

Differential Induction of Extracellular Glutathione Peroxidase and Nitric Oxide Synthase 2 in Airways of Healthy Individuals Exposed to 100% O₂ or Cigarette Smoke

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Reactive oxygen species (ROS) is increased in the airway during the inhalation of 100% O₂ or cigarette smoke and participates in the development of tracheobronchitis. We hypothesized that inhaled ROS upregulates local extracellular ROS scavenging systems or reactive molecules, e.g., nitric oxide (NO). Extracellular glutathione peroxidase (eGPx) is synthesized by airway epithelium and alveolar macrophages, secreted into the surface epithelial lining fluid, and functions as a first-line defense against inhaled ROS. NO, produced by NO synthase 2 (NOS2), combines rapidly with ROS to form reactive nitrogen species (RNS). In this study, human airway epithelial cells and alveolar macrophages from healthy individuals before and after exposure to 100% O₂ for 12 h, or from cigarette-smoking individuals, were evaluated for eGPx and NOS2 messenger RNA (mRNA) expression. Hyperoxia increased NOS2 mRNA in airway epithelial cells by 2.5-fold but did not increase eGPx mRNA. In contrast, cigarette smoke upregulated eGPx mRNA over 2-fold in airway epithelial cells and alveolar macrophages but did not affect NOS2 expression. *In vitro* exposure of respiratory epithelial cells to ROS or RNS also increased eGPx expression. These findings define distinct molecular responses in the airway to different inhaled ROS, which likely influences the susceptibility of the airway to oxidative injury.

Cigarette smoke contains high levels of reactive oxygen species (ROS), which participate in the development of chronic tracheobronchitis (1, 2). High levels of ROS also occur during hyperoxia exposure and result in acute tracheobronchitis, usually developing within 12 h of 100% O₂ (3). The susceptibility of the airway to oxidative injury will depend in part on the ability to upregulate protective ROS scavenging systems. In general, antioxidants are present in the lung and protect against ROS. However, antioxidant enzyme systems in the lung may be overwhelmed by increased levels of ROS as occurs with cigarette smoke exposure or high inspired O₂ concentration. Unfortunately, the primary intracellular antioxidants copper-zinc (Cu,Zn-SOD) and manganese superoxide dismutase (MnSOD) or

catalase are expressed at low levels in the human airway and do not increase after exposure of healthy volunteers to 100% O₂ for 12 to 18 h (3). Similarly, prolonged exposure to cigarette smoke does not increase the major intracellular antioxidants in rat lungs (4). Although intracellular antioxidant enzymes are not induced, the response of extracellular antioxidant enzymes to oxidant stress are not known. Extracellular antioxidants are the critical primary defense against exogenous, inhaled ROS, which dissolves first in the epithelial lining fluid on the airway surface. Extracellular glutathione peroxidase (eGPx) is a major antioxidant in the lung epithelial lining fluid, which coupled with glutathione or *S*-nitrosoglutathione (GSNO) detoxifies lipid peroxides (1, 5, 6). Recently, we showed that eGPx is increased in epithelial lining fluid of cigarette-smoking individuals (5). Although the mechanism by which eGPx protein increases is unknown, cigarette smoke contains numerous compounds, including ROS, reductants, and bioactive unsaturated aldehydes, that may contribute to the eGPx induction in the airway of smoking individuals (1, 2). We propose that exogenously inhaled ROS upregulates protective extracellular ROS scavenging systems such as eGPx. To determine the effect of acute or chronic oxidative stress on eGPx expression, we quantitated eGPx messenger RNA (mRNA) expression in human airway epithelial cells and alveolar macrophages from healthy individuals exposed to chronic cigarette smoke or 100% O₂ for 12 h. Because ROS may also be rapidly scavenged by other reactive molecules, such as nitric oxide (NO), to yield reactive nitrogen species (RNS), we also evaluated NO synthase 2 (NOS2) mRNA expression. Finally, oxidant mechanisms of eGPx induction are evaluated in respiratory epithelial cells exposed directly to ROS and RNS *in vitro*.

Materials and Methods

Study Population

To evaluate epithelial and macrophage gene expression *in vivo*, 13 healthy, nonsmoking volunteers and 11 healthy, smoking individuals were studied. All had normal histories, physical examinations, chest roentgenograms, and lung function tests (3, 5). Exclusion criteria for the two groups included age under 18 yr or over 65 yr, pregnancy, human immunodeficiency virus infection, and history of respiratory infection in the previous 6 wk. Additional exclusion criteria for nonsmoking individuals included current tobacco use, prolonged exposure to second-hand smoke at home or at work, exposure to dusty environments or known pulmonary disease-producing agents, and history of recurrent episodes of breathlessness, chest tightness, cough, and/or sputum production. Smoking individuals must have smoked for a minimum of 5 pack-years and be current smokers.

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Abbreviations: activator protein, AP; bronchoalveolar lavage, BAL; complementary DNA, cDNA; copper-zinc superoxide dismutase, Cu,ZnSOD; extracellular glutathione peroxidase, eGPx; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; *S*-nitrosoglutathione, GSNO; manganese superoxide dismutase, MnSOD; messenger RNA, mRNA; nitric oxide, NO; NO synthase 2, NOS2; nuclear factor κ B, NF- κ B; polymerase chain reaction, PCR; reactive nitrogen species, RNS; reactive oxygen species, ROS; *S*-nitroso-*N*-acetyl-D,L-penicillamine, SNAP.

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Study Design

Five healthy volunteers underwent bronchoscopy with cytology brushings from the right lung to obtain bronchial epithelial cells, and bronchoalveolar lavage (BAL) to obtain alveolar macrophages. They returned 2 wk later and were exposed to 100% O₂ for 12 h after which all individuals underwent bronchoscopy immediately for sampling of bronchial epithelium and BAL from the left lung (3, 5). In addition, 11 healthy, smoking individuals and eight healthy, nonsmoking individuals underwent bronchoscopy to collect human airway epithelial cells and alveolar macrophages to determine the effects of cigarette smoke on NOS2 and eGPx mRNA expression. The study was approved by the Institutional Review Board, and written informed consent was obtained from all individuals enrolled in the study.

Isolation of Bronchial Epithelial Cells

Bronchial epithelial cells were obtained by cytology brushings from second- and third-order bronchi with a 1-mm cytology brush (Microvasive, Watertown, MA) as previously described (3, 5). The brush sample was immediately placed in RPMI 1640 (GIBCO-BRL, Grand Island, NY) and an aliquot was taken for cytology and cell differential determination. RNA was immediately extracted from cells.

Alveolar Macrophages

The BAL fluid obtained by bronchoscopy was filtered through a Y-blood filter (Drip Chamber pump flashball Divia; Baxter, Deerfield, IL) and cellular components were separated by centrifugation (700 × *g* for 10 min) (5). Cells were washed once with Hanks' balanced salt solution (GIBCO-BRL) and counted with a hemacytometer. Cell differential was performed after a Giemsa-type staining (Diff-Quik; American Scientific Products, Stone Mountain, GA).

Polymerase Chain Reaction and Cloning

The human eGPx complementary DNA (cDNA) was obtained by polymerase chain reaction (PCR) of normal human lung cDNA. PCR primers were based upon the known eGPx cDNA (7). PCR was performed using the following two nested reactions (F, forward primer; R, reverse primer): The first nested PCR:

F-eGPx1, 5'-CGCCATGGCCCGGCTGCTGCAG-3';
R-eGPx2, 5'-GGACGTCAGTCATAGT-3';

at 94°C for 2 min; 40 cycles of 94°C for 40 s, 50°C for 40 s, 72°C for 90 s; and 72°C for 5 min.

The second nested PCR:

F-eGPx1, 5'-CGCCATGGCCCGGCTGCTGCAG-3';
R-eGPx4, 5'-GACGGCCTTCAGTTACTTCT-3';

at 94°C for 2 min; 25 cycles of 94°C for 40 s, 50°C for 40 s, 72°C for 90 s; and 72°C for 5 min.

The 671-bp PCR product was cloned into a TA cloning vector (Invitrogen, Carlsbad, CA) to create plasmid pCCF 33 and sequenced using Sequenase 2.0 (United States Biochemical) and/or using 373 DNA sequencing system (Applied Biosystems, Foster City, CA) (Genbank accession no. AF217787).

Cell Culture

BET1A, a human bronchial epithelial cell line transformed by the SV40 virus, was cultured in serum-free Lechner and LaVeck medium (LHC-8; Biofluids, Inc., Rockville, MD) with additives of 0.33 nM retinoic acid and 2.75 μM epinephrine, on plates pre-coated with coating media containing 29 μg/ml collagen (Vitrogen; Collagen Corp., Palo Alto, CA), 10 μg/ml bovine serum albumin (Biofluids), and 10 μg/ml fibronectin (Calbiochem, La Jolla, CA) for 5 min (8). To evaluate the response to ROS and RNS, the cells were stimulated at 70% confluence with menadione (Sigma-

Aldrich Co., St. Louis, MO), an intracellular hydrogen peroxide generating compound, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) (Alexis, San Diego, CA), or GSNO (Alexis) in a dose- and time-dependent manner.

RNA Extraction and Northern Blot Analysis

Total RNA from freshly obtained human airway epithelial cells and alveolar macrophages was extracted by the GTC ([4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0], 0.5% sarkosyl, and 0.1 M β-mercaptoethanol)-CsCl gradient method and evaluated by Northern blot analysis using a [³²P]-labeled eGPx probe (pCCF33) and a [³²P]-labeled 1.9-kb NOS2 cDNA probe (pCCF21) (9), or a control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (10) and γ-actin cDNA (pHFγA-1) (11), and then subjected to autoradiography. Quantitation of eGPx mRNA relative to GAPDH mRNA and NOS2 mRNA relative to γ-actin or GAPDH was accomplished using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

All data are expressed as the mean and standard error of the mean (SEM). Comparisons were made using two-tailed Student's *t* test. A value of *P* < 0.05 was considered significant.

Results

Patient Characteristics

Control and smoking individuals were similar in terms of race and sex distribution (age [yr]: healthy, nonsmoking individuals, 27 ± 2; healthy, smoking individuals, 36 ± 5; *P* > 0.05; sex (M/F): healthy, nonsmoking individuals, 8/5; healthy, smoking individuals, 5/6; *P* > 0.05). As previously shown, the total cell counts in BAL were increased in smokers in comparison to control volunteers (*P* < 0.05) (5). However, smokers' BAL cell differentials were similar to those of control volunteers (5).

Increased NOS2 mRNA in Airway Epithelia of Healthy Individuals Exposed to 100% O₂

Brushing cells were comprised of > 92% airway epithelial cells, and BAL cells were > 95% alveolar macrophages (1, 8). No significant change in cell differentials occurred with hyperoxia. NOS2 mRNA is present in freshly obtained airway epithelial cells as a prominent signal at 4.5 kb using a [³²P]-labeled NOS2 cDNA (pCCF21) (Figure 1). Strikingly, NOS2 mRNA increased in the epithelial cells of all individuals exposed to 100% O₂ (NOS2/GAPDH mRNA: basal levels, 4 ± 1; 100% O₂, 10 ± 2; *n* = 5 paired observations, *P* < 0.05) (Figure 1). Unlike murine macrophages, NOS2 mRNA is not detected in human macrophages before or after O₂ exposure (Figure 1). For the first time, rapid induction of gene expression in the human airway is shown in response to acute hyperoxia *in vivo*.

Lack of eGPx Gene Induction with Hyperoxia

eGPx mRNA is present at 1.9 kb in all samples using a [³²P]-labeled eGPx cDNA (pCCF31). eGPx mRNA is significantly more abundant in alveolar macrophages (eGPx mRNA/GAPDH mRNA: human airway epithelial cells, 8 ± 1; alveolar macrophages, 28 ± 8; *P* < 0.05). Exposure to 100% O₂ did not upregulate the eGPx gene (eGPx mRNA/GAPDH mRNA: human airway epithelial cells 100% O₂, 5.6 ± 0.8, *n* = 5 paired observations, *P* > 0.05; alveolar

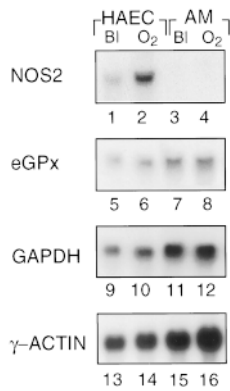


Figure 1. NOS2 and eGPx mRNA expression in human airway epithelial cells and alveolar macrophages in response to 100% O₂ exposure *in vivo*. Representative Northern blot analysis (1 μg total RNA/lane) of NOS2 expression in human airway epithelial cells (HAEC) obtained from a healthy control volunteer at baseline (Bl, lane 1) and after 12 h exposure to 100% O₂ (O₂, lane 2), and in alveolar macrophages (AM) obtained from the same individual at baseline (Bl, lane 3) and after 12 h exposure to 100% O₂ (O₂, lane 4). Representative Northern blot

analysis (5 μg total RNA/lane) of eGPx mRNA in HAEC, obtained from a healthy control volunteer at baseline (Bl, lane 5) and after 12 h exposure to 100% O₂ (O₂, lane 6), and in AM obtained from the same individual at baseline (Bl, lane 7) and after 12 h exposure to 100% O₂ (O₂, lane 8). Human [³²P]-labeled γ-actin cDNA and GAPDH hybridization is shown as control for RNA loading (lanes 9–16).

macrophages 100% O₂, 22 ± 7, *n* = 5 paired observations, *P* > 0.05) (Figure 1). These data show that antioxidant gene expression of eGPx is not upregulated by hyperoxia.

Upregulation of eGPx mRNA in Smoking Individuals

Previous studies have shown that cigarette smoke exposure increases eGPx protein levels in lung epithelial lining fluid (8). To investigate if the increased protein is related to increased eGPx gene expression, human airway epithelial cells and alveolar macrophages were obtained from 12 healthy controls and 11 smoking individuals. Northern blot analyses showed that human airway epithelial cells and alveolar macrophages of smoking individuals have increased expression of eGPx mRNA (eGPx mRNA/GAPDH mRNA: human airway epithelial cells, healthy controls, 8.0 ± 0.8 versus smoking individuals, 18 ± 3; *P* < 0.05; human alveolar macrophages, healthy controls, 24 ± 3 versus smoking individuals, 44 ± 10; *P* < 0.05) (Figure 2). Thus, the eGPx gene expression is induced by chronic cigarette smoke exposure but not acutely by hyperoxia. In contrast, NOS2 mRNA expression is not increased by cigarette smoke (NOS2 mRNA/γ-actin mRNA: human airway epithelial cells, healthy controls, 20 ± 4 versus smoking individuals, 14 ± 2; *P* < 0.05). NOS2 is not detectable in alveolar macrophages from cigarette-smoking individuals at the level of Northern blot analyses (data not shown).

Upregulation of eGPx in Response to ROS and RNS

In Vitro

BET1A cells were exposed to various ROS and RNS *in vitro* to investigate whether these agents increase eGPx in a time- and dose-dependent manner. Northern blot analysis showed that BET1A cells express the eGPx gene in culture as the expected 1.9-kb mRNA transcripts. Furthermore, eGPx mRNA transcripts increase after exposure to menadione, GSNO, and SNAP. Quantification of eGPx mRNA levels relative to GAPDH mRNA show that men-

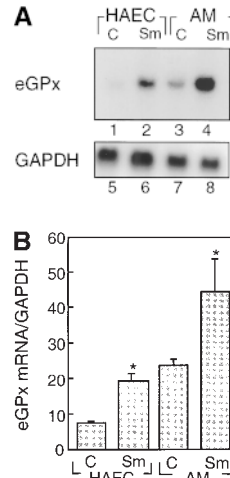


Figure 2. eGPx expression in human airway epithelial cells (HAEC) and alveolar macrophages (AM) in response to cigarette smoke. (A) Representative Northern blot analysis (5 μg total RNA/lane) of eGPx expression in HAEC obtained from a healthy control nonsmoker (C, lane 1) and from a smoking individual (Sm, lane 2), and in AM obtained from healthy nonsmoker and smoking individuals (lanes 3 and 4). Human [³²P]-labeled GAPDH cDNA hybridization is shown as control for RNA loading (lanes 5–8). (B) eGPx mRNA relative to GAPDH mRNA quantitated by Northern blot analysis is significantly increased in HAEC and AM of smoking individuals. **P* < 0.05.

adione (10 μM) increases eGPx mRNA levels at 24 h (*P* = 0.01) (Figure 3A). Exposure to NO donors, SNAP and GSNO, produce a significant increase in eGPx mRNA (1 mM SNAP or GSNO, 48 h; *P* < 0.05) (Figure 3B). These data show that the eGPx gene is upregulated by ROS or RNS.

Discussion

Antioxidant enzymes are traditionally responsible for the protection of the lungs against ROS. For example, increases in antioxidant enzymes in lungs after exposure to hyperoxia have been postulated to play a major role in allowing animals to survive a subsequent exposure to a lethal concentration of oxygen (12). Furthermore, rats overexpressing Cu,ZnSOD and catalase are resistant to toxic effects of hyperoxia (13), and mice genetically deficient in the extracellular superoxide dismutase are more susceptible to hyperoxia (14). Information about the effect of oxygen on the molecular regulation of human antioxidant enzymes is complex and far less understood than that in experimental animals. Although the evidence that species from unicellular organisms to primates are capable of upregulating antioxidant genes in response to oxidant stress (9), the epithelium of the large airways of the normal human lung is not able to upregulate the major antioxidants with hyperoxia (3). For example, *in vitro* studies with transformed bronchial epithelial cells exposed to hyperoxia for 48 h show that hyperoxia has no effect on intracellular GPx mRNA and activity, or on other antioxidant enzymes such as Cu,ZnSOD, MnSOD, or catalase (10). Similar to the intracellular antioxidant enzymes, eGPx expression in the airway is not increased in individuals exposed to 100% O₂ *in vivo*. In contrast, we recently reported that exposure to chronic oxidant stress of cigarette smoke increases the levels of eGPx protein in human lung epithelial lining fluid (5). Here, chronic cigarette smoke exposure is shown to induce eGPx mRNA in human airway epithelial cells and alveolar macrophages, providing a mechanism for the increased protein levels in epithelial lining fluid. In contrast, chronic exposure of rat lungs to cigarette smoke does not lead to induction of NOS2 mRNA or protein levels (11). Similarly, NOS2 expression

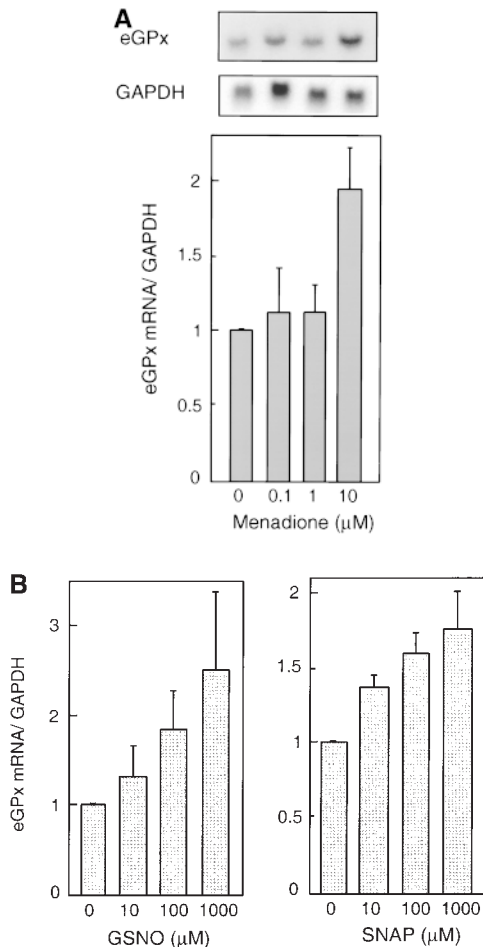


Figure 3. (A) Induction of eGPx mRNA in transformed bronchial epithelial cells (BET1A) by ROS. BET1A cells were exposed to the hydrogen peroxide generating compound menadione (0 to 10 μ M) for 24 h. The representative Northern blot analysis of total RNA (10 μ g/lane) demonstrates increase of eGPx mRNA with 10 μ M menadione (lane 4). GAPDH is shown as control (lanes 5–8). Relative units of eGPx mRNA/GAPDH is summarized in the graph, with means \pm standard deviation (SD) demonstrated. (B) Induction of eGPx mRNA in BET1A cells by RNS. BET1A cells were exposed to NO generating compounds SNAP or GSNO for 48 h. Northern blot analysis of total RNA (10 μ g/lane) with [32 P]-labeled eGPx cDNA and GAPDH cDNA was performed to quantitate changes in mRNA expression. Results are means \pm SD of a minimum of three experiments.

is not increased in the airway of cigarette-smoking individuals in this study.

As previously shown (8), NOS2 is continuously expressed in the airway epithelial cells of normal individuals, whereas lung macrophages do not tonically express NOS2. In this study, NOS2 expression in airway epithelial cells is increased in all individuals exposed to 100% O₂ *in vivo*. We and others (15, 16) have shown that increasing inspired oxygen leads to increasing NO in exhaled air from healthy individuals through kinetic effects on NOS2 activity (15). Interestingly, whereas NO may contribute to tissue injury, it has also been ascribed a protective antioxidant role against hyperoxic lung injury. Specifically, inhaled NO

administered exogenously with hyperoxic gas mixtures protects against lung injury (17), whereas NO synthase inhibitor in animals during hyperoxia results in increased toxicity and earlier death (18). Anti-inflammatory effects of NO may be mediated by several mechanisms, including inhibition of gene expression and secretion of proinflammatory cytokines (19, 20), or by protection against programmed cell death through inactivation of the proteolytic enzymes responsible for apoptosis, i.e., caspases (21). Thus, endogenous NO synthesis during hyperoxia may be an important early physiologic defense mechanism against oxygen toxicity. However, high levels of NO synthesis may also lead to RNS formation, e.g., nitrated proteins, and accentuate tissue injury (1, 22, 23). For example, NOS2 upregulation is causally linked to development of the acute tracheobronchitis in respiratory viral infections (24, 25).

Interestingly, previous reports have shown that GPx also function as peroxynitrite reductases, preventing both oxidation and nitration reactions caused by RNS (25). In this context, NO/RNS generating compounds and ROS generating compound all lead to eGPx induction in respiratory epithelial cells *in vitro*. Taken together, the cigarette-smoke induction of eGPx mRNA *in vivo* is likely due to oxidative and/or nitrosative mechanisms. Although *in vivo* hyperoxia did not induce eGPx mRNA, upregulation of eGPx *in vitro* occurs only after 24 h of ROS exposure. Thus, the 12-h time of hyperoxia exposure may have been inadequate for eGPx induction. Alternatively, although hyperoxia and cigarette smoke both lead to oxidant stress, the signaling mechanisms activated are different (17, 26). In general, ROS regulates the expression of numerous genes through effects on several redox-sensitive transcription factors, such as nuclear factor κ B (NF- κ B) and activator protein (AP)-1 (27, 28). Hyperoxia activates NF- κ B but decreases AP-1 in the lungs of rats exposed to hyperoxia for 24 h (17). In contrast, cigarette smoke activates AP-1 in human epithelial cells *in vivo* (26). Notably, the 5' flanking region of the eGPx gene contains a consensus DNA sequence element for AP-1 binding (25). Based upon this work and others (26–28), ROS and RNS induction of the eGPx mRNA may involve the activation of AP-1, whereas hyperoxia induction of NOS2 may be related to NF- κ B (27). However, further studies are necessary to determine the signaling mechanisms involved in ROS and RNS induction of eGPx expression.

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