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Dual Oxidase 1 and 2 Expression in Airway Epithelium of Smokers and Patients with Mild/Moderate Chronic Obstructive Pulmonary Disease

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ABSTRACT

Dual oxidase (Duox) 1 and Duox2 are important sources of hydrogen peroxide production and play a role in host defense in airways. Little is known about their regulation in association with smoking or chronic obstructive pulmonary disease (COPD). We investigated the epithelial expression of Duox1 and Duox2 in the airways of smokers, and the relationship between this expression and COPD at early stage. First, using bronchoscopy, we harvested tracheal and bronchial epithelium from individuals who have never smoked and current smokers. Duox1 expression in brushed tracheal and bronchial epithelium was significantly downregulated, whereas Duox2 was upregulated, in current smokers as compared to individuals who have never smoked. Second, laser capture microdissection and microscope-assisted manual dissection were performed in surgically resected lung tissues to collect bronchiolar epithelium and alveolar septa. Subjects with mild/moderate COPD, who were all former smokers, exhibited downregulation of bronchiolar Duox1 and Duox2 when compared to individuals who have never smoked, whereas a difference between former smokers, with and without COPD, was observed only for Duox1. Alveolar Duox1 and Duox2 expression was low and did not differ among the groups. These results imply that the airway expression of Duox1 and Duox2 is diversely associated with smoking and COPD. *Antioxid Redox Signal* 10, 705–714.

INTRODUCTION

Partially reduced metabolites of molecular oxygen, superoxide and hydrogen peroxide (H_2O_2), are detected in respiratory tract lining fluid, and it is assumed that these are key components of innate immunity (43). Phagocytic cells produce by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, which contributes to the killing of invading microorganisms. Dual oxidase (Duox), a newly identified NADPH oxidase homolog, is expressed in the barrier epithelia, including epithelial surfaces of colon, rectum, salivary gland ducts, and bronchi (9, 11, 15). Adult flies in which *Drosophila* Duox expression is silenced have a markedly increased mortality rate after a minor infection caused by the ingestion of microbe-contaminated food; this finding suggests that Duox plays an indispensable role in antimicrobial activities (16). Duox contains two well-characterized domains, an NADPH oxidase domain and a heme peroxidase domain (7). The two isoforms of Duox, Duox1 and Duox2, share >83% sequence homology at the amino acid level (4). Duox1 is constitutively expressed in ciliated epithelium along the respiratory tract (11, 15, 33), although Duox2 expression is inducible in response to exogenous stimuli (17). Both Duox1 and Duox2 expressions are calcium dependent (6, 8). Duox1 mRNA expression is modestly upregulated in response to Th2-specific cytokines interleukin (IL)-4 and IL-13, whereas Duox2 mRNA expression is markedly induced by the Th1-specific cytokine interferon-γ (IFN-γ) (17, 18). However, many questions remain

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regarding the regulation and function of each Duox isoform in human airway epithelium.

ROS has been suggested to play a role in smoking-induced COPD (31). ROS could be exogenously driven by air pollutants or cigarette smoke, or endogenously produced through metabolic reactions (32). The sources of ROS in patients with COPD have been thought to be not only cigarette smoke itself, but also increased inflammatory cells such as leukocytes and macrophages (25) in the lungs, where NADPH oxidase is one of the key enzymes to generate ROS. We speculate that newly identified epithelial Duox may contribute to the generation of endogenous ROS in airway epithelial cells. Cigarette smoke exposure elicits a variety of stimuli on airway epithelium. COPD associated with chronic cigarette smoking eventually involves all levels of the airways, although the earliest smoking-induced changes occur in the small airway epithelium (19, 37). So far, it remains to be elucidated whether Duox1 and Duox2 are differently regulated by chronic smoking *in vivo* and whether the changes in their gene expression are associated with COPD. Therefore, in the present study we compared the mRNA expression of Duox1 and Duox2 in tracheal and bronchial epithelial cells, which were harvested by bronchoscopic brushing, from individuals who have never smoked and age-matched, healthy, current smokers. Second, to study the roles of Duox1 and Duox2 in the pathophysiology of COPD, the Duox1 and Duox2 mRNA expression levels were examined in bronchiolar epithelial cells and alveolar septa from individuals who have never smoked and former smokers with and without COPD.

MATERIALS AND METHODS

Subjects

We recruited two sets of subjects: one set for the bronchoscopic study and the other set for the surgical tissue study. For the bronchoscopic study, tracheal and bronchial epithelial cells were harvested by brushing, and epithelial lining fluid was collected by microsampling (20, 45). The bronchoscopic study had 11 subjects, consisting of 6 individuals who have never smoked and 5 healthy current smokers. All healthy current smokers abstained from smoking for 12 h prior to bronchoscopy in order to maintain a consistent amount of time from the last exposure to cigarette smoke.

Thirty patients who had lung resection for small peripheral tumors were recruited for the surgical tissue study. Ten were individuals who have never smoked, 10 were former smokers without COPD (smoking cessation; 7 days to 27 years), and 10 were former smokers with COPD (smoking cessation; 5 days to 9 years). The subjects with COPD were classified according to the guidelines of the Global Initiative for Obstructive Lung Disease (GOLD) (10) into stage I ($n = 8$) and stage II ($n = 2$). Eight subjects had emphysema on computed tomography scans, including the two subjects with stage II COPD. Two subjects with stage I COPD had no emphysema on computed tomography scans. Some patients were subjects in our previous study (14).

For both studies, pulmonary function tests were performed as previously described (26). None of the subjects had a history of asthma or other allergic disorders; also, none of the subjects had a chronic productive cough. All subjects gave written informed consent, and the study was approved by the Ethics Committee of Hokkaido University School of Medicine, Sapporo, Japan.

Collection of tracheal and bronchial epithelial cells by bronchoscopic brushing

Bronchoscopic brushing was conducted through a flexible fiberoptic bronchoscope (Model BF-1T200; Olympus, Tokyo, Japan) to collect tracheal and bronchial epithelial cells around carina and at the fourth or fifth branches of subsegmental lower lobe bronchus, respectively. The cells were immediately collected by vortexing the brush in RPMI 1640 medium (GIBCO, Grand Island, NY). The cells were centrifuged for 5 min at 1,000 rpm, and the red blood cells were removed with red blood cell lysing buffer (Sigma, St. Louis, MO). The recovered cells were washed twice in Hanks' balanced salt solution without calcium and magnesium (Gibco). The cytospin preparations were stained by Diff-Quik stain (Kokusai Shiyaku, Kobe, Japan). Differential counts were performed by examining >300 cells, using a standard light microscope as previously described (2). For detection of keratin in the cells, the specimens were stained with antikeratin antibody (KL-1; Immunotech, Marseille, Cedex, France). The cytospin preparations were also stained by periodic acid-Schiff (PAS) stain for the detection of secretory granules. Cells were adjusted to 1×10^6/ml and used for preparation of total RNA.

Collection of epithelial lining fluid by bronchoscopic microsampling

During bronchoscopy, microsampling was performed as previously described (20), with slight modification prior to the brushing procedure. Using a special cotton probe (model BC-402C, Olympus), sampling of epithelial lining fluid was repeated three times at sites adjacent to the location of brushing. The absorbed epithelial lining fluid (using three microsampling probes per given site) was extracted into 3 ml of saline and the mean weight of lining fluid per probe was calculated as previously described (20).

Quantification of IFN-γ in epithelial lining fluid

IFN-γ was quantified using a human IFN-γ enzyme-linked immunoassay kit II (BD Biosciences, San Diego, CA) according to the supplied protocol. A standard curve was obtained by log–log using serial dilutions of the supplied recombinant IFN-γ (minimal detection limit 1 pg/ml). The concentrations of IFN-γ were converted using the respective dilution factors and were expressed as those in epithelial lining fluid, as previously described (20).

Sampling of bronchiolar epithelial cells, alveolar septa for lung specimen

Six or more blocks of peripheral lung tissue were collected in areas remote enough from the tumors and were frozen. Laser capture microdissection (LCM) of bronchiolar epithelial cells was performed by using a PixCell II System (Arcturus Engineering, Mountain View, CA) (Figs. 1a, b, and c), as described

previously (14). At least six bronchioles were randomly selected, and a total of 40,000 laser bursts were used to collect cells from each subject. Alveolar septa were identified, and adjacent unwanted tissues were removed using an 18-gauge fine sterile needle; the remaining tissue was harvested from hematoxylin-stained lung sections (Figs. 1d, e, and f).

RNA purification and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

We used the epithelial cell samples collected by bronchial brushing for RT-PCR only when the samples contained <5%

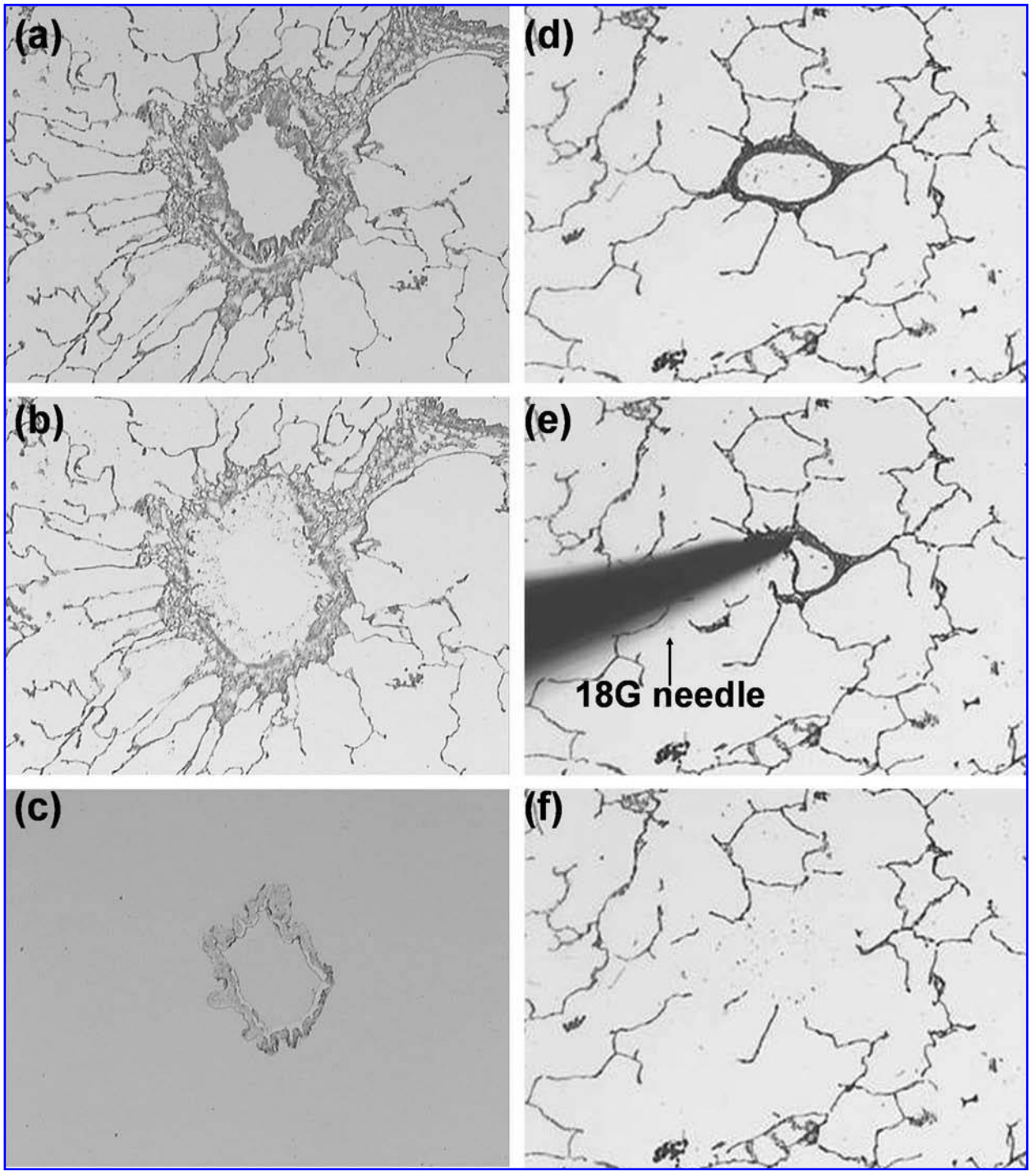

FIG. 1. Sampling of bronchiolar epithelial cells, alveolar septa for lung specimen. Lung tissue section (**a**) before and (**b**) after laser capture microdissection of bronchiolar epithelial cells, and (**c**) captured bronchiolar epithelium cells on a transfer film. Lung tissue sections (**d**) before dissection, **(e)** manual dissection of unwanted tissue, and (**f**) remained alveolar tissue.

nonepithelial cells, as determined by Diff-Quik staining and keratin staining. Total RNA was extracted by using an RNeasy mini kit (Qiagen, Hilden, Germany) with guanidium thiocyanate. The quantity and quality of the RNA were determined by using a LabChip kit (Agilent Technologies, Palo Alto, CA). Reverse transcription was performed in the presence of Maloney leukemia virus reverse transcriptase (Epicentre Technologies, Madison, WI), and 5′ exonuclease-based fluorogenic PCR was performed with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA), as described previously (1). TaqMan Gene Expression Assay probes (Applied Biosystems) were used for the human Duox1 (Hs00213694_m1, GenBank accession number NM 000619) and Duox2 gene sequence (Hs00204187_m1, GenBank accession number NM 014080), and the levels were normalized against glyceraldehyde-3-phosphatase-dehydrogenase (GAPDH) mRNA.

Data presentation and statistical analysis

Demographic data were expressed as means ± standard error (SE). Data were analyzed by using an unpaired *t* test for the bronchoscopic study. For the surgical tissue study, a single factor analysis of variance followed by Fisher's protected least significant difference test as a *post hoc* test. The other data were expressed as median and range, considering that the values in each group were widely ranged and not evenly distributed. Differences between two groups were analyzed by using the Mann–Whitney U test, and more than two groups were compared by using the Kruskal–Wallis test, followed by the Mann–Whitney U test. All tests were performed using the StatView J 5.0 System (SAS Institute Inc., Cary, NC). P values <0.05 were considered statistically significant.

RESULTS

Subjects

Eleven subjects underwent bronchoscopy for epithelial brushing and microsampling. There was no difference in age between the individuals who have never smoked and the current smokers (68 ± 3 *vs.* 61 ± 2 years, mean ± SE). The number of pack-years of smoking for the current smokers was 48 ± 9 (mean ± SE). All subjects had normal pulmonary function tests. There was no difference between the individuals who have never smoked and current smokers in the percent predicted value of the forced expiratory volume in one second (FEV_1;122 ± 10% *vs.* 112 ± 11%) and in FEV_1/forced vital capacity (79 ± 4% *vs*. 78 ± 2%).

The clinical characteristics of the subjects enrolled and the pathological diagnosis of the resected tumors in the surgical tissue study are summarized in Table 1. Both groups of former smokers (with and without COPD) had a similar number of pack-years of smoking; these two groups had various durations of smoking and cessation.

Differential cell counts for brushed epithelial cells

The percentage of epithelial cells was no <95% for all specimens. The total cell number, viability, and cell differential counts were not statistically different at either site between individuals who have never smoked and current smokers (Table 2). Cell viability did not differ between the two groups either at the trachea (43 ± 4% *vs.* 31 ± 7%, NS) or at the subsegmental bronchus (31 ± 3% *vs*. 23 ± 2%, NS). Very few PAS-positive epithelial cells were observed at either site in individuals who have never smoked and current smokers.

Duox1 and Duox2 mRNA expression are differently regulated in large airways of current smokers

To investigate the effects of current smoking on the transcriptional regulation of Duox1 and Duox2 in airway epithelial cells *in vivo*, we examined these mRNA expression levels in epithelial cells harvested by bronchoscopic brushing. We compared these levels between the six individuals who have never smoked and the five healthy, current smokers. Duox1 expression was significantly downregulated in the current smokers, as

Table 1. Clinical Characteristics of Subjects in the Surgical Tissue Study

	Individuals who have never smoked	*Former smokers without COPD*	*Former smokers with COPD*
n (M/F)	10 (2/8)	10 (7/3)	10 (8/2)
Age (yr)	62 ± 5	63 ± 6	70 ± 2
Pack-years	0	54 ± 15	68 ± 11
FEV_1/FVC (%)	81 ± 2	81 ± 2	62 ± 2*†
FEV_1 (% predicted value)	114 ± 6	115 ± 7	95 ± 6*†
Pathological diagnosis of resected tumor	8 adenocarcinoma 1 carcinoma of cystic dust 1 osteosarcoma	6 adenocarcinoma 2 squamous cell carcinoma 2 small cell carcinoma	6 adenocarcinoma 3 squamous cell carcinoma 1 large cell carcinoma

*$p < 0.05$ *vs.* individuals who have never smoked; †$p < 0.05$ *vs.* former smokers without COPD; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second.

Data are presented as mean ± SE.

TABLE 2. DIFFERENTIAL COUNTS OF BRUSHED CELLS COLLECTED BY BRONCHOSCOPY

	Never smoked	*Current smokers*
At trachea		
Total number, $\times 10^6$	2.3 ± 0.5	1.2 ± 0.4
Epithelial cells, %	97.3 ± 0.6	98.1 ± 0.7
Neutrophils, %	0.3 ± 0.2	0.3 ± 0.2
Lymphocytes, %	1.1 ± 0.4	0.5 ± 0.3
Macrophages, %	1.3 ± 0.3	1.1 ± 0.5
Eosinophils, %	0.0 ± 0.0	0.0 ± 0.0
At subsegmental bronchus		
Total number, $\times 10^6$	4.0 ± 1.3	4.0 ± 0.5
Epithelial cells, %	97.1 ± 0.8	97.0 ± 0.7
Neutrophils, %	1.3 ± 0.8	0.8 ± 0.3
Lymphocytes, %	0.6 ± 0.1	0.8 ± 0.5
Macrophages, %	1.0 ± 0.4	1.3 ± 0.4
Eosinophils, %	0.1 ± 0.1	0.1 ± 0.1

Data are presented as mean ± SE.

compared to the individuals who have never smoked, both at the trachea (median, range: 4.6, 2.5–5.4 *vs*. 7.2, 4.3–9.8, $p = 0.0285$) (Fig. 2a) and at the subsegmental bronchus (5.7, 3.1–6.3 *vs*. 10.0, 5.7–19.7, $p = 0.0285$) (Fig. 2b). In contrast, Duox2 was significantly upregulated in current smokers, as compared to the individuals who have never smoked, both at the trachea (12.1, 7.1–31.5 *vs*. 3.7, 2.8–6.1, $p = 0.0062$) (Fig. 3a) and at the subsegmental bronchus (8.6, 4.7–25.6 *vs*. 4.0, 3.5–6.6, $p = 0.0446$) (Fig. 3b). These results imply that chronic smoking diversely affects the epithelial expression of Duox1 and Duox2 at large airways.

Upregulation of epithelial Duox2 mRNA is not accompanied by increased IFN-γ in epithelial lining fluid in large airways

Since a Th1-specific cytokine, IFN-γ, is the only molecule known to dramatically induce Duox2 transcription *in vitro* (18), we hypothesized that IFN-γ might be increased in the lining fluid which overlays airway epithelial cells, leading to Duox2-specific upregulation in those cells in current smokers. However, there was no significant difference in the levels of IFN-γ in the epithelial lining fluid from individuals who have never smoked and current smokers either at the trachea (471 pg/ml trachea lining fluid; range: 0–2088 *vs*. 0; 0–1243, NS) (Fig. 4a) or at subsegmental bronchus (512 pg/ml bronchial lining fluid; range: 0–1118 *vs*. 0; 0–778, NS) (Fig. 4b). Furthermore, there was no positive correlation between epithelial Duox2 expression and IFN-γ levels in epithelial lining fluid at either site (data not shown), suggesting no direct link between airway Duox2 expression and IFN-γ in airways.

Expression of Duox1 and Duox2 mRNA is downregulated in bronchiolar epithelium in COPD

To determine if altered expression of Duox in bronchiolar epithelium might be linked to smoking histories and/or COPD, we compared the expression of Duox1 and Duox2 among the three groups: 10 individuals who have never smoked, 10 former smokers without COPD, and 10 former smokers with mild or moderate COPD. Duox1 mRNA levels in the bronchiolar epithelium were significantly decreased in former smokers with mild or moderate COPD as compared to individuals who have never smoked and former smokers without COPD (2.0, 1.0–3.2 *vs*. 4.0, 2.0–7.8 and 2.9, 1.6–6.3, $p = 0.0015$, $p = 0.0034$, respectively) (Fig. 5). Surprisingly, levels of Duox2 expression were also significantly decreased in the groups of former smokers, both with and without COPD, compared to individuals who have never smoked (2.5, 0.9–3.6 and 2.8, 1.3–6.0, *vs*. 5.4, 2.6–10.3, $p = 0.0019$, $p = 0.0126$, respectively) (Fig. 6).

No change in mRNA expression of alveolar Duox1 and Duox2

Duox was also present in the alveolar septa, although to a lesser extent than in the bronchiolar epithelium. Using a microscope-assisted manual dissection technique, we harvested not only alveolar epithelial cells, but also the cells that com-

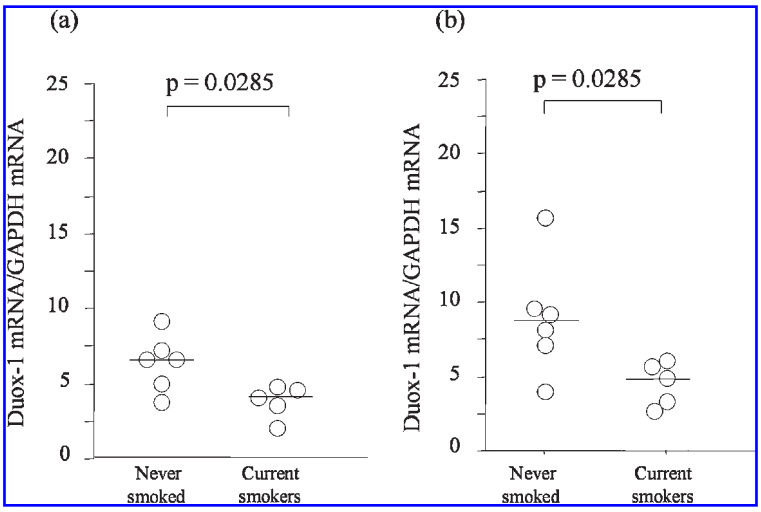

FIG. 2. Duox1 mRNA expression in tracheal and subsegmental bronchial epithelial cells. (**a**) Tracheal Duox1 mRNA; (**b**) Bronchial Duox1 mRNA. Duox1 expression at trachea and at subsegmental bronchus was significantly downregulated in current smokers as compared to individuals who have never smoked. Medians are indicated by *horizontal lines*.

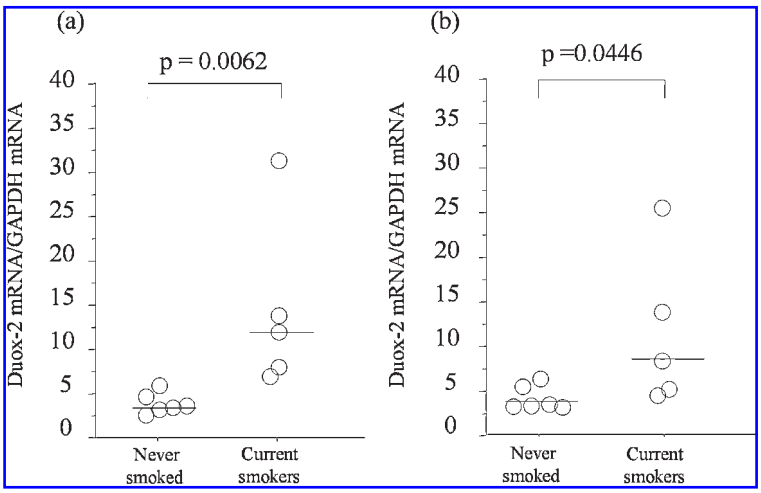

FIG. 3. Duox 2 mRNA expression in tracheal and subsegmental bronchial epithelial cells. (**a**) Tracheal Duox2 mRNA; (**b**) Bronchial Duox2 mRNA. Duox2 expression at the trachea and subsegmental bronchus was significantly upregulated in current smokers as compared to individuals who have never smoked. Medians are indicated by *horizontal lines*.

pose the alveolar septa, including capillary endothelial cells and inflammatory cells. It remains to be elucidated which cell types are the source of alveolar Duox. However, Duox1 and Duox2 mRNA expression levels in the alveolar septa did not significantly differ among the three groups (Figs. 7 and 8).

DISCUSSION

We report that Duox1 is decreased while Duox2 is increased in large airways of current smokers. On the other hand, both Duox1 and Duox2 are downregulated in bronchiolar epithelium, but not in alveolar septa, of the former smokers as compared to individuals who have never smoked, although Duox1 did not reach statistical significance. In subjects with mild or moderate COPD, their expressions appear to be further decreased compared with the smoking controls, although only Duox1 reaches a statistical difference. These preliminary data indicate that airway epithelial expressions of Duox1 and Duox2 might be diversely affected by chronic smoking and/or its cessation, and the development of early stage COPD.

The effects of cigarette smoking on airway epithelial cells over time may result from several processes that have different time frames. Duox1 mRNA levels in normal, human bronchial epithelial cells in culture are unchanged by 2 h exposure to cigarette-smoke condensates, indicating that acute exposure to cigarette smoke has no effect on the transcriptional levels of Duox1 *in vitro* (22); however, no study has been conducted to assess a possible link between Duox2 and cigarette smoke exposure. Affymetrix arrays were recently used for global gene expression analysis in bronchial brushings, revealing the downregulation of Duox1 and upregulation of Duox2 in current smokers compared to nonsmokers (29); this finding is consistent with our current findings on large airways. However, it remains unclear whether these altered Duox1 and Duox2 mRNA expressions in large airways of healthy current smokers are related to the development of COPD because large airways might not directly contribute to airflow obstruction. We have, therefore, extended our studies to analysis of these gene expressions in bronchiolar epithelium.

In former smokers with mild or moderate COPD, the bronchiolar expression of both Duox1 and Duox2 was significantly downregulated. Perhaps, a decrease in Duox1 is detected in re-

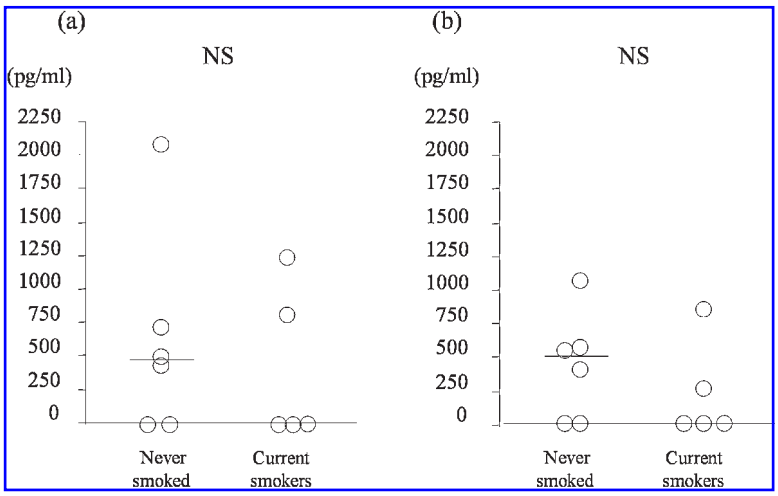

FIG. 4. Levels of IFN-γ in epithelial lining fluid. (**a**) IFN-γ in epithelial lining fluid at the trachea; (**b**) IFN-γ in epithelial lining fluid at bronchus. There was no significant difference in the levels of IFN-γ in epithelial lining fluid at the trachea or subsegmental bronchus of individuals who have never smoked and current smokers. Medians are indicated by *horizontal lines*.

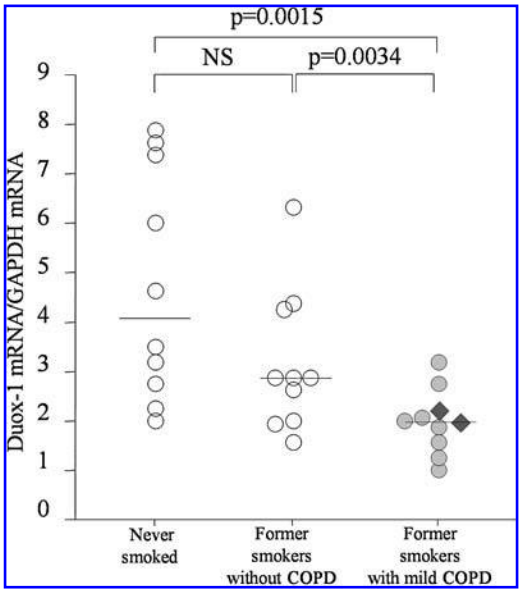

FIG. 5. Duox1 mRNA expression in bronchiolar epithelial cells. Duox1 mRNA levels in the bronchiolar epithelium were significantly decreased in former smokers with COPD as compared to individuals who have never smoked and former smokers without COPD. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

sponse to repeated smoke exposure; but, after smoking cessation, the levels go back to normal in subjects who are not susceptible to COPD. However, the Duox1 levels in subjects who stopped smoking but had already developed COPD, even an early stage of COPD, could not rebound properly and the decrease was amplified, suggesting that there is a link between COPD and decreased Duox1.

On the other hand, bronchiolar Duox2 mRNA was significantly decreased, even in former smokers without COPD, which is in sharp contrast to the increase of tracheal and bronchial Duox2 mRNA observed in current smokers. We cannot address the question in this study whether this discrepancy in the regulation of Duox2 might be due to regional differences along the airways, large airways *versus* small airways, or differences in the smoking status on examination, current smokers *versus* former smokers.

To get further insight into the mechanism of epithelial Duox2 mRNA upregulation in current smokers, we quantified the levels of IFN-γ in epithelial lining fluid. Although IFN-γ transgenic mice develop inflammation and emphysema (23, 41), cigarette smoke exposure reportedly suppresses IFN-γ (3, 28). In the present study, the levels of IFN-γ in tracheal and bronchial lining fluid did not differ between individual who have never smoked and current smokers. Also, the IFN-γ levels and epithelial Duox2 mRNA expression at given sites were uncorrelated. Therefore, a direct link between epithelial Duox2 and IFN-γ in epithelial lining fluid in large airways was not established in the present study.

Bacterial colonization in airway epithelium is a prominent feature of cystic fibrosis. When Wright *et al*. performed microarray analysis of nasal respiratory epithelium to investigate the molecular basis of variability in cystic fibrosis phenotype, they found that Duox2 is one of the genes most reduced in cystic fibrosis patients (44). Long-term cigarette smoking also appears to disrupt these innate immune mechanisms, and as a consequence, microbial pathogens are able to persist in the lower airways (38). Some of these mechanisms might be further amplified, but not in a linear fashion, in individuals who develop COPD (19, 24, 29, 34, 35). Sethi *et al*. demonstrated the relationship between bacterial colonization and increased levels of IL-8 in bronchoalveolar lavage fluid in COPD patients (35). We also reported that increased levels of IL-8 in bronchoalveolar lavage fluid were found in smokers with subclinical emphysema, but not in smokers without emphysema (39). Also, the expression of IL-8, macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 was upregulated in LCM-retrieved bronchiolar epithelium (14), in which both Duox1 and Duox2 were reversely downregulated in patients with early COPD. Wesley *et al*. recently demonstrated that Duox1-targeted small interfering RNA suppressed wound closure and epithelial cell migration, suggesting another role for Duox1 as an important component of airway epithelial repair in response to injury (42). Taken together, these studies indicate that decreased Duox expression and upregulation of inflammatory chemokines in bronchiolar epithelium may contribute to persistent inflammation, impaired wound repair, and progressive airway obstruction in COPD patients, and these problems persists even after cessation of smoking.

Although Duox1 and Duox2 likely serve specific functions (*e.g*., host defenses and signaling for cell migration), they also might damage or adversely affect airway epithelial cells by overproducing ROS as an endogenous oxidant. Shao *et al*.

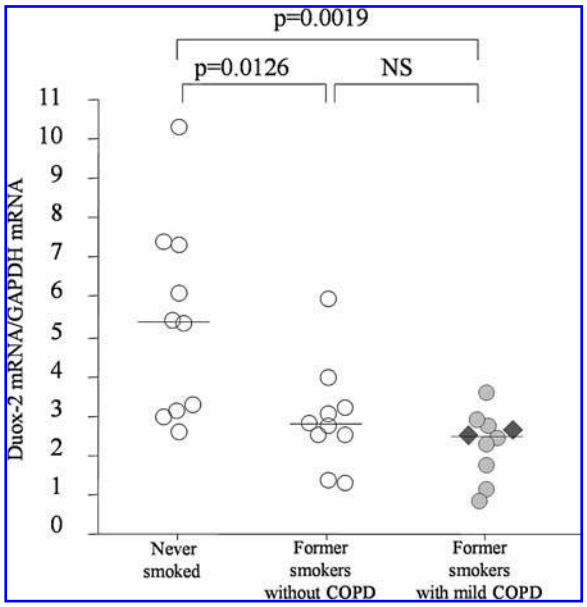

FIG. 6. Duox2 mRNA expression in bronchiolar epithelial cells. Levels of Duox2 expression were significantly decreased in smokers with and without COPD as compared to individuals who have never smoked. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

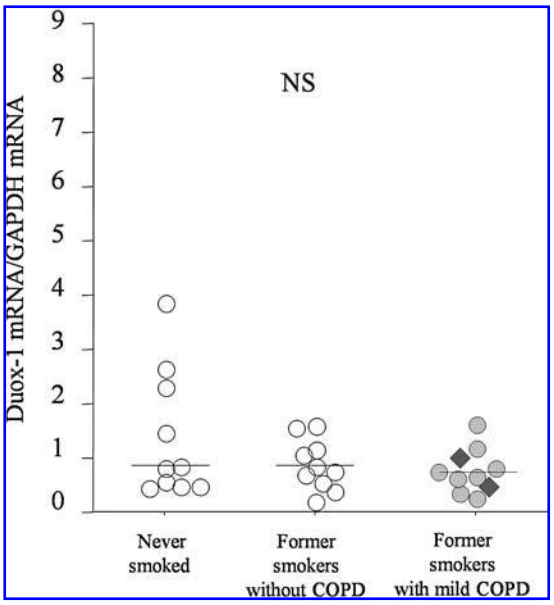

FIG. 7. Duox1 mRNA expression in alveolar septa. Duox 2 mRNA expression levels in the alveolar septa did not significantly differ among the three groups. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

showed that ROS produced by Duox1 in airway epithelial cells are involved in mucus hypersecretion (36). Knockdown of Duox1 by small interfering RNA reduced lipopolysaccharide-induced H_2O_2 generation and IL-8 production through a neutrophil elastase/tumor necrosis factor-α-converting enzyme/epidermal growth factor pathway (27). Duox is possibly involved in chronic airway inflammation with mucus hypersecretion, providing novel mediators of these disease processes. It should be noted, however, that individuals with productive coughs were not included in the present study. Moreover, very few PAS-positive cells were found in epithelial cells obtained by brushing, nor was goblet cell hyperplasia observed in surgical tissue specimens (data not shown). It is unlikely that upregulated Duox2 contributes to airway hypersecretion in healthy current smokers.

Recently, it has been reported that emphysema is developed either by overexpression or deficiency of NADPH in mice (21, 46), although the precise mechanism remains to be elucidated. Accordingly, alteration of Duox expression in airway epithelium might have some causative relationship with COPD.

Although we collected epithelial cells with >95% purity by bronchoscopic brushing, and the LCM specifically targeted bronchiolar epithelium (14), it should be noted that the cells harvested were likely a mixture of various epithelial cell types that were present at a given level. Epithelial heterogeneity in the airways could be important under certain circumstances, such as in long-term smokers. In the same context, the analyses of gene expression in alveolar septa also have limitations.

There are also some limitations with respect to the study population. The study of the effects of current smoking on Duox1 and Duox2 is limited only in large airways. We could not examine the effects of current smoking on the bronchiolar and alveolar expressions of Duox1 and Duox2 because surgical tissues were only available from former smokers, and there was a considerable variation in the length of smoking cessation among the former smokers. In the group of smokers without COPD, four subjects had stopped smoking for <1 month, two subjects had stopped smoking for many years. In the surgical tissue specimens, the bronchiolar gene expression might be influenced by the small tumor located within the same lobe. However, it could be negligible because the six or more tissue specimens were randomly sampled from tumor-free peripheral lung and no metastasis was found in the lungs of those patients. Another study limitation is that most of the COPD patients in the surgical tissue study had only mild or moderate COPD. To obtain a better understanding of the involvement of Duox in the progression of COPD, a larger sample of patients with more advanced disease should be assessed.

The present study demonstrates that current smoking diversely regulates the epithelial Duox1 and Duox2 expression only at the transcriptional levels. An attempt to correlate Duox1 and Duox2 gene expression with protein translation should be made, and the relative importance of these isoforms should be further investigated at their protein levels. However, to the best of our knowledge, Duox1- or Duox2-specific antibodies do not currently exist, as the Duox antibodies that are currently available recognize motifs on both Duox proteins (5). According to the previous reports using that antibody recognizing both Duox1 and Duox2 (11, 15), the diverse regulation of Duox1 and Duox2 should exclusively occur within epithelial cells.

Very few studies have examined the regulation of other oxidases by smoking. Higher traces of xanthine oxidase (XO) substrates were detected in BAL fluid from COPD patients compared to the controls, suggesting the increased activity of XO in COPD patients (30). On the other hand, the two subtypes of monoamine oxidase (MAO) A and MAO B were both downregulated by cigarette smoking (12, 13). Impaired induction of

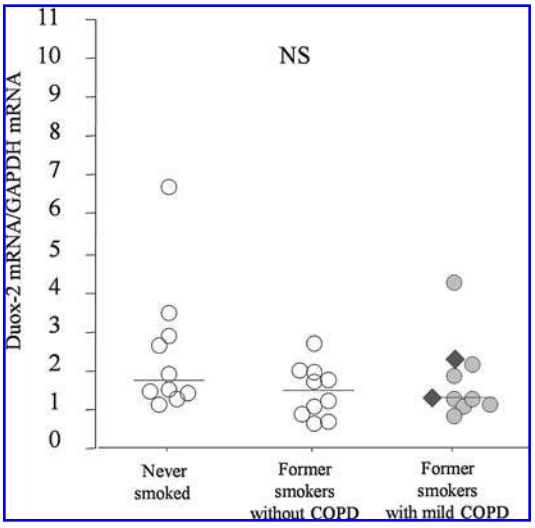

FIG. 8. Duox2 mRNA expression in alveolar septa. Duox 2 mRNA expression levels in the alveolar septa did not significantly differ among the three groups. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

Duox may cause an unwanted host defense imbalance that may contribute to smoking-induced lung disorders, including COPD. Our findings suggest bronchiolar epithelium as a possible cellular target for the development of new host defense approaches (40) that may help protect cells from the accumulation of smoking-related damage.

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ABBREVIATIONS

COPD chronic obstructive pulmonary disease; Duox, dual oxidase; FEV_1, forced expiratory volume in one second; GOLD, Global Initiative for Obstructive Lung Disease; GAPDH, lyceraldehyde-3-phosphatase-dehydrogenase; H_2O_2, hydrogen peroxide; IFN-γ, interferon-γ; IL, interleukin, MAO, monoamine oxidase; NADPH, nicotinamide adenine dinucleotide phosphate; PAS, periodic acid-Schiff; ROS, reactive oxygen species; SE, standard error; XO, xanthine oxidadase.

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