Catalase. Catalase is a homotetrameric protein and has a high molecular weight (240 kDa). This antioxidant enzyme decomposes hydrogen peroxide into water and oxygen as shown in the following equation (32):

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is ubiquitous in most animal cells. Catalase is located in peroxisomes and in the cytosol and is specially localized in type II pneumocytes and alveolar macrophages (49). A high concentration of catalase exists in the epithelial lining fluid and it is thought to play a protective role against oxidative stress in the lungs (31). When sensitized sheep were exposed to antigen, airway hyper-responsiveness to carbacol was observed. Pretreatment with catalase suppressed the increased airway hyper-responsiveness, suggesting that hydrogen peroxide plays a role in the airway hyper-responsiveness in asthma and catalase may protect against oxidant-induced airway hyper-responsiveness (54).

Glutathione peroxidase. Glutathione peroxidases are a family of selenium-dependent and -independent antioxidant enzymes. These antioxidant enzymes are divided into two forms, cellular and extracellular. Glutathione peroxidase is a 85 kDa protein with a tetrameric structure. It requires four atoms of selenium bound as seleno-cycteine moieties which confer catalytic activity. Glutathione peroxidase reduces hydrogen peroxide to water in the presence of GSH. Glutathione peroxidase has four isoforms. Three are selenium-dependent glutathione peroxidases and the other one is selenium independent. These glutathione peroxidases have been identified in a variety of cells (73) and are ubiquitously present in the cytosol. In asthmatic patients, the levels of glutathione peroxidase in blood are decreased compared to healthy subjects (30). There is increasing evidence that reactive nitrogen species including peroxynitrite are associated with the pathophysiology of bronchial asthma (41, 89). Because selenium-related antioxidants are powerful scavengers of peroxynitrite, glutathione peroxidase may reduce the inflammation of airways in asthmatic patients (98).

Heme oxygenase. Heme oxygenase is a member of the heat-shock protein family that catalyses the degradation of the heme molecule into biliverdin in a reaction that generates carbon monoxide and iron (113). This antioxidant enzyme has cytoprotective effects against oxidative stress. It has been reported that heme oxygenase knockout mice were markedly sensitive to oxidative stress (81). In an asthmatic model, heme oxygenase-1 induced by repeated administration of hemin suppressed the inflammation of the airways (114). The carbon monoxide concentration in the exhaled air from asthmatic patients is significantly elevated compared with healthy subjects (119), suggesting that heme oxygenase may have a protective effect against the oxidative stress in bronchial asthma.

Ascorbic acid. Ascorbic acid is a low molecular weight and water-soluble radical scavenger. Administration of ascorbic acid to asthmatic patients is reported to improve their airway hyper-responsiveness to methacholine and partially block exercise-induced bronchoconstriction (71).

Sources of ROS in lungs

Recruited inflammatory cells and lung resident cells including epithelial cells can produce oxidants. The univalent reaction of oxygen to superoxide anion is the most important step in the production of oxidants. Three major sources of superoxide anion have been identified as follows.

NADPH oxidase system. The generation of superoxide anion is mediated by NADPH as an electron donor in the one-electron reduction of oxygen to produce superoxide anion. NADPH oxidase is a multicomponent enzyme complex, part of which is located in the plasma membrane. Cell fractionation experiments have shown that the dormant oxidase is located in the plasma membrane and that it requires a cytosolic component for activation. The plasma membrane components include a 99 kDa glycosylated subunit and a 22 kDa nonglycosylated subunit. Together, the two subunits are tightly associated and contain a heme group, likely bound to the 22 kDa subunit. These two subunits and heme act as a unit to form the terminal component of the NADPH oxidase, since it directly reduces oxygen to superoxide anion. Another membrane component is a 67 kDa protein, likely a flavoprotein, requiring flavin adenine dinucleotide to function, and it probably acts to convert intracellular NADPH to NADP+. In addition to the membrane components, there are at least two, and probably more, cytoplasmic subunit components. A 47 kDa protein is phosphorylated following stimulation. The second is a 65 kDa protein which migrates from the cytosol to the plasma and phagolysosomal membranes following NADPH activation. Because eosinophils and neutrophils from asthmatic patients can produce more superoxide anion compared to those of healthy subjects (25, 94), NADPH oxidase in asthmatic subjects may be activated.

Xanthine oxidase. Xanthine oxidoreductase (XOR), first identified a century ago in milk, is a highly conserved member of the molybdoenzyme family. XOR has two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). Both forms catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid (UA), the terminal two reactions of the purine degradation pathway. Basal expression of the XOR gene is mediated by several transcription factors including C/EBP, ETS-1, AP-1, AP-2, and TF-IID (115). Although the basal expression of XOR is low, a variety of factors including hypoxia, lipopolysaccharide (LPS), IFN-γ, IL-1, IL-6, TNF-α, and steroids upregulate the transcription (27). In an asthmatic model, the XOR activity was upregulated in the lung during the late allergic response phase (105). Moreover, an XOR inhibitor, {4-amino-6-hydroxypyrazolo(3,4-d)pyrimidine}(AHPP), suppressed allergen-induced microvascular hyperpermeability during LAR, suggesting that XOR may be associated with the extravasation induced by allergen challenge.

Mitochondrial respiration chain. The third major pathway for the generation of superoxide anion is the mitochondrial respiration chain. Mitochondrial superoxide anion can be generated from several sites in the respiration chain. The matrix side of the organelle associated with complex I, complex III, and the Q pool can generate superoxide anion in mitochondria. Superoxide may have a direct interaction with some targets such as aconitase that may contribute to cell signaling through the release of iron from the enzyme. In bronchial asthma, the role of mitochondrial superoxide anion has not been well clarified. Because eosinophils from asthmatic patients can generate more superoxide anion than those from healthy subjects, the generation of superoxide anion from mitochondrion may be upregulated.

RNS AND BRONCHIAL ASTHMA

NO in the respiratory system

NO has a variety of physiological effects in mammalian cells (43, 72). In the respiratory system, endogenous NO plays a key role in the physiological regulation of the airway function. NO and related compounds are produced by a wide variety of residential and inflammatory cells in the respiratory system. NO is generated via a five-electron oxidation of a terminal guanidinium nitrogen on the amino acid L-arginine. The reaction is both oxygen- and NADPH-dependent and yields the coproduct L-citrulline. This reaction is catalyzed by three isoforms of NO synthase (NOS). Neural NOS (NOS-I or nNOS) and endothelial NOS (NOS-III or eNOS) are both the constitutive type of NOS. The inducible type of NOS (NOS-II or iNOS) is upregulated by various cytokines, such as TNF-α, IFN-γ, and IL-1β, and can generate more NO compared with the constitutive type of NOS. The NO-generating cell types in lungs are listed in Table 2. In inflammatory sites, superoxide anion can be generated at the same time. It has been reported that NO reacts with superoxide anion very rapidly ($k = 6.7 \times 10^9$ $M^{-1}S^{-1}$) and forms peroxynitrite (10, 82). RNS are also formed via the H2O2/peroxidase-dependent nitrite oxidation pathway (28). These RNS cause airway inflammation (105), namely the activation of matrix metalloproteinase (MMP) (75), and an enhancement of the production of the proinflammatory cytokine TNF-α (65). Therefore, RNS may be involved in the pathophysiology of the inflammatory process of bronchial asthma.

TABLE 2. LOCALIZATION OF NITRIC OXIDE SYNTHASES (NOS) IN HUMAN LUNGS

Isoforms	*Localization*
NOS I	Neurons (ganglion, trachea, and bronchus) Airway epithelial cells Neutrophils
NOS II	Macrophages Airway epithelial cells Type II pheumocytes Endothelial cells Fibroblasts Vascular smooth muscle cells Neutrophils Eosinophils Mast cells
NOS III	Endothelial cells Airway epithelial cells Platelets

Role of NOS in bronchial asthma

NO derived from cNOS plays a physiological role in the respiratory system. NO derived from NOS I is thought to partly regulate the airway smooth muscle tone. In the pulmonary vasculature, the role of NO from NOS III has been controversial. Conversely, NO derived from NOS II is thought to play a critical role in the airway inflammation. The role of each NOS is reviewed in this section.

NOS I. The inhibitory nonadrenergic noncholinergic (iNANC) nerve is the only neural bronchodilator system in human airways, where its bronchodilatory function has been demonstrated *in vitro* by electrical field stimulation (EFS) (108), as well as *in vivo* by reflex stimulation (42). The neurotransmitter of this nervous system has been suggested to be vasoactive intestinal peptide (VIP) or other related peptides in several species, since these peptides have a potent bronchodilatory action. NO has also been recognized as a transmitter of iNANC nerves distributed in various organs (110), including airways (110), by functional studies using inhibitors for NOS. Histochemically, colocalization of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity, a marker for the nervous system, was used to demonstrate the existence of NOS I in guinea pig airways. In guinea pig airways, both NO and VIP have been reported to functionally mediate iNANC relaxation (57). In human airways, NO is thought to mainly mediate iNANC responses (11). This bronchodilatory function of iNANC may be impaired in asthmatic airways. According to our previous report, the bronchodilatory function of iNANC by EFS in the tracheas from antigen-challenged guinea pigs was impaired, compared to control animals (70). Furthermore, the impaired bronchodilatory function of iNANC was restored by pretreatment with SOD, suggesting that excessively produced superoxide anion inactivates the bronchodilatory action of NO derived from NOS I (70).

NOS III. Some studies showed that NO derived from NOS III regulated the pulmonary vascular tone in various species (34). These findings are based on studies using NOS inhibitors. However, other studies showed that even the highest doses of NOS inhibitor did not show any vasoconstriction in pulmonary vasculatures. In human, Stamler *et al.* reported that L-NMMA caused pulmonary vasoconstriction in healthy human volunteers (101). However, the effect of NOS inhibitors was more dominant in the systemic circulation than in the pulmonary circulation (34). Data obtained from *in vitro* human preparations are also inconclusive (23). Taken together, the role of NO derived from NOS III has not been fully elucidated yet.

NOS II. In various inflammatory lung diseases including asthma, chronic obstructive pulmonary diseases (COPD), idiopathic pulmonary fibrosis (IPF), and cystic fibrosis, iNOS is reported to be upregulated in the airways and lungs. iNOS gene is stimulated by various proinflammatory cytokines. In asthmatic individuals, increased levels of exhaled NO have been found (47). In asthmatic airways, the expression of iNOS was enhanced in pathological examinations (33). Increased expression of iNOS was seen in infiltrated inflammatory cells and macrophages, and the increased expression was corticosteroid sensitive (91). Because steroids can suppress both iNOS expression and the exhaled NO levels, the increasing NO levels in expired air from asthmatic patients is thought to be derived from iNOS. This result is supported by the fact that inhalation of a relatively specific inhibitor of iNOS, SC-51, reduced the expired NO levels in asthmatic individuals (35). We previously showed that iNOS immunopositive cell counts in induced sputum had a significantly positive correlation with the NO levels in exhaled air in asthmatic patients (Fig. 2) (41). These results suggest that the enhanced iNOS expression in the airways of asthmatic patients is responsible for the increased NO levels in expired air. According to previous studies, exhaled NO may reflect the clinical control of asthma, particularly during exacerbations (64).

Nitrative stress and airway inflammation in bronchial asthma

Our previous report showed that nitrative stress could induce airway inflammation during the late allergic phase in an antigen-challenged guinea pig model (105). During the late phase, the NO levels in antigen-challenged animals significantly increased compared to control animals. A nonspecific NOS inhibitor, L-NAME, and the peroxynitrite scavenger, ebselen, inhibited the antigen-induced microvascular hyperpermeability, suggesting that nitrative stress may contribute to the airway inflammation in bronchial asthma. Moreover, exogenously administered peroxynitrite stimulated the release of toxic granules from eosinophils (95). In addition, because eosinophil cationic protein and major basic protein from eosinophils can cause tissue injury (22) and muscarinic 2 (M2) receptor dysfunction (56), RNS may be responsible for the airway inflammation in asthma through eosinophil activation.

Nitrative stress and airway hyper-responsiveness in bronchial asthma

To clarify the role of iNOS in the airway hyper-responsiveness in asthmatic airways, we investigated further by using an asthmatic mouse model. We showed that iNOS was upregulated after antigen challenge in sensitized mice (Fig. 3) (50). Furthermore, we showed that both pharmacological blockade of iNOS (50) and depletion of iNOS gene (51) also inhibited the antigen-induced airway hyper-responsiveness in mice. In iNOS knockout mice, nitrotyrosine formation, which is a footprint of RNS, was completely diminished. Furthermore, Sadeghi–Hashjin *et al.* showed that exogenously administered peroxynitrite induced airway hyper-responsiveness *in vivo* and *in vitro* (95). Taken together, nitrative stress appears to cause airway hyper-responsiveness in bronchial asthma. In humans, we reported that the exhaled NO level significantly correlated with the baseline FEV1 values in steroid naïve asthmatic patients (40). Treatment with inhaled corticosteroids was associated with a reduction in the NO levels in exhaled air and an improvement in FEV1 and airway hyper-responsiveness, suggesting that exhaled NO can serve as a marker of airway inflammation and is associated with the airway caliber and hyper-responsiveness induced by the airway inflammation.

Nitrative stress and airway remodeling in bronchial asthma

Excessive production of NO during inflammatory and immune processes leads to the formation of RNS including peroxynitrite and nitrogen dioxide (NO_2). These RNS are formed from NO and superoxide anion or via the H_2O_2/peroxidase-dependent nitrite oxidation pathway. In inflammatory condi-

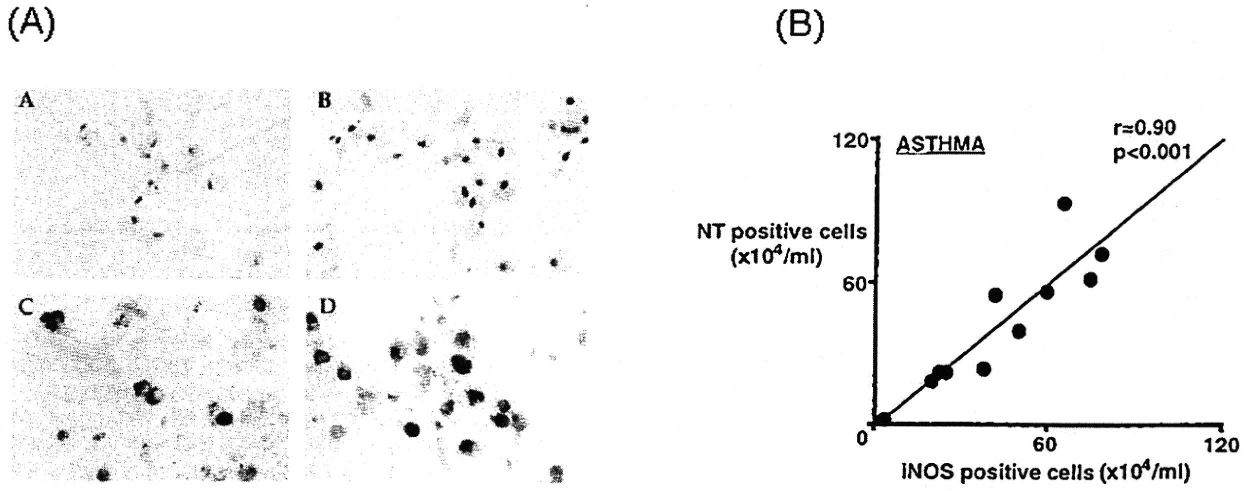

FIG. 2. Immunocytostaining of the inducible type of nitric oxide synthase (iNOS) and nitrotyrosine. (**A**) Representative photographs of immunocytostaining of iNOS (*left panels*) and nitrotyrosine (*right panels*) from healthy (*upper panels*) and asthmatic subjects (*lower panels*) are shown. Relation of iNOS-positive cell counts to nitrotyrosine (NT)-positive cell counts in induced sputum from asthmatic subjects (**B**). R is correlation coefficient; the line and *p* value correspond to the fitted regression equation. Excerpted with permission Ichinose M, Sugiura H, Yamagata S *et al. Am J Respir Crit Care Med* 2000; 162: 701–706. Copyright 2000 American Thoracic Society.

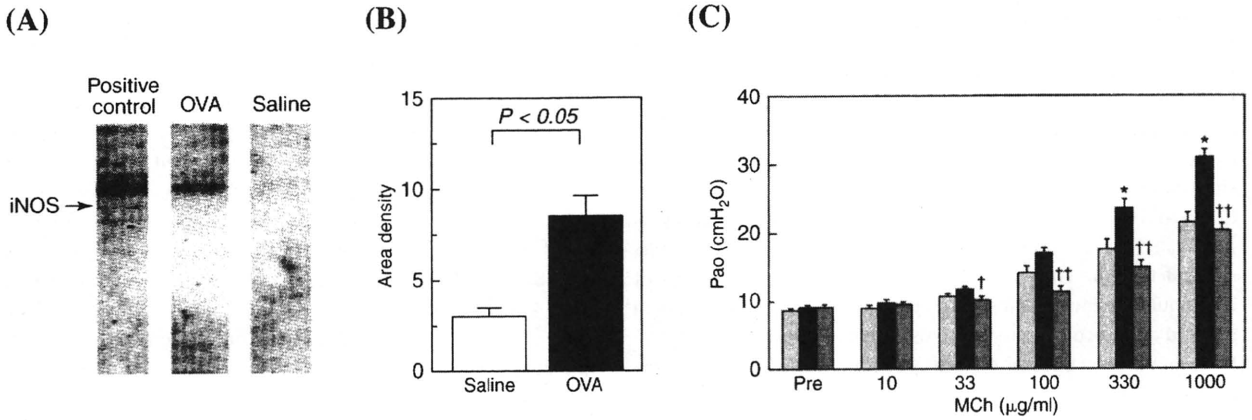

FIG. 3. Inducible type of nitric oxide synthase (iNOS) expression during late phase allergic reaction in mice and effect of the specific iNOS inhibitor, 1,400 W, on airway hyper-responsiveness. (A) Immunoblot analysis of the expression of iNOS in airway tissue isolated from sensitized mice. Positive control: sample of mice macrophage lysates stimulated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS); OVA: 24 h after ovalbumin (OVA) challenge; saline: 24 h after saline exposure. **(B)** Quantification of the intensity of the bands by densitometry. Lanes are the same as in (A). (C) Effect of 1,400 W on airway hyper-responsiveness after OVA challenge in sensitized mice. Airway responsiveness was assessed by means of airway opening pressure (Pao) measurement after intravenous methacholine (MCh) administration. $*p < 0.01$ compared with the saline-pretreated saline challenged mice. $+p < 0.05$ and $++p < 0.01$ compared with the saline-pretreated OVA-challenged mice. Each value indicates mean + SEM. *Open bars*: saline-pretreated saline-challenged; *filled bars*: saline-pretreated OVA-challenged; *hatched bars*: 1,400 W-pretreated OVA-challenged. Excerpted with permission, Koarai A, Ichinose M, Sugiura H *et al. Pulm Pharmacol Ther* 2000; 13: 267–275. Copyright 2000 Elsevier Ltd.

tions where superoxide anion is generated, NO is rapidly consumed by reacting with superoxide to produce highly reactive peroxynitrite. Peroxynitrite is an extremely powerful oxidant and is presumed to be largely responsible for many of the adverse effects of the excessive generation of NO. Excessive production of RNS causes tissue injury, lipid peroxidation, and nitration of tyrosine residues. Consistent with the role of this pathway in disease, excessive production of 3-nitrotyrosine has been observed in various inflammatory lung diseases, including bronchial asthma (33, 91, 103), COPD (37, 103), and IPF (90). Inflammatory processes are frequently accompanied by alterations in the tissue structure. Such alterations may result from tissue damage due to active proteases or toxic moieties released by inflammatory cells. In addition, mediators released at inflammatory sites are capable of directly altering the cell function, leading to tissue repair and remodeling. The production of RNS causes tissue injury, but whether RNS can affect tissue repair and remodeling remains unknown. Recently, we reported the effect of peroxynitrite on tissue remodeling by using a collagen gel contraction assay model mediated by human lung fibroblasts *in vitro* (104). As shown in Fig. 4, exogenously administered peroxynitrite augmented fibroblast-mediated collagen gel contraction in a dose-dependent manner. Peroxynitrite also stimulated the production of TGF-β_1, fibronectin, and vascular endothelial growth factor (VEGF), which are thought to play critical roles in lung tissue remodeling (104). Furthermore, treatment with peroxynitrite augmented the chemotaxis of fibroblasts toward fibronectin through augmenting the expression of integrins, which are receptors for fibronectin (104). The augmented collagen gel contraction, mediator production, and chemotaxis were reversed by treatment with neutralizing anti-TGF-β antibody (104). These results suggest that peroxynitrite or other RNS may cause tissue remodeling through TGF-β_1 activation. In fact, TGF-β_1 is a key mediator in a variety of physiological and pathological processes, including fibroblast repair responses. TGF-β

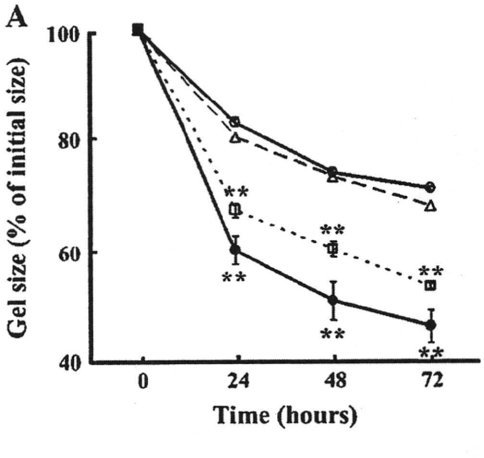

FIG. 4. Effect of authentic peroxynitrite on collagen gel contraction by human fetal lung fibroblasts (HFL-1). Fibroblasts were cast into three-dimensional collagen gels and floated in medium containing various concentrations of peroxynitrite. Gel size was measured daily. When the gel size becomes smaller, the fibroblast-mediated tissue remodeling is augmented. *Vertical axis*: gel size (% of initial size); *horizontal axis*: time. $**p < 0.01$; compared with the values of control. *Open circles*, control; *triangles*, 0.1 μM peroxynitrite; *squares*, 1 μM peroxynitrite; *filled circles*, 10 μM peroxynitrite. Excerpted with permission, Sugiura H, Liu X, Kobayashi T *et al. Am J Respir Cell Mol Biol* 2006; 34: 592–599. Copyright 2006 American Thoracic Society.

TABLE 3. OXIDATIVE AND NITRATIVE STRESS IN LUNG TISSUES

Target tissue and cells	*Effects of ROS and RNS*
Epithelial cells	Injury Proinflammatory mediators production
Mucus glands	Mucus hypersecretion
Airway micro vessels	Plasma leakage Edema Vasodilatation Neovascularization
Sensory nerves	Activation
Inhibitory nonadrenergic noncholinergic nerves	Inactivation
Fibroblasts	Activation Augmentation of migration
Airway smooth muscle cells	Contraction
Inflammatory cells	Mediator production and secretion MMP activation

ROS, reactive oxygen species; RNS, reactive nitrogen species; MMP, matrix metalloproteinase.

regulates the fibroblast migration, proliferation, differentiation, and production of matrix and soluble factors. In addition, TGF-β stimulates the fibroblast-mediated contraction of the extracellular matrix. Through these actions, TGF-β is believed to be a major regulator of tissue remodeling. Among the mediators induced by TGF-β are fibronectin (117) and VEGF (112). Fibronectin can form a provisional extracellular matrix following injury and is a potent chemoattractant for fibroblasts. VEGF is a multifunctional cytokine that stimulates endothelial cell mitogenesis and migration and modulates the endothelial permeability. The production of VEGF, therefore, could play a role in the neovascularization that characterizes tissue repair following injury. In fact, the production of these mediators was augmented in asthmatic airways and excessive extracellular matrix deposition was observed in the basement membrane in the airways of asthmatic patients (29). Therefore, nitrative stress may contribute to the formation of the airway remodeling observed in asthmatics, especially in refractory asthmatic patients.

NITRATIVE STRESS AND REFRACTORY ASTHMA

Asthma is a disorder characterized by chronic inflammation of the airways, airflow obstruction, and airway hyperreactivity. Most asthmatic patients are well controlled by low doses of anti-inflammatory agents with or without bronchodilators. However, 5–10% of asthmatic patients have more troublesome disease, reflected by a higher medication requirement to maintain good disease control or persistent symptoms and disease exacerbation, airflow obstruction, or low quality of life in spite of using high doses of steroids (3). To describe this subgroup of asthmatic patients with troublesome disease, the term "refractory asthma" has been used (3). It has been reported that individuals with refractory asthma are 15 times more likely to use emergency medical care than well-controlled asthmatic patients and are 20 times more likely to require hospital admission (97). Furthermore, these individuals with refractory asthma also have greater absenteeism from work on account of their

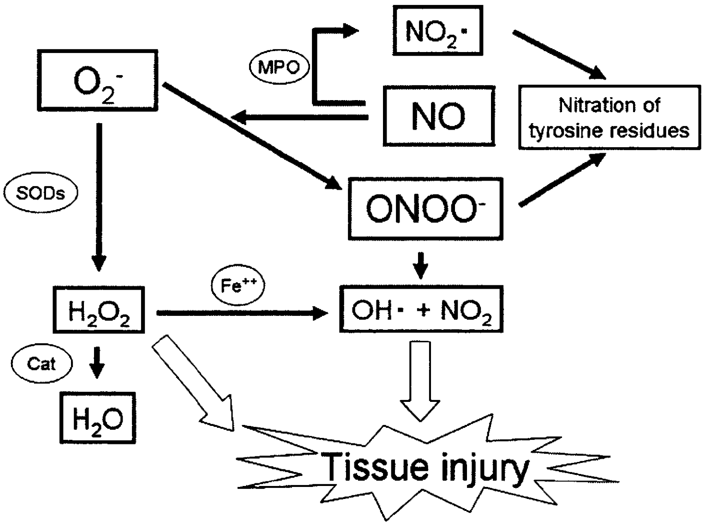

FIG. 5. Crosstalk between reactive oxygen species and reactive nitrogen species. Cat, catalase; Fe^{++}, ferrous ion; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; NO, nitric oxide; $NO_2^{\bullet}$, nitrogen dioxide radical, NO_2, nitrogen dioxide; $OH^{\bullet}$, hydroxyl radical; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite, SODs, superoxide dismutases.

disease. However, there has been little information on the pathophysiological mechanisms responsible for refractory asthma. Understanding the differences in the airway inflammation between refractory and well-controlled asthma could therefore be important for determining the mechanisms responsible for refractory asthma. Recently, we investigated the differences in oxidative and nitrative stress among healthy subjects, well-controlled asthmatic subjects, and refractory asthmatic subjects. Oxidative stress and nitrative stress were enhanced in the refractory asthmatic group (unpublished data). Steroids have a number of anti-inflammatory actions, including the suppression of iNOS expression. In this study, steroids could not suppress iNOS expression or 3-nitrotyrosine formation in the sputum from patients with refractory asthma even at high doses. Recently, Ito and co-workers reported that peroxynitrite reduced the histone deacetylase 2 (HDAC 2) activity in epithelial cells through the nitration of tyrosine residues in HDAC 2 (45). A reduction of HDAC 2 activity could contribute to the worsening of inflammation through the excessive production of proinflammatory cytokines including IL-1β and IL-8 (1). They also showed that the HDAC 2 activity was decreased in asthmatic patients and patients with COPD (1). Inhibition of HDAC 2 was reported to interfere with glucocorticoid receptor-activated transcription (1). Taken together, it may be possible that an inactivation of HDAC 2 by excessive RNS occurs in refractory asthma. Moreover, since RNS may be associated with tissue remodeling as mentioned before, oxidative and nitrative stress may contribute to the refractoriness to steroid therapy in refractory asthmatic patients.

SUMMARY

The composite role of ROS and RNS in the lung pathology of asthma is shown in Table 3. Asthma is associated with increased levels of ROS and RNS due to the activation of infiltrated inflammatory cells and resident cells stimulated by proinflammatory mediators. Numerous reports have shown that oxidative and nitrative stress can cause airway inflammation and hyper-responsiveness which are the most important features of asthma. Because of excessive oxidative and nitrative stress, an imbalance of oxidants and antioxidants occurs in the airways of asthma. Interactions between ROS and RNS are summarized in Fig. 5. ROS and RNS may stimulate some transcription factor activities and change the transcription of proinflammatory cytokines. ROS and RNS appear to be related to the airway remodeling and refractoriness to steroids. Blockers or scavengers of ROS and RNS may become therapeutic targets in the future.

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ABBREVIATIONS

ACE, angiotensin converting enzyme; BALf, bronchoalveolar lavage fluid; BK, bradykinin; COPD, chronic obstructive pulmonary disease; COX, cyclooxygenase; EFS, electrical field stimulation; GSH, glutathione; HDAC, histone diacetylase; HFL, human fetal lung fibroblast; IFN, interferon; IL, interleukin; iNANC, inhibitory nonadrenergic noncholinergic; IPF, idiopathic pulmonary fibrosis; LAR, late allergic response; LPS, lipopolysaccharide; LT, leukotrien; MCh, methacholine, MMP, matrix metalloproteinase; NEP, neutral endopeptidase; NF-kB, nucleus factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; NT, nitrotyrosine; OVA, ovalbumin; PAF, platelet activating factor; Pao, airway opening pressure, PG, prostaglandin; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SP, substance P; TGF, transforming growth factor; TNF, tumor necrosis factor; Tx, thromboxane; UA, uric acid; VEGF, vasoactive endothelial growth factor; VIP, vasoactive intestinal peptide; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxide reductase.

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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 %FEV_1 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

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

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 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) oxidase[9] 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.

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

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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 (μg/d). Patients with refractory asthma were treated with bronchodilators in addition to high doses of inhaled steroid (μg/d) or high doses of inhaled steroid (μg/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.

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 μg/d beclomethasone dipropionate, or equivalent doses of other ICSs, or a combination of ICSs and oral corticosteroids. Because the values of FEV_1 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 μg/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 790*g* 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.

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.

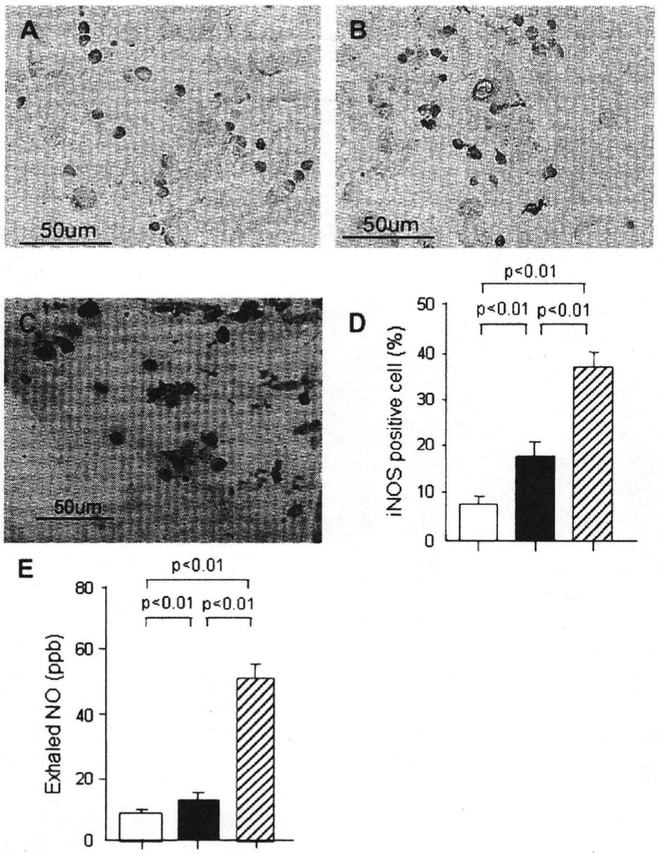

FIG 1. Immunochemical detection of iNOS in induced sputum cells and exhaled NO levels in healthy subjects **(A)**, subjects with well-controlled asthma **(B)**, and subjects with refractory asthma **(C)**. **D**, The percentages of iNOS-positive cells (%) in the sputum from healthy subjects (*open bar*, 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.

Immunocytostaining

Immunocytostaining 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 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 ± 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.

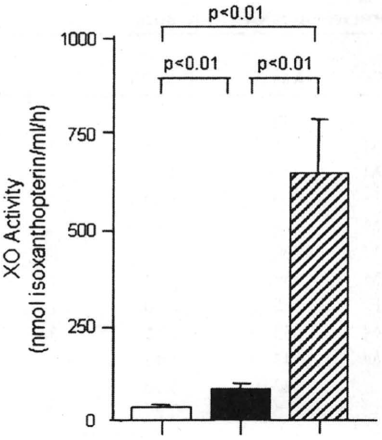

FIG 2. XO activities in the supernatant of the sputum from healthy subjects, subjects with well-controlled asthma, and subjects with refractory asthma. The activities of XO (nmol isoxanthopterin/mL/h) in the supernatant of the sputum from healthy subjects (*open bar*, n = 10), subjects with well-controlled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

RESULTS

The values of FEV_1, FEV_1/forced vital capacity, and $\%FEV_1$ 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 ± 3.4%; Fig 1, *D, filled bar*) and refractory asthma groups (36.6 ± 3.3%; Fig 1, *D, hatched bar*) were higher than those in the healthy group (7.5 ± 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 ± 4.3 ppb; Fig 1, *E, hatched bar*) were significantly higher than in the healthy (9.1 ± 0.9 ppb; Fig 1, *E, open bar*) or well-controlled asthma groups (13.3 ± 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 ± 140 nmol isoxanthopterin/mL/h; Fig 2, *hatched bar*) were significantly higher than in the healthy (33.5 ± 5.6 isoxanthopterin/mL/h; Fig 2, *open bar*) or well-controlled asthma groups (83.0 + 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 ± 2.6%; Fig 3, *D, filled bar*) and refractory asthma groups (35.1 ± 3.5%; Fig 3, *D, hatched bar*) were higher than those in the healthy group (7.1 ± 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–

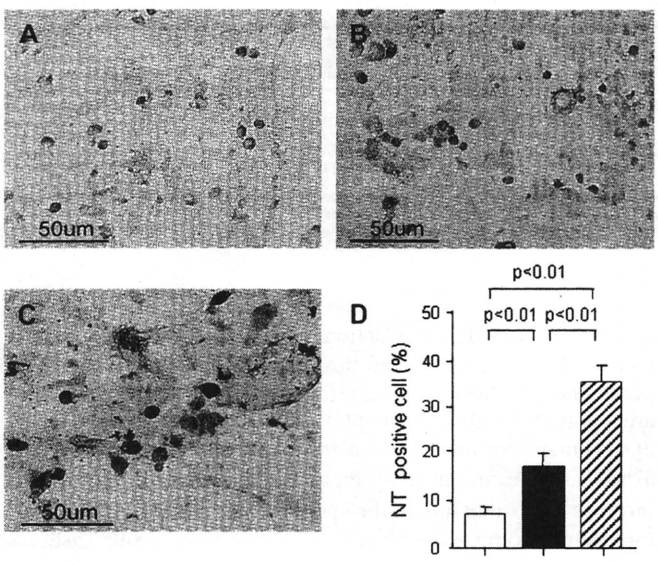

FIG 3. Immunochemical detection of 3-nitrotyrosine of induced sputum cells. Preparations of sputum from healthy subjects **(A)**, subjects with well-controlled asthma **(B)**, and subjects with refractory asthma **(C)** were stained with antinitrotyrosine antibody. **D,** The percentage of 3-nitrotyrosine–positive 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.

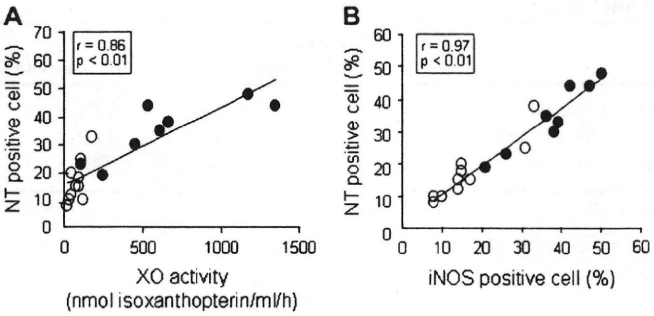

FIG 4. Correlation between values of 3-nitrotyrosine–positive cells and values of XO activities or iNOS-positive cells in sputum from subjects with asthma. Correlations between the values of 3-nitrotyrosine–positive cells and XO activities **(A)** or iNOS-positive cells **(B)** from all patients with 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.

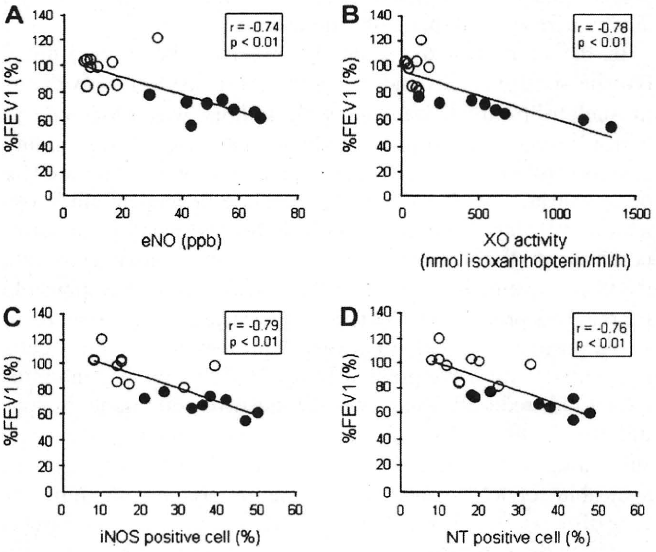

FIG 5. Correlation between airway caliber and each marker of oxidative/nitrative stress in subjects with asthma. Correlation between %FEV$_1$ values and values of exhaled NO **(A)**, activities of XO **(B)**, iNOS-positive cells **(C)**, or 3-nitrotyrosine–positive cells **(D)** was analyzed (*open circles,* subjects with well-controlled asthma; *closed circles,* subjects with refractory asthma). *r* values were Pearson correlation coefficients. *NT,* 3-Nitrotyrosine.

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 %FEV$_1$ (Fig 5). Sputum neutrophils in the refractory asthma group (22.5 + 2.4%) were significantly higher than those in the healthy (13.0 ± 1.3%; $P < .01$) or well-controlled asthmatic groups (13.7 ± 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 nitrative stress between refractory asthma and well-controlled asthma because RNSs induce airway inflammation[12] and remodeling.[13] We demonstrated that patients with refractory asthma had more nitrative stress compared with patients with well-controlled asthma. Nitrative 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-κB 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 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

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.

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 peroxynitrite[11] or via the H_2O_2/ 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-nirotyrosine–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 al[25] 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-1β 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 neutrophilic[15] or persistent eosinophilic inflammation[30] 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-nitrotyrsine–positive cells were significantly increased in refractory asthma compared with well-controlled asthma. Each parameter of iNOS-positive cells, eNO, XO activities, 3-nitrotyrsine 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.

We thank Dr M. Tomaki and H. Ogawa for discussions on the current study. We thank Dr G. Tamura for recruiting patients with asthma into the current study. We also thank Mr B. Bell for reading the manuscript.

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

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Peroxynitrite augments fibroblast-mediated tissue remodeling via myofibroblast differentiation

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Ichikawa T, Sugiura H, Koarai A, Yanagisawa S, Kanda M, Hayata A, Furukawa K, Akamatsu K, Hirano T, Nakanishi M, Matsunaga K, Minakata Y, Ichinose M. Peroxynitrite augments fibroblast-mediated tissue remodeling via myofibroblast differentiation. *Am J Physiol Lung Cell Mol Physiol* 295: L800–L808, 2008. First published September 12, 2008; doi:10.1152/ajplung.90264.2008.—Irreversible airflow limitation in asthma is associated with airway remodeling in which the differentiation of fibroblasts to myofibroblasts plays a pivotal role. In asthmatic airways, excessive production of reactive nitrogen species (RNS) has been observed. The aim of this study is to evaluate whether peroxynitrite, one of the RNS, can affect the differentiation of fibroblasts to myofibroblasts. Human fetal lung fibroblasts were treated with various concentrations of authentic peroxynitrite or a peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1), and the expressions of α-smooth muscle actin (α-SMA) and desmin, markers of myofibroblast differentiation, were evaluated. The releases of transforming growth factor-β_1 (TGF-β_1) and ECM proteins including fibronectin and collagen I were assessed. To clarify the mechanism in this differentiation, the effect of anti-TGF-β antibody or NF-κB inhibitors on the α-SMA expression and ECM production was assessed. Peroxynitrite and SIN-1 significantly augmented the α-SMA expression compared with control in a concentration-dependent manner ($P < 0.01$ and $P < 0.05$, respectively). Peroxynitrite significantly increased desmin and TGF-β_1 production ($P < 0.01$). Peroxynitrite enhanced the translocation of NF-κB into the nucleus confirmed by immunocytostaining and immunoblotting. Peroxynitrite-augmented α-SMA expression was blocked by NF-κB inhibitors, MG132 and caffeic acid phenethyl ester (CAPE), and anti-TGF-β antibody. CAPE completely inhibited the peroxynitrite-augmented TGF-β_1 release. The production of fibronectin and collagen I was significantly increased by peroxynitrite ($P < 0.01$) and inhibited by anti-TGF-β antibody. These results suggest that RNS can affect the differentiation to myofibroblasts and excessive ECM production via a NF-κB-TGF-β_1-dependent pathway.

reactive nitrogen species; airway remodeling; asthma; α-smooth muscle actin; nuclear factor-κB

ASTHMA IS A DISORDER CHARACTERIZED by chronic inflammation of the airways, airflow limitation, airway hyperresponsiveness (AHR), and changes in the airway architecture sometimes termed airway remodeling (9). Airway remodeling plays a pivotal role in increasing the severity and irreversible airflow limitation in asthma and leads to the refractoriness of this disease in spite of treatment with high doses of corticosteroids (1a, 8). The parenchymal cells of the airway, including epithelial cells, airway smooth muscles, endothelial cells, and fibroblasts are responsible for the maintenance of the airway structure and are involved in the progression of airway remodeling (27). In airway remodeling, subepithelial fibrosis is one of the pathological features and is characterized by excessive deposition of ECM protein including collagens I, III, and V, fibronectin, and tenascin in the lamina reticularis of the basement membrane (12). In an in vitro model, epithelial injury reportedly differentiated lung fibroblasts into myofibroblasts, which leads to excessive ECM production (24). In a chronic allergen-challenged murine model, the differentiation of fibroblasts to myofibroblasts in the airway was observed (33). In addition, there were more myofibroblasts within the airway walls of asthmatic patients (10, 30), especially refractory asthmatic patients, compared with healthy subjects in pathological examinations suggesting that the differentiation of fibroblasts to myofibroblasts is a key process of airway remodeling in asthma (14).

Excessive production of nitric oxide (NO) during inflammatory and immune processes leads to the formation of reactive nitrogen species (RNS) including peroxynitrite and nitrogen dioxide (4). These RNS are formed from NO and superoxide anion or via the hydrogen peroxide/peroxidase-dependent nitrite oxidation pathway (35). Especially peroxynitrite is an extremely powerful oxidant and is presumed to be largely responsible for many of the adverse effects of excessive NO generation. Excessive RNS production has been reported to cause tissue injury, lipid peroxidation, and nitration of tyrosine residues (35). Furthermore, peroxynitrite caused airway inflammation (31) and AHR (28) in asthmatic animal models. In fact, excessive production of 3-nitrotyrosine, which is a footprint of RNS production, has been observed in the airways of asthmatic patients (15, 29). These data suggest that increased RNS production can be an important contributor to the pathophysiology of asthma.

Transforming growth factor-β_1 (TGF-β_1) is a key mediator in a variety of pathological processes, including fibroblast-mediated repair responses. TGF-β_1 is believed to be a major regulator of tissue remodeling (5) and is reportedly overexpressed in asthmatic airways (23, 36). It has been reported that the expression of TGF-β_1 is modulated by transcriptional factor complexes including NF-κB (7, 18, 19) and activator protein-1 (AP-1) (11, 19). These pathways potentially play a central role in the progression of airway remodeling in asthmatic airways.

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The effect of RNS on airway remodeling process has not been fully understood. It has been reported that RNS augment fibroblast-mediated collagen gel contraction and fibroblast chemotaxis toward fibronectin (34), suggesting that RNS can affect the tissue repair process. However, the contribution of RNS to myofibroblast differentiation, which plays a pivotal role in airway remodeling observed in asthma, has not been elucidated yet. In addition, the molecular mechanism involved in the RNS-mediated tissue remodeling process also remains unclear.

The present study was therefore designed first to determine whether RNS could affect the differentiation of lung fibroblasts to myofibroblasts. Next, we assessed the effect of peroxynitrite on the release of TGF-β_1 and ECM proteins including fibronectin and collagen I in lung fibroblasts. Finally, we investigated the mechanism of differentiation of lung fibroblasts to myofibroblasts by peroxynitrite.

MATERIALS AND METHODS

Materials. Commercially available reagents were obtained as follows: anti-TGF-β_1 antibody (clone: 9016.2), TGF-β_1, biotinylated anti-TGF-β_1, neutralizing anti-TGF-β antibody, and anti-IgG were from R&D Systems (Minneapolis, MN); peroxynitrite was from

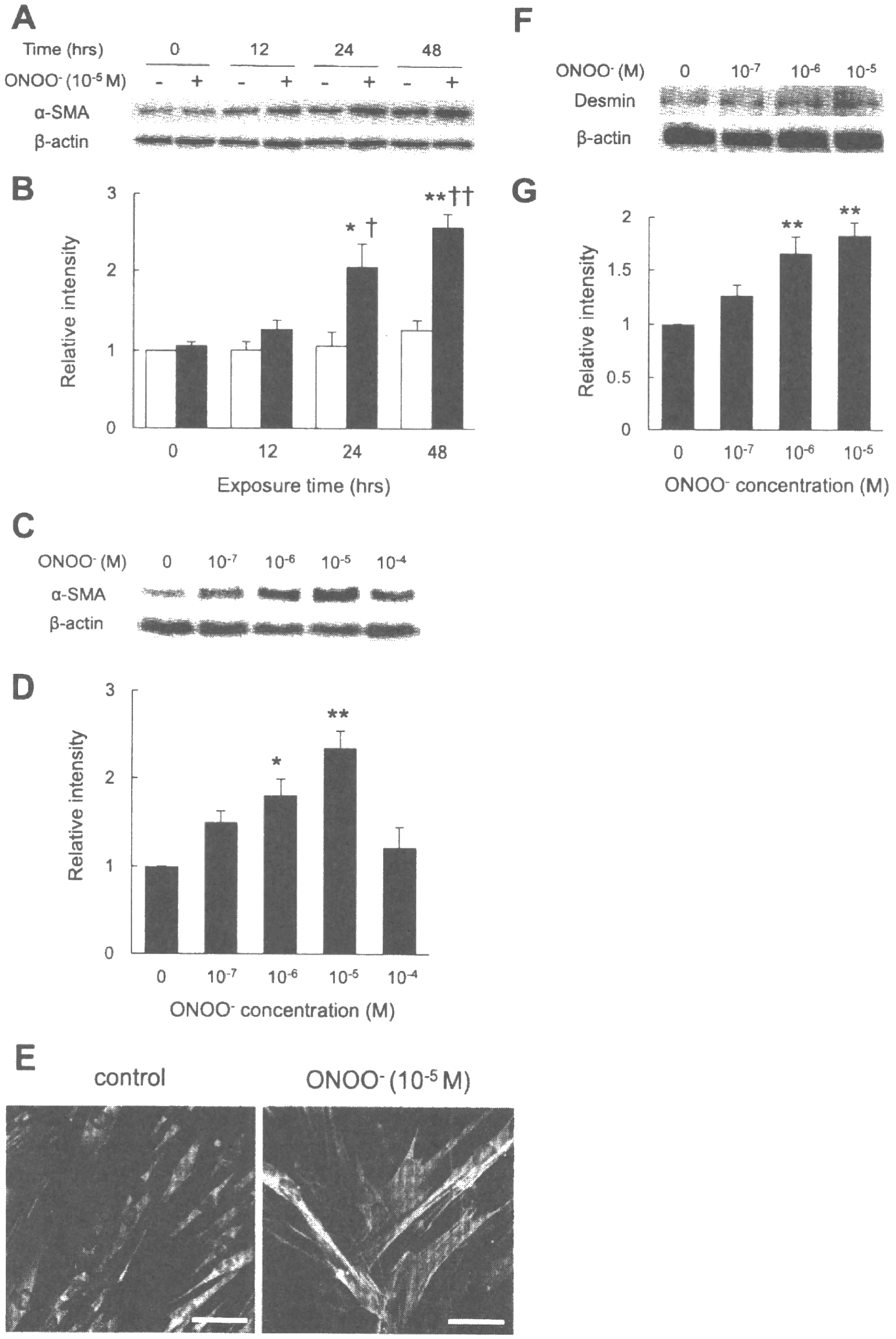

Fig. 1. Effect of authentic peroxynitrite ($ONOO^-$) on α-smooth muscle actin (α-SMA) and desmin expression in human fetal lung fibroblasts (HFL-1). Cultured cells were treated with 10^{-5} M authentic peroxynitrite (filled bars) or vehicle (open bars) and harvested at various time points. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Cultured cells were treated with various concentrations of authentic peroxynitrite for 48 h. α-SMA expression was analyzed by Western blotting (*C*) and quantified by densitometry (*D*). Cells were treated with 10^{-5} M authentic peroxynitrite (*right*) or vehicle (*left*) for 48 h. α-SMA expression was determined by immunocytostaining. Bars = 50 μm (*E*). Cultured cells were treated with various concentrations of authentic peroxynitrite for 48 h. Desmin expression was analyzed by Western blotting (*F*) and quantified by densitometry (*G*). Each band intensity of α-SMA or desmin was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 4 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, compared with the values of vehicle-treated control.

Upstate Biotechnology (Temecula, CA); 3-morpholinosydnonimine hydrochloride (SIN-1), 3,3′,5,5′-tetramethylbenzidine (TMB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), monoclonal anti-human fibronectin antibody, polyclonal anti-human fibronectin antibody, anti-rabbit IgG antibody, and ebselen, a peroxynitrite scavenger, were from Sigma (St. Louis, MO); MG132, a proteasomal inhibitor, and caffeic acid phenethyl ester (CAPE), a specific NF-κB inhibitor (25), were from Calbiochem (La Jolla, CA); DMEM, FCS, and antibiotic-antimycotic were purchased from Invitrogen Life Technologies (Grand Island, NY).

Cell culture. Human fetal lung fibroblasts (HFL-1) were obtained from American Type Culture Collection (Rockville, MD). Normal human adult lung fibroblasts (NHLF) were obtained from Cambrex (Walkersville, MD). The cells were cultured on tissue culture dishes (Falcon; Becton-Dickinson, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 μg/ml penicillin, 250 μg/ml streptomycin, and 2.5 μg/ml fungizone. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. HFL-1 and NHLF cells were passaged every 4–5 days at a 1:4 ratio. HFL-1 cells were used between the 14th and 18th passages and NHLF cells were used between the 4th and 6th passages. To evaluate mediator production in the monolayer culture, cells were seeded in six-well tissue culture plates at a cell density of 1×10^5 per milliliter. At 90% confluence, cells were treated with various concentrations of peroxynitrite in serum-free DMEM (SF-DMEM). For the investigation of the effect of neutralizing anti-TGF-β antibody on peroxynitrite-modulated mediator release, neutralizing anti-TGF-β antibody (10 μg/ml) was also added to the media. The supernatants were harvested after 48-h treatment with peroxynitrite and stored at −80°C until later assay.

Determination of cell viability. For monitoring cell viability, peroxynitrite or vehicle-treated cells were incubated with MTT solution at a final concentration of 1 mg/ml for 4 h at 37°C. After incubation, DMSO was added into each well. The absorbance of each sample at 570 nm was determined by a spectrophotometer using a reference wavelength of 630 nm.

Western blotting. Cells were seeded in 60-mm dishes at a density of 1×10^5 per milliliter. At 90% confluence, cells were starved with SF-DMEM for 24 h. Then, cells were treated with various concentrations of authentic peroxynitrite or SIN-1 in the presence or absence of ebselen, MG132, CAPE, or anti-TGF-β neutralizing antibody for 48 h. Cells were washed with 4°C PBS and homogenized in cell lysis buffer (35 mM Tris·HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 1 μM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 1 μg/ml leupeptin). To obtain the nuclear fraction, a Nuclear Extraction Kit (Active Motif, Carlsbad, CA) was used according to the manufacturer's instructions. Samples were solubilized in SDS-PAGE sample buffer. Equal amounts of protein were loaded and separated by electrophoresis on 12.5% SDS-polyacrylamide gels. After electrophoresis, the separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The following antibodies were used for detection of the target protein: mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (1:5,000 dilution; Sigma), mouse monoclonal anti-desmin antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-β-actin antibody (1:10,000 dilution; Sigma), rabbit polyclonal anti-collagen I antibody (1:5,000 dilution; Rockland Immunochemicals, Gilbertsville, PA), mouse monoclonal anti-NF-κB p65 antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or mouse monoclonal anti-lamin A/C antibody (1:400 dilution; Santa Cruz Biotechnology). Bound antibodies were visualized using peroxidase-conjugated appropriate second antibodies and enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, United Kingdom) with a chemiluminescence imaging system (Luminocapture AE6955; Atto, Tokyo, Japan). For detection of NF-κB p65 and desmin, SuperSignal West Femto (Pierce, Rockford, IL), a higher sensitivity substrate, was used. Each band intensity was quantified by densitometry (ImageJ; National Institutes of Health, Frederick, MD).

Measurement of TGF-β₁ and fibronectin. TGF-β_1 and fibronectin in the media of the monolayer culture were determined by ELISA (34). Quantification of TGF-β_1 was performed as follows: plates were coated with monoclonal anti-TGF-β_1 antibody at 4°C overnight. After being washed three times (5 min each), standards and samples were added and incubated at room temperature for 2 h. To measure TGF-β_1, all samples were assayed both with and without acidification and neutralization to convert the latent form of TGF-β_1 to the active form. To accomplish this, a 500-μl sample was mixed with 100 μl of 1 N HCl and, after 10 min at room temperature, neutralized with 100 μl of 1.2 N NaOH/0.5 M HEPES. Bound antigen was detected after adding biotinylated anti-TGF-β_1 antibody for 1 h at room temperature. Horseradish peroxidase (HRP)-streptavidin (1:20,000 dilution) was added for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H_2SO_4, and the product was quantified at 450 nm with a microreader. Fibronectin was assayed with an ELISA that specifically detects human but not bovine fibronectin (34). Plates were coated with monoclonal anti-fibronectin antibody at 4°C overnight. After being washed three times, standards and samples were added and incubated at room temperature for 2 h. Bound antigen was detected after adding polyclonal anti-human fibronectin antibody (1:2,000 dilution) at room temperature for 1 h. HRP-conjugated anti-rabbit IgG antibody (1:10,000 dilution) was added at room temperature for 1 h. Bound HRP was detected with TMB. The reaction was stopped with 1 M H_2SO_4, and the product was quantified at 450 nm with a microreader.

Immunochemical localization of α-SMA and NF-κB p65. HFL-1 cells were seeded in an 8-well chamber slide at a density of 1×10^5 per milliliter and cultured for 24 h, and then the medium was replaced with SF-DMEM for 24 h. The cells were incubated with 10^{-5} M peroxynitrite for various time points for NF-κB assessment and for 48 h for α-SMA assessment. After washing, cells were fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were then permeabilized with 0.1% Triton

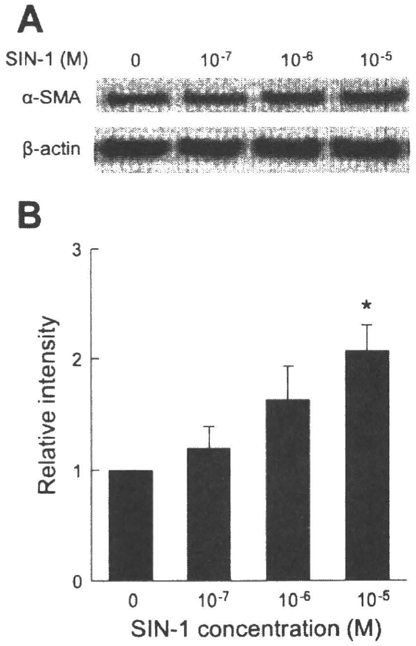

Fig. 2. Effect of the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1) on α-SMA expression in HFL-1. Cultured cells were treated with various concentrations of SIN-1 for 48 h and harvested. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. All values are expressed as means ± SE for 4 separate experiments. $^*P < 0.05$, compared with the values of control.

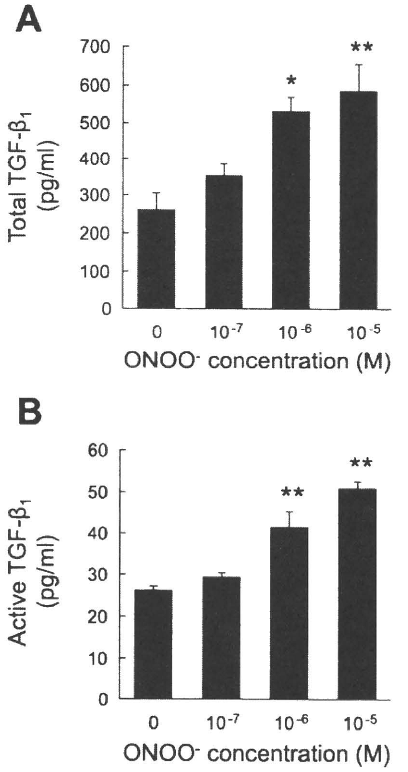

Fig. 3. Effect of peroxynitrite on transforming growth factor-β1 (TGF-β1) release by HFL-1. Cultured cells were treated with various concentrations of peroxynitrite. After 48 h, media were harvested and assayed for total or active TGF-β1 by ELISA. All values are expressed as means ± SE for 4 (*A*; total TGF-β1) or 3 (*B*; active TGF-β1) separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control.

X-100 in PBS for 10 min at room temperature and blocked with 1% skim milk in PBS for 1 h at room temperature and rinsed with PBS. Then, they were incubated with mouse monoclonal anti-α-SMA antibody (1:400 dilution; Sigma) or mouse monoclonal anti-NF-κB p65 antibody (1:100 dilution; Santa Cruz Biotechnology) in 1% skim milk at 4°C overnight. After washing, the cells were incubated with FITC-conjugated anti-mouse IgG antibody (1:1,000 dilution; Sigma) in 1% skim milk for 60 min at room temperature and then viewed with an epifluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) and photographed with a digital camera (DMX1200C; Nikon) under ×400 magnification.

Statistical analysis. Data were expressed as means ± SE. Experiments with multiple comparisons were evaluated by one-way analysis of variance followed by Scheffé test to adjust for multiple comparisons. An unpaired two-tailed Student's *t*-test was used for single comparisons. Probability values of <0.05 were considered significant.

RESULTS

To determine whether peroxynitrite induces the differentiation of fibroblasts to myofibroblasts, we assessed the expression of α-SMA, most commonly used as a molecular marker of myofibroblasts, in HFL-1 cells by Western blotting treated with various concentrations of peroxynitrite. Authentic peroxynitrite significantly augmented α-SMA expression compared with the control in a time-dependent manner (at 48 h, 2.6-fold increase; $P < 0.01$; Fig. 1, *A* and *B*). Peroxynitrite also significantly augmented α-SMA expression at 48 h in a concentration-dependent manner (at 10^{-5} M, 2.4-fold increase; $P < 0.01$; Fig. 1, *C* and *D*). Furthermore, peroxynitrite induced morphological changes in HFL-1 cells. More stress fibers, estimated for α-SMA immunostaining, were observed in the peroxynitrite-treated cells (Fig. 1*E*). We also confirmed the peroxynitrite-mediated myofibroblast differentiation by assessing the expression of desmin, which is reportedly another marker of the differentiation (37). The expression of desmin was significantly upregulated by peroxynitrite in a concentration-dependent manner (at 10^{-5} M, 1.8-fold increase: $P < 0.01$; Fig. 1, *F* and *G*). The peroxynitrite donor SIN-1 also significantly augmented α-SMA expression in a concentration-dependent manner (at 10^{-5} M, 2.1-fold increase; $P < 0.05$; Fig. 2).

To confirm whether the increased α-SMA expression was directly mediated by peroxynitrite, we assessed the effect of ebselen, a peroxynitrite scavenger, on the peroxynitrite-augmented α-SMA expression. Ebselen completely inhibited the augmentation (at 5 μM; $P < 0.01$; Supplemental Fig. 1, *A* and *B*, available in the data supplement online at the *AJP-Lung Cellular and Molecular Physiology* web site). Furthermore, we investigated the effect of peroxynitrite on cell viability and

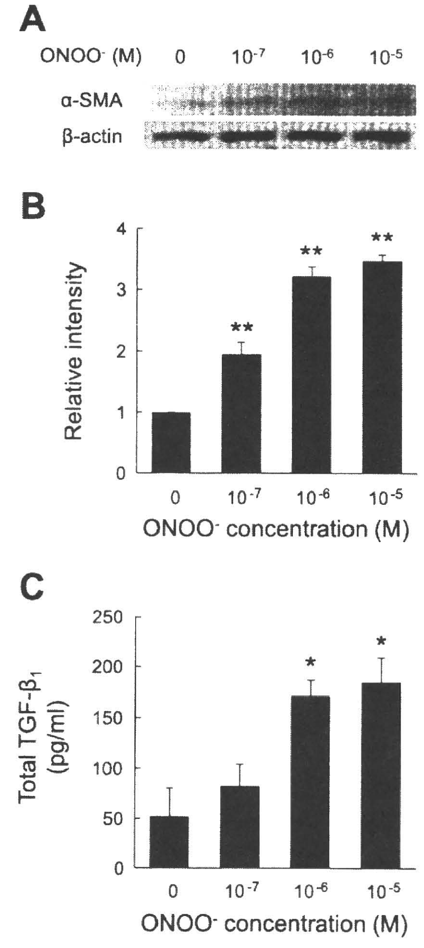

Fig. 4. Effect of peroxynitrite on α-SMA expression and TGF-β1 release in normal human adult lung fibroblasts (NHLF). NHLF cells were treated with various concentrations of peroxynitrite for 48 h. Cells and media were harvested. α-SMA expression was analyzed by Western blotting (*A*) and quantified by densitometry (*B*). Each α-SMA band intensity was normalized with the corresponding β-actin band intensity. Media were assayed for total TGF-β1 by ELISA (*C*). All values are expressed as means ± SE for 3 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with the values of control.

proliferation because high doses of peroxynitrite have been reported to have cytotoxic effect. A concentration of 10^{-5} M or less peroxynitrite has no cytotoxic effects, and cell proliferation was not affected by 10^{-5} M peroxynitrite (Supplemental Fig. 1, *C* and *D*).

To determine whether peroxynitrite augments TGF-β_1 release by HFL-1 cells, we measured the TGF-β_1 concentration in the media. Both total and active TGF-β_1 amounts were accumulated in the media in a time-dependent manner (data not shown). Peroxynitrite significantly increased total TGF-β_1 (at 10^{-5} M, 587 ± 69 vs. 251 ± 44 pg/ml; $P < 0.01$; Fig. 3*A*) and active TGF-β_1 release(at 10^{-5} M, 51.0 ± 1.7 vs. 26.3 ± 0.9 pg/ml; $P < 0.01$; Fig. 3*B*) from HFL-1 cells in a concentration-dependent manner.

We assessed the effect of peroxynitrite on α-SMA expression and TGF-β_1 release in NHLF. Peroxynitrite significantly augmented α-SMA expression (at 10^{-5} M, 3.5-fold increase; $P < 0.01$; Fig. 4, *A* and *B*) and total TGF-β_1 release (at 10^{-5} M, 184 ± 25 vs. 52 ± 28 pg/ml; $P < 0.05$; Fig. 4*C*).

To clarify how peroxynitrite augments TGF-β_1 release by HFL-1 cells, we assessed the translocation of NF-κB into the nucleus, which is thought to regulate TGF-β_1 expression. Translocation of NF-κB p65 into the nucleus was assessed by immunocytostaining and Western blotting. After treatment

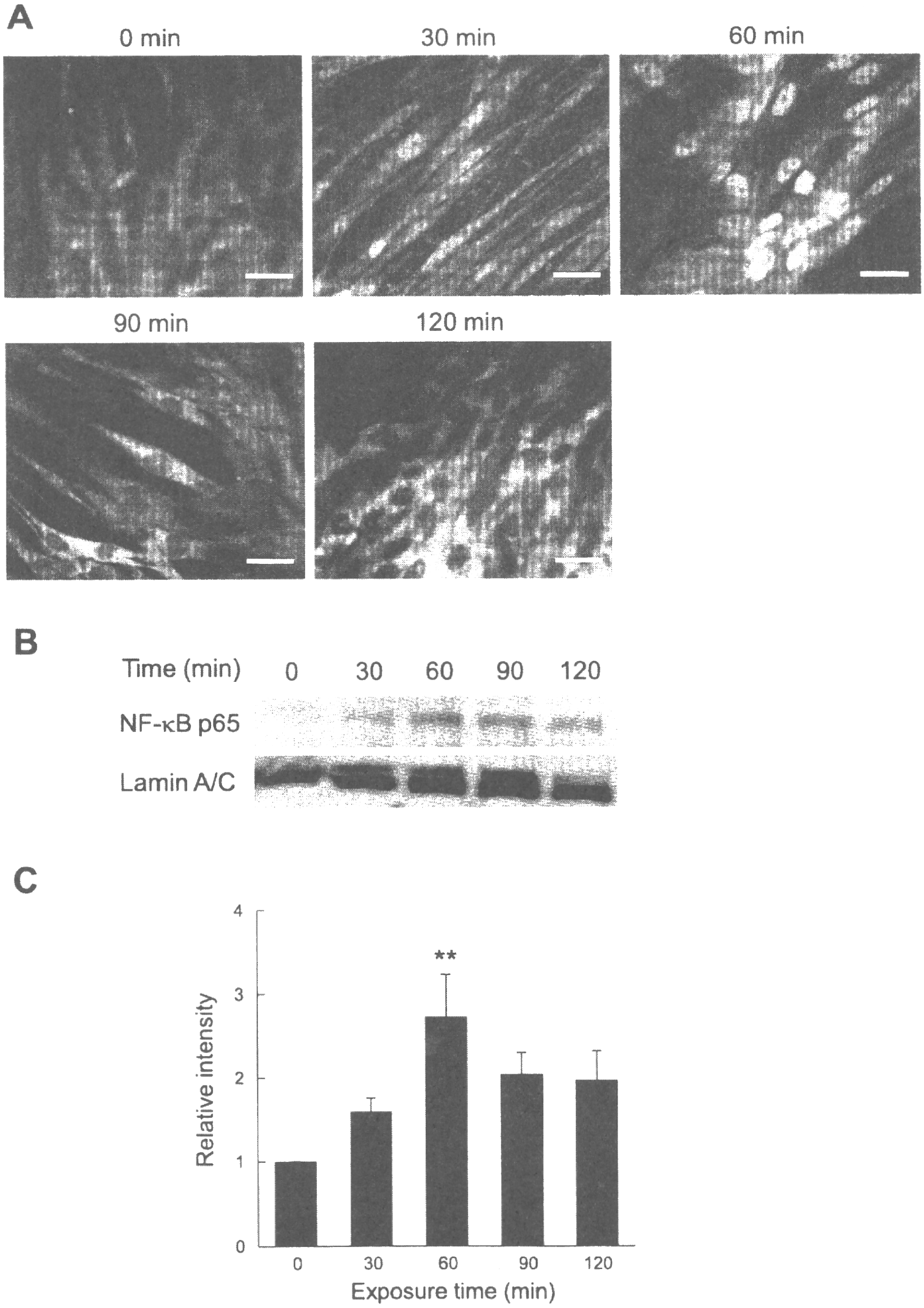

Fig. 5. Effect of peroxynitrite on translocation of NF-κB p65 into nucleus in HFL-1. HFL-1 cells were treated with 10^{-5} M peroxynitrite for 0, 30, 60, 90, and 120 min, and the intracellular localization of NF-κB p65 was determined by immunocytostaining (*A*). The amount of NF-κB p65 in the nuclear fraction was analyzed by Western blotting (*B*) and quantified by densitometry (*C*). Each NF-κB p65 band intensity was normalized with the corresponding lamin A/C band intensity. All values are expressed as means ± SE for 4 separate experiments. $^{**}P < 0.01$, compared with the values of control. Bars = 50 μm.