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Platelet-Activating Factor Receptor Develops Airway Hyperresponsiveness Independently of Airway Inflammation in a Murine Asthma Model1

Satoshi Ishii,2,* 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 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 aeroallergen 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)1 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-O-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 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

1 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; EC50, effective concentration of methacholine required to double the baseline Rl.

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5 Materials and Methods
the corresponding wild-type (paf−/−) 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 Committee for Animal Experiment of the University of Tokyo (Tokyo, Japan).

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 distance between these genes is ~4.3 cM. Both C57BL/6 and 129/Ola inbred mice are deficient in group IIA phospholipase A2 due to a congenital disruption of the gene, whereas BALB/c inbred mice have an intact gene 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−/− 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 40-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′-TATGGGCTACCTGCTGTTCTCCTGAT-3′, and reverse, 5′-TTATGGAACATGTTGTTGGAGATG-3′; for detecting the intact PAF receptor allele; and forward, 5′-GCCCTCTGCCGCGATATCATGGTGGAAAAT-3′, and reverse, 5′-AGATGAGCTAGCACAATGAAACATGATC-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 690–900 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, Kyoto, 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 5 min. The primers were as follows: forward, 5′-TGTACCTGCTCTTCAAGACGTGAC-3′, and reverse, 5′-TCACTCTTTCTACGGCGCTTTGAGC-3′, producing 673- and 674-bp DNA fragments from genomic DNA with the intact or mutant allele, respectively. RFLP 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 modifications. 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) and aluminum hydroxide (Alum; Pierce, Rockland, IL) in 200 μl of 0.9% sterile saline (Otsuka, Tokyo, Japan). Nonsensitized mice received only 1 mg of aluminum hydroxide in 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) in 0.9% saline for 30 min. The nonsensitized mice received saline only. The aerosol with a mass median diameter of 3.8 μm was generated at 20 μl/min by a nebulizer (Pariboy; Pari, Shanghai, China). The inhaled aerosol was delivered through the inspiratory line into the trachea. Measurement of airway responsiveness
A 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 (R L) of a mechanically ventilated mouse was measured as previously described (34). Saline and methacholine (acetyl-L)-methylcholine chloride; Wako, Osaka, Japan) were inhaled at a positive end-expiratory pressure of 3 cm H 2 O. 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). 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 inhibitor mixture (Complete: Roche, Mannheim, Germany) at room temperature. The initial lavage was instilled and retrieved one time, whereas the second lavage was instilled twice. This procedure allowed for a greater number of lung washes with less dilution. In total, ~1.6 ml of BAL fluid was consistently recovered. The sample was centrifuged at 1000 rpm for 10 min at 4°C, and the supernatant was collected and stored at ~80°C. The cell pellet was resuspended in 200–250 μl of cold saline containing 0.1% fatty acid-free BSA (Serologicals Proteins, Kankakee, IL). After an appropriate dilution (2–20-fold) of the cell suspension with Turk solution (Mutoh Chemical, Tokyo, Japan), the total cell number was counted with a hemocytometer. Slides of BAL fluid cells were prepared by placing 3 × 10 7 cells in 3% glutaraldehyde (Cytospan; Shandon, Pittsburgh, PA) at 350 rpm for 2 min, and staining with Diff-Quik (International Reagents, Kobe, Japan). The percentages of eosinophils, lymphocytes, macrophages/monocytes, and neutrophils were determined by counting their number in a total of 300 cells.

Determination of Ab levels in serum
The total IgE, and OVA-specific IgE and IgG1 levels in appropriately sensitized sera were measured by ELISA as previously described (27). The lower limit of detection for total IgE was 50 ng/ml.

Determination of cytokine and cysteinyi leukotriene levels in BAL fluid
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 proteinase 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 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 (LTC 4 , LTD 4 , and LTE 4 ) 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 cells. A semiquantitative score (0–4) was assigned to each lung to represent 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 McMillan 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 S.I.
required to double the basal $R_L$ ($EC_{200}R_L$), which was calculated by interpolation.

**Binding assay for muscarinic receptors**
Each membrane fraction was prepared from four lung tissues of two male and two female mice as previously described (35). The binding assays were performed in triplicate using 100 μg of membrane protein.

**Statistical analysis**
Mann-Whitney’s U test (for nonparametric analysis) or unpaired t test (for parametric analysis) was used to determine the levels of difference between two groups. A value of $p < 0.05$ was considered to have statistical significance. For four groups, the difference was evaluated by ANOVA. When the ANOVA showed significant differences, pairwise comparisons were tested by Bonferroni-Dunn posthoc test, where $p < 0.0083$ was considered to be significant. All statistical calculations were performed with Statistical View-J, version 5.0 (Abacus Concepts, Berkeley, CA). The values for all measurements were expressed as the mean ± SEM.

**Results**

**Serum Ig levels**
In both $pafr^{+/+}$ and $pafr^{-/-}$ 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 $pafr^{+/+}$ and $pafr^{-/-}$ 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 $pafr^{+/+}$ and $pafr^{-/-}$ mice (data not shown).

**Inflammatory cell recruitment in BAL fluid**
The recovery of cells from the BAL fluid of saline-aerosolized $pafr^{+/+}$ and $pafr^{-/-}$ 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 $pafr^{+/+}$ and $pafr^{-/-}$ mice given OVA (Table I). These data imply that $pafr^{-/-}$ mice were capable of recruiting significant numbers of inflammatory cells into the airway lumen after OVA challenge in a manner similar to $pafr^{+/+}$ 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 $pafr^{+/+}$ and $pafr^{-/-}$ 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 $pafr^{+/+}$ mice compared with $pafr^{-/-}$ 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 $pafr^{+/+}$ and $pafr^{-/-}$ mice ($p = 0.38$, t test).

**Lung histology**
The lung tissue fixed after OVA inhalation revealed dense peri-bronchial 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Total Cell Counts ($\times 10^6$)</th>
<th>Eosinophils</th>
<th>Lymphocytes</th>
<th>Macrophages</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pafr^{+/+}$ 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>$pafr^{-/-}$ 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>$pafr^{+/+}$ 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>$pafr^{-/-}$ 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BAL Fluid (pg/ml)</th>
<th>Lung Homogenate (pg/g lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>IL-5</td>
<td>IL-13</td>
</tr>
<tr>
<td>$pafr^{+/+}$ SAL</td>
<td>12.0 ± 3.8</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>$pafr^{-/-}$ SAL</td>
<td>9.1 ± 0.9</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>$pafr^{+/+}$ OVA</td>
<td>66.4 ± 15.4</td>
<td>53.8 ± 19.9</td>
</tr>
<tr>
<td>$pafr^{-/-}$ OVA</td>
<td>32.7 ± 5.7</td>
<td>20.7 ± 6.0</td>
</tr>
</tbody>
</table>

* Data are the means ± SEM. $pafr^{+/+}$, Wild-type mice; $pafr^{-/-}$, 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 $pafr^{+/+}$ OVA and $pafr^{-/-}$ 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.
sections failed to elucidate a significant difference in the degree of airway inflammation between pafr<sup>+</sup><sup>+</sup> and pafr<sup>−/−</sup> mice (p = 0.34, Mann-Whitney’s U test; Fig. 1E). Similarly, as shown in Fig. 1, A and C, the histological findings after saline treatment were unremarkable, with no observable differences between pafr<sup>+</sup><sup>+</sup> and pafr<sup>−/−</sup> 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 asthmatics. Semiquantification of goblet cells stained with PAS revealed similar mucus scores in pafr<sup>−/−</sup> mice compared with pafr<sup>+</sup><sup>+</sup> 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 R<sub>L</sub> and airway responsiveness to an inhaled spasmogen, methacholine, were determined. Several aeroallergen-challenged mice had an increased baseline R<sub>L</sub> compared with mice treated with saline. However, when analyzed as a group, aeroallergen-challenged mice exhibited no significant difference in the basal R<sub>L</sub> in either saline-treated pafr<sup>+</sup><sup>+</sup> mice (0.55 ± 0.06 vs 0.39 ± 0.05 cmH<sub>2</sub>O/ml/s; p = 0.09, ANOVA with Bonferroni-Dunn test) or saline-treated pafr<sup>−/−</sup> mice (0.50 ± 0.06 vs 0.40 ± 0.04 cmH<sub>2</sub>O/ml/s; p = 0.30) (Fig. 2A). The inhalation of methacholine showed that pafr<sup>+/−</sup> mice aerosolized with OVA developed AHR compared with pafr<sup>−/−</sup> mice treated with aerosolized saline, because OVA-treated pafr<sup>+/−</sup> mice required a significantly lower dose of methacholine to achieve a 100% increase of the baseline R<sub>L</sub> (EC<sub>200</sub>R<sub>L</sub>) than saline-treated pafr<sup>+/−</sup> mice (logEC<sub>200</sub>R<sub>L</sub> = −0.15 ± 0.09 vs 1.01 ± 0.12; p < 0.0001, ANOVA with Bonferroni-Dunn test) (Fig. 2B). Likewise, aerosol challenge of pafr<sup>−/−</sup> mice with OVA induced a significantly greater responsiveness to methacholine challenge compared with pafr<sup>−/−</sup> mice given saline (logEC<sub>200</sub>R<sub>L</sub> = 0.55 ± 0.16 vs 1.13 ± 0.08; p = 0.0001 and p = 0.0063, respectively).

FIGURE 1. Histological analysis of lung sections. A, Nonsensitized pafr<sup>+/−</sup> mice exposed to an aerosol of saline. B, Sensitized pafr<sup>+/−</sup> mice exposed to OVA. C, Nonsensitized pafr<sup>−/−</sup> mice exposed to saline. D, Sensitized pafr<sup>−/−</sup> 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, pafr<sup>−/−</sup> mice develop lung inflammation at a comparable level to pafr<sup>+/−</sup> mice, as determined in sections stained with H&E (E). The levels of OVA-induced mucus production in pafr<sup>+/−</sup> and pafr<sup>−/−</sup> mice are identical, as determined in sections stained with PAS (F).
Because methacholine is an agonist of muscarinic acetylcholine receptors, we examined the muscarinic receptor-binding activities of lung tissues from OVA-treated mice using a radiolabeled antagonist, \([N\text{-methyl-}^3\text{H}]\text{scopolamine}\). The lung membranes of \(\text{pafr}^{+/+}\) and \(\text{pafr}^{-/-}\) mice aerosolized with OVA bound similar amounts of this nonselective antagonist (Fig. 3), indicating comparable expression of muscarinic receptor-binding activity \(B_{\text{max}} = 42.7 \pm 2.7\) and 44.8 \pm 1.8 fmol/mg protein, respectively; \(n = 3, p = 0.54, t\) test. The binding was saturable with similar calculated \(K_d\) values of 237 \pm 15 and 197 \pm 19 pM (\(n = 3, p = 0.16, t\) test) in \(\text{pafr}^{+/+}\) and \(\text{pafr}^{-/-}\) 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 \(\text{pafr}^{+/+}\) and \(\text{pafr}^{-/-}\) 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 \(\text{pafr}^{+/+}\) and \(\text{pafr}^{-/-}\) 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 \(\text{pafr}^{+/+}\) and \(\text{pafr}^{-/-}\) mice. However, another study of \(\text{pafr}^{-/-}\) 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 \(\text{pafr}^{-/-}\) 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 and airway inflammation after allergen challenge (48). IL-4 and IL-5 are thought to be central to the development of asthmatic symptoms, because IL-5 regulates the differentiation, recruitment, and activation of eosinophils (49), and IL-4 drives IgE synthesis by B cells (50). Another Th2 cytokine, IL-13, is also hypothesized to play a pivotal role in the pathogenesis of asthma by activating B cells, eosinophils, and airway smooth muscle cells (51). OVA sensitization/challenge of \(\text{pafr}^{-/-}\) mice resulted in serum Ab responses (data not shown) and airway Th2 responses (Table II). In all of these aspects, however, they were indistinguishable from \(\text{pafr}^{+/+}\) 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
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 paf

-/- and paf

+/- mice. We found that paf

-/- mice had airway responsiveness similar to paf

+/- mice after saline-aerosolized treatment (Fig. 2B), indicating that the basal airway responsiveness is not different between paf

+/- and paf

-/- mice. Following Ag challenge, paf

-/- mice developed significantly increased airway responsiveness compared with their saline-treated controls. Furthermore, their responsiveness proved to be significantly lower than that of paf

+/- mice given OVA. Thus, PAF receptor is critical for the development of AHR following repeated allergen 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 allergen-induction-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 (56, 60) indicating dissociation of AHR from airway inflammation because of the lack of muscarinic cholinergic receptor. However, PAF receptor, suggesting that, as a complement anaphylatoxin C3a hydrolase 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 of the report may be attributed to the difference of sensitization model (65). The apparent discrepancy between our data and those 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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