mice showed elevated levels of the Th2 cytokines in the BAL fluid compared with their respective nonsensitized controls. Although there were trends toward higher levels of all three Th2 cytokines in pafrr++/+ mice compared with pafrr−/− mice, these differences did not reach statistical significance (p = 0.10 for IL-4, p = 0.20 for IL-5, and p = 0.12 for IL-13; Mann-Whitney’s U test). Whole-lung homogenates of the allergen-challenged mice also contained similar levels of IL-5 and IL-13 in both genotypes (p = 0.87 for IL-5, and p = 0.07 for IL-13; Mann-Whitney’s U test). Next, the total level of cysteinyl leukotrienes (LTC4, LTD4, and LTE4) in the BAL fluid was evaluated (Table II), because these lipid mediatorS are also involved in airway inflammation in mice as well as humans (36–38). In saline-treated mice of either genotype, the levels of cysteinyl leukotrienes were below the limit of detection. Aeroallergen challenge resulted in comparable elevation of the cysteinyl leukotriene levels in pafrr++/+ and pafrr−/− mice (p = 0.38, t test).

#### Lung histology

The lung tissue fixed after OVA inhalation revealed dense peribronchial and perivascular accumulation of inflammatory cells as well as gross alterations in the structural integrity of the airway walls (Fig. 1, B and D). However, semiquantitative grading of the

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**Table I. Total cell and differential counts obtained from BAL fluid**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Total Cell Counts (×10⁴)</th>
<th>Eosinophils</th>
<th>Lymphocytes</th>
<th>Macrophages</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>pafrr++/+ SAL</td>
<td>4</td>
<td>1.6 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>2.7 ± 1.5</td>
<td>97.2 ± 1.5</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>pafrr−/− SAL</td>
<td>4</td>
<td>2.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>2.6 ± 1.2</td>
<td>97.0 ± 1.4</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>pafrr++/+ OVA</td>
<td>7</td>
<td>40.9 ± 6.3</td>
<td>69.1 ± 3.0</td>
<td>17.7 ± 2.3</td>
<td>12.2 ± 1.9</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>pafrr−/− OVA</td>
<td>7</td>
<td>47.3 ± 4.1</td>
<td>63.5 ± 1.4</td>
<td>21.0 ± 2.0</td>
<td>13.7 ± 1.3</td>
<td>1.7 ± 0.5</td>
</tr>
</tbody>
</table>

* Data are the means ± SEM. SAL, Saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized and OVA-aerosolized treatment.

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**Table II. Th2 cytokine and cysteinyl leukotriene levels in lung**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BAL Fluid (pg/ml)</th>
<th>Cysteinyl leukotrienes</th>
<th>Lung Homogenate (pg/g lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-4</td>
<td>IL-5</td>
<td>IL-13</td>
</tr>
<tr>
<td>pafrr++/+ SAL</td>
<td>12.0 ± 3.8</td>
<td>5.0 ± 0.0</td>
<td>1.5 ± 0.0</td>
</tr>
<tr>
<td>pafrr−/− SAL</td>
<td>9.1 ± 0.9</td>
<td>5.0 ± 0.0</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>pafrr++/+ OVA</td>
<td>66.4 ± 15.4</td>
<td>53.8 ± 19.9</td>
<td>248.0 ± 45.2</td>
</tr>
<tr>
<td>pafrr−/− OVA</td>
<td>32.7 ± 5.7</td>
<td>20.7 ± 6.0</td>
<td>156.4 ± 30.2</td>
</tr>
</tbody>
</table>

* Data are the means ± SEM. pafrr++/+, Wild-type mice; pafrr−/−, PAF receptor-deficient mice; SAL, saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized OVA-aerosolized treatment. BAL fluid and whole-lung homogenate were centrifuged, and the resulting supernatant was subjected to ELISA. There was no significant difference between pafrr++/+ OVA and pafrr−/− OVA mice (Mann-Whitney’s U test) in any mediators. The lower limits of detection for IL-4, IL-5, IL-13, and cysteinyl leukotrienes were 5.0, 5.0, 1.5, and 15 pg/ml, respectively.
After aeroallergen challenge, scores for individual mice are presented. Bars depict means of groups.

**Materials and Methods**

were semiquantitatively scored as described in Materials and Methods, and scores for individual mice are presented. Bars depict means of groups. After aeroallergen challenge, mice develop lung inflammation at a comparable level to saline-treated or OVA-treated group, respectively. Scale bar, shown are representative of six lung sections per mouse, from four or seven mice exposed to OVA. Comparisons of goblet cell hyperplasia fully occurs in the absence of the PAF signaling. Excessive production of airway mucus glycoproteins by goblet cells in airway epithelium is a consistent finding in the lung of asthma. Semiquantification of goblet cells stained with PAS revealed similar mucus scores in mice exposed to OVA. The H&E-stained sections failed to elucidate a significant difference in the degree of airway inflammation between mice and mice (score: 0 in E). Excessive production of airway mucus glycoproteins by goblet cells in airway epithelium is a consistent finding in the lung of asthma. Semiquantification of goblet cells stained with PAS revealed similar mucus scores in mice compared with mice (Fig. 1F). Taken together, these results suggest that airway inflammation and goblet cell hyperplasia fully occurs in the absence of the PAF signaling.

**Airway responsiveness**

To assess aeroallergen-induced physiologic changes, both baseline and airway responsiveness to an inhaled spasmogen, methacholine, were determined. Several aeroallergen-challenged mice had an increased baseline compared with mice treated with saline. However, when analyzed as a group, aeroallergen-challenged mice exhibited no significant difference in the baseline compared with saline-treated mice (0.55 ± 0.06 vs 0.39 ± 0.05 cmH2O/ml; p = 0.09, ANOVA with Bonferroni-Dunn test) or saline-treated mice (0.50 ± 0.06 vs 0.40 ± 0.04 cmH2O/ml; p = 0.30) (Fig. 2A). The inhalation of methacholine showed that mice aerosolized with OVA developed AHR compared with mice treated with aerosolized saline, because OVA-treated mice required a significantly lower dose of methacholine to achieve a 100% increase of the baseline (EC200 Rl) than saline-treated mice (logEC200 Rl = 0.15 ± 0.09 vs 1.01 ± 0.12; p < 0.0001, ANOVA with Bonferroni-Dunn test) (Fig. 2B). Likewise, aerosol challenge of mice with OVA induced a significantly greater responsiveness to methacholine challenge compared with mice given saline (logEC200 Rl = 0.55 ± 0.16 vs 1.13 ± 0.08; p = 0.0001, ANOVA with Bonferroni-Dunn test). OVA-challenged mice aerosolized with saline were signiﬁcantly more responsive than their respective saline-treated controls (p < 0.0001 and p = 0.0063, respectively).

**FIGURE 1.** Histological analysis of lung sections. A, Nonsensitized paf−/− mice exposed to an aerosol of saline. B, Sensitized paf−/− mice exposed to OVA. C, Nonsensitized paf−/− mice exposed to saline. D, Sensitized paf−/− mice exposed to OVA. The H&E-stained sections shown are representative of six lung sections per mouse, from four or seven mice in each saline-treated or OVA-treated group, respectively. Scale bar, 200 μm. E and F, Assessments of lung inﬂammation. The stained sections were semiquantitatively scored as described in Materials and Methods, and scores for individual mice are presented. Bars depict means of groups. After aeroallergen challenge, paf−/− mice develop lung inﬂammation at a comparable level to paf−/− mice, as determined in sections stained with Haematoxylin and Eosin (H&E). The levels of OVA-induced mucus production in paf−/− and paf−/− mice are identical, as determined in sections stained with PAS (F).

**FIGURE 2.** PAF receptor-regulated development of AHR in sensitized and aeroallergen-challenged mice. A, Baseline Rl. SAL, Saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized and OVA-aerosolized treatment. Values for individual mice are presented. Bars depict means of groups. Before methacholine inhalation, no signiﬁcant differences were observed among the four groups despite a nonsigniﬁcant trend toward an increased baseline Rl in the aeroallergen-challenged groups. B, Airway responsiveness to methacholine. Airway responsiveness was assessed by EC200 Rl. The logarithmic values of EC200 Rl for individual mice are presented. Bars depict the means of the groups. paf−/− OVA mice have signiﬁcantly lower responsiveness to methacholine than paf−/− OVA (p < 0.0001, ANOVA with Bonferroni-Dunn test). OVA-challenged paf−/− and paf−/− mice were signiﬁcantly more responsive than their respective saline-treated controls (p < 0.0001 and p = 0.0063, respectively).
ing was saturable with similar calculated 
\( K_{\text{d}} \) values of 237 \pm 15 and 197 \pm 19 pM \((n = 3; p = 0.16, t \text{ test})\) in pafr\(^{+/+}\) and pafr\(^{-/-}\) mice, respectively.

**Discussion**

The murine asthma model recapitulates many of the features of human asthma, including the abundant eosinophilic and lymphocytic infiltration. PAF is chemotactic for eosinophils as well as macrophages/monocytes and neutrophils, all of which are also able to produce PAF (13, 14). Thus, it was reasonable to assume that PAF receptor may contribute to the induction of the airway inflammation associated with asthma. Unexpectedly, however, our studies indicate that the lack of PAF receptor did not alter the recruitment of inflammatory cells (i.e., total numbers of cells, or proportions of eosinophils, lymphocytes, macrophages/monocytes, and neutrophils) in the BAL fluid in this asthma model (Table I). Consistently, we found no significant histological differences in the degree of inflammation in the lung between pafr\(^{+/+}\) and pafr\(^{-/-}\) mice (Fig. 1). These data strongly suggest that PAF is dispensable for the airway inflammation, at least under our murine asthma model. Alternate chemoattractants, such as chemokines and leukotrienes (39–42), may recruit inflammatory cells to the airways. Indeed, BAL fluids from pafr\(^{+/+}\) and pafr\(^{-/-}\) mice contained comparable levels of cysteinyl leukotrienes, which are reported as important mediators for airway inflammation (36–38) (Table II). The present observations are consistent with our previous studies of thioglycolate-elicited peritoneal exudate macrophages (43), casein-elicited peritoneal exudate neutrophils (44), and acid-elicited neutrophils in the lung (28), where no differences were detected in cell numbers and differentials between pafr\(^{+/+}\) and pafr\(^{-/-}\) mice. However, another study of pafr\(^{-/-}\) mice demonstrated diminished eosinophil recruitment in a murine model of allergic pleurisy where the s.c. sensitized mice were challenged once with OVA by intrapleural injection (45). The sensitization/challenge protocol of the pleurisy model is substantially different from that of the asthma model regarding route of Ag sensitization/challenge and frequency of Ag challenge; in this study, the i.p. sensitized mice were repeatedly challenged with OVA aerosols. Therefore, the lack of any differential recruitment of inflammatory cells in pafr\(^{-/-}\) airways is likely due to the nature of the chronic inflammatory responses in the asthma model.

Elevated serum IgE levels have been reported to be important in the development of asthmatic responses (46, 47). Mice passively sensitized with IgG1 as well as IgE were reported to develop AHR (as not shown) and airway Th2 responses (Table II). In all of these aspects, however, they were indistinguishable from pafr\(^{+/+}\) controls. These findings suggest that a deficiency in PAF receptor did not affect the ability to induce humoral immune responses or Th2-directed cytokine responses to Ag.

AHR is a cardinal feature of asthma. PAF has been reported to induce AHR in animals and humans (18, 19), although the mechanisms are not fully understood. Moreover, we previously reported

**FIGURE 3.** Muscarinic cholinergic receptor expression in lungs from sensitized and aeroallergen-challenged mice. A, \([N\text{-methyl}-^3\text{H}]\)Scopolamine binding to lung membrane fractions. The lungs were excised and aerallergen-challenged mice were incubated with increasing concentrations of \([N\text{-methyl}-^3\text{H}]\)Scopolamine for the detection of total binding. Nonspecific binding was determined by incubation in the presence of 20 \(\mu\text{M\ choline. After incubation, free and bound antagonists were separated by rapid filtration on glass microfiber filters. Each point is the mean \pm \text{SEM of triplicate determinations, and the data are representative of three independent experiments. B, Scatchard analysis of the specific binding of \([N\text{-methyl}-^3\text{H}]\)Scopolamine to the lung membrane fractions shown in A. The specific binding was calculated as the difference between the total and nonspecific values. The data of pafr\(^{+/+}\) and pafr\(^{-/-}\) mice are shown. The mean values of \(K_{d}\) and \(B_{\text{max}}\) from the three independent experiments had no statistical significance between pafr\(^{+/+}\) and pafr\(^{-/-}\) mice given OVA (see Results).
that transgenic mice overexpressing PAF receptor showed AHR to inhaled methacholine under physiological conditions (52). To determine whether a lack of PAF receptor has an effect on the development of airway dysfunction, AHR was assessed in \( \text{pafr}^{+/+} \) and \( \text{pafr}^{-/-} \) mice. We found that \( \text{pafr}^{-/-} \) mice had airway responsiveness similar to \( \text{pafr}^{+/+} \) mice after saline-aerosolized treatment (Fig. 2B), indicating that the basal airway responsiveness is not different between \( \text{pafr}^{+/+} \) and \( \text{pafr}^{-/-} \) mice. Following Ag challenge, \( \text{pafr}^{-/-} \) mice developed significantly increased airway responsiveness compared with their saline-treated controls. Furthermore, their responsiveness proved to be significantly lower than that of \( \text{pafr}^{+/+} \) mice given OVA. Thus, PAF receptor is critical for the development of AHR following repeated aerollogen challenge in sensitized mice, and AHR develops by PAF receptor-dependent and -independent pathways.

As reviewed by Drazen et al. (2) and Gali (3), two pathways involving mast cells and eosinophils have been elucidated to mediate aerollogen-induced AHR. Recently, Hogan et al. (9) proposed a novel pathway to the development of AHR intimately mediated by CD4\(^+\) T cells independently of IL-4 and IL-5, although the details of this pathway remain unknown. The relative contribution of these three cellular pathways to the induction of AHR is likely to be dependent on a number of factors including the strain of mouse, the choice of Ag, and the protocols for Ag sensitization and challenge, which may account for the apparent conflict observed among the mouse asthma models used by different investigators (9, 32, 39, 53–56). In the present study, we induced AHR in BALB/c mice with the procedure of Foster and coworkers (9, 32, 54, 57, 58) who have provided corroborative evidence of the important role of IgE, eosinophils, Th2 cytokines, and CD4\(^+\) T cells. They immunized and boosted mice with OVA by i.p. injection of aluminum hydroxide-absorbed OVA, followed by repeated exposure to aerosolized OVA.

It is interesting that after such a strong sensitization/challenge procedure, little or no obligatory role of mast cells in AHR was observed in mast cell-deficient W/W\(^{v}\) mice (56, 59, 60). Consistently, AHR occurred normally with the sensitization/challenge procedure in IL-4-deficient BALB/c mice (9). Thus, in our study, the mast cell-dependent pathway could be excluded from the possible cellular mechanisms leading to the induction of AHR. Hence, it still remains unclear whether PAF is involved in the mast cell pathway. By using other procedures for sensitization/challenge to yield relatively attenuated airway responses, an even more pronounced contribution of PAF receptor to the mast cell pathway may be observed (56, 60).

\( \text{pafr}^{-/-} \) mice, which have BALB/c genetic background, showed partially but significantly attenuated AHR despite a robust airway inflammation with infiltration of eosinophils and lymphocytes, indicating dissociation of AHR from airway inflammation in the mice. As described above, CD4\(^+\) T cells regulate two distinct pathways that have been proposed to regulate aerollogen-induced AHR: one is dependent on eosinophils, and another acts independently of IL-4 and IL-5. In BALB/c mice, the latter pathway is reported to play a major role in the development of AHR without eosinophilic inflammation and morphologic changes in the airways (9). Thus, it is possible that dissociation between AHR and airway inflammation observed in \( \text{pafr}^{-/-} \) BALB/c mice is due to the involvement of PAF in the latter pathway. However, PAF may also be responsible for the development of AHR through the former (eosinophil) pathway. Although this lipid mediator was shown to be dispensable for eosinophil recruitment in this asthma model, it is possible that the infiltrated eosinophils in \( \text{pafr}^{-/-} \) mice are not fully activated at the site of inflammation because of the lack of PAF stimulation. This is reminiscent of the results obtained in the murine acute lung injury model using \( \text{pafr}^{-/-} \) mice in that PAF was essential for the activation of neutrophils but not for their recruitment (28).

The alternative possible target of PAF is smooth muscle. Our data demonstrate that the deficiency of PAF receptor is not associated with a detectable change in either the expression level (\( B_{\text{max}} \)) or ligand affinity (\( K_{d} \)) of muscarinic receptors in the lung, as measured by the nonspecific antagonist [N-methyl-\(^{3}H\)]scopolamine (Fig. 3). Although change of a minor pool of receptors cannot be ruled out, it is likely that the impaired muscarinic cholergic response is due to a postreceptor event. PAF increases the susceptibility of smooth muscle to cholergic stimulation, possibly by modulating the function of M\(_3\) muscarinic receptor, a primary receptor for smooth muscle contraction (61). Indeed, we reported that the AHR to methacholine in transgenic mice overexpressing PAF receptor is mediated by a pathway sensitive to a PAF receptor antagonist (52). Similarly to the present data, the muscarinic receptor-binding activities (\( B_{\text{max}} \) and \( K_{d} \)) of the PAF receptor transgenic mice were indistinguishable from those of wild-type control mice (35). Because PAF receptor mRNA was detected in airway smooth muscle in human peripheral lung (62), it is possible for PAF to modulate the M\(_3\) receptor-evoked smooth muscle contraction at the level of intracellular signal transduction. Whereas M\(_3\) receptor on smooth muscle cells couples to phosphoinositide turnover through G\(_{\text{q}11}\) (61), PAF receptor is capable of coupling to G\(_{\text{q}11}\) and G\(_{\text{q}14}\) (14), suggesting a stimulatory cross talk between the intracellular signals from the two distinct receptors (63).

In most cases, AHR is strongly associated with airway inflammation (64–67), and anti-inflammatory drugs are currently used for bronchial asthma (6, 7). However, the PAF-mediated AHR appears to be independent of inflammation, because \( \text{pafr}^{-/-} \) mice showed a reduction of AHR without diminishment of airway inflammation as shown in this study. This notion is further supported by our previous findings that the PAF receptor-overexpressing mice had AHR without obvious inflammatory responses (52). Recombinant plasma-type PAF acetylhydrolase abrogated airway responsiveness and inflammation concomitantly in a mouse asthma model (65). The apparent discrepancy between our data and those of the report may be attributed to the difference of sensitization procedure. In addition, it is notable that substrates for PAF acetylhydrolase and agonists for PAF receptor do not overlap completely (14, 68).

In summary, the present study demonstrates an important role for PAF receptor in the development of AHR after allergic sensitization/challenge in mice despite the normal expression density and ligand affinity of muscarinic cholinergic receptor. Furthermore, the airway inflammation was not affected by the absence of PAF receptor, suggesting that, as a complement anaphylatoxin C3a (69), PAF only acts downstream of the airway inflammation in bronchial asthma.

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