

Nitrative stress in refractory asthma

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Background: Most asthma is mild and moderate and can be well controlled by low-dose inhaled steroid with or without bronchodilators. However, 5% to 10% of patients with asthma have more troublesome disease despite using such medication. Recent reports showed that nitrative stress induced tissue remodeling *in vitro*, which is associated with a component of refractoriness in asthma. However, there is no report that nitrative stress is involved in refractory asthma.

Objective: The aim of this study is to evaluate whether patients with refractory asthma have more nitrative stress.

Methods: Ten healthy subjects, 10 patients with well-controlled asthma, and 8 patients with refractory asthma took part in the current study. Exhaled nitric oxide, xanthine oxidase activity in the supernatant of the sputum, immunostaining for the inducible type of nitric oxide synthase, and 3-nitrotyrosine in induced sputum from the subjects were assessed.

Results: All nitrative markers including exhaled nitric oxide ($P < .01$), immunopositivities for inducible nitric oxide synthase ($P < .01$), xanthine oxidase activities ($P < .01$), and 3-nitrotyrosine ($P < .01$) in sputum from the refractory asthma group were enhanced compared with the well-controlled group. All these nitrative markers in the sputum had a significant negative correlation with the %FEV₁ values ($P < .01$).

Conclusion: These results suggested that patients with refractory asthma have more nitrative stress in their airways compared with patients with well-controlled asthma.

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Key words: Refractory asthma, induced sputum, steroids, nitrotyrosine, reactive oxygen species

Asthma is a disorder characterized by chronic inflammation of the airways, airflow obstruction, and airway hyperreactivity.¹⁻³ Most asthma is well controlled by low doses of anti-inflammatory agents with or without bronchodilators.⁴ However, 5% to 10% of patients with asthma have more troublesome disease reflected by a higher medication requirement to maintain good disease control. They have persistent symptoms, disease exacerbation, airflow obstruction, and low quality of life in spite of using

Abbreviations used

eNO:	Exhaled nitric oxide
HDAC 2:	Histone deacetylase 2
ICS:	Inhaled corticosteroid
iNOS:	Inducible nitric oxide synthase
NO:	Nitric oxide
RNS:	Reactive nitrogen species
XO:	Xanthine oxidase

bronchodilators in addition to high doses of steroids.⁵ For describing this subgroup of patients with asthma with troublesome disease, the term *refractory asthma* has been used.⁵ It has been reported that individuals with refractory asthma are 15 times more likely to use emergency medical care than patients with well-controlled asthma and are 20 times more likely to require hospital admission.⁶ In view of the effect of refractory asthma on healthcare resources and the need to understand the mechanisms involved in refractory asthma, elucidating the pathophysiological features of refractory asthma could be important.

Recent reports showed that patients with severe asthma had more oxidative stress in their airways.^{7,8} In inflammatory conditions, excessive nitric oxide (NO) derived from the inducible type of NO synthase (iNOS) was produced as well as superoxide anion from nicotinamide adenine dinucleotide (NADPH) oxidase⁹ or xanthine oxidase (XO).¹⁰ In general, NO is rapidly reacted with superoxide anion to produce the highly reactive nitrogen species (RNSs) such as peroxynitrite.¹¹ In addition, excessive RNSs cause tissue injury, lipid peroxidation, and nitration of tyrosine residues.¹¹ RNSs can also cause the inflammation in asthmatic airway^{12,13} and moreover are reported to induce airway remodeling, which may be associated with a component of airflow obstruction in patients with refractory asthma,⁵ mediated by fibroblasts.¹⁴

A recent cross-sectional, multicenter study showed that patients with more severe refractory asthma treated with oral corticosteroids had NO levels that were almost 3 times higher than those treated only with inhaled corticosteroids (ICSs),¹⁵ suggesting that NO or NO-related molecules such as RNSs may contribute to the pathogenesis of refractory asthma. However, there is little information on nitrative stress in the airways of refractory asthma.

The aim of the current study, therefore, was to evaluate the degree of nitrative stress in refractory asthma. To accomplish this, healthy subjects, patients with well-controlled asthma, and patients with refractory asthma took part in the current study, and the nitrative stress markers including exhaled NO (eNO), iNOS expression, XO activity, and 3-nitrotyrosine formation were assessed. Furthermore, we assessed the correlation between lung function and each marker of nitrative stress.

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TABLE I. Characteristics of study subjects

Subject no.	Sex	Age (y)	FVC (L)	FEV ₁ (L)	FEV ₁ /FVC (%)	%FEV ₁ (%)	Exacerbation rates (times/y)	Inhaled steroid (oral steroid)	Other medications
H-1	F	49	3.30	2.67	80.9	118			
H-2	M	21	5.01	4.21	84.0	99.0			
H-3	M	67	3.34	2.55	76.3	90.0			
H-4	F	26	3.89	3.33	85.6	116			
H-5	M	19	3.88	3.33	85.8	111			
H-6	M	60	3.81	3.00	78.7	98.0			
H-7	M	51	3.54	3.13	88.4	97.4			
H-8	F	50	3.70	3.18	85.9	100			
H-9	M	33	4.11	3.54	86.1	92.0			
H-10	M	44	4.43	3.41	77.0	110			
Mean		42.0	3.90	3.24	82.9	103			
SE		5.5	0.2	0.2	1.4	3.3			
W-1	F	35	3.36	2.76	82.1	103	0	400	
W-2	M	26	4.16	3.03	72.8	82.9	0	400	
W-3	F	30	3.62	2.54	70.2	84.4	1	400	
W-4	F	27	3.91	3.31	84.7	120	0	400	
W-5	F	65	2.24	1.62	72.3	80.3	1	400	
W-6	F	68	2.58	1.84	71.3	101	0	400	
W-7	F	49	3.04	2.33	76.6	98.0	0	400	
W-8	M	35	5.29	4.25	80.3	97.4	0	400	
W-9	M	33	4.83	3.89	80.5	103	1	400	
W-10	M	32	4.88	3.93	80.5	102	0	400	
Mean		40.0	3.79	2.95	77.1	97.2	0.3		
SE		5.1	0.3	0.3	1.7	4.0	0.2		
R-1	F	55	2.90	1.65	56.9	71.5	3	1600	LABA, T
R-2	M	53	2.49	1.63	65.5	54.0	4	1600	LABA, T, LT
R-3	F	38	3.68	2.21	60.1	76.6	2	1600 (5)	LABA, T, LT
R-4	M	35	3.57	2.37	66.4	59.6	2	1600	LABA, T
R-5	M	24	4.22	2.76	65.4	70.6	3	1600 (10)	LABA, T, LT
R-6	M	35	3.96	2.48	62.6	63.8	2	1600	LABA, T
R-7	F	32	3.65	1.88	51.5	66.2	3	1600	LABA, T, LT
R-8	F	45	3.34	1.76	52.7	73.4	2	1600 (5)	LABA, T, LT
Mean		39.6	3.48	2.09*†	60.1*‡	67.0*‡	2.6‡		
SE		3.6	0.2	0.2	2.0	2.8	0.3		

Patients with well-controlled asthma were treated with low doses of inhaled steroid ($\mu\text{g}/\text{d}$). Patients with refractory asthma were treated with bronchodilators in addition to high doses of inhaled steroid ($\mu\text{g}/\text{d}$) or high doses of inhaled steroid ($\mu\text{g}/\text{d}$) plus oral steroid (mg/d).

F, Female; FVC, forced vital capacity; H, healthy subjects; LABA, long-acting β -agonist; LT, leukotriene receptor antagonist; M, male; R, patient with refractory asthma; T, theophylline; W, patient with well-controlled asthma.

* $P < .01$ compared with values of healthy subjects group; † $P < .05$, ‡ $P < .01$ compared with values of well-controlled asthma group.

METHODS

Subjects

Ten healthy subjects, 10 patients with well-controlled asthma, and 8 patients with refractory asthma took part in the current study. Patients with asthma had their disease diagnosed on the basis of recurrent episodes of wheezing, airway reversibility by β_2 -agonist, airway hyperresponsiveness, and eosinophilia as assessed by sputum examination. All subjects had never smoked and had not had a respiratory tract infection during the month preceding the test. The patients were divided into those with refractory and those with well-controlled disease according to the criteria of the American Thoracic Society Workshop.⁵ Briefly, refractory asthma was not well controlled despite continuous treatment more than half the year with long-acting β -agonists, theophylline, and leukotriene antagonist in addition to more than 1260 $\mu\text{g}/\text{d}$ beclomethasone dipropionate, or equivalent doses of other ICSs, or a combination of ICSs and oral corticosteroids. Because the values of FEV₁ percent predicted in all patients with refractory asthma were less than 80%, they had persistent airway obstruction (Table I). All patients with refractory asthma satisfied both the major criteria and more than 2 minor criteria, and they did not have any other diseases such as chronic obstructive pulmonary disease. Healthy subjects with normal lung function, no

abnormality in chest x-ray, and no respiratory symptoms were enrolled in the current study as the healthy subjects. Inclusion in the well-controlled asthma group required that there had been no asthma exacerbation in the past year and normal lung function treated with low doses of inhaled steroid (400 $\mu\text{g}/\text{d}$). The study was conducted with the approval of the Tohoku University Committee on Clinical Investigation. Written informed consent was obtained from all subjects.

Sputum induction and processing

Sputum was induced and processed according to the method described in our previous study.¹⁶ Briefly, after inhalation of salbutamol, the subjects inhaled 4% hypertonic saline. The sputum induction was safely performed even in the subjects with refractory asthma by treating them with salbutamol. Obtained sputum samples were treated with Sputasol (Oxoid Ltd, Basingstoke, United Kingdom) and then centrifuged at 790g for 10 minutes. All supernatant samples were stored at -80°C . The obtained cell pellet was resuspended and the total cell count of leukocytes was obtained, and then the cells were stained using Hansel stain (Torii Pharmaceutical, Tokyo, Japan) to assess the cell differential.

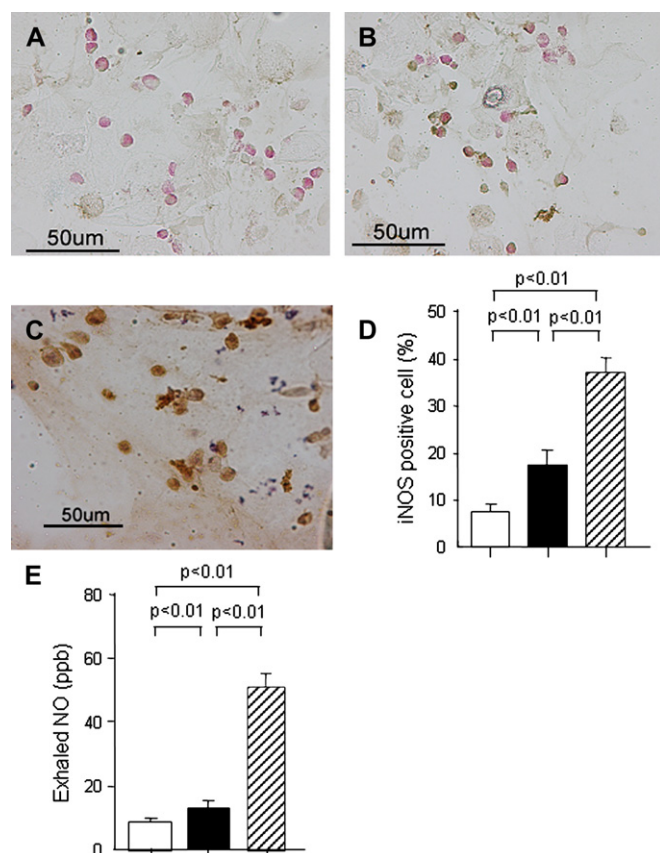


FIG 1. Immunocytochemical detection of iNOS in induced sputum cells and exhaled NO levels in healthy subjects (A), subjects with well-controlled asthma (B), and subjects with refractory asthma (C). D, The percentages of iNOS-positive cells (%) in the sputum from healthy subjects (open bar, $n = 10$), subjects with well-controlled asthma (filled bar, $n = 10$) and subjects with refractory asthma (hatched bar, $n = 8$) were counted. E, Exhaled NO levels (ppb) in healthy subjects (open bar, $n = 10$), subjects with well-controlled asthma (filled bar, $n = 10$) and subjects with refractory asthma (hatched bar, $n = 8$) were measured.

Measurement of eNO

A rapid response chemiluminescent analyzer (280NOA; Sievers Instruments Inc, Boulder, Colo) was used for the analysis of eNO.¹⁶ The subjects expired at a constant flow rate (100 mL/min), and the eNO was measured by an online system.

Immunocytochemical staining

Immunocytochemical staining for iNOS or 3-nitrotyrosine was performed as previously described.¹⁶ In the current study, we used 3-nitrotyrosine formation as a stable marker for RNS production. Briefly, after fixation, blocking of endogenous peroxidase, and nonspecific binding of antibodies, the preparations were incubated with primary antibody (anti-iNOS rabbit antisera, 1:200 dilution; Wako Pure Chemical Industries, Osaka, Japan; or antinitrotyrosine rabbit polyclonal IgG, 1:100 dilution; Upstate Biotechnology, Lake Placid, NY). The immunoreactions were visualized by using ENVISION polymer reagent (DAKO Japan Ltd, Kyoto, Japan). The diaminobenzidine reaction was performed, followed by counterstaining with Hansel stain. Two investigators examined and counted more than 500 leukocytes and iNOS or 3-nitrotyrosine immunopositive cells without previous knowledge of the treatment. The mean values from 2 investigators were registered.

Measurement of XO activity

Xanthine oxidase activity in the supernatant of the sputum was measured according to the previous study.¹² Pterin was added to each sample as a

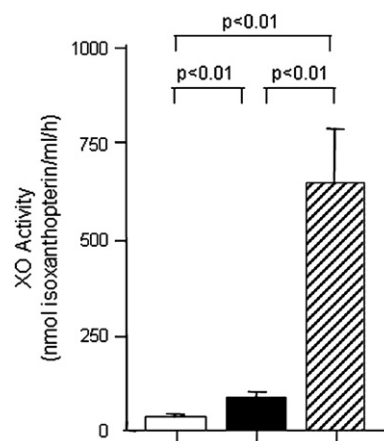


FIG 2. XO activities in the supernatant of the sputum from healthy subjects, subjects with well-controlled asthma, and subjects with refractory asthma. The activities of XO (nmol isoxanthopterin/mL/h) in the supernatant of the sputum from healthy subjects (open bar, $n = 10$), subjects with well-controlled asthma (filled bar, $n = 10$), and subjects with refractory asthma (hatched bar, $n = 8$) were measured.

substrate for XO. All samples were assayed for their XO activity by using a spectrofluorometer (model 650-40; Hitachi Ltd, Tokyo, Japan). The activity was expressed as the formation of isoxanthopterin.

Statistical analysis

Data were expressed as means $\pm$ SEMs. Experiments with multiple comparisons were evaluated by 1-way ANOVA followed by the Scheffé test. Pearson correlation analysis was performed to assess the correlation. Probability values of less than .05 were considered significant.

RESULTS

The values of FEV₁, FEV₁/forced vital capacity, and %FEV₁ in the refractory asthma group were significantly lower than those in the healthy or well-controlled asthma groups (Table I). Exacerbations rates were more frequent in the refractory asthma group than in the well-controlled asthma group (Table I).

Cells positive for iNOS were observed mainly in neutrophils, macrophages, and eosinophils (Fig 1, A-C). The percentages of iNOS-positive cells in both the well-controlled (17.1 $\pm$ 3.4%; Fig 1, D, filled bar) and refractory asthma groups (36.6 $\pm$ 3.3%; Fig 1, D, hatched bar) were higher than those in the healthy group (7.5 $\pm$ 1.6%; $P < .01$; Fig 1, D, open bar). Moreover, there were significantly more iNOS-positive cells in the refractory asthma group than in the well-controlled asthma group ($P < .01$; Fig 1, D). The values of eNO in the refractory asthma group (50.9 $\pm$ 4.3 ppb; Fig 1, E, hatched bar) were significantly higher than in the healthy (9.1 $\pm$ 0.9 ppb; Fig 1, E, open bar) or well-controlled asthma groups (13.3 $\pm$ 2.5 ppb; $P < .01$; Fig 1, E, filled bar). The activities of XO in the sputum, which is an enzyme generating superoxide anion, in the refractory asthma group (643 $\pm$ 140 nmol isoxanthopterin/mL/h; Fig 2, hatched bar) were significantly higher than in the healthy (33.5 $\pm$ 5.6 isoxanthopterin/mL/h; Fig 2, open bar) or well-controlled asthma groups (83.0 $\pm$ 16 isoxanthopterin/mL/h; $P < .01$; Fig 2, filled bar). 3-Nitrotyrosine positive cells were observed mainly in neutrophils, macrophages, and eosinophils. The percentages of 3-nitrotyrosine positive cells in both the well-controlled (16.6 $\pm$ 2.6%; Fig 3, D, filled bar) and refractory asthma groups (35.1 $\pm$ 3.5%; Fig 3, D, hatched bar) were higher than those in the healthy group

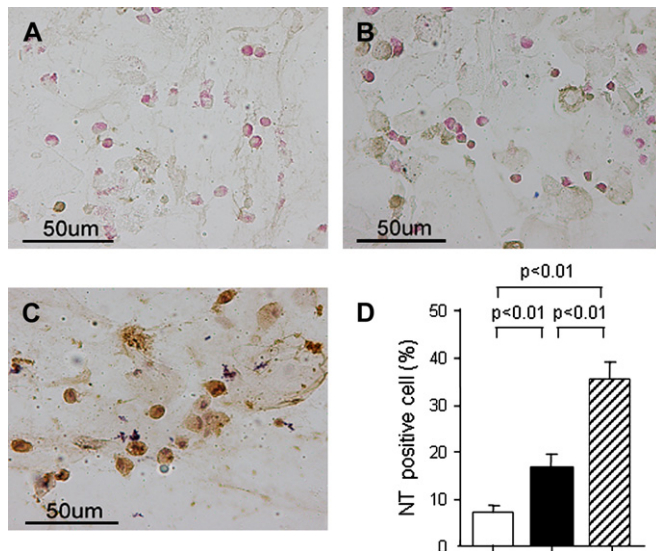


FIG 3. Immunohistochemical detection of 3-nitrotyrosine of induced sputum cells. Preparations of sputum from healthy subjects (A), subjects with well-controlled asthma (B), and subjects with refractory asthma (C) were stained with antinitrotyrosine antibody. D, The percentage of 3-nitrotyrosine-positive cells (%) in the sputum from healthy subjects (open bar, $n = 10$), subjects with well-controlled asthma (filled bar, $n = 10$), and subjects with refractory asthma (hatched bar, $n = 8$) were counted. NT, 3-Nitrotyrosine.

($7.1 \pm 1.6\%$; $P < .01$; Fig 3, D, open bar). Moreover, there were significantly more 3-nitrotyrosine-positive cells in the refractory asthma group than in the well-controlled asthma group ($P < .01$; Fig 3). We also investigated the correlation between the XO activities or iNOS expression and 3-nitrotyrosine formation. There were significantly positive correlations between 3-nitrotyrosine-positive cells and XO activities ($r = 0.86$; $P < .01$; Fig 4, A) or iNOS-positive cells ($r = 0.97$; $P < .01$; Fig 4, B). Each parameter of eNO ($r = -0.74$; $P < .01$), XO activities of sputum ($r = -0.78$; $P < .01$), percentage of iNOS-positive cells ($r = -0.79$; $P < .01$), and 3-nitrotyrosine-positive cells ($r = -0.76$; $P < .01$) had a significantly negative correlation with %FEV₁ (Fig 5). Sputum neutrophils in the refractory asthma group ($22.5 \pm 2.4\%$) were significantly higher than those in the healthy ($13.0 \pm 1.3\%$; $P < .01$) or well-controlled asthmatic groups ($13.7 \pm 1.0\%$; $P < .01$; Table II). The eosinophil counts in the well-controlled ($P < .01$) or refractory asthmatic groups ($P < .01$) were significantly increased compared with those in the healthy group. The eosinophil counts in refractory asthma group had a tendency to increase compared with those in the well-controlled group; however, there was no significant difference between the 2 groups (Table II).

DISCUSSION

Although the patients with refractory asthma have high medication requirements to maintain good disease control, they had an airflow obstruction or low quality of life despite treatment with high doses of steroids.⁵ Such patients have a disproportionate effect on healthcare utilization, accounting for at least half of the direct and indirect costs of treating asthma.⁶ In view of the effect of refractory asthma on healthcare resources, it is important to clarify the mechanisms involved in refractory asthma.

To explore the mechanisms underlying refractory asthma, we investigated the degree of nitrate stress between refractory

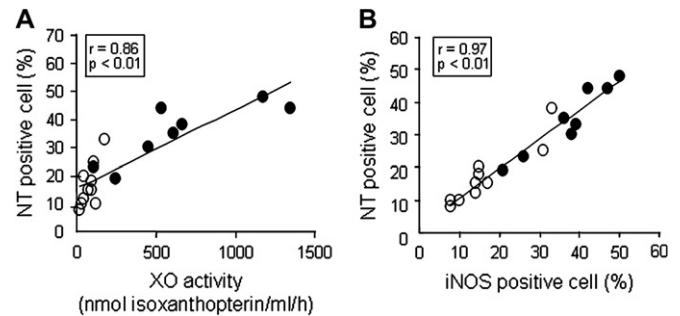


FIG 4. Correlation between values of 3-nitrotyrosine-positive cells and values of XO activities or iNOS-positive cells in sputum from subjects with asthma. Correlations between the values of 3-nitrotyrosine-positive cells and XO activities (A) or iNOS-positive cells (B) from all patients with asthma were analyzed (open circles, subjects with well-controlled asthma; closed circles, subjects with refractory asthma). r values were Pearson correlation coefficients. NT, 3-Nitrotyrosine.

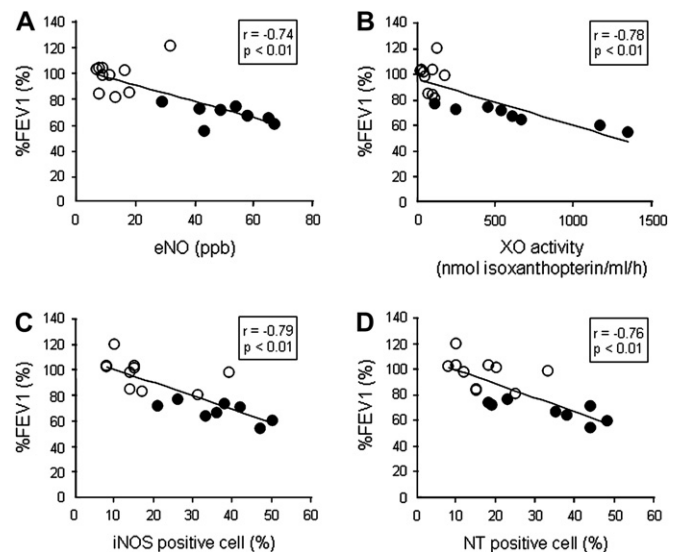


FIG 5. Correlation between airway caliber and each marker of oxidative/nitrative stress in subjects with asthma. Correlation between %FEV₁ values and values of exhaled NO (A), activities of XO (B), iNOS-positive cells (C), or 3-nitrotyrosine-positive cells (D) was analyzed (open circles, subjects with well-controlled asthma; closed circles, subjects with refractory asthma). r values were Pearson correlation coefficients. NT, 3-Nitrotyrosine.

asthma and well-controlled asthma because RNSs induce airway inflammation¹² and remodeling.¹³ We demonstrated that patients with refractory asthma had more nitrate stress compared with patients with well-controlled asthma. Nitrate stress has been reported to stimulate the production of proinflammatory cytokines, adhesion molecule expression, and chemokine production through the stimulation of transcription factors including NF- κ B and activator protein 1.^{17,18} Furthermore, it has been reported that RNSs had inflammatory effects on the airways of asthmatic models,¹² and RNSs caused airway hyperresponsiveness.^{19,20} These findings suggested that excessive RNSs could play a role in the pathogenesis of refractory asthma.

Many patients with refractory asthma have impaired lung function. Such an impairment is associated with a component of airflow obstruction that appears either irreversible or, at best, difficult to reverse. This irreversible airflow limitation in patients

TABLE II. Cell differential counts of sputum in study subjects

	Total cell number (10 ⁴ cells/mL)	Neutrophil (%)	Macrophage (%)	Eosinophil (%)	Lymphocyte (%)
Healthy subjects	95.9 ± 26	13.7 ± 1.0	78.2 ± 2.0	1.0 ± 0.3	7.1 ± 1.2
Well-controlled asthma	176 ± 60	13.0 ± 1.3	71.6 ± 2.0	7.5 ± 1.9*	7.9 ± 1.0
Refractory asthma	201 ± 54	22.5 ± 2.4**++	61.1 ± 2.1**++	10.0 ± 1.9**	6.4 ± 1.0

***P* < .01 compared with values of healthy subjects group; +*P* < .05, ++*P* < .01 compared with values of well-controlled asthma group.

with asthma has been reported to be associated with airway remodeling.⁵ Recently, we reported that peroxynitrite, one of the RNSs, enhanced fibroblast-mediated collagen gel contraction and chemotaxis toward fibronectin *in vitro*.¹³ We also reported that peroxynitrite stimulated fibroblast-mediated mediator production including TGF- β_1 , fibronectin, and vascular endothelial growth factor, which are key mediators responsible for airway remodeling.¹³ In the current study, we did not perform the airway reversibility test using a short-acting β -agonist or airway tissue examination by bronchial biopsy. Therefore, it was unclear whether airway remodeling occurred in the airways of the subjects with refractory asthma. However, each parameter of exhaled NO, XO activities, iNOS-positive cells, or 3-nitrotyrosine positive cells was significantly correlated with the airway caliber, suggesting that RNS-mediated mechanisms may be related to the airway narrowing of patients with asthma.

In the current study, a good correlation between 3-nitrotyrosine-positive cells and XO activities or iNOS-positive cells in sputum from all patients with asthma was observed. 3-Nitrotyrosine is a footprint of RNS production. A free amino acid form of tyrosine or tyrosine residues of proteins is nitrated by peroxynitrite¹¹ or via the H₂O₂/peroxidase-dependent nitrite oxidation pathway.²¹ As shown in Fig 4, there was very good correlation (*r* = 0.97) between 3-nitrotyrosine-positive cells and iNOS-positive cells, suggesting that iNOS might be responsible for the RNS production observed in the current study. Also, there was a positive correlation between 3-nitrotyrosine-positive cells and XO activities. We previously reported that the XO inhibitor allopurinol reduced 3-nitrotyrosine-positive cell counts in sputum from patients with chronic obstructive pulmonary disease,²² suggesting that superoxide anion derived from XO might be responsible for RNS production in the airways of inflammatory lung diseases. Taken together, both iNOS and XO may be associated with the generation of RNSs in the airways of patients with asthma.

Steroids have a number of anti-inflammatory actions including the suppression of iNOS expression.^{23,24} In the current study, steroids were not enough to suppress iNOS expression and 3-nitrotyrosine production completely in the sputum from patients with refractory asthma even at high doses. Recently, Ito et al²⁵ reported that peroxynitrite reduced the histone deacetylase 2 (HDAC 2) activity in epithelial cells through the nitration of tyrosine residues in HDAC 2. A reduction of HDAC 2 activity could contribute to the worsening of inflammation through the excessive production of proinflammatory cytokines including IL-1 β and IL-8.²⁵ They also showed that the HDAC 2 activity was decreased in patients with asthma and patients with COPD.²⁶ Inhibition of HDAC 2 was reported to interfere with glucocorticoid receptor-activated transcription.²⁷ It may be possible that inactivation of HDAC 2 by excessive RNSs occurs in refractory asthma.

There was a significant increase of neutrophils in the sputum from patients with refractory asthma compared with patients with well-controlled asthma. Our results are compatible with a previous multicenter study.¹⁵ Although the precise mechanisms responsible for the increased neutrophilia in refractory asthma are still uncertain, there are a few possibilities. First, RNSs can stimulate airway IL-8 production, which is a potent chemoattractant for neutrophils,^{26,28} and this may account for the increased neutrophilia in the airways of refractory asthma. Second, steroids are reported to inhibit the apoptosis of neutrophils.²⁹ These mechanisms may contribute to the neutrophilia in refractory asthmatic airways. In the current study, there was no statistically significant increase of eosinophils in the sputum from patients with refractory asthma compared with patients with well-controlled asthma, as shown in Table II. It remains controversial whether neutrophilic¹⁵ or persistent eosinophilic inflammation³⁰ occurs in the airways of refractory asthma, and further study is needed to clarify this issue.

A limitation of the current study is that the cellular expression of iNOS and the formation of 3-nitrotyrosine were only investigated in leukocytes of the sputum. In the airways of patients with asthma, many types of cells including bronchial epithelial cells, endothelial cells, and neurons express nitric oxide synthase.³¹ Especially bronchial epithelial cells have been reported to express iNOS strongly and to be one of the main sources of RNSs.³¹ However, the bronchial epithelial cells in the sputum were very scarce (less than 1%) in the current study, which is compatible with a previous report.³² Furthermore, it was hard to analyze the staining in the bronchial epithelial cells because most part of the cells were overlapped on the preparation. Thus, bronchoscopic biopsies of the airway are needed to assess the degree of nitrative stress in the bronchial epithelial cells precisely.

In summary, our data demonstrate that iNOS-positive cells, eNO, XO activities, and 3-nitrotyrosine-positive cells were significantly increased in refractory asthma compared with well-controlled asthma. Each parameter of iNOS-positive cells, eNO, XO activities, 3-nitrotyrosine positive-cells, and neutrophils in sputum had a significant negative correlation with the airway caliber. Because RNSs have inflammatory and remodeling actions in tissues, these mediators may be related to the refractoriness of asthma. Further study is needed to clarify the precise mechanisms. Specific inhibitors or scavengers of RNSs may become a therapeutic target for refractory asthma.

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Clinical implications: More nitrative stress could be involved in the refractoriness of bronchial asthma.

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