Platelet-Activating Factor Receptor Develops Airway Hyperresponsiveness Independently of Airway Inflammation in a Murine Asthma Model[1]

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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)[3] 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 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; R_L, total lung resistance; $EC_{200}R_L$, effective concentration of methacholine required to double the basal R_L.

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the corresponding wild-type (*pafr*$^{+/+}$) 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 University of Tokyo Ethics Committee for Animal Experiments.

During the course of the backcrossing, we selected mice homozygous for the intact allele of the group IIA phospholipase A_2 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 A_2 due to a congenital disruption of the gene, whereas BALB/c inbred mice have an intact gene for group IIA phospholipase A_2 (29, 31). Thus, our selection was able to exclude the possible effects of group IIA phospholipase A_2 deficiency, which may cause an abnormal metabolism of PAF, on the phenotypes of *pafr*$^{-/-}$ 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′-TATGGCTGACCTGCTCTTCCT GAT-3′, and reverse, 5′-TATTGGGCACTAGGTTGGTGGAGT-3′, for detecting the intact PAF receptor allele; and forward, 5′-GCCTGCTTGCCG AATATCATGGTGGAAAAT-3′, and reverse, 5′-AGGATGCAGTAGC CACAATGGATAC-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 A_2 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 3 min. The primers were as follows: forward, 5′-TGTACCTGT CCTTCACAGAGCTGAC-3′, and reverse, 5′-TCCACTTTTCTCCAGG CGCTTGTAGC-3′, producing 673- and 674-bp DNA fragments from genomic DNA with the intact or mutant allele, respectively. *Hin*fI 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)/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 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 l/min by a nebulizer (Pariboy; Pari, Starnberg, Germany) into a plastic desiccator of 19 liters, whose internal pressure was maintained at atmospheric pressure by an aspirator. 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 a serum separator tube coated with a coagulant (Seraquick Super; Azwell, Osaka, Japan), and then allowed to clot at room temperature for 1 h. The serum was recovered by centrifugation at 2,000 rpm for 10 min at room temperature. After a subsequent centrifugation at 12,000 rpm for 10 min at 4°C in a microcentrifuge, the supernatant was stored at −80°C until use.

Once bleeding had ceased, the trachea of a tracheostomized mouse was cannulated with an 18-gauge metal cannula with a beveled tip. After opening of the thorax by a wide incision of the diaphragm, the lung was lavaged twice with 1 ml of Ca^{2+}- and Mg^{2+}-free PBS containing a proteinase 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 diluent. 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- to 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 3×10^5 cells into a cytocentrifuge (Cytospin 3; 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 randomly selected areas, and dividing these numbers by the total cell count (at least 300 cells).

Determination of Ab levels in serum

The total IgE, and OVA-specific IgE and IgG1 levels in appropriately diluted 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 cysteinyl 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 (Physcotron; 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 inflammatory foci. The total lung 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 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.

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 (R_L) of a mechanically ventilated mouse was measured as previously described (34). Saline and methacholine (acetyl-β-methylcholine chloride; Wako, Osaka, Japan) were inhaled at a positive end-expiratory pressure of 3 cmH_2O. 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) and 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

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

Treatment	n	Total Cell Counts ($\times 10^5$)	Differential Counts (% Total Cells)			
			Eosinophils	Lymphocytes	Macrophages	Neutrophils
pafr$^{+/+}$ SAL	4	1.6 ± 0.1	0.0 ± 0.0	2.7 ± 1.5	97.2 ± 1.5	0.1 ± 0.0
pafr$^{-/-}$ SAL	4	2.0 ± 0.1	0.0 ± 0.0	2.6 ± 1.2	97.0 ± 1.4	0.3 ± 0.2
pafr$^{+/+}$ OVA	7	40.9 ± 6.3	69.1 ± 3.0	17.7 ± 2.3	12.2 ± 1.9	1.0 ± 0.2
pafr$^{-/-}$ OVA	7	47.3 ± 4.1	63.5 ± 1.4	21.0 ± 2.0	13.7 ± 1.3	1.7 ± 0.5

[a] Data are the means ± SEM. SAL, Saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized and OVA-aerosolized treatment. After BAL, the recovered cells were counted, and a portion of the cells were centrifuged onto microscope slides using a cytocentrifuge. The slides were stained with Diff-Quik, and differential cell counts were obtained. For the total cell counts and the percentages of the designated cell types, no significant differences were found between *pafr*$^{+/+}$ OVA and *pafr*$^{-/-}$ OVA mice (*t* test).

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 StatView-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 (LTC_4, LTD_4, and LTE_4) 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 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

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

Treatment	BAL Fluid (pg/ml)					Lung Homogenate (pg/g lung)		
	IL-4	IL-5	IL-13	Cysteinyl leukotrienes	n	IL-5	IL-13	n
pafr$^{+/+}$ SAL	12.0 ± 3.8	5.0 ± 0.0	1.5 ± 0.0	15 ± 0	4	ND	ND	
pafr$^{-/-}$ SAL	9.1 ± 0.9	5.0 ± 0.0	2.2 ± 0.5	15 ± 0	4	ND	ND	
pafr$^{+/+}$ OVA	66.4 ± 15.4	53.8 ± 19.9	248.0 ± 45.2	239 ± 45	10	393 ± 32	2152 ± 203	13
pafr$^{-/-}$ OVA	32.7 ± 5.7	20.7 ± 6.0	156.4 ± 30.2	192 ± 26	10	388 ± 27	1747 ± 101	14

[a] 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.

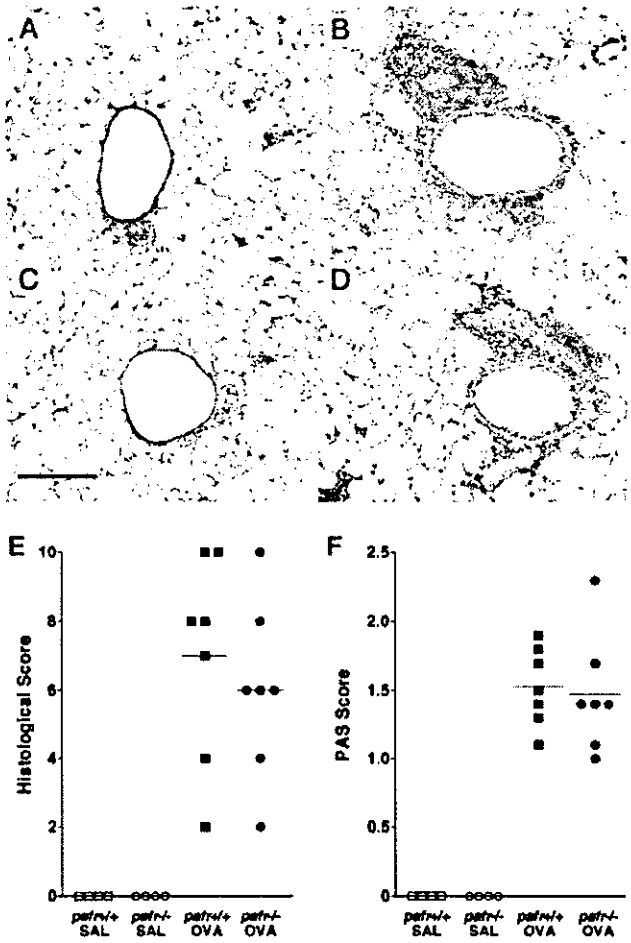

FIGURE 1. Histological analysis of lung sections. *A*, Nonsensitized $pafr^{+/+}$ mice exposed to an aerosol of saline. *B*, Sensitized $pafr^{+/+}$ mice exposed to OVA. *C*, Nonsensitized $pafr^{-/-}$ mice exposed to saline. *D*, Sensitized $pafr^{-/-}$ mice exposed to OVA. The H&E-stained sections 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, $pafr^{-/-}$ mice develop lung inflammation at a comparable level to $pafr^{+/+}$ mice, as determined in sections stained with H&E (*E*). The levels of OVA-induced mucus production in $pafr^{+/+}$ and $pafr^{-/-}$ mice are identical, as determined in sections stained with PAS (*F*).

sections failed to elucidate a significant difference in the degree of airway inflammation between $pafr^{+/+}$ and $pafr^{-/-}$ mice (p = 0.34, Mann-Whitney's U test; Fig. 1*E*). Similarly, as shown in Fig. 1, *A* and *C*, the histological findings after saline treatment were unremarkable, with no observable differences between $pafr^{+/+}$ and $pafr^{-/-}$ 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 $pafr^{-/-}$ mice compared with $pafr^{+/+}$ mice (Fig. 1*F*). 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_L and airway responsiveness to an inhaled spasmogen, methacholine, were determined. Several aeroallergen-challenged mice had an increased baseline R_L compared with mice treated with saline. However, when analyzed as a group, aeroallergen-challenged mice exhibited no significant difference in the basal R_L in either saline-treated $pafr^{+/+}$ mice (0.55 ± 0.06 vs 0.39 ± 0.05 $cmH_2O/ml/s$; p = 0.09, ANOVA with Bonferroni-Dunn test) or saline-treated $pafr^{-/-}$ mice (0.50 ± 0.06 vs 0.40 ± 0.04 $cmH_2O/ml/s$; p = 0.30) (Fig. 2*A*). The inhalation of methacholine showed that $pafr^{+/+}$ mice aerosolized with OVA developed AHR compared with $pafr^{+/+}$ mice treated with aerosolized saline, because OVA-treated $pafr^{+/+}$ mice required a significantly lower dose of methacholine to achieve a 100% increase of the baseline R_L ($EC_{200}R_L$) than saline-treated $pafr^{+/+}$ mice ($\log EC_{200}R_L$ = −0.15 ± 0.09 vs 1.01 ± 0.12; $p < 0.0001$, ANOVA with Bonferroni-Dunn test) (Fig. 2*B*). Likewise, aerosol challenge of $pafr^{-/-}$ mice with OVA induced a significantly greater responsiveness to methacholine challenge compared with $pafr^{-/-}$ mice given saline ($\log EC_{200}R_L$ = 0.55 ± 0.16 vs 1.13 ± 0.08; p =

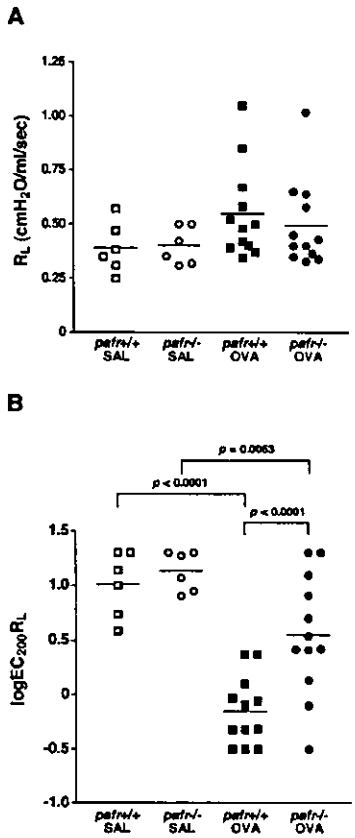

FIGURE 2. PAF receptor-regulated development of AHR in sensitized and aeroallergen-challenged mice. *A*, Baseline R_L. SAL, Saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized and OVA-aerosolized treatment. Values for individual mice are presented. Bars depict means of groups. Before methacholine inhalation, no significant differences were observed among the four groups despite a nonsignificant trend toward an increased baseline R_L in the aeroallergen-challenged groups. *B*, Airway responsiveness to methacholine. Airway responsiveness was assessed by $EC_{200}R_L$. The logarithmic values of $EC_{200}R_L$ for individual mice are presented. Bars depict the means of the groups. $pafr^{-/-}$ OVA mice have significantly lower responsiveness to methacholine than $pafr^{+/+}$ OVA ($p < 0.0001$, ANOVA with Bonferroni-Dunn test). OVA-challenged $pafr^{+/+}$ and $pafr^{-/-}$ mice were significantly more responsive than their respective saline-treated controls ($p < 0.0001$ and p = 0.0063, respectively).

0.0063), but lower responsiveness than $pafr^{+/+}$ mice given OVA ($p < 0.0001$). These findings indicate that aeroallergen-induced AHR develops in $pafr^{-/-}$ mice to a significantly lower degree than $pafr^{+/+}$ mice.

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-methyl*-^{3}H]scopolamine. The lung membranes of $pafr^{+/+}$ and $pafr^{-/-}$ mice aerosolized with OVA bound similar amounts of this nonselective antagonist (Fig. 3), indicating comparable expression of muscarinic receptor-binding activity (B_{max} = 42.7 ± 2.7 and 44.8 ± 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 ± 15 and 197 ± 19 pM ($n = 3$; $p = 0.16$, t 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 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 $pafr^{-/-}$ 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 $pafr^{+/+}$ 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.

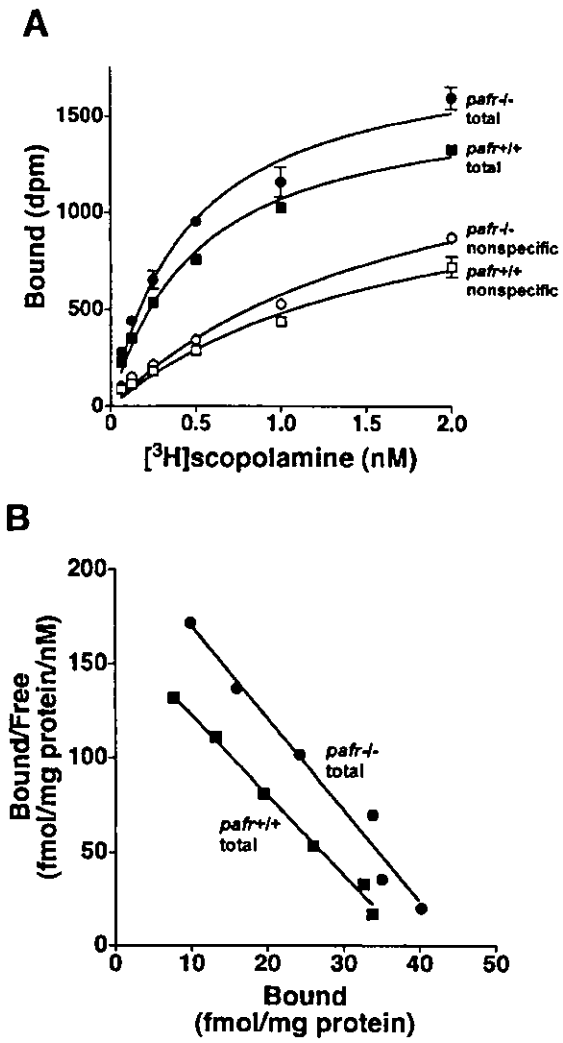

FIGURE 3. Muscarinic cholinergic receptor expression in lungs from sensitized and aeroallergen-challenged mice. *A*, [*N-methyl*-^{3}H]Scopolamine binding to lung membrane fractions. The lungs were excised and homogenized in a buffer, and the membrane fraction was prepared by ultracentrifugation. Aliquots of the resuspended membranes from $pafr^{+/+}$ and $pafr^{-/-}$ mice were incubated with increasing concentrations of [*N-methyl*-^{3}H]scopolamine for the detection of total binding. Nonspecific binding was determined by incubation in the presence of 20 μM methacholine. After incubation, free and bound antagonists were separated by rapid filtration on glass microfiber filters. Each point is the mean ± SEM of triplicate determinations, and the data are representative of three independent experiments. *B*, Scatchard analysis of the specific binding of [*N-methyl*-^{3}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_{max} from the three independent experiments had no statistical significance between $pafr^{+/+}$ and $pafr^{-/-}$ mice given OVA (see *Results*).

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 *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice. We found that *pafr*$^{-/-}$ mice had airway responsiveness similar to *pafr*$^{+/+}$ mice after saline-aerosolized treatment (Fig. 2*B*), indicating that the basal airway responsiveness is not different between *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice. Following Ag challenge, *pafr*$^{-/-}$ mice developed significantly increased airway responsiveness compared with their saline-treated controls. Furthermore, their responsiveness proved to be significantly lower than that of *pafr*$^{+/+}$ mice given OVA. Thus, PAF receptor is critical for the development of AHR following repeated aeroallergen 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 aeroallergen-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).

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 aeroallergen-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 *pafr*$^{-/-}$ BALB/c mice is due to the involvement of PAF in the latter pathway. However, PAF also may 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 *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 *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_{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 cholinergic response is due to a postreceptor event. PAF increases the susceptibility of smooth muscle to cholinergic 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_{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 phosphoinositol turnover through $G_{q/11}$ (61), PAF receptor is capable of coupling to $G_{i/o}$ and $G_{q/11}$ (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 *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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New therapeutic key for cystic fibrosis: a role for lipoxins

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One of the hallmarks of cystic fibrosis is the propensity of patients to develop lung infections with *Pseudomonas aeruginosa*, which eventually compromises lung function. New data suggest loss of CFTR impairs lipoxin production, thus preventing resolution of lung inflammation and creating an environment susceptible to further infection.

Cystic fibrosis (CF) is an autosomal recessive disorder characterized by progressive lung disease and multiple organ dysfunction caused by abnormal electrolyte transport. CF patients show pancreatic insufficiency, increased sweat electrolyte concentrations and lung pathologies with recurrent bacterial infections. The CF transmembrane conductance regulator (*CFTR*) was identified as the causal gene[1]. Soon after the identification of *CFTR*, the molecule encoded was found to be a cyclic AMP–activated chloride ion transporter[2] whose altered function after *CFTR* mutation can account for the dysfunction of most organs affected in CF. However, this specific molecular defect cannot fully explain the lung pathology, as CF patients maintain normal chloride ion concentrations in their lungs[3]. No medication can complement the functional loss of CFTR molecule. Several trials of gene therapy to transfer wild-type *CFTR* nucleotide sequences into the airway epithelium have faced many difficulties, resulting in insufficient *CFTR* expression to achieve clinical benefit. In this issue of *Nature Immunology*, Karp *et al.*[4] provide insight into another CF therapeutic approach from the viewpoint of the lipid mediators that regulate inflammatory responses.

CFTR maps to the long arm of chromosome 7, where it spans 250 kilobases (kb), and consists of 27 exons. The gene is transcribed into a 6.5-kb mRNA that encodes a 1,480–amino acid membrane protein. Although the clinical course of the disease varies with the type of mutation in *CFTR* (800 independent mutations have been identified so far), the estimated median survival age of patients of CF was still only 33.4 years in the US in 2001 (ref. 2). More than 70% of CF patients have homozygous deletion of the three base pairs encoding phenylalanine at position 508 (ΔF508)[2]. In the ΔF508 mutation, mutant CFTR protein is misfolded, leading to rapid ubiquitinylation and protein degradation. Thus, affected cells completely lack CFTR molecules. In some types of mutations (for example, R347P), mutant molecules can reach the cell membrane and show partial chloride conductance. Hence, the clinical manifestations of CF are thought to be related to the type of mutation in *CFTR*.

That CF patients maintain normal chloride concentrations in their lungs indicates another mechanism must exist to explain how the functional loss of CFTR in affected cells results in the pathophysiology leading to progressive lung disease. CF patients are especially prone to chronic airway bacterial infection with pathogens such as *Pseudomonas aeruginosa* and neutrophilic bronchitis and bronchiolitis.

To understand the new findings from Karp *et al.*[4], a discussion of how the onset and resolution of inflammation is regulated by lipid mediators is required. A group of eicosonoid lipid mediators known as lipoxins are anti-inflammatory molecules similar to prostaglandin E_2 that are synthesized during multicellular responses such as inflammation, atherosclerosis and thrombosis (reviewed in ref. 5). In the airway system, activated epithelial cells generate 15*S*-hydroxyeicosatetraenoic acid (HETE) from arachidonic acid with 15-lipoxygenase and release 15*S*-HETE[6]. Polymorphonuclear neutrophils (PMNs) quickly take up 15*S*-HETE and convert it to 15*S*-epoxytetraene with 5-lipoxygenase. The 15*S*-epoxytetraene is then hydrated into 5*S*,6*R*,15S-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid (LXA_4) and its positional isomer 5*S*,14*R*,15*S*-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid (LXB_4). In addition to these native lipoxins, aspirin (acetylsalicylic acid) triggers the endogenous generation of 15-epimeric lipoxins (15-epi-LXA_4 and 15-epi-LXB_4) by the enzyme cyclooxygenase-2.

LXA_4 and LXB_4 have an inhibitory effect on PMNs and eosinophils and also activate monocytes and macrophages. Thus, these lipoxins are proposed to be involved in resolving inflammation. LXA_4 and LXB_4 avoid unwanted effects elicited by PMNs such as PMN-dependent vascular injury and further release of chemoattractants after the initial steps of inflammation. The 15-epi-lipoxins have also inhibitory effect on PMNs. The lipoxin receptor was identified as a seven-transmembrane receptor (ALX/FPRL1) that belongs to the G_i-coupled chemoattractant receptor family.

Recently, another class of anti-inflammatory lipid mediators, resolvins, which are produced from ω-3 polyunsaturated fatty acids such as eicosapentaenoic acid or docosahexaenoic acid, was identified[7]. Although the production of resolvins seem to be regulated through the availability of ω-3 polyunsaturated fatty acids, thus using a different mechanism than the tightly regulated production of lipoxins, competition with 'pro-anti-inflammatory' mediators among chemicals sharing structural similarity indicates that inflammatory reaction is highly regulated and balanced by interactions among cells participating in the inflammation process at each stage. A similar well-known example of such balancing interaction occurs between prostacyclin and thromboxane: both are produced from the same precursor but have diametrically opposite functions in platelet activation, vasoconstriction and possibly in immunolo-gical reactions[8,9].

The enzyme phospholipase A_2 (PLA_2) is essential in the production of proinflammatory mediators, which also include various eicosanoids. Although many distinct types of PLA_2 have been reported, cytosolic PLA_2 is thought to be particularly important. Cytosolic PLA_2 preferentially hydrolyzes phospholipids containing

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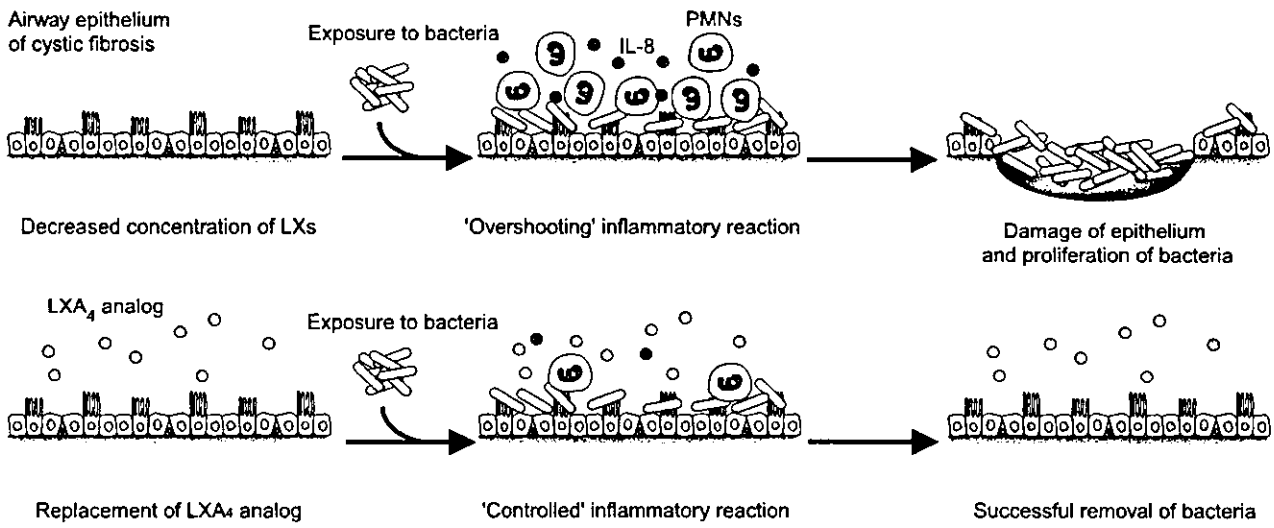

Figure 1 The pathophysiology of CF-affected airway systems and bacterial infection. Top: In the CF airway, because of decreased concentrations of lipoxins, bacterial infection results in an 'overshooting' of inflammation, with excessive numbers of PMNs and interleukin 8 (IL-8), tissue damage and failure to remove bacteria. Bottom: After supplementation with LXA_4 analog, bacterial infection causes 'controlled' inflammation with a moderate number of PMNs, and successful removal of the bacteria results.

arachidonic acid and is activated by submicromolar concentration of calcium and by phosphorylation of serine residues. Lung inflammatory diseases, including acute lung injury and pulmonary fibrosis, are attenuated in mice with disruptions in the gene encoding cytosolic PLA_2 (refs. 10,11). Given that lipoxins belong to the 'downstream' mediators of cytosolic PLA_2, these observations may indicate the complexity of the regulation of lipid mediators in the lung. Is the chronic airway infection and inflammation of CF due to disruption of this regulation?

Karp *et al.*[4] found decreased concentrations of LXA_4 in airway fluids of CF patients. In a mouse airway infection model, supplementation with LXA_4 analogs attenuates PMN accumulation in airway and improves resolution of *P. aeruginosa* infections[4], although those authors[4] used C57BL/6 mice, not $Cftr^{-/-}$ mice, which are susceptible to *P. aeruginosa*–induced inflammation. These results together demonstrate a previously unknown pathophysiology in the airway system in CF: because of decreased concentrations of lipoxins, bacterial infection results in 'overshooting' of inflammation, tissue damage and failure to remove bacteria (**Fig. 1**, top). However, after supplementation with LXA_4 analogs, bacterial infection causes inflammation but is well controlled and bacteria are successfully removed (**Fig. 1**, bottom). Karp *et al.* also demonstrated increased lymphocyte recruitment by LXA_4 analogs[4]. This result is consistent with the hypothesis that lipoxins and resolvins make regional inflammation mature from the 'acute' phase to the 'chronic' phase[7]. Ibuprofen, a nonsteroidal anti-inflammatory drug like aspirin, has been used clinically for the management of CF. There was a substantial reduction in the rate of decrease in lung function in children with CF who were treated with high-dose ibuprofen[12], although this drug does not produce 15-epi-LXA_4.

Although lipoxins' antagonistic effects on cysteinyl leukotriene receptors have been clearly demonstrated, a complete view of the anti-inflammatory mechanisms of lipoxins and resolvins has not been achieved. Do they regulate small GTPase proteins, protein kinase A or transcription factor NF-κB in inflammatory cells? Karp *et al.*[4] did not demonstrate such an 'exorbitant' connection of an ion channel to a lipid mediator in this study. However, electrolyte and/or pH imbalance might affect the production, export and uptake of lipid mediators. The catalytic activities of various enzymes (such as leukotriene A_4 hydrolase–aminopeptidase, a zinc-containing chloride-activated enzyme) might be affected by local electrolyte concentrations. Alternatively, mutant CFTR molecules might directly regulate the expression of 5-lipoxygenase and/or 15-lipoxygenase, which are essential for production of lipoxins. Nevertheless, the present observations and the authors' interpretation of CF-associated lung disease as a deficiency of maturation of regional inflammation might produce new possibilities for therapeutic intervention in this intractable disease. Furthermore, in a more fundamental and broader sense, this study provides a hint for the elucidation of the molecular 'switch' governing how acute inflammation matures and finally resolves. Clearly, these findings bear clinical relevance for the modulation of lipid mediators in CF-affected airways and for our understanding of the relationship between CFTR deficiency and selective inhibition of anti-inflammatory lipid mediators.

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Voltage-gated sodium channel expressed in cultured human smooth muscle cells: involvement of SCN9A

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Abstract **Voltage-gated Na^+ channel (I_{Na}) is expressed under culture conditions in human smooth muscle cells (hSMCs) such as coronary myocytes. The aim of this study is to clarify the physiological, pharmacological and molecular characteristics of I_{Na} expressed in cultured hSMCs obtained from bronchus, main pulmonary and coronary artery. I_{Na}, was recorded in these hSMCs and inhibited by tetrodotoxin (TTX) with an IC_{50} value of approximately 10 nM. Reverse transcriptase/polymerase chain reaction (RT-PCR) analysis of mRNA showed the prominent expression of transcripts for SCN9A, which was consistent with the results of real-time quantitative RT-PCR. These results provide novel evidence that TTX-sensitive Na^+ channel expressed in cultured hSMCs is mainly composed of $Na_v1.7$.**

Keywords: Voltage-gated sodium channel; Cultured human smooth muscle cell; SCN9A; $Na_v1.7$; RT-PCR; Tetrodotoxin

1. Introduction

The voltage-gated Na^+ channel (I_{Na}) exists in a variety of excitable cells including nerves, heart and skeletal muscle. In response to depolarizing stimuli, the channels open and play an essential role in the rising phase of action potential, which is important for impulse generation and conduction. I_{Na} is an integral membrane protein composed of α and auxiliary β-subunits [1–3]. Several α-subunit Na^+ channel genes have been cloned and functionally analyzed in heterologous expression systems [1,2]. Until now, ten types of α-subunit Na^+ channel genes denoted as SCN1A to SCN11A [1,3] have been identified, and are distributed among mammalian cells.

I_{Na} is also identified in several types of freshly isolated smooth muscle cells (SMCs) including vascular, urinary and gastrointestinal SMCs [4–8]. The jejunal circular human SMCs (hSMCs) express a tetrodotoxin (TTX)-insensitive I_{Na} [9], probably $Na_v1.5$ known as the Na^+ channel gene in heart. However, I_{Na} expressed in SMCs [4–8] is a TTX-sensitive type, indicating that different types of α-subunit Na^+ channel genes may be involved. Esophageal hSMCs express TTX-sensitive I_{Na}, $Na_v1.4$ [10]. Existence of I_{Na} has also been reported in cultured hSMCs such as bronchial (hBSMCs) [11], coronary arterial (hCASMCs) [12,13], pulmonary arterial (hPASMCs) and aortic SMCs [14–16], but the types of I_{Na} expressed in cultured hSMCs remain unknown.

Therefore, we investigated the molecular, pharmacological and physiological characteristics of I_{Na}, expressed under culture conditions in hSMCs. Here, we show that cultured hSMCs possess TTX-sensitive I_{Na} mainly composed of $Na_v1.7$.

2. Materials and methods

2.1. Cell preparation

Culture cells isolated from normal human bronchus (hBSMCs), main pulmonary artery (hPASMCs) and large coronary artery (hCASMCs) were purchased from Clonetics Corporation (San Diego, USA). The hSMCs were cultured in medium supplemented with 5% fetal calf serum, human epidermal growth factor (0.5 μg/ml), insulin (5 mg/ml), human fibroblast growth factor (1 μg/ml), gentamycin (50 μg/ml), and amphotericin B (0.05 μg/ml) [SmGM-2 Buffer-Kit, Clonetics] in an atmosphere of 5% CO_2 and 95% air at 37 °C. At confluence, the cells were passaged using 0.05% trypsin in 0.02% EDTA, and they were subcultured in the same medium. Cells isolated by trypsin just before confluence at passages 3–7 were used for later experiments. The cells were identified as SMCs by the expression of α-actin as shown previously [17].

2.2. Solutions and drugs

The composition of control Tyrode solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and HEPES–NaOH buffer 5.5 (pH 7.4). To block K^+ currents, the patch pipette contained (in mM): CsCl 140, EGTA 10, $MgCl_2$ 2, Na_2ATP 3, guanosine-5′-triphosphate (GTP, sodium salt; Sigma) 0.1, and HEPES–CsOH buffer 5 (pH 7.2). 4-aminopyridine (4-AP, 4 mM), a voltage-dependent K^+ channel blocker, tetraethylammonium (2 mM), a Ca^{2+}-activated K^+ current blocker, and Ba^{2+} (1 mM), a blocker of an inwardly rectifier K^+ current [18], were added to the control Tyrode solution. In addition, nifedipine (1 μM) was included into the bathing solution to block the voltage-dependent L-type Ca^{2+} currents. In the $NMDG^+$ solution, extracellular Na^+ was replaced with equimolar *N*-methyl-D-glucamine ($NMDG^+$). All experiments were performed at room temperature (20–25 °C).

2.3. Recording technique and data analysis

Membrane currents were recorded with tight-seal whole-cell clamp techniques using a patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany) as previously described [19–21].

The steady-state inactivation was estimated using double-pulse protocol. Conditioning voltage pulses (500 ms in duration) of various membrane potentials were applied from a holding potential of −80 mV. At 10 ms after the end of each conditioning pulse, a test pulse of +0 mV

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Abbreviations: I_{Na}, voltage-gated Na^+ channel; SMCs, smooth muscle cells; TTX, tetrodotoxin; RT-PCR, reverse transcriptase/polymerase chain reaction

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Table 1
PCR primers used for amplification of voltage-gated Na^+ channel genes

Gene symbol	Channel	Size (bp)		Sequence (5′–3′)
SCN1A	$Na_v1.1$	298	Sense	GACAGCATCAGGAGGAAAGG
			Antisense	TGGTCTGACTCAGGTTGCTG
SCN2A	$Na_v1.2$	194	Sense	ATCCAGAGGGCTTACAGACG
			Antisense	ATCATACGAGGGTGGAGACG
SCN3A	$Na_v1.3$	354	Sense	AATTCTGTGGGGGCTCTAGG
			Antisense	AGCAGCAAGGTTGTCTGAGC
SCN4A	$Na_v1.4$	502	Sense	CAGGCATCTTCACAGCAGAG
			Antisense	ACCATGAGGAAGACGGTGAG
SCN5A	$Na_v1.5$	618	Sense	ACCATCGTGAACAACAAGAGCC
			Antisense	GGCAGCCAGCTTGACAATACAC
SCN6A	Na_x	449	Sense	AAGAGGTGTCTGGGCAGGAT
			Antisense	GACCAGCATCTGTCCTGTTG
SCN8A	$Na_v1.6$	599	Sense	GAGGTGAAGCCTCTGGATGA
			Antisense	CGGATGGTCTTTCTCTGCTC
SCN9A	$Na_v1.7$	403	Sense	GAGGCCTGTTTCACAGATGG
			Antisense	TGGGGCCAAGATCTGAGTAG
SCN10A	$Na_v1.8$	453	Sense	CTTGGGCTTTCCTCTCACTG
			Antisense	AGGCGAGGCCIAGAAAAGAC
SCN11A	$Na_v1.9$	343	Sense	GGCTGTGCGTTAAGAAGGTC
			Antisense	ACCCTGAGCACTCTGAAGGA

was applied. The ratio of I_{Na} amplitude with and without conditioning pulses was plotted against each conditioning voltage. From current–voltage (I–V) data, the steady-state activation curve was derived by using the following equation: $gNa = I_{Na}/[(V_m - E_{Na})]$, where I_{Na} is the peak current amplitude at each membrane potential (V_m), gNa is the chord conductance, and E_{Na} is the Na^+-equilibrium potential. E_{Na} was obtained from I–V curve, where the I–V curve crossed over the zero line. The time course of recovery from inactivation was measured by double-pulse protocols. The first (PI, 50 ms) and the second pulse (PII, 50 ms) with variable interpulse intervals were applied from −80 to +0 mV.

Data were expressed as the means ± S.E. Student's t-test was used for statistical analysis and $P < 0.05$ was significant.

2.4. RNA extraction, RT-PCR and real-time quantitative RT-PCR

Total cellular RNA was extracted using ISOGEN (Nippon Gene, Tokyo). For reverse transcriptase/polymerase chain reaction (RT-PCR), complementary DNA (cDNA) was synthesized from 1 μg of total RNA with reverse transcriptase with random primers (Toyobo, Osaka) as shown previously [18]. The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 30 cycles consisting of heat denaturation, annealing, and extension. PCR products were size-fractionated on 2% agarose gels and visualized under UV light. Primers were chosen based on the sequence of human SCN1-6A and 8A–11A as shown in Table 1. Table 1 also shows the Na^+ channel protein created by each SCN gene. Total RNA of human fetal brain, skeletal muscle and heart (Toyobo, Osaka) was used for positive control.

Real-time quantitative RT-PCR was performed with the use of real-time Taq-Man technology and a sequence detector (ABI PRISM® 7000, Applied Biosystems, Foster City, CA) [22]. Gene-specific primers and Taq-Man probes were used to analyze transcript abundance. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript abundance of SCN family genes. We performed six independent experiments.

3. Results

3.1. Voltage-gated Na^+ channels expressed in cultured hSMCs

Fig. 1A shows a typical recording of I_{Na} expressed in hBSMCs. The cell was held at −80 mV and the command voltage pulses were applied to various membrane potentials. At potentials more positive than −40 mV, a transient inward current was elicited (Fig. 1A). The current–voltage (I–V) relations measured at the peak of the inward current are shown in B. The peak amplitude of the inward current was observed at approximately −10 mV. The time course of the inactivation of the current was well fitted as a single exponential with τ value of 1.1 ± 0.2 ms ($n = 5$) at +0 mV. Replacement of Na^+ with $NMDG^+$, an impermeable cation, completely abolished the inward current (Fig. 1C). Nifedipine (10 μM, $n = 3$) did not inhibit it, but TTX (1 μM) completely blocked it (Fig. 1D). TTX inhibited it concentration-dependently with an IC_{50} value of 5.9 nM ($n = 5$) in hBSMCs as shown in Fig. 1E. I_{Na} was detected in 38% of the total cells tested($n = 95$).

Similarly, I_{Na} was recorded in cultured hPASMCs (Fig. 2A, 20%, 10 out of 50 cells examined) and hCASMCs (Fig. 2B, 15%, 6 out of 40 cells). TTX inhibited I_{Na} expressed in cultured hCASMCs with an IC_{50} value of 6.5 nM ($n = 4$).

3.2. Voltage-dependent characteristics of I_{Na}

Fig. 2C shows typical examples of I_{Na} recordings used to obtain the steady-state inactivation protocol in hBSMCs (see Section 2). The relation between membrane potentials and the V_h value (Fig. 2C) was fitted to the following equation (Boltzmann equation) using the least-squares methods: $I(V)/I_{max} = I/\{1 + \exp[(V - V_h)/k]\}$, where V is the membrane potential in mV, V_h is the membrane potential at half maximum, and k is the slope factor. The value of V_h and k was −37 ± 5 mV and 11.6 ± 3 mV ($n = 4$), respectively. The steady-state activation curves were obtained from the conductance (gNa) and also fitted by Boltzmann equation (Fig. 2D). The value of V_h and k was −16 ± 5 and −5.3 ± 1.0 mV ($n = 4$), respectively. The overlap of the steady-state activation and inactivation curve at potentials more positive than −40 mV determines the I_{Na} window currents. Fig. 2E and F show the time courses of recovery from the inactivation of I_{Na}. By fitting

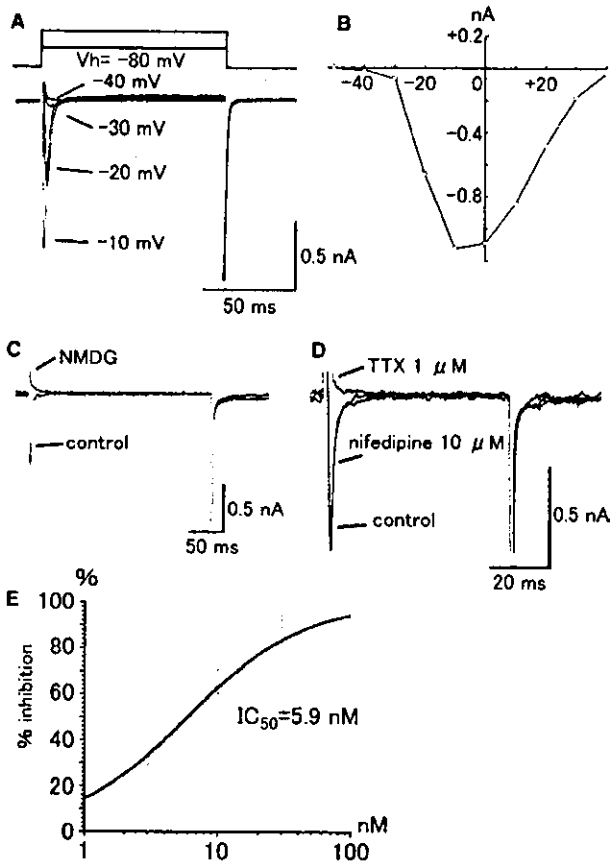

Fig. 1. I_{Na} expressed in cultured human bronchial SMCs. The original current traces elicited by depolarizing pulses are indicated in A. The current–voltage (I–V) relations measured at the peak are illustrated in B. The I–V relations are shown after the leakage currents were subtracted. (C) Effects of replacement of extracellular Na^+ with $NMDG^+$. The current traces in C were elicited from a holding potential of −80 to +0 mV. (D) Effects of nifedipine and TTX on the transient inward current. (E) Concentration-dependent inhibition of I_{Na} by TTX. The cells were held at −80 mV, and command voltage-pulses to +0 mV (100 ms in duration) were applied at 0.2 Hz. The inhibitory effect of TTX on the current amplitude measured at the peak is plotted against various concentrations of TTX Data are shown as means ± S.E. (n = 5) and fit by a Michaelis–Menten simple bimolecular model: % inhibition = 100/{1 + (IC_{50}/[TTX])}, where IC_{50} is 50% inhibitory concentration for TTX. The data were best fit with an IC_{50} value of 5.9 nM.

a single exponential function to the data, the recovery time constants were calculated as 24 ± 5 ms (n = 4).

3.3. Expression of voltage-gated Na^+ channel (SCN) in cultured hSMCs

We investigated the expression of SCN channel family members (SCN1A-11A) except SCN7A mRNA in cultured hSMCs, because SCN6A and 7A are probably the same gene [23]. The amplitude of SCN cDNA fragments was of predicted molecular size, identical to cDNA fragments amplified from reversely transcripted mRNA. The transcript of SCN5A was only detected in hPASMCs, and that of SCN3A was detected only in hCASMCs. The transcripts of SCN1A, 2A, 4A, 6A, 10A, and 11A (data not shown) were not detected in these cells. The transcript of SCN8A was detected weakly in hBSMCs and hPASMCs. The definite transcript of SCN9A was detected in all of the cells. A positive control for SCN1A–

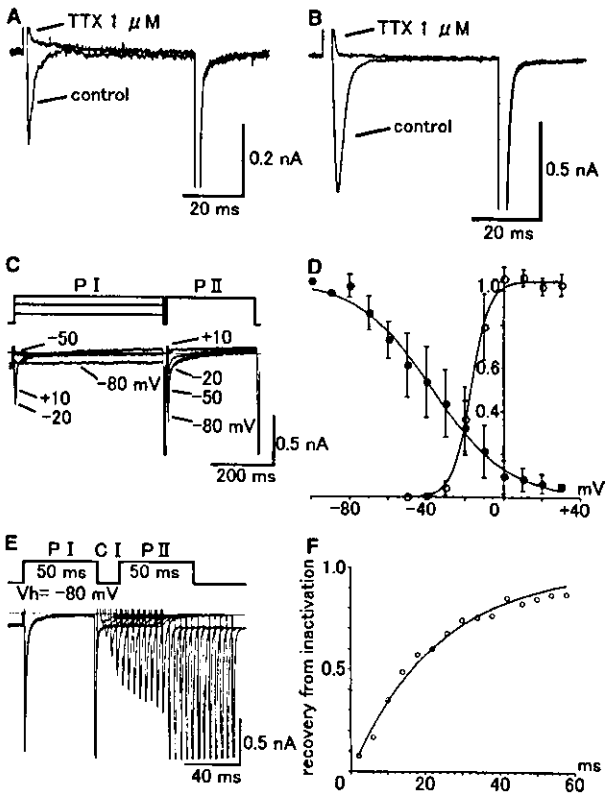

Fig. 2. (A) and (B) I_{Na} expressed in cultured hPASMCs (A) and hCASMCs (B). The cells were held at −80 mV and command voltage pulses to +0 mV were applied. (C) and (D) Steady-state activation and inactivation curves for I_{Na} expressed in hBSMCs. The typical original current traces for obtaining the steady-state inactivation curves are indicated in C and the data were fitted by Bolzmann equation (D). In D, the data represent means ± S.E. value obtained from four different cells. Open circles (steady-state activation curve), closed circles (steady-state inactivation curve). (E) and (F) Recovery from inactivation for I_{Na}. The typical original current traces are shown in E. The data were fitted by single exponential function, where the time constant of recovery from inactivation was 23.9 ms in this cell (F).

3A, SCN4A and SCN5A–6A was observed in human fetal brain, skeletal muscle and heart, respectively.

Expression of SCN channel family member genes (SCN4A–5A, 8A–9A) was also investigated by real-time quantitative RT-PCR. Transcript levels were normalized to 18S ribosomal housekeeping gene. As shown in Fig. 3B, expression levels of SCN9A mRNA were much higher than those of SCN8A. The transcripts of SCN4A and SCN5A were much less than those of SCN9A.

4. Discussion

The present study demonstrated the presence of I_{Na} under culture conditions in hSMCs, which was consistent with the previous papers [11–14]. TTX inhibited it concentration-dependently with an IC_{50} value of approximately 10 nM. These findings indicate that I_{Na} expressed in cultured hSMCs closely resembles TTX-sensitive I_{Na} found in human brain and skeletal muscle, but is different from that found in human heart [1]. Similar TTX-sensitive I_{Na} has been reported in

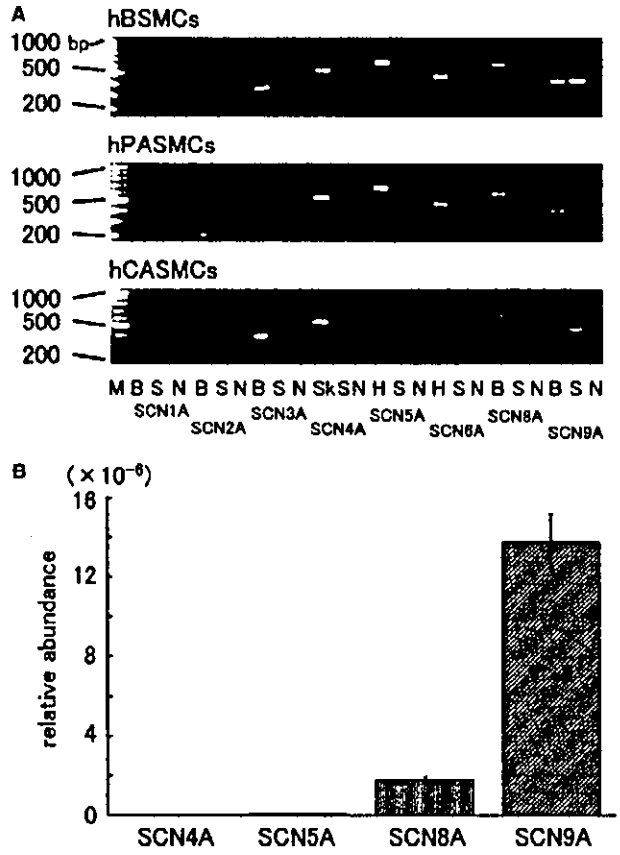

Fig. 3. Expression of I_{Na} genes detected by RT-PCR in cultured hSMCs. (A) RT-PCR. As a positive control, SCN1A–3A were observed in human fetal brain. SCN4A and 5A–6A were observed in human skeletal muscle and human heart, respectively. N, negative control; B, human fetal brain; H, human heart; Sk, human skeletal muscle; S, cultured hSMCs (hBSMCs, hCASMCs and hPASMCs). (B) Real-time quantitative RT-PCR in hBSMCs. The expression levels of SCN channel genes were normalized to those of the 18S ribosomal RNA levels. Data are means ± S.E. from six independent samples.

several types of freshly isolated SMCs [4–8]. Recently, using molecular techniques, human jejunal circular and esophageal SMCs have been shown to express a TTX-insensitive I_{Na} gene, SCN5A [9], and a TTX-sensitive Na^+ channel gene, SCN4A [10], respectively. In the present studies using RT-PCR, SCN5A was detected in hPASMCs. However, it is unlikely that $Na_v1.5$ contributes to form I_{Na} in these cells because low concentration of TTX could completely inhibit I_{Na}. And, SCN4A did not exist. Alternatively, the prominent expression of SCN9A was detected in all types of cultured hSMCs examined. TTX-sensitivity of Na^+ channels is determined by whether one amino acid in SS2 regions of repeat I is aromatic or not, and the amino acid (Y374) is aromatic, suggesting that $Na_v1.7$ belongs to TTX-sensitive I_{Na} [24]. Thus, these results suggest that $Na_v1.7$ is mainly responsible for I_{Na} expressed in cultured hSMCs, which was consistent with the results of real-time quantitative RT-PCR analysis.

I_{Na} expressed in cultured hSMCs was activated at potentials greater than −40 mV and displayed a half inactivation voltage (V_h) of approximately −37 mV, and the window current was observed at potentials more positive than −40 mV. During the repolarization period of 10 ms used in our protocol, parts of I_{Na} can recover, and the residual current may affect the inactivation curves. But, it is negligible at potentials negative than −30 mV due to the small amplitude of I_{Na}. Thus, these values are more positive than those reported for I_{Na} reported in muscle, nerves [23,25]. and freshly isolated SMCs [7–9], proposing that it has atypical characteristics of I_{Na}. But, since the cultured SMCs used here had a membrane potential of approximately −40 mV as described previously [18], it is reasonable that I_{Na} expressed in cultured hSMCs contributes to form membrane potentials and muscle excitability in these cells. In addition, it is likely that small depolarizing stimuli open I_{Na} furthermore, thereby inducing an increase of Na influx and intracellular Na^+ concentration ($[Na^+]_i$). The increase in $[Na^+]_i$ alters the driving force for Na^+, the rate of movement of Na^+ ions by the Na^+/K^+ pump and then the Na^+/Ca^{2+} exchanger, which may subsequently raise intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells.

Expression of $Na_v1.7$ has been found at high levels in peripheral nervous systems and neuroendocrine cells [26,27], which is involved in cell excitability and secretion. Recently, it has been reported in rat and human prostate cancer cell lines, pheochromocytoma and thyroid gland tumor [28]. Physical significance of I_{Na} expressed in these tumor cells remains unclear, but it may be related to the tumor invasion, metastasis and cell proliferation. I_{Na} has been identified in various types of phasic SMCs [8], which usually generate action potential. On the other hand, it is not expressed in freshly isolated tonic SMCs such as human coronary artery and bronchial muscle [11–13], in contrast to the cultured cells [11–14], though expression of I_{Na} has been reported only in rabbit pulmonary arterial cells [6]. Thus, it is likely that I_{Na} expressed in cultured hSMCs is limited to the culture conditions, where cellular dedifferentiation and proliferation may be involved [13,15]. Expression of I_{Na} could be found in parts of the cells examined in this study, which may also support this notion. However, these changes may be occurred under various pathophysiological conditions such as vascular injury, atherosclerosis and asthma [29–32]. Therefore, the possible expression of I_{Na} under these conditions is worth considering, and further studies are necessary to clarify the physiological significance of the channel.

In conclusion, the present study provides novel evidence that TTX-sensitive Na^+ channel is expressed under culture conditions in hSMCs, mainly composed of $Na_v1.7$.

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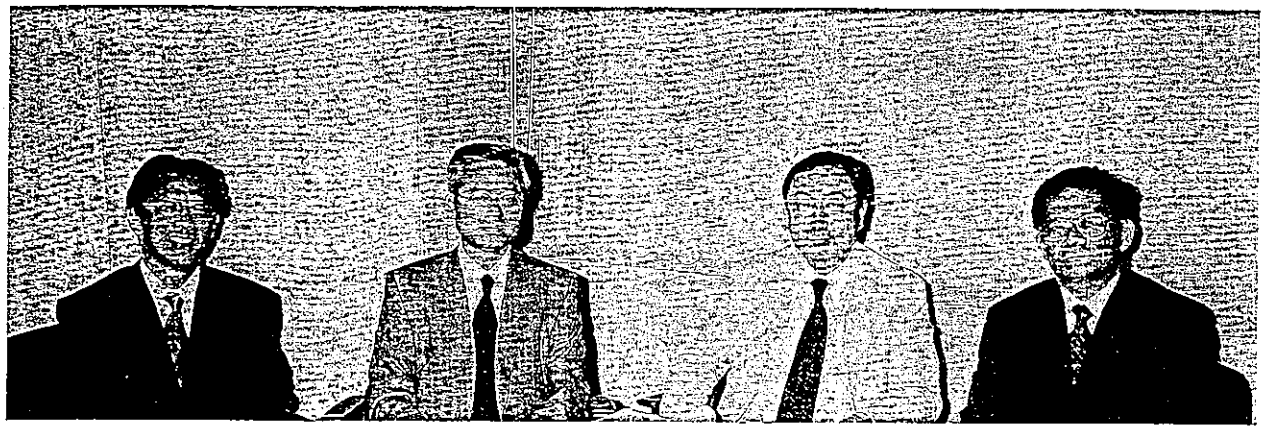
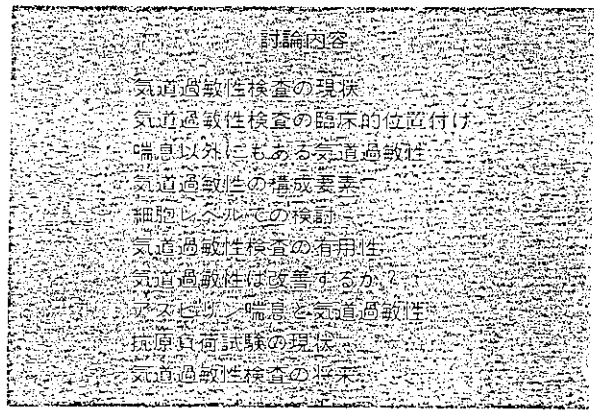
座談会

気道過敏性検査の臨床の現状



一ノ瀬正和[1)](司会)
秋山　一男[2)]
榊原　博樹[3)]
塚越　秀男[4)]



Round table talk：Airway responsiveness measurement in clinical setting
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気道過敏性検査の現状

一ノ瀬　それでは「気道過敏性検査の臨床の現状」ということで，『呼吸』の座談会をはじめさせていただきたいと思います。本日は，気道過敏性検査に関してわが国で第一人者でいらっしゃる秋山先生，榊原先生，塚越先生の3人においでいただきました。

まず，気道過敏性検査に関してですが，これは気管支喘息の病態として非常に重要であるということがもともと指摘されていまして，GINAのガイドラインで「炎症に付随する」という書き方をしていますが，気道の不安定さ，

これは外からの刺激とか自分の持っている炎症細胞の過敏性とか色々あるわけですけれども，それを気道過敏性(airway hyperresponsiveness)と定義しているわけです。実際の臨床上の検査として行われており有用性も示されていますが，問題点も幾つかあります。今回は気道過敏性検査の現状と将来の方向性についてお話ができればと思います。

まず，気道過敏性検査が実際にどの程度なされているのかという点について塚越先生のほうからお話し下さい。

塚越　今回，気道過敏性の実施状況を調査させていただきました。日本では気道過敏性測定法としては標準法とアストグラフ法が主流です。このアストグラフの納入先大学を調べさせていただきました。日本の 68 施設の大学にアストグラフが納入されています。

さらにインターネットで検索可能なホームページを有する，アストグラフ施行可能な上記以外の施設が 17 施設ありました。したがって，アストグラフ納入先の約 85 施設においてアストグラフ法による気道過敏性検査が実施可能ということです。群馬県におきましては群馬大学がその 1 つですが，その他にも約 8 施設にアストグラフが納入されていまして，その裾野は広いと考えられます。

一ノ瀬　アストグラフが 68 施設の大学にあるということは，8 割以上の大学でその検査が行われており，それ以外の病院でも，例えば群馬県で 8 つですから裾野はかなり広いということだと思います。

アストグラフ法というのは瀧島任先生が発案された，連続的に安静換気でメサコリンを吸入し，呼吸抵抗の上昇を直記式に記入するというもので，非常に解析しやすいということで普及しているかと思います。他にも幾つかの方法があると思いますが，榊原先生からそれぞれの利点と欠点についてお話しいただけますか。

榊原　アストグラフ法は，先程いわれた特徴の他に，測定に強制呼出を要さないというメリットがあります。強制呼出をさせますと，気道攣縮が誘発されますので，それが起きないというのはメリットであろうと思います。ただ，装置がかなり大がかりですし，高価であるという点はデメリットでしょう。

一般的に，日本で広く行われているのは標準法ではないかと思います。私たちの施設も 20 年以上前からこの方法を使っています。これは特別な道具立ては必要なく，スパイロメーターあるいはフローボリューム測定器があれば，どこでも実施可能です。ただ，強制呼出させますので，その影響の出る可能性があります。

それから，ドシメータ法というのは，日本では行う施設はあまりないと思いますが，吸入する薬剤の量がコントロールできるという面で，定量性に優れていると考えられます。

一ノ瀬　日本で気道過敏性検査を実際行って経時的にみている施設というと，やはり秋山先生のところの国立相模原病院だと思うのですが，先生のところはどの方法でされていますか。

秋山　うちは標準法で，基本的にはアセチルコリンです。しかし最近は，全部の患者さんに行っているわけではないですけれども，ヒスタミンとアセチルコリンの両方をみるようにして，それはある意味ではまだ研究的な面がありますが，比較しながらみています。

榊原　いまお話の出た，薬剤に何を使うかということは考えなければいけないですね。アセチルコリンかメサコリンかヒスタミン。

気道過敏性検査の臨床的位置付け

一ノ瀬　そうですね。代表的に 3 つですね。標準法は確かアセチルコリンが多いかと思うのですが，アストグラフ法は安定性の面からメサコリンを使っていて，ヒスタミンは実験的な目的などで使いますけれども，咳が出たり，血管作動性で低血圧が問題になります。

実際に患者さんがいらしたときに，例えば喘息疑いであるとか，あるいは喘息を除外するためでもよいのですが，気道過敏性検査の位置付けを，秋山先生から話していただきたいと思います。例えば患者さんがいらして，病歴をとって，色々な検査を行いますが，まずレントゲンとスパイログラムは呼吸器の患者さんであればほぼ 100％行うと思うのですが，気道過敏性検査というのは実際問題，喘息が考えられても全部の方には多分できないと思うのですが，先生のところはどういう流れでしょうか。

秋山　一応我々は，喘息の診断の三種の神器ではありませんが，気道過敏性と可逆性と痰の好酸球，これはぜひやりたいと思うのです。ところが，ご承知のように過敏性を行うには前値が一応ちゃんとした呼吸機能がないと，発作ではまずできません。逆に可逆性は，呼吸機能がよいときにはみることができませんから，これはどちらかというと相反してしまうわけです。ですから，それを同時期に一緒にというのはなかなか難しい面があります。我々は，初診の日には痰は出していただいて好酸球をみるのと，可逆性は呼吸機能検査のキャパシティによりけりですが，できればその日にやってしまおうと。過敏性は，薬を使っている人などの場合には一応薬をやめなければいけないということもありますので，予約制にしています。

一応この 3 つをできるだけ初診に近い時期に行って，

病歴と合わせて診断します。3つ全部そろえば確実例といいますが，そうではなくて2つでも，場合によっては1つでもということで，一応，初診の患者さんで喘息疑いには全員実施するというスタンスです。

一ノ瀬　基本的に可逆性であるとか，痰中の好酸球は比較的すぐにできますが，気道過敏性に関しては予約制になるということですね。

秋山　薬をやめなければいけないということがありますから。

一ノ瀬　榊原先生のところは，大体週に何人とかありますか。

榊原　気道過敏性検査は2人か3人ぐらいが平均です。

一ノ瀬　どうしても負荷試験ですし，1人行うのに時間がかかるということと，検査技師というか医師の立会いが必要になってきますから，マンパワーの問題で色々なリミテーションがかかるかと思うのですが，塚越先生のところはどうでしょうか。

塚越　私どもは大体週に1人程度に実施しています。

一ノ瀬　意外と少ないですね。

塚越　そうですね。実際問題なかなか…。

一ノ瀬　マンパワーですね。呼吸器科医の多いところは色々な病態に対する検索目的で行うのでしょうけれどもね。

塚越　もう少し増やしたいと思います。

一ノ瀬　気道過敏性があるということは，刺激で気道が不安定で縮んだり開いたりするということですから，最近ではピークフローのバリエーション，20％以上で「気道過敏性あり」としようとか，そういう報告もあると思うのですけれども，実際それであれば患者さん自身がモニタリングすればよいわけです。ただ，きちんと診断するにはやはり気道過敏性をみたいというところだと思います。

次に，疾患の弁別性について榊原先生にうかがいたいのですが，先程から喘息ということで話を進めていますが，気道過敏性というのは喘息に特異的ではないと思いますが，その点について他の疾患でもみられるということと，健常者でもどの程度いるかということについて，お話しいただけますか。

喘息以外にもある気道過敏性

榊原　健常な一般住民を対象にした気道過敏性調査に関しては幾つかの論文があります。そういった論文によりますと，おしなべてかなりの高頻度で気道過敏性を持っている方がみつかっています。その気道過敏性調査の方法や使用する薬剤は一定していませんが，気道過敏性の基準としては，同じ方法を用いたときに喘息と診断できる基準を用

一ノ瀬　正和先生

いています。その結果，大体20％，多い報告では30％を超えるような頻度で，一般健常者のなかに気道過敏性を持った人がみつかります。これらはいずれも数百名を超える大規模な調査で，信頼性は高いと思います。

我々も数年間にわたって大学生に協力していただいて調査をしてみました。メサコリンによる標準法を用いました。

当初PC_{20}を使っていたのですが，健常者の1秒量を20％を超すレベルまで落とすと非常に苦しがるのです。それでPC_{10}を使うことにしました。PC_{20}とPC_{10}の相関をとると，PC_{20}で8,000 $\mu g/ml$が，PC_{10}では5,000 $\mu g/ml$に相当するものですから，5,000をカットオフにして，それ以下を「気道過敏性あり」と診断しました。その結果，5,000 $\mu g/ml$以下の頻度が553人中150人，27％ということでした。かなり高頻度に潜在的に気道過敏性を持っている方がいるということが分りました。

一ノ瀬　オランダのcohort studyですと，もともと呼吸器疾患がなくても，潜在的に気道過敏性が亢進している人は将来的には喘息とかCOPDになっていくということも報告されています。あるから必ず病気というわけではないし。ただ，気道過敏性がある場合には何らかのリスクファクターかもしれないので，経過をみることが重要だと考えます。

秋山　榊原先生，そういう人たちというのは，やはり何かallergicな素因があるというか，何かありましたね。

榊原　そうですね。気道過敏性を持っている人と持っていない人を2群に分けて比較すると，持っている人たちはアレルギー性疾患の既往があったり，IgEが高かったり，好酸球が有意に高かったり，1秒量が既に有意に下がっていたり，ということがあります。ですからアトピーあるいは潜在的なアレルギーが気道過敏性とリンクしてい

るという可能性はあります。

一ノ瀬　以上の測定法というか実施状態をまとめると，大学病院の8割以上ではその器械を完備しているし，それ以外の裾野も広いということで，実際されてはいるのですが，やはりマンパワーというか時間がかかるということで，なかなか完璧には行われていないというのが実状と思われます。

気道過敏性検査はアストグラフ法にしろ標準法にしろ時間がかかるわけですから，可逆性検査であるとか痰の好酸球の検査とか，あるいはピークフローの日内変動あるいは日間変動で代用していくということが有用かと思います。

さらに榊原先生からご指摘があったように，健常者でも気道過敏性検査をすると陽性の方が1〜2割，あるいは報告によってはもっとおられて，何らかの呼吸器疾患のバックグラウンドあるいはアトピーとか，そういったものとの関連があるようですが，気道過敏性があるから必ず喘息というわけではないということが，いままでのところのまとめかと思います。

では，その気道過敏性の機序というか，なぜそういったものが認められるのかという点について，秋山先生，お話しいただけますか。

気道過敏性の構成要素

秋山　定義では，気道に色々な炎症があって，前はそれで反応性の亢進を起こすというような表現だったのですが，いまは伴うとかいうことで，原因が気道の炎症であるとは必ずしもとれないような書き方になっています。しかし，どちらにしても我々の理解では，やはり好酸球性の炎症があるために起こるということで，我々もアセチルコリンでの気道過敏性の強さと，あるいは末梢のT細胞のIL-5の産生能をみると，確かに過敏性が高い人はIL-5の産生能が高いということがあるのです。

しかしそれとは別に，我々は喘息で3年以上無治療，無症状の人を寛解といっていますが，その寛解者のなかで，T細胞からのIL-5の産生能をみてみると，例えばダニに対して感作されているような人で，ダニ特異的あるいはPMA+イオノマイシンといったいわゆる非特異的なIL-5産生能をみると，両方とも抑えられている人と，どちらか一方は抑えられていてもう一方は抑えられていない人，両方ともまだまだ非常に敏感というか，沢山産生する人がいる。臨床的には全く症状はないのですが，それでもその人たちのアセチルコリンの過敏性をみると，症状はなくてもそういうIL-5の産生能が高まっている人では，やはり過敏性はまだまだ高いということがあるのです。

ですから，T細胞，IL-5，好酸球，そして過敏性というような流れは，抗IL-5の臨床スタディはありますが，やはり基本的にはそれが正しいのではないかと臨床的には思っています。

一ノ瀬　気道の炎症があって，恐らくそれは好酸球が主体でしょうが，その炎症に付随して，遺伝的な素因も一部は関与しているということですね。確かにガイドラインでも「炎症に付随して」というようになっていますね。

秋山　炎症と過敏症を並列して記載している場合と，原因・結果のような記載とありますね。

一ノ瀬　炎症が原因で気道過敏性が亢進するというのがガイドラインの主旨のようですが，異常は確かにありますね。

秋山　その過敏性といったときに，普通我々が気道過敏性というと，抗原非特異的な過敏性という意味でいいますが，そのときにアセチルコリンなりヒスタミンなりメサコリンなり，どれを使っても非特異的な過敏性をみるにはよいということになっていますけれども，やはりそれぞれに特異的な過敏性があると思います。

一ノ瀬　GINAのガイドラインには色々な物質が気道平滑筋の直接刺激だけでなく，炎症細胞や神経からのメディエーターの遊離を起こし，最終的には気道の内腔を狭窄させるという図が記載されています。喘息患者は刺激に対しても反応するということで非特異的といっているのであって，刺激自体はすべてが特異的だと思います。

一般的には平滑筋を直接縮めるような，アセチルコリンやメサコリンを使うわけですが，確か榊原先生のところでは咳の気道過敏性でカプサイシンをお使いだと先程聞いたのですけれども，それはどういった患者さんにどの程度されているのでしょうか。

榊原　喘息の患者さんも含めてかなりやりましたが，カプサイシンの咳閾値でみる限り，喘息の患者さんと健常者に差はありません。確かに一部の慢性咳嗽の患者さんに閾値の低い人がいますが，ただそれもはっきり健常者と区別できないのです。経過を追ってみて，咳がよくなると閾値が上がるという形で，はじめて咳が出ているときは過敏だったということが分るというような状況です。臨床的にどのように利用すべきか，私自身はっきり確立したものはできていないのです。

細胞レベルでの検討

一ノ瀬　気道過敏性といった場合に，瀧島先生がよくおっしゃっていたのですが，気道の平滑筋だけではなくて気道の微小血管とか神経とか，炎症細胞それぞれに過敏性

があるはずなので，それぞれを臨床レベルで全部分けるのは難しいでしょうけれども，少なくとも研究レベルでは分けて検討するべきだというご意見でした。塚越先生のところは基礎的なこともされていると思うのですが，それに対して何かコメントがありますか。

塚越　気道過敏性の構成要素は，ご指摘のように複雑です。そこで私どもは気道のリモデリングに着目して，気道過敏性の評価を行っています。具体的にはアストグラフ法を用いて，約20名の患者さんで約2年ほどの経過で経年的に気道過敏性の変化を検討してみました。

その解析方法は私どもが10年以上前に動物実験で用いたもので気道の肥厚が気道抵抗に及ぼす影響を除外し，気道平滑筋の収縮率そのものがどのように推移するかを評価するものです。この方法を患者さんで応用してみました。

その結果，気道壁の肥厚は2年間の経過において変化がありませんでした。ただし，平滑筋の収縮率にしてみると，同じ濃度のメサコリンに対して約46％気道過敏性が改善しました。このことは気道過敏性評価における気道の形態の変化の重要性を示唆する結果と考えられました。

一ノ瀬　要するに気道壁の浮腫と平滑筋のショートニングの速さですね。先生は気道壁の厚さは初期抵抗ですべて代用し，ショートニングは抵抗上昇の傾きですか。

塚越　そうです。初期抵抗値の経年的変化を考慮して，気道抵抗の上昇率で，解析してみたのです。

気道過敏性の局在に対して，1つは気道のリモデリングが重要とされていますので，その関与を検討するためにこのような方法を試してみました。

一ノ瀬　確かに臨床だけではなくて，色々な病態を研究する目的からは，気道過敏性の閾値だけではなくて昔，瀧島先生がいわれたreactivity，反応曲線の傾きで検討することも重要かと思います。

塚越　この他，気道過敏性は，罹病期間が長いほど改善しづらいという結果を得ています。

一ノ瀬　それは幾つか報告がありましたね。

塚越　また治療期間が罹病期間に対して長いほど，即ち治療を早期に開始したほど，気道過敏性は改善しやすいという結果が得られました。

気道過敏性検査の有用性

一ノ瀬　次に，「臨床上の有用性」についてお話しいただきたいと思います。ここで秋山先生にお尋ねしたいのですが，有用性というとやはり疾患を診断することと，あとは治療がどの程度うまくいっているかという効果判定の2点が挙げられると思うのですが，診断に関しては榊原先生

秋山　一男先生

から，健常者でも気道過敏性がある人がいるという話と，あとCOPDにも認められるので，必ずしも喘息特異的ではないけれども，診断的価値は高いと。

秋山　それだけでは，ということですね。

一ノ瀬　そういうことですね。あとは診断の2点目としては，ふだんは軽いけれども，ひどい重積発作を起こしやすいとか，そういった人を気道過敏性で見分けるということはできるのですか。

秋山　ちょっと話は違うのですが，いまはまだ確定的なものではないのですけれども，Churg-Strauss症候群の人たちというのは重症喘息なのです。ところが，過敏性は非常に軽いのです。うちでいま30例くらいみていますが，重症喘息ではあるのですが，過敏性の検査をすると例えば閾値が10,000 μg/ml とか20,000 μg/ml とか。ですから，あれはいわゆる気道炎症からくる気道過敏性ではなくて，血管炎からくる過敏性ではないかと。それはまだはっきりはしないのですが，そういうことがあります。

それから，確かに重症度と過敏性というのはきれいなデータがよくありますが，実際問題として重症の人はなかなか過敏性の検査はできないですね。薬を止めるのも難しいし。ですから，私たちのところも軽症とか寛解とか，中等症までのデータはあっても，重症のデータがどうしても少ないですから，重症の人の過敏性が本当にひどいのかどうかということは，大体傾向としてはもちろんそうだと思いますが，必ずしもそうはいえないということはあると思うのです。

それから，そういう過敏性から患者さんがいわゆる突然の大発作になるかどうかということに関しては，少なくとも私たちもそういう目であまりみたことがないので，分りませんが。