CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Inhibition of reactive nitrogen species production in COPD airways: comparison of inhaled corticosteroid and oral theophylline

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Background: Reactive nitrogen species (RNS) are thought to be one of the important factors in the pathogenesis of chronic obstructive pulmonary disease (COPD). A study was undertaken to examine the effects of theophylline and fluticasone propionate (FP) on RNS production in subjects with COPD.
Methods: Sixteen COPD subjects participated in the study. Theophylline (400 mg/day orally) or FP (400 μg/day inhalation) were administered for 4 weeks in a randomised crossover manner with a washout period of 4 weeks. Induced sputum was collected at the beginning and end of each treatment period. 3-nitrotyrosine (3-NT), which is a footprint of RNS, was quantified by high performance liquid chromatography with an electrochemical detection method as well as by immunohistochemical staining.
Results: Theophylline significantly reduced the level of 3-NT in the sputum supernatant as well as the number of 3-NT positive cells (both $p<0.01$). FP also reduced 3-NT formation, but the effect was smaller than that of theophylline. Theophylline also significantly reduced the neutrophil cell counts in the sputum ($p<0.01$), while FP treatment had no effect on the number of inflammatory cells in the sputum, except eosinophils.
Conclusions: Theophylline reduces nitrative stress and neutrophil infiltration in COPD airways to a larger extent than inhaled corticosteroid.

Airway inflammation is the pathophysiological feature of chronic obstructive pulmonary disease (COPD). Although a number of cells and mediators are involved in the pathophysiology of COPD, neutrophilic airway inflammation[1–4] and oxidative stress[5 6] in the lung are thought to play an important part in its development.

Reactive oxygen/nitrogen species (ROS/RNS) have a potent pro-inflammatory action[7] causing airway inflammation,[8] and therefore are thought to be one of the important factors in the pathogenesis of COPD, which results in airway epithelial injury, neutrophil migration and protease/antiprotease imbalance.[9] 3-Nitrotyrosine (3-NT) is a footprint of RNS. We have previously shown that the number of 3-NT positive cells and the level of 3-NT are increased in COPD airways, and these increases are correlated with the airflow limitation of COPD.[10 11] These data strongly suggest that ROS/RNS could have a key role in the pathogenesis of COPD, and that a reduction in ROS/RNS would lead to an anti-inflammatory effect.

More recently we have shown that inhaled corticosteroid can cause a small but significant reduction in RNS production in COPD airways.[12] On the other hand, a recent paper has reported that theophylline reduces the number of neutrophils via a reduction of interleukin (IL)-8 in COPD airways.[13] However, it is still unclear whether theophylline can suppress nitrative stress in COPD airways.

This study was undertaken to assess the anti-inflammatory effects of oral theophylline and inhaled corticosteroid in COPD using a crossover design. Neutrophilic airway inflammation and production of RNS were quantified by measuring 3-NT immunoreactivity in induced sputum. In addition, in order to evaluate the production of RNS in COPD airways in more detail, the levels of 3-NT were measured using high performance liquid chromatography (HPLC) with electrochemical detection (HPLC/ECD) analysis.

METHODS

Subjects

Sixteen patients with COPD regularly visiting Wakayama Medical University Hospital were recruited after giving informed consent. All patients satisfied the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria.[14] None of the patients had either asthma or atopy. The study was approved by the local ethics committee. All patients had a smoking history of at least 10 pack-years. The basal lung function of the patients is shown in table 1. All patients were stable and had had no exacerbation including viral or bacterial infection for at least 3 months before the study. None of the patients had been treated with inhaled or oral corticosteroid for at least 4 weeks before the study.

Study design

The study was a randomised crossover design to compare the effects of theophylline 200 mg twice daily with fluticasone propionate (FP) 200 μg twice daily. Each treatment was administered for 4 weeks with a 4 week washout period between treatments. All patients were assessed at the start and end of the treatment period (fig 1). Nitrotyrosine immunoreactivity, differential cell counts, and protein bound 3-NT levels in induced sputum were measured at that time.

Lung function testing

Lung function was evaluated using a dry rolling seal spirometer (System 7; Minato Medical Science, Osaka, Japan). Before and after treatment with theophylline or FP,

Abbreviations: COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 second; FP, fluticasone propionate; FVC, forced vital capacity; HDAC, histone deacetylase; HPLC/ECD, high performance liquid chromatography with electrochemical detection; IL, interleukin; 3-NT, 3-nitrotyrosine; RNS, reactive nitrogen species

Table 1 Characteristics of study subjects

Age (years)	71 (2)
Sex (F/M)	1/15
Smoking (current/ex)	4/12
Pack-years	68 (8)
FVC (l)	3.17 (0.19)
FEV_1 (l)	1.58 (.16)
FEV_1/FVC (%)	46.8 (3.9)
FEV_1 (% predicted)	53.8 (6.6)

Values are mean (SD).
FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 second.

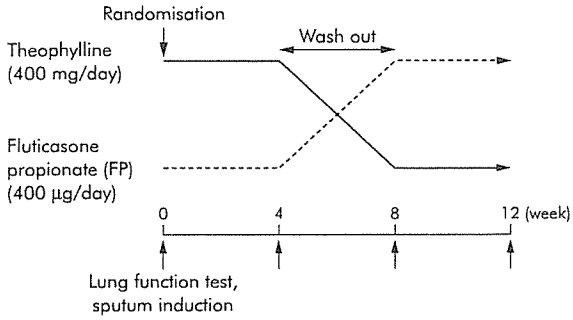

Figure 1 Study design.

forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were measured. Each measurement was performed 15 minutes after inhalation of 400 μg salbutamol via a metered dose inhaler.

Sputum induction and processing

Sputum was induced and processed as described in previous studies.[10 15] Briefly, after 15 minutes pretreatment with 400 μg salbutamol, all patients inhaled 4% hypertonic saline using an ultrasonic nebuliser (UN-701; AICA Co Ltd, Tokyo, Japan). Contamination of saliva was eliminated by visual inspection and examination with an inverted microscope. Hypertonic saline inhalation was performed for 15–30 minutes until the sputum volume was approximately 1 ml. The sputum sample was immediately treated with dithiothreitol (4 mg/g sputum) to dissociate the sulfide bonds of the mucus. Cell viability was determined by the trypan blue exclusion method. The total and absolute number of cells per ml processed sputum was calculated using a haemocytometer. 100 μl of the cell suspension adjusted to 1.0×10^6/ml were centrifuged in a Cytospin 4 cytocentrifuge (ThermoShandon, ThermoBioAnalysis, Tokyo, Japan) at 30*g* for 5 minutes. The preparation was stained with Hansel's stain (Torii Pharmaceutical, Tokyo, Japan) to assess the cell differential counts and stored at −80°C until immunocytochemical analysis.

Immunocytostaining

Samples were immunostained with antisera against 3-NT as described in previous studies.[10] Briefly, the preparation was fixed in 4% paraformaldehyde fixative solution for 30 minutes. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 15 minutes at room temperature. After washing in PBS, the preparations were incubated with anti-nitrotyrosine rabbit polyclonal IgG (1:100 dilution; Upstate Biotechnology, Lake Placid, NY, USA) for 12 hours at 4°C. Non-specific binding to the antibody was prevented by preincubation with 4% skimmed milk in PBS containing 0.3% Triton-X for 30 minutes. The immunoreactions were visualised by the indirect immunoperoxidase method using Envision polymer reagent which is goat anti-rabbit IgG conjugated with peroxidase labelled dextran (Dako Japan Ltd, Kyoto, Japan) for 1 hour at room temperature. Diaminobenzidine reaction was performed, followed by counterstaining with Hansel's stain. The numbers of immunopositive cells were counted by two blinded investigators and the mean of the two values was registered. Cell types were distinguished by cell size, cell form, nuclear segmentation, and nuclear-cytoplasmic ratio.

Quantification of serum IL-8

The levels of serum IL-8 were measured using a commercially available ELISA kit (DuoSet ELISA Development Systems, R&D Systems, Minneapolis, MN, USA) according to the instructions provided by the manufacturer. The minimum detectable concentration of IL-8 was 31.2 pg/ml. A standard curve was obtained with serial dilution of the supplied recombinant human IL-8 by linear regression. The concentration of IL-8 in each sample was obtained by interpolation of its absorbance from a standard curve, and the mean value of the duplicate samples was then taken as the representative value.

Quantification of 3-nitrotyrosine

The levels of 3-NT in the cell-free supernatant were measured by HPLC/ECD as described previously.[11] Briefly, the cell debris was removed by additional centrifugation of the sputum at 3000*g* for 15 minutes at 4°C and, to condense the samples, 400 μl of supernatant were centrifuged using an Ultrafree-MC centrifugal filter (Millipore Corp, Bedford, MA, USA) at 9000*g* for 30 minutes at 4°C. This filter can collect protein of over 10 kDa. After centrifugation, the protein concentration of the sample was determined by the Lowry method.[16] After recovering the sputum protein, it was hydrolysed at 50°C for 18 hours with a freshly prepared solution of *Streptomyces griseus* Pronase (Calbiochem, Darmstadt, Germany) to liberate tyrosine and 3-NT residues. The hydrolysate was centrifuged at 9000*g* with filtration for 30 minutes with an Ultrafree-MC centrifugal filter and the filtrates were then analysed by HPLC/ECD.

50 μl of the sample were injected into a reverse phase column (C18: 3×150 mm; Eicom, Kyoto, Japan) at a flow rate of 0.5 ml/min. Eluents consisting of 5% methanol and 5 mg/l EDTA-2Na in 100 mM sodium phosphate buffer (pH 5.0) were continuously applied to the analytical electrochemical cells. The upstream electrochemical cell (coulometric cell) was used at −900 mV of applied potential for the reduction of 3-NT. The downstream cell (amperometric cell) was used at an oxidation potential of +300 mV for the detection of the reduced form of 3-NT. 3-NT was detected at a 13.5 minute retention time by the response at the oxidation cell on the basis of a standard curve of electrochemical responses as a function of the authentic 3-NT (Sigma Chemical Co, St Louis, MO, USA) concentration. We checked whether this peak was 3-NT as follows:[11] (1) there was no difference in the retention time of the peak between the standard 3-NT and the sputum samples under these HPLC conditions; and (2) when the reduction potential was changed from −900 mV to −600 mV, only the peak at 13.5 minutes disappeared.

The effect of treatment with dithiothreitol on the 3-NT level was determined. Levels of 3-NT with and without treatment with dithiothreitol showed quite good correlation ($r = 0.998$, $p<0.0001$), so it was considered that the processing of induced sputum with dithiothreitol had no influence on the measurement of 3-NT.

Table 2 Differential cell counts in induced sputum

	Theophylline		Fluticasone propionate	
	Before	After	Before	After
Total cells	2.53 (1.79–3.26)	1.63 (1.01–2.24)†	2.53 (1.86–3.19)	1.65 (0.70–2.60)
Neutrophils	1.89 (1.35–2.42)	1.15 (0.80–1.49)†	1.87 (1.33–2.42)	1.16 (0.46–1.87)
(%)	74.7	70.6	73.9	70.3
Macrophages	0.46 (0.18–0.74)	0.42 (0.10–0.74)	0.48 (0.24–0.72)	0.42 (0.08–0.76)
(%)	18.2	25.8	19.0	25.5
Eosinophils	0.06 (0.03–0.07)	0.03 (0.01–0.04)†	0.05 (0.04–0.07)	0.02 (0.01–0.04)†
(%)	2.4	1.8	2.0	1.2

Values are median (interquartile range) $\times 10^6$/ml.
†$p<0.01$ *v* pretreatment values.

The amount of tyrosine in the same sample was also determined in a separate process using HPLC analysis. Briefly, 1 µl of each sample was injected into a reverse phase column (Wakopak C30.5: 4.6 mm×300 mm, Wako Pure Chemical, Osaka, Japan) at a flow rate of 0.8 ml/min maintaining the temperature at 37°C. The eluents consisted of 5% methanol in 50 mM sodium acetate buffer (pH 4.7). Tyrosine was detected at a retention time of 8.47 minutes with the electrochemical response set at +600 mV. The amount of tyrosine in a sputum sample was determined based on the peak area compared with the standard curve of tyrosine (Wako Pure Chemical). The level of 3-NT was shown as a ratio to the total tyrosine concentration.

As shown in our previous report, the spike recovery analysis indicated that the percentage of recovery of 3-NT and tyrosine was more than 90%.[11] In addition, the coefficient of variation of 3-NT measurement in sputum samples previously performed in triplicate was 5–10%, indicating that the determination of 3-NT by this technique is highly reproducible.[11]

Statistical analysis

All data were expressed as median (interquartile range). Comparison of outcomes between the theophylline and FP groups was performed using repeated measures ANOVA. Wilcoxon's signed rank sum test was used to compare the effect of treatment on the total and differential cell counts and pulmonary function. Pearson's correlation analysis was used to assess the correlations between changes in the RNS marker and those in the differential cell counts. A value of $p<0.05$ was considered to be significant.

RESULTS

The mean (SD) plasma theophylline level during theophylline administration was 6.32 (0.9) mg/l, which is lower than the clinically recommended concentration as a bronchodilator (10–20 mg/l). Because of this low concentration of theophylline, neither FP nor theophylline had a significant effect on FVC and FEV_1 after 4 weeks of administration (FVC: before theophylline 3.34 (2.64–4.03) l; after theophylline 3.49 (2.81–4.16) l; before FP 3.41 (2.86–3.96) l; after FP 3.48 (2.89–4.07) l; FEV_1: before theophylline 1.51 (1.11–1.91) l; after theophylline 1.60 (1.09–2.11) l; before FP 1.48 (0.91–2.05) l; after FP 1.59 (1.06–2.11) l).

Theophylline administration significantly reduced the total number of inflammatory cells in the sputum from 2.53 (1.79–3.26)$\times 10^6$/ml to 1.63 (1.01–2.24)$\times 10^6$/ml ($p<0.01$, table 2, fig 2A). Consistent with this, the number of neutrophils in the sputum also decreased significantly from 1.89 (1.35–2.42)$\times 10^6$/ml to 1.15 (0.80–1.49)$\times 10^6$/ml ($p<0.01$, table 2,

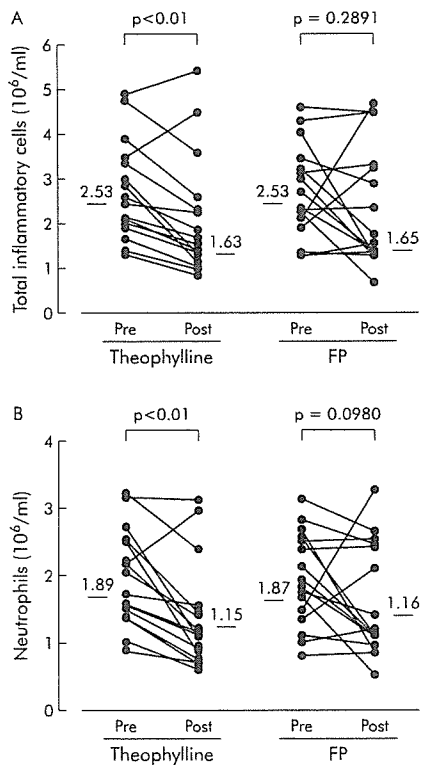

Figure 2 Effect of theophylline and fluticasone propionate (FP) on inflammatory cells. Theophylline significantly reduced the total number of inflammatory cells (A) and neutrophils (B), whereas FP had no apparent effect. Bars indicate median values.

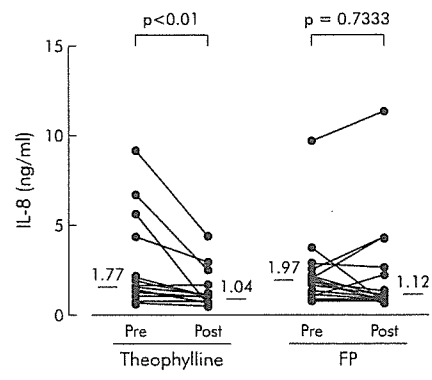

Figure 3 Effect of theophylline and fluticasone propionate (FP) on the concentration of interleukin (IL)-8. The IL-8 concentration in the sputum supernatant was significantly decreased by theophylline while FP had no effect. Bars indicate median values.

Table 3 Positive cell counts of 3-nitrotyrosine (3-NT) in induced sputum

	Theophylline		Fluticasone propionate	
	Before	After	Before	After
Total cells	1.24 (0.75–1.72)	0.73 (0.28–1.18)†	1.35 (0.86–1.83)	0.83 (0.25–1.41)*
Neutrophils	0.97 (0.52–1.42)	0.53 (0.22–0.83)†	0.89 (0.47–1.32)	0.52 (0.12–0.93)
(%)	78.2	72.6	65.9	44.6
Macrophages	0.31 (0.07–0.55)	0.18 (0.02–0.34)	0.40 (0.19–0.61)	0.42 (0.08–0.76)
(%)	23.8	24.7	29.6	50.6

Values are median (interquartile range) $\times 10^6$/ml.
†$p<0.01$; *$p<0.05$ v values before treatment.

fig 2B), while FP treatment did not affect the numbers of any inflammatory cells in the sputum with the exception of eosinophils (table 2, fig 2A, B). Neither theophylline nor FP had any effect on the number of macrophages in the sputum. The number of eosinophils was quite small, but significant decreases were seen with both theophylline and FP (table 2).

To determine the mechanism of the decrease in neutrophils, we next measured the concentration of IL-8 in the sputum supernatant which is one of the well known chemoattractants of neutrophils. As shown in fig 3, theophylline significantly reduced the level of IL-8 in the sputum supernatant (from 1.77 (0.03–3.50) ng/ml before treatment to 1.04 (0.39–1.70) ng/ml after treatment ($p<0.01$), while FP did not. There was no apparent correlation between the serum levels of IL-8 and the number of neutrophils.

We then compared the effects of theophylline and FP on nitrative stress in the airway inflammation of COPD. As shown in table 3 and fig 4A–C, after 4 weeks of treatment with theophylline the total number of 3-NT positive cells in the induced sputum was decreased from 1.24 (0.75–1.72) $\times 10^6$/ml to 0.73 (0.28–1.18) $\times 10^6$/ml. Theophylline also decreased the number of immunopositive neutrophils for 3-NT from 0.97 (0.52–1.42) $\times 10^6$/ml to 0.53 (0.22–0.83) $\times 10^6$/ml ($p<0.01$, table 3, fig 4D). In contrast, although FP also decreased the total number of 3-NT positive cells (from 1.35 (0.86–1.83) $\times 10^6$/ml to 0.83 (0.25–1.41) $\times 10^6$/ml, $p<0.05$), the effect was milder than that of theophylline and there was no apparent effect on the number of 3-NT positive neutrophils (0.89 (0.47–1.32) $\times 10^6$/ml before treatment, 0.52 (0.12–0.93) $\times 10^6$/ml after treatment; table 3, fig 4C, D).

We next measured the levels of 3-NT in sputum. There was a possibility that current smoking may affect the levels of 3-NT, but no significant difference in 3-NT levels was seen between current smokers and ex-smokers, at least in the

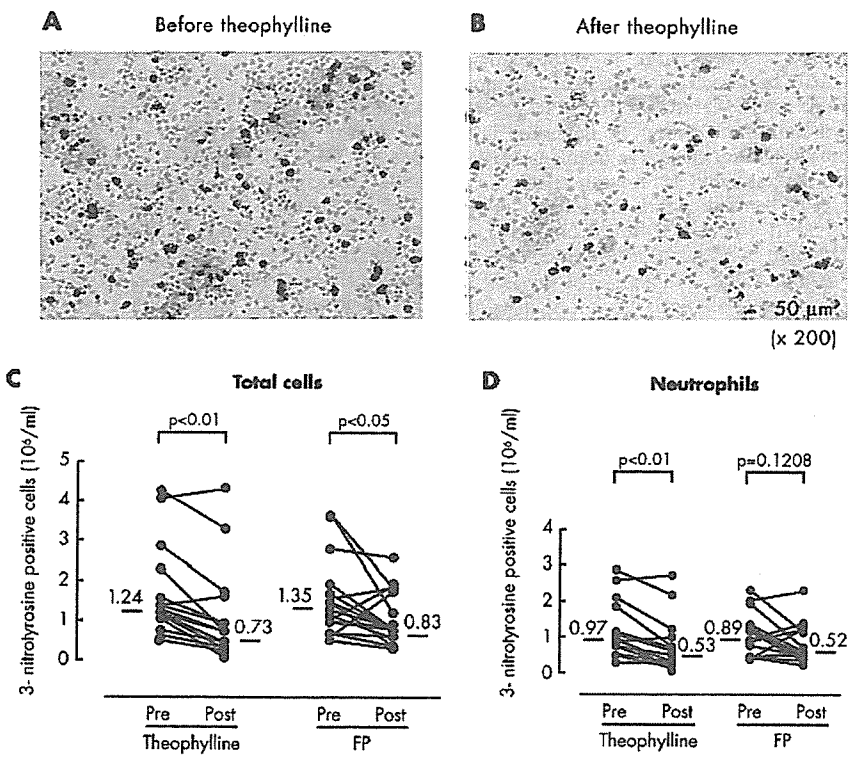

Figure 4 Effect of theophylline and fluticasone propionate (FP) on immunocytochemical staining against 3-nitrotyrosine (3-NT). Immunopositive inflammatory cells for 3-NT in the induced sputum were reduced after treatment with theophylline (B) compared with before treatment (A). Theophylline significantly reduced the total immunoreactivity of 3-NT in inflammatory cells (C) and neutrophils (D). FP also reduced the total immunoreactivity of 3-NT in inflammatory cells, but not in neutrophils. Bars indicate median values.

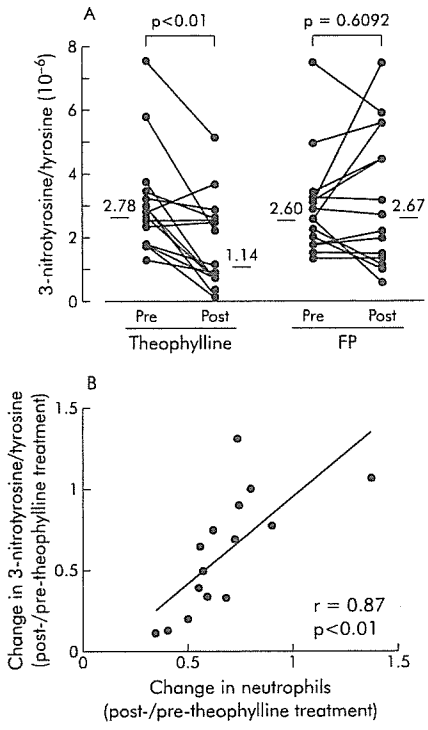

Figure 5 Effect of theophylline and fluticasone propionate (FP) on 3-nitrotyrosine (3-NT) levels (A) Theophylline significantly reduced the level of 3-NT in induced sputum as measured by HPLC/ECD, while FP had no effect. (B) There was a significant correlation between the decrease in neutrophils and the decrease in the 3-NT level after theophylline treatment. Bars indicate median values.

present study (current smokers 2.14×10^{-6}, ex-smokers 3.43×10^{-6} 3-NT/tyrosine). Consistent with the results of immunocytochmeical staining, the level of 3-NT in the sputum supernatant was significantly decreased after 4 weeks of treatment with theophylline ($p<0.01$, fig 5A), while no apparent effect was seen after treatment with FP. As shown in fig 5B, there was a significant positive correlation between the decrease in the number of neutrophils in the induced sputum and the reduction in 3-NT levels in the sputum supernatant in those treated with theophylline ($r=0.87$, $p<0.01$). There was no significant correlation between the serum IL-8 levels and the levels of 3-NT in sputum.

DISCUSSION

We have shown for the first time that treatment with low dose theophylline significantly reduces RNS production and neutrophil infiltration to a greater extent than inhaled corticosteroid in COPD airways.

We have previously reported the effect of inhaled corticosteroid on the suppression of nitrative stress in COPD airways. In that study, administration of inhaled corticosteroid for 4 weeks significantly reduced total 3-NT immunoreactivity of inflammatory cells, macrophages and neutrophils in the induced sputum.[12] Inhaled corticosteroid also reduced the inducible nitric oxide synthase (iNOS) immunoreactivity in those cells. The formation of nitrotyrosine depends on the oxidation of nitric oxide (NO), which reacts with superoxide anion to produce the more potent RNS, peroxynitrite.[17] Peroxynitrite causes tyrosine nitration. Because iNOS is one of the main sources of NO production, it is suggested that the mechanism of nitrative stress inhibition by inhaled corticosteroid, as in our present study, could be due mainly to the reduction of iNOS.

Low dose theophylline reduced nitrative stress in COPD airways to a larger extent than inhaled corticosteroid. Theophylline also inhibited neutrophilic inflammation in COPD airways. In addition, as shown in fig 5, there was a significant positive correlation between the reduction in 3-NT levels and the decrease in the number of neutrophils after theophylline administration. An alternative pathway for the formation of 3-NT is via the neutrophil myeloperoxidase (MPO) effect on NO.[17 18] Nitrite produced by the reaction of NO with oxygen is oxidised by MPO which results in the formation of reactive nitrogen intermediates. These products are also involved in tyrosine nitration.[17] It is therefore possible that the theophylline induced inhibition of nitrative stress seen in the present study was due to the inhibition of neutrophil infiltration.

We also observed a significant reduction in IL-8 production after theophylline administration, which is a possible mechanism for the inhibition of neutrophilic inflammation by theophylline. This is compatible with the findings of a previous study.[13] The precise mechanism of IL-8 reduction by theophylline is unclear. However, it has recently been shown that theophylline can inhibit the release of IL-8 from respiratory epithelial cells in vitro.[19] The direct effect of theophylline on respiratory epithelial cells might therefore be one possible mechanism.

A new anti-inflammatory mechanism by theophylline in the treatment of COPD has recently been proposed by Barnes and co-workers.[20-22] The activity of histone deacetylases (HDACs), which mediate inflammatory gene repression, is reduced in patients with COPD.[23] Although the precise mechanism of this inactivation of HDACs is not yet clear, oxidative/nitrative stress might be involved via the nitration of tyrosine residues in the active centre of HDACs by peroxynitrite or other RNS.[24 25] Theophylline has been reported to restore the decreased HDAC activity in patients with COPD.[20] In this study we have shown, for the first time, the reduction in tyrosine nitration by theophylline using electrochemical as well as immunohistochemical techniques. Our results support the hypothesis of Barnes and colleagues. It is considered that combined evaluation of the HDAC activity and nitrative stress by HPLC/ECD could clarify the precise mechanism of action of theophylline.

Recent investigations have shown that RNS has an important role in the pathogenesis of COPD, causing cell injury,[26] activation of metalloproteinases,[27] inactivation of α_1-antiproteinase,[28] and enhanced IL-8 production.[29] Neutrophilic airway inflammation is another important feature of COPD,[1-3] and neutrophils are an important source of RNS.[10] It is considered that both neutrophilic inflammation and oxidative/nitrative stress could have critical roles in the development of COPD. The findings of our study suggest that theophylline might be a useful therapeutic tool for COPD treatment by inhibiting both neutrophilic airway inflammation and nitrative stress.

In conclusion, treatment with theophylline reduces nitrative stress as well as neutrophilic inflammation in COPD. Because there is a significant positive correlation between the decrease in the number of neutrophils and the reduction in 3-NT levels, the reduction in nitrative stress is considered to be due mainly to the inhibition of neutrophilic inflammation. Since the suppression of nitrative stress seems to be effective in inhibiting the inflammatory process and subsequent obstructive changes in COPD airways, theophylline may slow the progression of airway obstruction in COPD. Further large, long term, placebo controlled studies with a range of concentrations of theophylline and different severities of COPD are needed to confirm this hypothesis.

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IMPORTANCE OF ASSISTANCE BY CAREGIVERS FOR INHALED CORTICOSTEROID THERAPY IN ELDERLY PATIENTS WITH ASTHMA

To the Editor: Complications accompany asthma in elderly patients more often than in other age groups, and some complications could interfere with the procedure of inhalation therapy.[1–3] Even in such cases, regular and accurately calibrated inhaled corticosteroid (ICS) therapy can be made possible with the assistance of a caregiver.[3] Although assisted inhalation therapy is currently in practice, no clinical studies have evaluated its effects. This objective of this study was to investigate the usefulness of assistance by caregivers during the inhalation procedure in elderly patients with asthma whose pulmonary functions were not improved by self-administered ICS therapy.

Patients aged 65 and older with severe asthma and less than 60% of percentage peak expiratory flow (PEF) received self-administered ICS therapy (equivalent to 800 μg beclomethasone dipropionate (BDP)/d) for 8 weeks. Of the patients who received the above therapy, 10 cases with PEF improvement of less than 15% were enrolled. While investigating the causes of the poor responsiveness to self-administered ICS therapy, we conducted a survey on the caregivers' preferences concerning the devices used for assistance and their reasons by giving them three types of ICS devices for training: one metered-dose inhaler (MDI; hydrofluoroalkane–BDP) and two dry powder inhalers (DPIs: fluticasone propionate and budesonide). After that, assisted ICS therapy (equivalent to 800 μg BDP/d) was administered for 8 weeks using the devices selected by the caregivers to prospectively evaluate the effects of assisted inhalation on PEF and forced expiratory volume in 1 second (FEV_1). Statistical analysis was performed using the multiple comparison Tukey tests.

The subjects consisted of five men and five women with an average age of 81.6. All the patients had severe asthma with a mean percentage FEV_1 of 58.0% and a mean percentage PEF of 43.2%. Poor responsiveness to self-administered ICS therapy was attributed to problems with the inhalation technique in seven cases and compliance in nine cases. The presence of one or more complications was observed in all patients. These complications consisted of rheumatoid arthritis, diabetic neuropathy, or cerebral infarction causing motor dysfunction in seven cases; Alzheimer's disease or cerebral infarction causing intellectual dysfunction in five cases; and chronic obstructive pulmonary disease (COPD) in four cases.

PEF was increased more than 15% in 70% of the study subjects with assisted ICS therapy. Significantly greater improvement was observed in PEF and FEV_1 at the end of assisted ICS therapy than with self-administered ICS therapy (Figure 1; $P < .001$ in both cases). With regard to the three cases in which the assisted ICS therapy did not bring about improvement in pulmonary function, one was attributed to failure in compliance because of the complication of stomatitis, and the other two were attributed to airflow limitation with less reversibility associated with COPD or the progress of asthmatic airway remodeling. Nine caregivers selected MDI inhalers for assisted inhalation. The reasons for selecting the MDI inhaler were that MDI inhalers were easy to use, because they enable one-step inhalation and that MDI inhalers could be used without anxiety, because the spraying and inhaling conditions can be observed visually.

It is often the case that functional disorders accompanying asthma in elderly patients interfere with their inhalation technique, and this has led to hesitation on the part of physicians to prescribe inhalation therapy for such patients. One study reported that the more complications an elderly

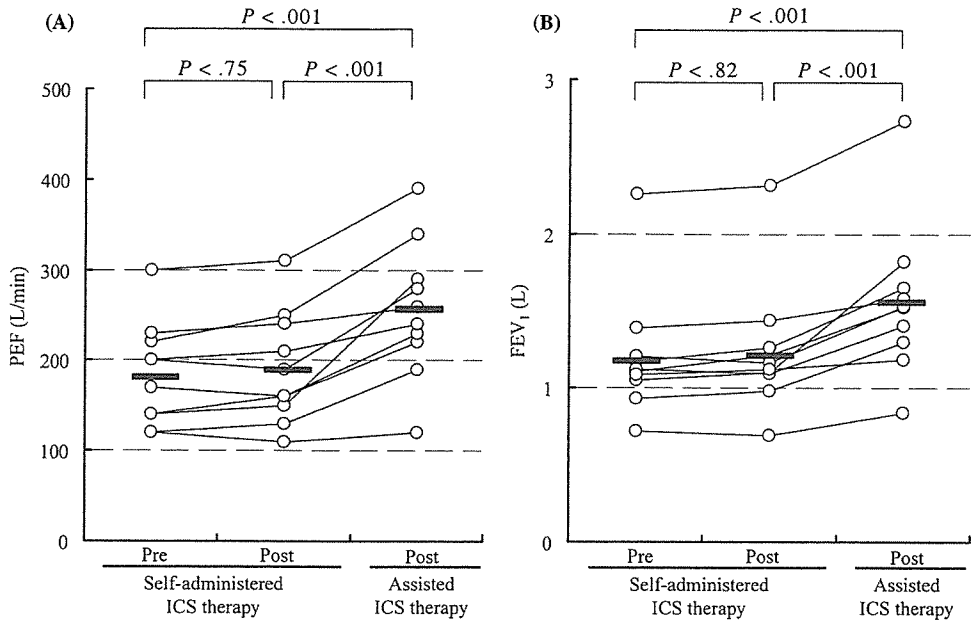

Figure 1. Changes in (A) peak expiratory flow (PEF) and (B) forced expiratory volume in 1 second (FEV_1) at the start and end of self-administered inhaled corticosteroid (ICS) therapy and at the end of assisted ICS therapy. Bold horizontal bars represent means of PEF and FEV_1 in each period.

asthma patient has, the lower the ICS prescription rate will be.[2] Intellectual dysfunction, such as Alzheimer's disease, can result in a poor understanding of the inhalation technique, and motor dysfunction, such as rheumatoid arthritis, will directly affect the physical ability to perform the inhalation procedure. It is assumed that, because the ICS therapy was not performed regularly, the improvement in asthma symptoms was limited, which in turn, led to poorer patient compliance. When asthma management is insufficient in spite of repeated inhalation guidance, assisted inhalation may be indicated. For assisted inhalation therapy, it is important to select inhalants that are easy for caregivers to use. Hydrofluoroalkane-BDP is considered appropriate for assisted ICS therapy, because it allows the caregiver to visually check the drug-spraying and inhalation conditions of the patient.

In conclusion, assistance by caregivers in ICS therapy is an important therapeutic strategy for elderly patients with asthma, especially those with complications that result in problems with the inhalation technique or compliance, and this strategy can be expected to expand the application of ICS therapy for elderly patients with asthma.

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目でみるページ

■ 鼻炎合併喘息の治療とロイコトリエン受容体拮抗薬 ■

The effectiveness of leukotriene receptor antagonists for bronchial asthma with allergic rhinitis

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はじめに

上気道と下気道におけるアレルギー性疾患の病態生理には密接な関連性があることが示されてきており，“One airway, one disease”という概念が提唱されている[1]。本稿ではアレルギー性鼻炎と気管支喘息との関連についてこれまでの知見を紹介し，鼻炎合併喘息の治療におけるロイコトリエン受容体拮抗薬の役割について概説する。

Ⅰ．喘息とアレルギー性鼻炎の合併

これまでの疫学的調査によりアレルギー性鼻炎患者の約30～40%に気管支喘息が合併し，気管支喘息患者の約30～80%にアレルギー性鼻炎が合併することが報告されている[1)2)]。アレルギー性鼻炎と気管支喘息の罹患率は先進国において高い傾向にあり，各国での両疾患の罹患率は似通った傾向を示す[3)]。またアレルギー性鼻炎は喘息発症に先行することが多く，喘息発症の危険因子の１つと認識されている。成人を対象とした23年間の追跡調査によれば，アレルギー性鼻炎を有する患者は有さない患者に比べ約３倍の頻度で喘息を発症したことが報告されている[4)]。

Ⅱ．喘息とアレルギー性鼻炎の病態生理

アレルギー性鼻炎と気管支喘息の基本病態はアレルギー性炎症であり，両者の炎症はTリンパ球，好酸球，肥満細胞などの免疫細胞やヒスタミン，ロイコトリエン，IL-4，IL-5，GM-CSF，RANTESなどの炎症性メディエーターが関与する共通の免疫機序により生じる。喘息においては抗原吸入負荷により即時相，遅発相の二相性に１秒量の低下が認められるが，アレルギー性鼻炎においては鼻炎症状スコアの悪化が同様に二相性に認められる。喘息患者における鼻粘膜中の好酸球は鼻炎合併の有無にか

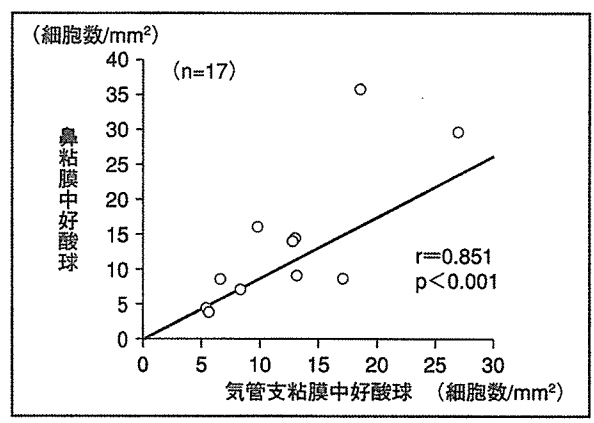

図１．喘息患者における鼻粘膜および気管支粘膜中の好酸球数の相関[5)]

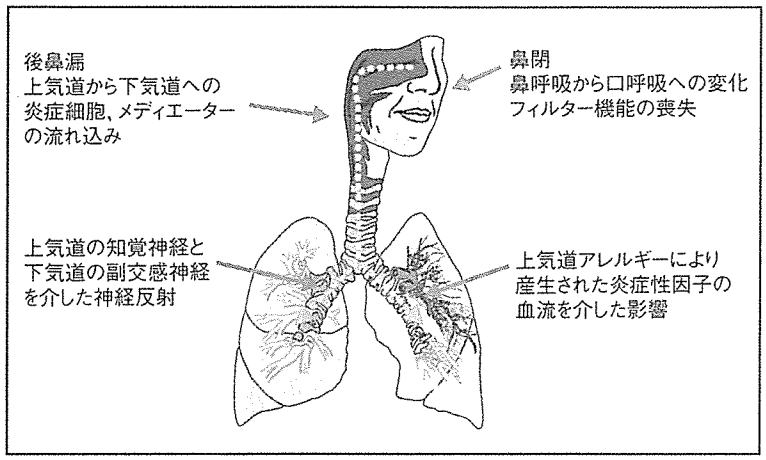

図2．上気道アレルギーが下気道に影響を及ぼす機序[6]

かわらず亢進しており，喘息患者の鼻粘膜と気管支粘膜中の好酸球数には有意な相関が認められる（図1）[5]。またアレルギー性鼻炎患者は花粉飛散期に気道過敏性が亢進することや，喘息のないアレルギー性鼻炎患者の気管支に抗原を滴下すると鼻粘膜の好酸球性炎症が誘導されることが示されている。これらの結果は上気道および下気道のアレルギー性炎症が連動して推移することを示唆している。

Ⅲ．One airway, one disease

以上述べてきたように気管支喘息とアレルギー性鼻炎は疫学的，病態生理学的に関連の深い気道の炎症性疾患であり，“One airway, one disease”と捉えて診断，治療することが提唱されている[1]。上気道アレルギーが下気道に影響を及ぼす機序の詳細は不明であるが，①後鼻漏による炎症細胞や炎症性メディエーターの上気道から下気道への流入，②上気道アレルギーにより産生された炎症性因子の血流を介した影響，③鼻閉に伴う口呼吸によるフィルター機能の喪失，④上気道の知覚神経と下気道の副交感神経を介した神経反射，などの因子が関与する可能性が推測されている（図2）[6]。

Ⅳ．鼻炎合併喘息の治療総論

喘息とアレルギー性鼻炎の基本病態は気道炎症である。両疾患のガイドラインにおいて，症状が通

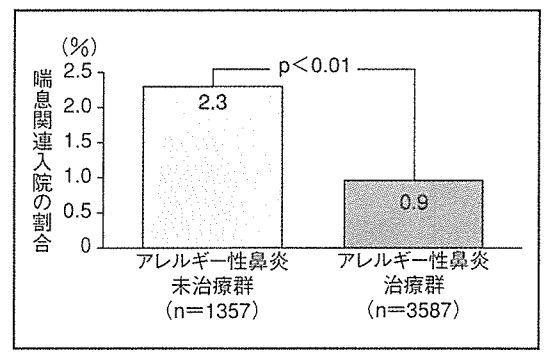

図3．アレルギー性鼻炎の治療による喘息関連入院の減少[7]

年性に認められる場合にはステロイド薬，ロイコトリエン受容体拮抗薬，抗アレルギー薬などの抗炎症薬による長期管理が重要であることが強調されている。喘息では吸入ステロイド薬が抗炎症治療の軸となるが，アレルギー性鼻炎では鼻閉などに伴い点鼻薬が鼻腔内に適切に分布しないことも多い。そのためアレルギー性鼻炎のガイドラインでは，すべての重症度における全身的な抗炎症治療が推奨されている。一方，気道閉塞の機序としてはアレルギー性鼻炎では豊富な血管網の充血が重要なのに対し，喘息では気管支平滑筋の収縮が重要である。そのため気道閉塞改善のためには，アレルギー性鼻炎ではα刺激薬などの血管収縮剤が，喘息ではβ刺激薬などの気管支拡張薬が有効である。鼻炎合併喘息においては喘息と鼻炎の両方を治療することが重要であり，アレルギー性鼻炎と喘息の両方

を治療した患者は喘息だけを治療した患者に比べ，喘息関連の入院が61%減少したことが報告されている(図3)[7]。

V．鼻炎合併喘息治療におけるロイコトリエン受容体拮抗薬

ロイコトリエン類(LT)は細胞膜のアラキドン酸から生成される脂質メディエーターであり，LTC_4, D_4, E_4などのシステイニルロイコトリエンには気道平滑筋収縮，好酸球遊走，血管透過性亢進などの生理活性がある。システイニルロイコトリエンはアレルギー性鼻炎患者の鼻汁中と喘息患者の喀痰中において産生が亢進している(図4)[8][9]。またヒト鼻粘膜と気管支粘膜にはシステイニルロイコトリエン受容体1が発現しており[10][11]，喘息患者における発現の亢進が確認されている(図5)[11]。ロイコトリエンの吸入刺激はアレルギー性鼻炎と喘息の症状を誘発し，ロイコトリエン受容体拮抗薬がその症状を抑制することからも，ロイコトリエンは両疾患の治療における重要な標的分子の1つと認識されている。

吸入ステロイド薬で喘息管理が不十分な症例において，吸入ステロイド薬を増量した群とロイコトリ

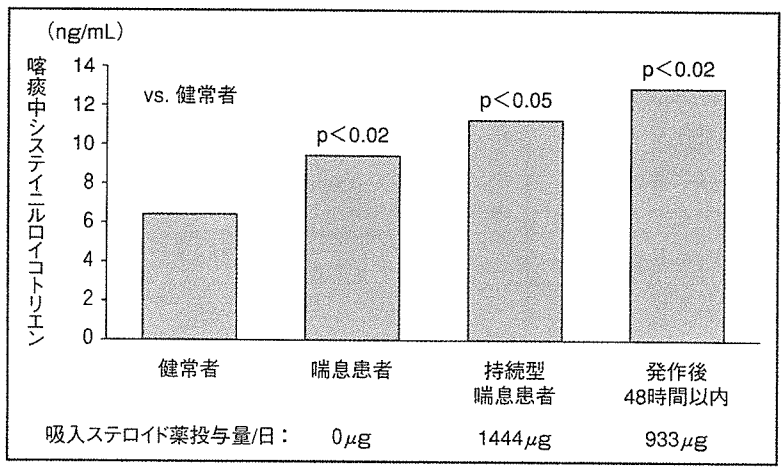

図4．喘息患者の誘発喀痰中のシステイニルロイコトリエン産生亢進[8]

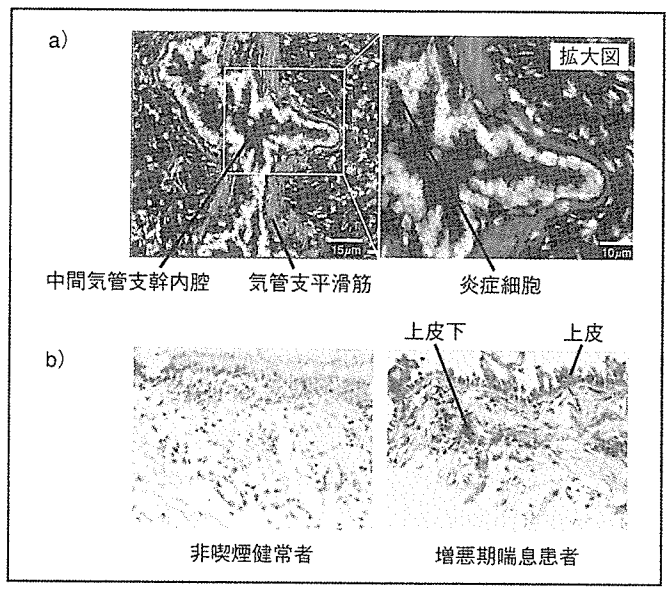

図5．気管支粘膜のシステイニルロイコトリエン受容体1発現状況[10][11]
a) 正常気管支における受容体発現
b) 喘息患者における気道上皮，上皮下の受容体発現亢進

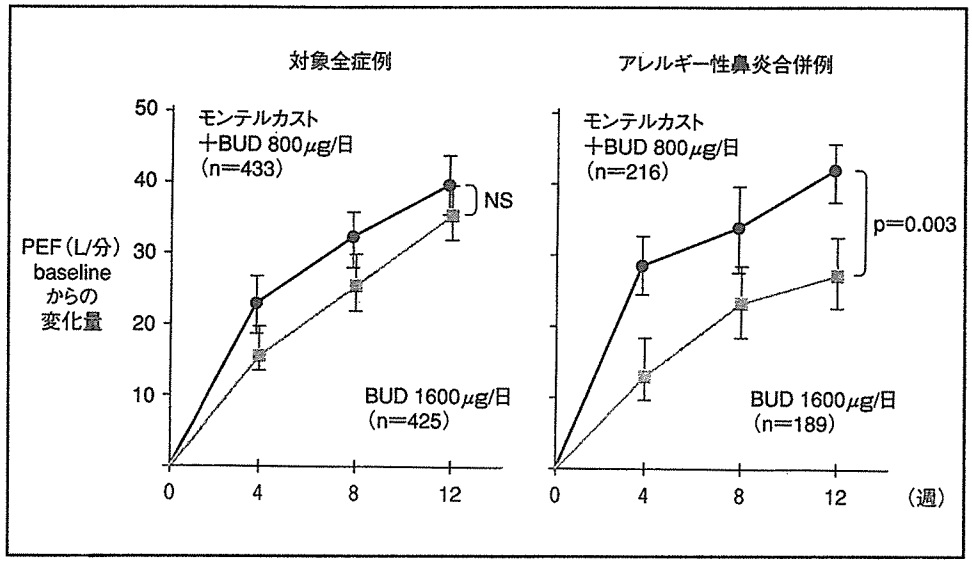

図6．鼻炎合併喘息に対するロイコトリエン受容体拮抗薬の併用効果[12]
BUD：ブデソニド

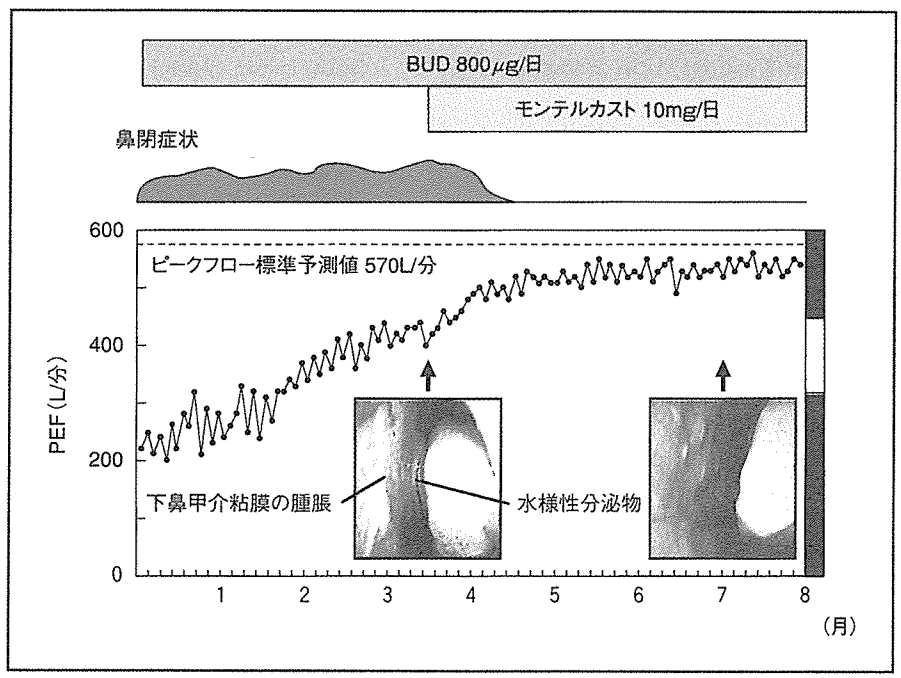

図7．ロイコトリエン受容体拮抗薬併用によりピークフロー値と鼻閉が改善した喘息症例
(48歳，男性)
BUD：ブデソニド

エン受容体拮抗薬(モンテルカスト)を上乗せした群とを比較した場合，全症例での検討では両治療群の効果は同等であったが，アレルギー性鼻炎合併患者を対象としたサブ解析では，モンテルカストを上乗せした群のほうが有意なピークフローの改善が認められたことが報告されている(図6)[12]。ステロイド薬は喘息気道におけるシステイニルロイコトリエンの産生を完全には抑制できないことも示されており，アレルギー性鼻炎を合併する喘息症例において吸入ステロイド薬の効果が不十分な場合には，ロイ

コトリエン受容体拮抗薬の併用が効果的であることが期待される。吸入ステロイド薬にモンテルカストを併用することにより，ピークフロー値，鼻炎症状と鼻閉所見(図7)が改善した症例を呈示する。

おわりに

関連の深い上気道と下気道のアレルギー性炎症の治療において，ロイコトリエンは共通の標的分子として重要である。鼻炎合併喘息では両疾患のアレルギー性炎症を同時に制御できる治療法が望ましく，吸入ステロイド薬に併用する薬剤としてロイコトリエン受容体拮抗薬の有効性が期待される。

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≪喘息の診断≫ 気道炎症の評価

南方良章　　一ノ瀬正和*

要旨

- 気管支喘息の気道炎症評価法として，誘発喀痰，呼気ガス，呼気凝縮液がある．
- 誘発喀痰は，細胞成分と液性成分の検討が可能で，前者では好酸球数が，後者では ECP，アルブミン，IL-5，RANTES，eotaxin，LTC4/D4/E4 などが炎症の指標となりうる．
- 呼気ガスでは，NO が気管支喘息で増加し，気流制限や気道過敏性との相関もみられ，炎症評価や病態管理の指標として用いうる．CO や炭化水素は，将来的に指標となりうる可能性はあるが，現状ではまだ不十分である．
- 呼気凝縮液は，まったくの無侵襲で将来有望な方法であることより，測定法の標準化や更なる報告の集積が期待される．

はじめに

喘息は気道の慢性炎症性疾患であり，病態の診断・管理には，この炎症をいかにして検出し評価するかが重要となる．喘息の炎症は，好酸球を中心とする細胞やサイトカイン，ケモカインなどが重要な役割を担っており[1]，これらを指標とすることで炎症を評価しうる可能性がある．従来，気道炎症の評価は病理学的手法，すなわち剖検組織あるいは軽症喘息患者に対する気管支鏡下生検組織によってなされていた．これに対し，近年誘発喀痰，呼気ガス，呼気凝縮液といった侵襲の少ない検査にて気道炎症を評価する試みが広がってきた．

本稿では，これら検査法による喘息の気道炎症評価の現状と可能性について述べる．

誘発喀痰(sputum induction)

1992 年 Pin ら[2]により開発され以来，喘息を含む炎症性気道疾患の病態解明に対する報告がみられるようになった．誘発喀痰はまったくの非侵襲的検査とはいえないが，気管支鏡下生検などに比較して低侵襲で，反復検査可能などの利点がある．

1．誘発喀痰採取法

採取 15 分前に短時間作動型 β_2刺激薬を吸入し気道を拡張したうえで，滅菌された 4% 程度の高張食塩水を超音波ネブライザーを用いて繰り返し吸入させる．吸入 5 分ごとに 1 回の咳嗽で喀痰を排出し，総量が約 1.0 m*l* になるまで 15～30 分間繰り返す．ただし，高張食塩水により気道攣縮をきたす可能性と，1 秒量 1*l* 未満の低肺機能患者に対する安全性が確認されていない点には注意が必要である．

採取した誘発喀痰は粘液を溶解し，細胞成分と液性成分に分離する．

2．誘発喀痰を用いた気道炎症の評価

細胞成分分析では，好酸球数の増加が気管支喘息患者で確認されている[3]．この好酸球数は気管支喘息の重症度と関連する(Fig. 1)[4]．ステロイド

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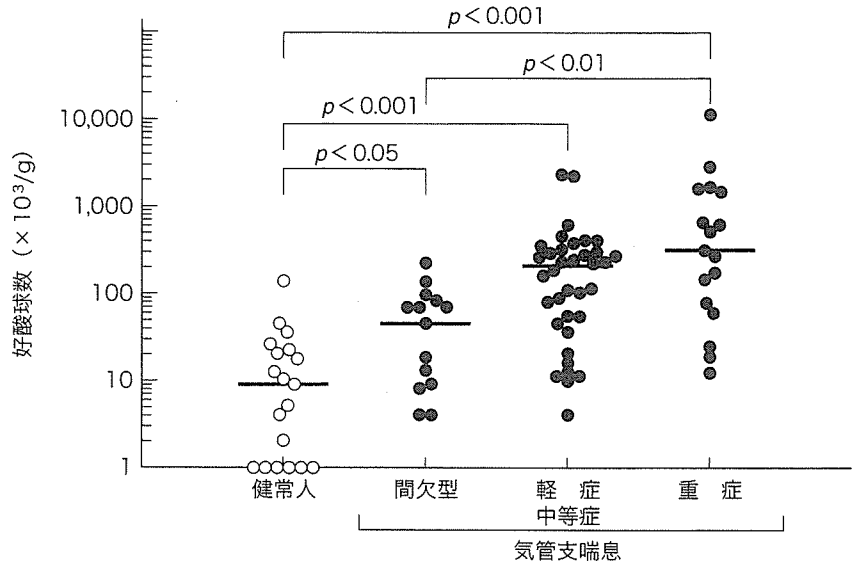

Fig. 1. 気管支喘息における誘発喀痰中好酸球数

気管支喘息患者では，健常人に比べ誘発喀痰中の好酸球数の有意な増加を認める．重症度が進むほど好酸球数の増加が顕著となる．

［文献 4）より引用］

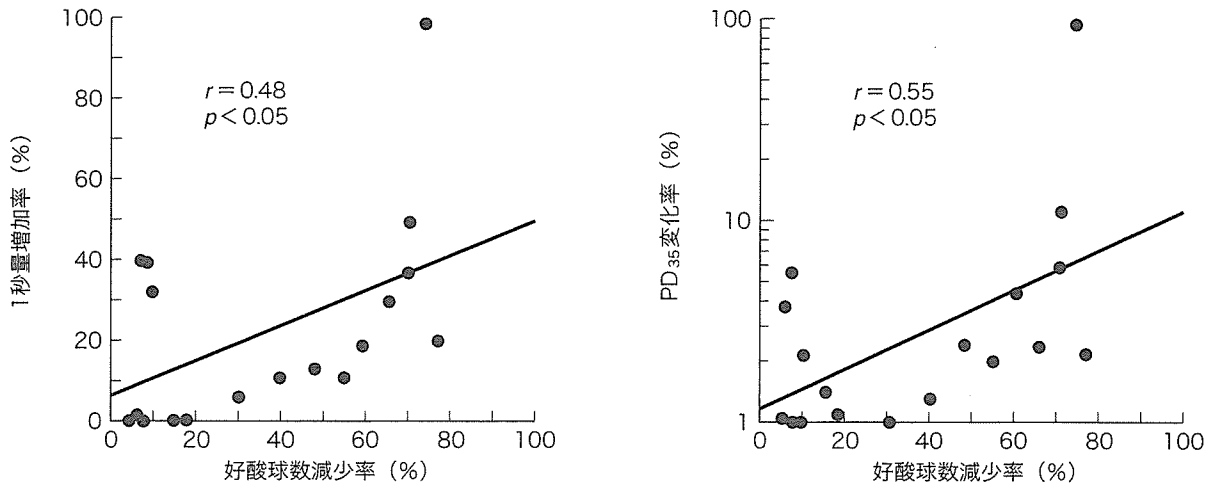

Fig. 2. ステロイド治療による好酸球数減少と気流制限・気道過敏性の改善率

誘発喀痰中の好酸球数は吸入ステロイド治療にて減少するが，その減少率は，1 秒量（気流制限），PD_{35}（気道過敏性）の改善率と有意な相関関係を示す．

［文献 3）より引用］

治療に反応して減少し，気流制限や気道過敏性の改善の程度と相関がみられることより（Fig. 2），診断ならびに治療効果の指標として有用である[3]．

また，急性発作時には顕著な好酸球増加とともに，好中球の増加も報告されている．

液性成分分析では，好酸球由来の蛋白質で，好酸球の活性化により放出される eosinophil cationic protein（ECP）が，喘息患者の誘発喀痰上清中で増加し，喘息の重症度との関連や，気流制限や気道過敏性の程度との相関も認められる[4]．微小血管の透過性亢進を示唆するアルブミン濃度は，上清中で高値を示す[4]．さらに，IL-5，RANTES，eotaxin などの増加や，leukotriene（LT）$C_4/D_4/E_4$の増加なども報告されている．

このように，誘発喀痰は細胞成分・液性成分両面から，気管支喘息の気道炎症評価ならびに治療効果の評価にとって有用な方法であると考えられる．

呼気ガス(exhaled gas)○

呼気中には生体由来の種々の揮発性ガス分子が含まれており，気道・肺の炎症病態が存在する状況では，その産生量や組成などが変化する．これらのガスを指標として用い，気道炎症を評価する報告がなされている．呼気ガス中で測定可能な物質としては，一酸化窒素(NO)，一酸化炭素(CO)，炭化水素類などがある．

1．呼気 NO 濃度とその測定

内因性の NO は NO 合成酵素(NO synthase：NOS)の働きにより，L-arginine が L-citrulline に変換される過程で産生される．末梢気道における NO の起源は明らかではないが，主として気道上皮やマクロファージなどの細胞に由来するものと考えられている．呼気 NO 濃度は，呼気流速，呼出時の肺気量位，鼻腔内で産生される NO の混入などにより影響を受けるため，ヨーロッパ呼吸器学会[5]や米国胸部疾患学会[6]が提唱した標準法に基づいた測定が重要である．

1）呼気 NO を用いた気道炎症の評価：気管支喘息患者の呼気 NO 濃度は健常人に比べ有意に高値である[3,7]．これは主に下気道に由来しており，気道上皮や炎症細胞における iNOS 活性化が関与していると考えられる[8]．実際，喘息患者では誘発喀痰中の炎症細胞においても iNOS の発現が増強しており，呼気 NO 濃度との相関が認められる[9]．

気管支喘息以外の原因の慢性咳嗽患者では呼気 NO 濃度は上昇せず，慢性咳嗽の鑑別にも有用である．また呼気 NO 濃度は気流制限や気道過敏性の程度，誘発喀痰中の好酸球浸潤の程度，喘息の重症度などと有意な相関がみられることより(Fig. 3)，疾患の診断や病態把握に有用である[9,10]．治療による呼吸機能や気道過敏性の改善との相関がみられることより(Fig. 4)，治療効果のモニタリングとして[3]，また増悪時や喘息コントロール不良の指標としても有用である[11]．

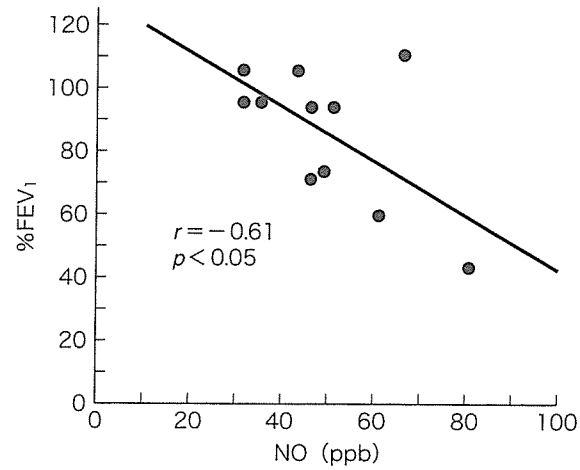

Fig. 3. 呼気 NO 濃度と気流制限重症度
呼気 NO 濃度は，気流制限の重症度の指標である $\%FEV_1$(1 秒量実測値の予測値に対する割合)と有意な相関関係を示す．
［文献 9)より引用］

吸入ステロイド薬の投与量設定とその増減は，ガイドラインでは症状・気管支拡張剤使用回数・肺機能検査などを指標にすることが推奨されている．これに対し，呼気 NO 濃度を指標として投与量を設定・増減を試みた場合，12ヵ月間での増悪率，増悪回数は従来の方法と比べ有意差はなく，むしろ低値で，しかも至適投与量決定時および12ヵ月後の時点での吸入ステロイド投与量は有意に少量であったと報告されている(Fig. 5)[12]．すなわち，非侵襲的に測定できる呼気 NO 濃度が，喘息コントロールにおける吸入ステロイド量調節の指標となりうる可能性が考えられる．

2．その他の呼気ガス

CO と炭化水素類が報告されている．CO は大部分が，ヘモグロビン中のヘムが酵素反応(heme oxygenase：HO)により代謝されることで産生され，呼気中に排出される．

一方，炭化水素類は，非特異的な脂質過酸化の指標であり，生体内での脂質過酸化の程度の評価や抗酸化作用をもつ薬剤の効果の評価として有用

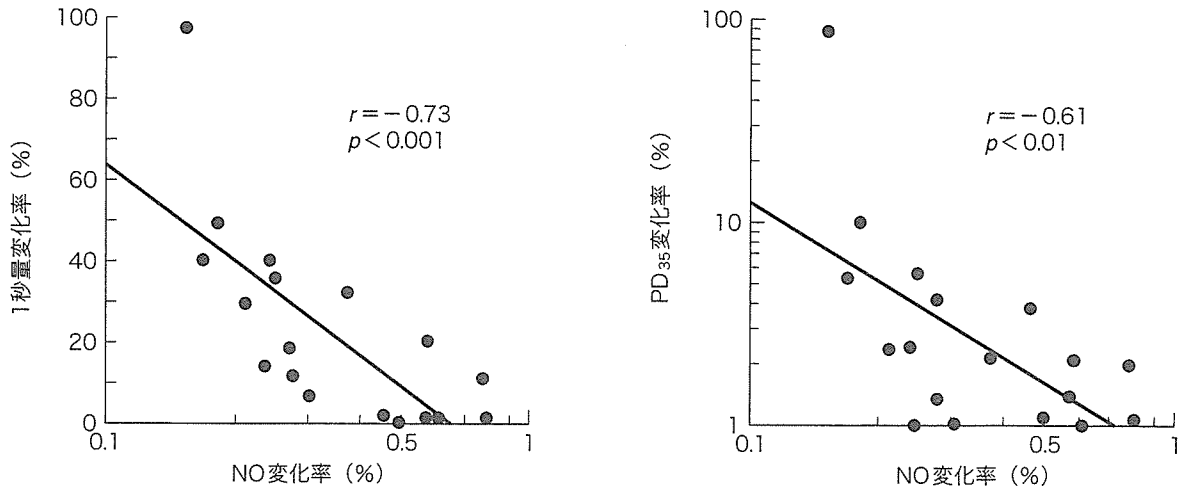

Fig. 4. ステロイド治療による呼気 NO 変化率と気流制限・気道過敏性の改善率

呼気 NO 濃度は，吸入ステロイド治療にて低下し，その低下率は 1 秒量(気流制限)，PD_{35}(気道過敏性)の改善率と有意な相関関係を示す．

［文献 3)より引用］

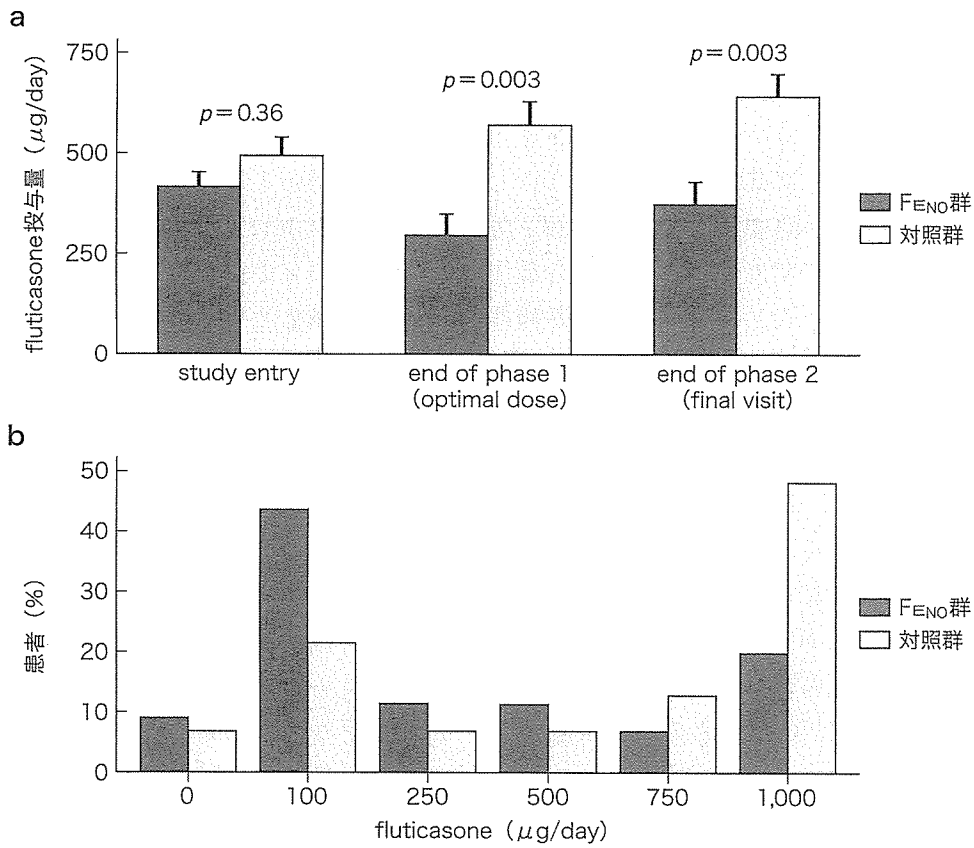

Fig. 5. 呼気 NO を指標とした場合の吸入ステロイド使用量

a：至適投与量決定時(end of phase 1)ならびに 12ヵ月後(end of phase 2)において，ガイドラインに準じた場合に比べ，呼気 NO 濃度(FE_{NO})を指標として調節したほうが吸入ステロイド使用量は有意に少ない．

b：12ヵ月後の吸入ステロイド使用量の分布は呼気 NO にて調節群で有意に少量側に分布している($p=0.008$)．

［文献 12)より引用］

Table 1. 呼気凝縮液中の炎症関連物質

	気管支喘息	
	安定期	不安定期
エイコサノイド		
8-イソプロスタン	↑	↑↑↑
ロイコトリエン $C_4/D_4/E_4$	↑	↑↑
ロイコトリエン B_4	↑	↑↑
プロスタグランジン	?	?
トロンボキサン	?	?
窒素関連産物		
ニトロタイロシン	↑	?
NO_2^-/NO_3^-	↑	↑↑
SNO	↑	↓
H_2O_2	↑	↑↑
lipid peroxidation 産物	↑	?
vasoactive amines	↑	?
アンモニア	↑	?
水素イオン(pH)	↔	↑↑
サイトカイン		?
IL-1β, IL-2, IL-6	↑	
電解質		?
Na, Cl	?	
Mg	↓	
Ca	↓	

な可能性がある．

1）呼気 CO を用いた気道炎症の評価：ステロイド未治療の気管支喘息では健常人に比べ呼気 CO 濃度は高値で，吸入ステロイド治療に伴う低下や，喀痰中の好酸球数の変動と相関した低下が報告されている[13]．しかし気管支喘息患者と健常人との差は呼気 NO 濃度でみられるほど顕著ではない．

また最近，十分量の吸入ステロイド治療によっても呼気 CO 濃度の低下がみられなかったとする報告もあり，喘息の病態把握における呼気 CO 濃度測定の意義はまだ確立していない．

2）呼気炭化水素類を用いた気道炎症の評価：気管支喘息増悪時に呼気ガス中ペンタン濃度が高値で，発作の改善とともに低下する．また呼気ガス中エタンはステロイド未治療の軽症喘息で健常人に比べ高値で，ステロイド治療により健常人レベルまで低下する．

炭化水素類については，現在報告が少なく指標としての意義もまだ確立していない．

呼気凝縮液(exhaled breath condensate：EBC)○

安静換気下において，吸気に伴い気道に生じた乱流により気道表面の液体がエロゾル化され，呼気の気流により排出される．この呼気を急速冷却し，呼気中の水蒸気・霧状粒子を液化させることで回収された検体が，呼気凝縮液である．

1．EBC を用いた気道炎症の評価

気管支喘息患者の EBC 中では，いくつかの炎症関連物質の検出が報告されている(Table 1)[14]．$LTC_4/D_4/E_4$は気管支喘息で有意な増加がみられ，重症度とも関連している．また，H_2O_2，8-イソプロスタン，NO_2^-，NO_3^-，*S*-ニトロソチオール，3-ニトロタイロシンなども気管支喘息患者の EBC 中で増加すると報告されている．

EBC は操作が簡便，非侵襲的，繰り返し採取が容易などの長所がある．またその反面，測定法が標準化されていないことより，現状ではいまだ臨床応用の段階ではない．今後の発展が期待される検査法である．

おわりに○

気管支喘息における気道炎症の評価法として，誘発喀痰・呼気ガス・EBC と各検査法で報告されている指標を概説した．現時点では，誘発喀痰により得られた指標と呼気 NO が炎症マーカーとしての有用性がほぼ確認されているが，他の呼気ガスや EBC も有用マーカーとなりうる可能性を秘めており，今後の検討が期待される．

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方，IOS法の指標では，R5, R20ともにNAC群を除く3群で有意に低下した。各指標の変化度をCVA群とNAC群の2群で比較すると，R5のみがCVA群でより大きな変化（13% vs 3%, $p=0.02$）を示した。

結論：安静呼吸下に非侵襲的に施行できるIOS法を用いたβ刺激薬吸入可逆性試験が，咳喘息と他の慢性咳嗽の鑑別に有用である可能性が示された。

副鼻腔気管支症候群患者のカプサイシン咳感受性に対するprostaglandin I_2誘導体経口投与の影響

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背景：副鼻腔気管支症候群は後鼻漏と湿性咳嗽を主症状とする慢性気道炎症性疾患である。われわれは本疾患の咳嗽の発生機序に種々の炎症性メディエーターが関与することを明らかにしたが，Prostaglandin I_2の咳感受性に対する作用は不明である。

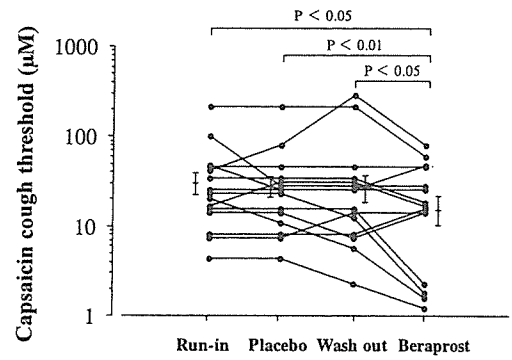
目的：本疾患患者のカプサイシン咳感受性に対するprostaglandin I_2投与の影響を検討する。

対象と方法：安定期副鼻腔気管支症候群患者15名を対象とした。既報の方法によりカプサイシン咳閾値を測定した後に，prostaglandin I_2 analogueであるberaprost 120 μg/日または対照薬を2週間cross-over法で投与した。

結果：beraprost投与により呼吸機能は変化しなかったが，カプサイシン咳閾値は有意に低下した。

考察：副鼻腔気管支症候群患者の気道において，prostaglandin I_2は咳受容体感受性を亢進させる方向に作用することが示唆された。

Effect of beraprost on capsaicin cough threshold in patients with stable sinobronchial syndrome



気道過敏性の簡便な臨床指標の検討

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目的：気道過敏性亢進は喘息の病態生理学的な特徴の一つであり，気道過敏性検査は喘息の診断や治療効果の判定に有用である。しかし気道収縮物質を吸入負荷し気道収縮反応を評価する気道過敏性検査は，侵襲的な負荷試験で簡便ではなく，安全性や適応禁忌が存在するなどの問題点もある。そこで気道過敏性検査に代わる簡便な気道過敏性の生理学的指標の確立を目的として検討する。

対象と方法：安定期喘息患者78例(男性/女性；28/50，喘息未治療群/喘息治療群；49/29，平均年齢；44.4±15.3歳，FVC（forced vital capacity）；3.30±0.77（L），FEV_1（forced expiratory volume in one second）；2.56±0.70（L），FEV_1%；77.5±9.3（%），%FEV_1；93.7±12.4（%））を対象とした。本試験はCross-sectional studyで，一回の外来受診時にスパイロメトリーおよび気道過敏性検査を実施した。また気道過敏性検査の前に2週間以上のPEF（peak expiratory flow）モニタリ