4-Hydroxy-2-Nonenal, a Specific Lipid Peroxidation Product, Is Elevated in Lungs of Patients with Chronic Obstructive Pulmonary Disease

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Cigarette smoking results in oxidative stress and inflammation in the lungs, which are involved in the pathogenesis of chronic obstructive pulmonary disease (COPD). 4-Hydroxy-2-nonenal (4-HNE), a highly reactive diffusible product of lipid peroxidation, is a key mediator of oxidant-induced cell signaling and apoptosis. 4-HNE has a high affinity toward cysteine, histidine, and lysine groups and forms direct protein adducts. We investigated the presence of 4-HNE–modified proteins in lung tissue obtained from subjects with and without COPD. We studied 23 current or ex-smokers with similar smoking histories with COPD (n = 11; $FEV_1 < 70\%$ predicted) or without COPD (n = 12; $FEV_1 > 84\%$ predicted) who had undergone lung resection. As 4-HNE and transforming growth factor-β_1 (TGF-β_1) can modulate γ-glutamylcysteine synthetase (γ-GCS) mRNA levels in lung cells, we assessed the relations between 4-HNE–modified protein levels, FEV_1, γ-GCS, and TGF-β_1. 4-HNE–modified protein levels were elevated in airway and alveolar epithelial cells, endothelial cells, and neutrophils in subjects with COPD, compared with the levels in subjects without COPD ($p < 0.01$). We also observed a significant inverse correlation between the levels of 4-HNE adducts in alveolar epithelium, airway endothelium, and neutrophils and FEV_1 ($p < 0.05$) and a positive correlation between 4-HNE adducts and TGF-β_1 protein and mRNA as well as γ-GCS mRNA levels in airway and alveolar epithelium ($p < 0.01$). The elevated levels of 4-HNE may play a role in the signaling events in lung inflammation leading to the imbalance of the expression of both proinflammatory mediators and protective antioxidant genes in COPD.

Keywords: 4-hydroxy-2-nonenal; glutathione; transforming growth factor-β_1; lungs; chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a condition characterized by airway inflammation and progressive and largely irreversible airway obstruction (1–3). Cigarette smoking is a major risk factor for COPD (1, 2). However, only 15 to 20% of cigarette smokers appear to be susceptible to its effects and show a rapid decline in FEV_1 and develop the disease (1). The reason why only some cigarette smokers are susceptible is unclear at present but may relate to differences between their responses to cigarette smoke. The creation of an imbalance between oxidants and antioxidants (oxidative stress) is considered to be an important event in the pathogenesis of COPD (4). This may be related to a "susceptibility" to the oxidative effects of cigarette smoke and hence to the inflammatory response that ensues.

The sources of the increased oxidative stress in patients with COPD derive from the increased burden of oxidants present in cigarette smoke (5) and from the increased amounts of reactive oxygen species (ROS) released from leukocytes and macrophages, both in the airspaces and in the blood (reviewed in [6–8]). A consequence of oxidative stress is membrane lipid peroxidation in the lungs, primarily involving polyunsaturated fatty acids. The levels of lipid peroxidation products are increased in exhaled air condensate in smokers and in patients with COPD (9–12) (reviewed in [6, 7]). There is increasing evidence that aldehydes, generated endogenously during the process of lipid peroxidation, are involved in many of the pathophysiologic effects associated with oxidative stress in cells and tissues (13). A specific and stable end product of lipid peroxidation, the aldehyde 4-hydroxy-2-nonenal (4-HNE), can diffuse within or even escape from the cell and attack targets far from the site of the original free radical event (14, 15). 4-HNE is a potent alkylating agent that reacts with DNA and proteins, generating various forms of adducts (cysteine, lysine, and histidine residues) (14) that are capable of inducing specific cellular stress responses such as cell signaling and apoptosis (14–16). The lipid aldehyde 4-HNE can be produced from arachidonic acid, linoleic acid, or their hydroperoxides in concentrations of 1 μM to 5 mM, in response to oxidative insults, and is believed to be responsible for many of the effects during oxidative stress *in vivo* (14). These include transcription of proto-oncogenes, including *c-jun*, and activation of activator protein 1 (AP-1) via mitogen-activated protein kinase pathways (17, 18). Studies in rat liver epithelial cells have shown that 4-HNE can enter cells and become bound to proteins within the cytosol (15). However, little is known about the localization of 4-HNE in the human lungs.

Our hypothesis is that the interaction of oxidative components of cigarette smoke with cell membrane phospholipids induces alteration in lipid peroxidation products in lung cells in COPD. The degree of formation of the lipid peroxidation product 4-HNE in response to smoking may be a factor in the susceptibility to the development of enhanced airspace inflammation in COPD.

Oxidative stress has been implicated in the expression of both proinflammatory and protective antioxidant genes. Transforming growth factor β1 (TGF-β_1) is a multifunctional growth factor that modulates cellular proliferation, differentiation, and tissue repair (19). Previously, we have shown an increase in TGF-β_1 expression in bronchiolar and alveolar epithelium in subjects with COPD (20). TGF-β_1 has been shown to decrease glutathione synthesis associated with increased ROS production in human alveolar epithelial cells

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TABLE 1. A SUMMARY OF THE CLINICAL CHARACTERISTICS OF SUBJECTS WITH AND WITHOUT CHRONIC OBSTRUCTIVE PULMONARY DISEASE*

Case	Sex (*M/F*)	Age	PY	FEV_1 % Pred	FEV_1/FVC (%)	Steroids
Non-COPD	9M, 3F	64 ± 4.5	37 ± 8.7	102 ± 13.1	0.72 ± 0.02	None
COPD	11M, 0F	65 ± 2.7	43 ± 4.0	66 ± 5	0.57 ± 0.02	3

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; PY = number of pack years.
* Data shown represent means ± SD.

and pulmonary artery endothelial cells *in vitro* (21–23). Hence, TGF-β_1 may cause oxidative stress or play a role in membrane lipid peroxidation. In contrast, 4-HNE has been shown to induce expression of the protective antioxidant gene γ-glutamylcysteine synthetase (γ-GCS) mRNA in alveolar epithelial cells (24). We have also shown increased γ-GCS expression in patients with COPD (25). Hence, 4-HNE may play a role in the regulation of TGF-β_1 and γ-GCS gene expression in patients with COPD. We hypothesized that the increased membrane lipid peroxidation (generation of 4-HNE) may be one factor that may provide a signal for the expression of TGF-β_1 and γ-GCS in lungs of patients with COPD. We therefore examined the relationship of levels of 4-HNE to TGF-β_1, γ-GCS, and airflow obstruction *in vivo* in human lung tissue obtained from subjects with and without COPD. We used immunohistochemistry to investigate the localization and differences in levels of 4-HNE adducts in lungs of subjects with and without COPD. This is the first study to demonstrate the localization of a specific lipid peroxidation molecule in the lungs in inflammatory lung disease.

METHODS

Subjects

In this study, we obtained lung tissue specimens from current or ex-smokers with or without COPD who underwent lung resection for lung cancer as described previously (20, 25). Compared with the previous study (20), we included five patients less as the tissue sections were completely used in previous studies. Eleven subjects with COPD ($FEV_1 < 70\%$ of predicted value before bronchodilatation; seven ex-smokers and four current smokers) and 12 subjects without COPD ($FEV_1 > 84\%$ predicted; eight ex-smokers and four current smokers) were studied. A summary of the data on lung function tests of these patients is presented in Table 1. The smoking history of the subjects with and without COPD was similar ($p > 0.05$) (Table 1). The ex-smokers stopped smoking at least 1 year before surgery. All subjects showed less than 13% reversibility of the FEV_1 after inhalation of 400

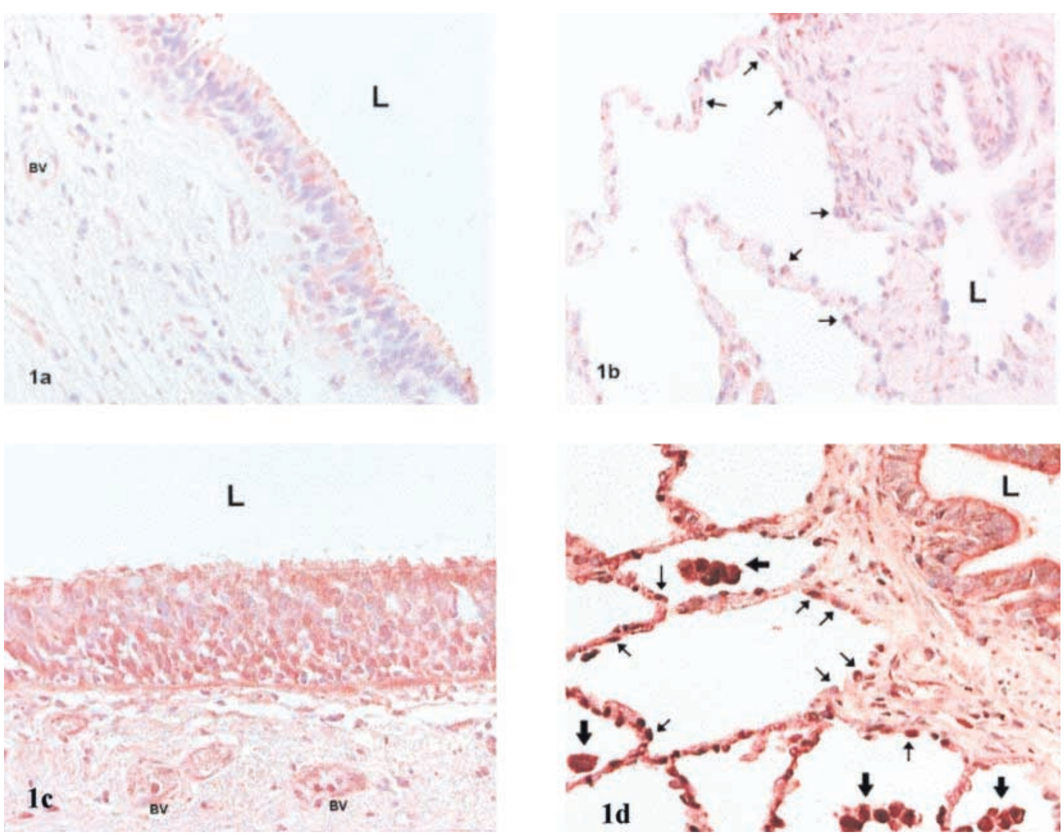

Figure 1. Photographs from immunostaining for 4-HNE in lung tissue from subjects with and without COPD. (*A*) Non-COPD, bronchial; (*B*) Non-COPD, alveolar; (*C*) COPD, bronchial; (*D*) COPD, alveolar. *Thin arrows* point to pneumocyte types I and II. *Thick arrows* indicate alveolar macrophages. *L* = airway lumen; *BV* = blood vessel. Note the intensely stained neutrophils in the blood vessels in Figure 1C. Original magnification: ×200.

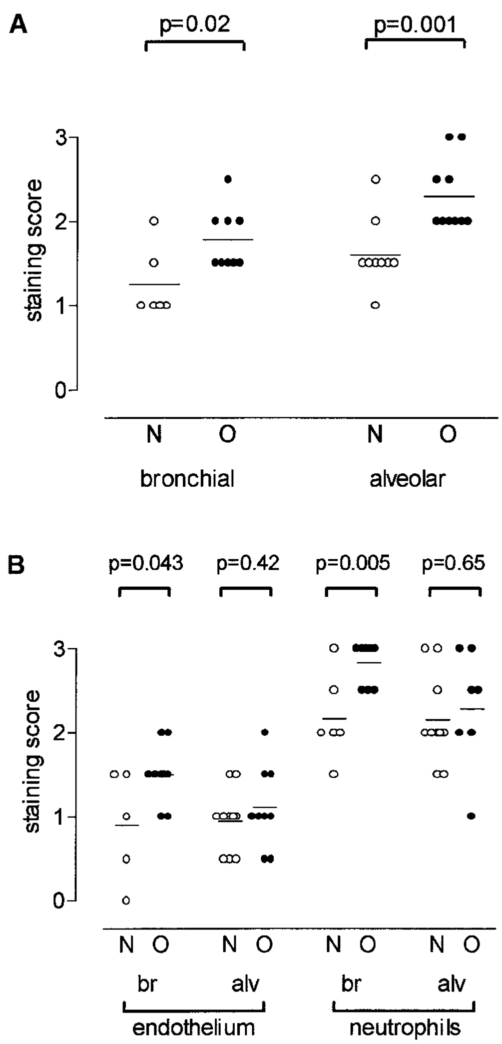

Figure 2. Immunostaining scores for 4-HNE adducts per subject and cell type in bronchial (*br*) and alveolar (*alv*) tissue. (*A*) Epithelial cells and (*B*) endothelial cells and neutrophils. *Open circles* represent subjects without COPD (*N*), and *closed circles* represent subjects with COPD (*O*). The mean is indicated; significance levels (p values) for differences between the indicated groups are also shown.

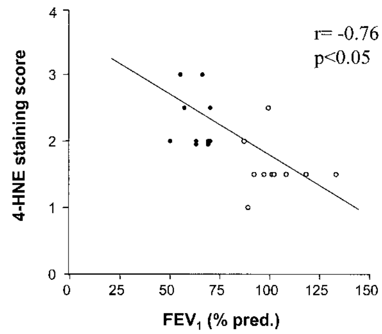

Figure 3. Correlations between the levels of 4-HNE adducts in alveolar epithelium with FEV_1 levels in subjects with (*closed circles*) and without (*open circles*) airway obstruction. Correlation (*r*) and significance level (p value) are indicated.

μg of salbutamol. Exclusion criteria included (*1*) diffuse pulmonary inflammation associated with lung fibrosis, (*2*) absence of tumor-free or pneumonia-free lung tissue specimens, and (*3*) obstruction of central bronchi due to the tumor. These exclusion criteria have been used in our previous studies (20, 25, 26). The histologic type of lung cancers was equally distributed in both subject groups. Preoperatively, none of the patients had clinical evidence of an upper respiratory tract infection and none had received antibiotics 4 weeks before operation or glucocorticoids 3 months before operation, with the exception of three patients who received oral glucocorticoids only perioperatively.

4-HNE Adduct Immunohistochemistry

Lung sections (3-μm thick) were deparaffinized, rehydrated, and pretreated before immunohistochemistry. The sections were refixed, and immunostaining was performed using a mouse monoclonal antibody specific for the 4-HNE adducts (obtained from Japan Institute for the Control of Aging, Fukuroi City, Shizuoka Prefecture, Japan) followed by the avidin–biotin–peroxidase complex (ABC) method, as described by Toyokuni (27). NovaRED (Vector Labs, Burlingame, CA) was used as chromogen. Lung tissue from a patient with adult respiratory distress syndrome was used as a positive control. Omitting the first antibody served as a negative control and resulted in no tissue staining.

The assessment of immunostaining intensity was performed semiquantitatively and in a blinded fashion as described previously (20, 25, 26): 0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = intense staining. Inflammatory cells were identified by immunostaining for CD68+ cells (for macrophages), CD3 (for T cells), or elastase (for neutrophils). Neutrophils were also identified by the presence of a three-lobed nucleus.

Statistical Analysis

The data are expressed as mean ± SEM. Differences between subject groups were compared by Student's *t* test. If variances differed, then Welch's correction was applied. Mann–Whitney analysis provided comparable data. For all the data, the distribution was Gaussian. Correlation of the 4-HNE adduct levels with the FEV_1, TGF-β_1 mRNA, or γ-GCS mRNA expression was performed using the Pearson correlation test. At p values less than 0.05, differences were considered to be statistically significant.

RESULTS

In all the subjects, 4-HNE adducts were localized predominantly in the cytoplasm of bronchial, bronchiolar and alveolar epithelial cells, endothelial cells, neutrophils, and CD68+ cells (which are regarded to be macrophages) (Figure 1). Subepithelial cells (fibroblasts, smooth muscle cells) and lymphocytes (CD3+) were less intensely stained or were not stained. The levels of 4-HNE–modified proteins were higher in bronchial (p = 0.02), but not bronchiolar, and in alveolar epithelial cells (both pneumocytes types I and II; p = 0.001) as well as in bronchial endothelial cells (p = 0.043) and neutrophils (p = 0.005) of subjects with COPD when compared with the levels in subjects without COPD (Figure 2). The increased level of 4-HNE adducts in alveolar epithelium was inversely correlated with the FEV_1 ($r = -0.76$, $p < 0.05$) if the results in all subjects were analyzed (Figure 3). However, a trend toward an inverse relationship was found between the levels of 4-HNE in bronchial epithelium and FEV_1 ($r = -0.51$; p = 0.075). Furthermore, the level of 4-HNE in bronchial endothelium and neutrophils correlated significantly with FEV_1 ($r = -0.61$, p = 0.028; $r = -0.56$, p = 0.048, respectively), in an analysis of the data from all subjects.

Recently, we showed in the same subject groups, on tissue sections adjacent to the sections used in this study, enhanced levels of TGF-β_1 and γ-GCS mRNA in bronchiolar and alveolar epithelium in patients with COPD (20, 25). For the present study, historical data for TGF-β_1 and γ-GCS expression from these studies were used. Upon correction for the number of patients included in the present study, these levels remained significantly enhanced in COPD for TGF-β_1 mRNA (1.6 times, $p < 0.001$) and protein (2.9 times, p = 0.005) as well as γ-GCS mRNA (1.9 times, p = 0.024) (data not shown). Because TGF-β_1 was reported to increase ROS production (21, 22) and ROS

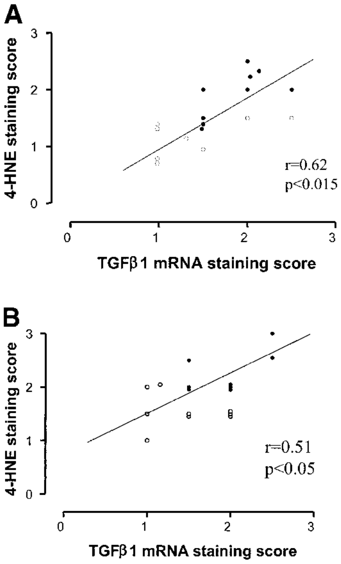

Figure 4. Correlations between the levels of 4-HNE adducts and TGF-β1 mRNA expression both in bronchial (*A*) and alveolar (*B*) epithelium. *Open circles* represent data from subjects without COPD, and *closed circles* represent data from subjects with COPD. Correlation (*r*) and significance level (p value) are given.

has been shown to induce γ-GCS-heavy subunit (HS) mRNA in alveolar epithelial cells (28), we examined the relationship of 4-HNE adduct levels with TGF-β_1 and γ-GCS mRNA levels in airway and in alveolar epithelium. We found a significant correlation between the levels of 4-HNE adducts in bronchial and alveolar epithelium and TGFβ_1 mRNA (r = 0.62 and r = 0.51, respectively, $p < 0.05$) (Figures 4A and 4B), and bronchial protein levels (r = 0.61, p = 0.015) (Figure 5), as well as alveolar γ-GCS mRNA (r = 0.63, $p < 0.004$) (Figure 6) in an analysis of data from all subjects. No significant correlation was found between 4-HNE and γ-GCS mRNA in bronchial epithelium (r = 0.42, p = 0.16). Similarly, there was no significant correlation between 4-HNE adducts and TGFβ_1 protein levels in alveolar epithelium (r = 0.47, p = 0.1).

DISCUSSION

The oxidant burden in the lungs is enhanced in smokers and in patients with COPD due to oxidants in cigarette smoke and by the release of ROS from airspace leukocytes (6, 7). A consequence of this increased burden may be lipid peroxidation in the lungs. 4-HNE is a highly reactive and specific diffusible end product of lipid peroxidation. In this study, we showed increased levels of 4-HNE adducts in lung epithelial and endothelial cells as well as neutrophils in subjects with COPD, compared with levels in subjects without COPD. We also found that the increased level of 4-HNE adducts in alveolar epithelium, airway endothelium, and neutrophils was inversely correlated with FEV_1. We have previously shown high levels of lipid peroxidation products (thiobarbituric acid reactive substances) in blood

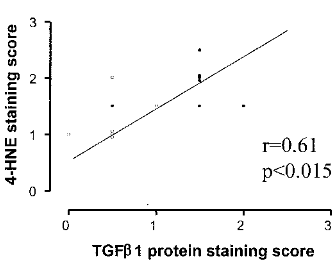

Figure 5. Correlations between the levels of 4-HNE adducts and TGF-β1 protein expression in bronchial epithelium. *Open circles* represent data from subjects without COPD, and *closed circles* represent data from subjects with COPD. Correlation (*r*) and significance level (p value) are given.

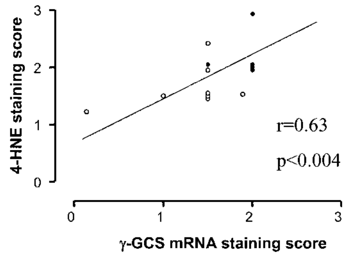

Figure 6. Correlations between the levels of 4-HNE adducts and γ-GCS mRNA expression in alveolar epithelium. *Open circles* represent data from subjects without COPD, and *closed circles* represent data from subjects with COPD. Correlation (*r*) and significance level (p value) are given.

and lung epithelial lining fluid of patients with COPD (29, 30). The levels of thiobarbituric acid reactive substances have also been shown to be elevated in breath condensate and lungs of smokers and patients with stable COPD (31–34). Further confirmation of oxidative stress and lipid peroxidation in patients with COPD comes from reports on elevated levels of 8-isoprostanes and hydrocarbons such as ethane and pentane in exhaled air condensate in healthy smokers and in patients with COPD (9–12). Isoprostanes (a member of F_2-isoprostane) are stable end products of nonenzymatic lipid peroxidation of arachidonic acid (35). The increased levels of F_2-isoprostane in exhaled breath condensate were found to be inversely correlated with airflow obstruction (9). Taken together, these data suggest a role for lipid peroxidation (specifically, the generation of 4-HNE adducts and F_2-isoprostane) in airway obstruction in COPD.

The results from the present study show that increased levels of 4-HNE adducts are associated with increased levels of both TGF-β_1 protein and mRNA. We, and others, have shown that TGF-β_1 is localized mainly in bronchiolar and alveolar epithelium and macrophages, and that the epithelial TGF-β_1 expression is higher in subjects with COPD, compared with those without COPD (20, 36). TGF-β_1 is a multifunctional growth factor that modulates cellular proliferation, differentiation, and tissue repair (19). TGF-β_1 is also a chemoattractant and mitogen for fibroblasts and fibroblast-like cells, and it stimulates the synthesis and deposition of extracellular matrix. TGF-β_1 has been suggested to increase oxidative stress, leading to the generation of lipid peroxidation products, on the basis of the observation that it increases the cellular release of hydrogen peroxide from endothelial cells (21, 22). Vice versa, lipid peroxidation products may also affect the expression of TGF-β_1, as has been shown in experiments with 4-HNE (37). 4-HNE has been shown to induce cellular stress responses such as cell signaling via the mitogen-activated protein kinase pathways leading to the induction of AP-1–mediated genes (16, 17). *In vitro* studies showed that 4-HNE increased TGF-β_1 expression by a mechanism dependent

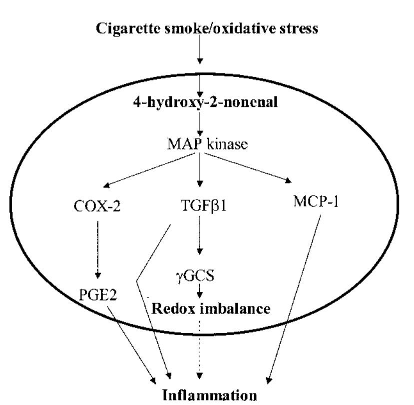

Figure 7. Proposed model of the mechanism of cigarette smoke–mediated oxidative stress (formation of 4-HNE) in lung inflammation.

on the activation of AP-1 in macrophages (37). Hence, it is likely that lipid peroxidation induces TGF-β_1 expression in lungs of patients with COPD. However, there are other confounding factors such as differences in local tumor necrosis factor-α levels, which have been shown to be higher in patients with COPD (38), that may be associated with increased oxidative stress (formation of 4-HNE) and γ-GCS expression (25, 28, 39).

In turn, TGF-β_1 also causes a marked decrease in glutathione levels in endothelial and alveolar epithelial cells and downregulates γ-GCS mRNA levels *in vitro* in alveolar epithelial cells (23). 4-HNE has also been shown to induce γ-GCS mRNA in alveolar epithelial cells (24). We also showed increased γ-GCS expression in lungs of patients with COPD (25). 4-HNE–mediated induction of γ-GCS gene was associated with the mitogen-activated protein kinase signaling pathways (24). It is also known that cigarette smoke induces γ-GCS gene expression via the activation of AP-1 in alveolar epithelial cells (40). In this study, we found a significant correlation between 4-HNE adduct levels and γ-GCS mRNA in airway or alveolar epithelium in subjects without or with COPD. The induction of γ-GCS may be an important adaptive response of the alveolar epithelium to oxidative stress. This suggests that 4-HNE is a second messenger that may play a role in the regulation of expression of the protective γ-GCS gene and also a variety of other genes like TGF-β_1, cyclooxygenase 2, and monocyte chemoattractant protein-1 that were reported to be implicated in the pathogenesis of COPD (41, 42) (Figure 7). An imbalance of an array of redox-regulated antioxidant versus proinflammatory genes might be associated with the susceptibility or tolerance to disease (34).

In conclusion, this study showed that in smokers with and without COPD, 4-HNE is formed in airway epithelial cells, endothelial cells, as well as neutrophils and macrophages. Higher 4-HNE adduct levels in epithelial cells were found in subjects with COPD, compared with levels in subjects without COPD. Thus, elevated levels of 4-HNE may be the end product of oxidant stress imposed by cigarette smoking, which appears to be more pronounced in those who developed COPD. Furthermore, we found a significant correlation of 4-HNE adduct levels with TGF-β_1 protein and mRNA, γ-GCS mRNA in airway or alveolar epithelium, and FEV_1 in subjects without or with COPD. This indicates that the generation of 4-HNE, TGF-β_1 mRNA, and γ-GCS may be associated with the imbalance of proinflammatory and protective antioxidant responses that occurs in lungs of patients with COPD. In turn, this points to a potential role for 4-HNE in the signaling events involved in lung inflammation leading to the development of COPD.

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