Extracellular glutathione peroxidase induction in asthmatic lungs: evidence for redox regulation of expression in human airway epithelial cells

SUZY A. A. COMHAIR,* PERCY R. BHATHENA,* CAROL FARVER,† FREDERIK B. J. M. THUNNISSEN,‡ AND SERPIL C. ERZURUM*,[1]

*Departments of Pulmonary and Critical Care Medicine, Cancer Biology and †Pathology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, USA; and ‡Department of Pathology, Canisius Wilhelmina Hospital, NL 6500 GS Nijmegen, The Netherlands

ABSTRACT **A critical first-line antioxidant defense on the airway epithelial surface against reactive oxygen and nitrogen species (ROS and RNS) is extracellular glutathione peroxidase (eGPx). Little is known about the regulation of eGPx or its role in ROS-mediated lung diseases such as asthma. Here we show that eGPx is increased in the asthmatic airway in comparison to healthy controls. Higher levels of eGPx mRNA in asthmatic airway epithelium verified bronchial epithelial cells as the source for the increased eGPx. The eGPx mRNA in bronchial epithelial cells *in vitro* increased eightfold after exposure to ROS and glutathione, an essential cofactor for eGPx activity. Alterations in intracellular and extracellular oxidized and reduced glutathione were temporally associated with eGPx induction, further supporting redox mechanisms in gene expression. Overexpression of superoxide dismutase, but not catalase, inhibited induction and identified superoxide as a key intermediary. The eGPx mRNA half-life was not affected by ROS, suggesting a transcriptional mechanism for eGPx regulation. Fusion genes of deletion fragments of the eGPx gene 5′ flanking region driving a reporter gene conclusively identified the ROS-responsive region, which contained the consensus DNA binding site for the redox-regulated transcription factor, activator protein 1.—Comhair, S. A. A., Bhathena, P. R., Farver, C., Thunnissen, F. B. J. M., Erzurum, S. C. Extracellular glutathione peroxidase induction in asthmatic lungs: evidence for redox regulation of expression in human airway epithelial cells. *FASEB J.* 15, 70–78 (2001)**

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The respiratory epithelium is frequently exposed to oxidants whether inhaled, as in cigarette smoke or air pollutants, or from reactive oxygen and nitrogen species (ROS, RNS) released from cells in the lungs during airway inflammation. Fortunately, the lung is endowed with an integrated defense system consisting of low molecular weight antioxidants such as glutathione and intracellular enzymes such as superoxide dismutase, catalase, and glutathione peroxidase to protect against the toxic effects of oxidants generated within cells (1, 2). However, a large portion of oxidative stress occurs on the extracellular surface of the lung epithelium. Thus, critical first-line antioxidant defenses are located in the epithelial lining fluid (ELF). The ELF contains the highest concentrations of reduced glutathione (GSH) found in the body and a specific secreted extracellular glutathione peroxidase (eGPx) (3–5). Glutathione peroxidases (EC 1.11.1.9, GPx) are a family of antioxidant enzymes that reduce hydrogen peroxide and/or lipid hydrogen peroxides by the oxidation of reduced glutathione or S-nitrosoglutathione (6–8). The eGPx is genetically distinct from the classical cellular GPx, membranous phospholipid hydroperoxidase GPx, or the gastrointestinal form of GPx (6). Lung cells are able to synthesize and secrete eGPx (9). However, little is known about the regulation of eGPx (10). Based on the knowledge that ROS and RNS play a role in regulation of a number of important genes (11–14), we hypothesized that increased oxidative stress leads to induction of eGPx in the lung. Studies show that increased generation of ROS and RNS occurs in asthmatic airways and plays a key role in the pathogenesis of asthma (15–22). If eGPx expression is upregulated by ROS, we reasoned that eGPx would be increased in asthmatic lungs. In the present study, we quantitate eGPx protein and mRNA in the human asthmatic airway in comparison to healthy controls *in vivo*. To define redox mechanisms of eGPx regulation in the lung, the effect of ROS and glutathione on eGPx expression in bronchial epithelial cells is defined *in vitro* and the 5′ flanking region of the eGPx gene necessary for transcriptional activation is identified. The results show that eGPx is increased in asthma and provide clear evidence of transcriptional activation of the gene by superoxide, which is strikingly augmented by glutathione.

[1] Correspondence: Department of Pulmonary and Critical Care Medicine, Cleveland Clinic Foundation, 9500 Euclid Ave./A90, Cleveland, OH 44195, USA. E-mail: erzurus@ccf.org

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MATERIALS AND METHODS

Study population

To evaluate eGPx in the respiratory system *in vivo*, the study population included 35 individuals: 22 healthy nonsmoking individuals and 13 asthmatic individuals. Exclusion criteria for the two groups included age under 18 years or over 65 years, pregnancy, human immunodeficiency virus infection, and history of respiratory infection in the previous 6 wk, current tobacco use, prolonged exposure to second hand smoke at home or at work, exposure to dusty environments or known pulmonary disease-producing agents. Asthma was defined by the American Thoracic Society guidelines, including episodic respiratory symptoms, reversible airflow obstruction, and/or a positive methacholine challenge test (a drop in FEV_1 20% or greater at the highest concentration administered). None of the subjects had a recent asthma exacerbation, hospitalization, or change in medications for 6 wk prior to the study. Finally, no subjects received systemic corticosteroids during the previous 6 months or inhaled corticosteroid in the preceding 6 wk. Asthma severity and temporal course in volunteers included mild intermittent and persistent asthma (23). Control individuals were nonsmokers, i.e., no cigarettes for > 5 years and < 5 pack years history. The Cleveland Clinic Foundation Institutional Review Board approved the study and written informed consent was obtained from all individuals.

Isolation of bronchial epithelial cells

Individuals underwent bronchoscopy to obtain samples of bronchial epithelial cells with cytology brushings from second- and third-order bronchi with 1 mm cytology brush (Microvasive, Watertown, Mass.) as described previously (1). The brush sample was immediately placed in RPMI 1640 (GIBCO-BRL, Grand Island, N.Y.) and an aliquot was taken for cytology and cell differential determination. RNA was extracted from cells as described previously (1).

BAL fluid

Bronchoalveolar lavage (BAL) was performed using fiberoptic bronchoscopy as described previously (4). Briefly, after local anesthesia with 2% lidocaine, a bronchoscope was wedged in a segmental bronchus of the right middle lobe or lingula. Three 50 ml aliquots of warm physiological saline were infused and recovered with manual suction. The BAL fluid was filtered through a Y blood filter (Drip Chamber Pump Fhashball Divia, Baxter, Morton Grove, Ill.) and cellular components were separated by centrifugation ($700 \times g$, 10 min). Cells were washed once with Hanks balanced salt solution (Life Technologies, Inc., Grand Island, N.Y.) and counted with a hemacytometer. Cell differential was performed after a Giemsa-type staining (Diff-Quick, American Scientific products, Stone Mountain, Calif.). Peripheral blood was obtained from study subjects on the same day as BAL; serum was then extracted by centrifugation of the whole blood ($1430 \times g$, 10 min). Urea was determined in BAL fluid and serum using the BUN (ENDPOINT) reaction (Sigma, St. Louis, Mo.) as described previously (4). Relative levels of ELF were estimated by using simple dilution principles of the urea concentration in serum and BAL fluid. Because the number of sample obtained in some individuals was limited, not all individuals were used for every measurement. The number of samples evaluated for each parameter is stated in the text.

eGPx

eGPx protein was measured by enzyme-linked immunosorbent assay (Calbiochem, La Jolla, Calif.). This method is based on a sandwich-type immunoassay, and is specific for eGPx. To determine the eGPx protein in the overlying media of the BET1A cells, the media was first concentrated 20 times by using 10,000 NMWL ultrafree MC filter unit (Millipore, Bedford, Mass.). The eGPx protein concentration present in the BAL fluid, BET1A cell lysates, and overlying media was based on 4-parameter curve fit generated from known standard concentrations of eGPx.

Cell culture

BET1A, a human bronchial epithelial cell line transformed by the SV40, was cultured in serum-free Lechner and LaVeck medium (LHC-8, Biofluids, Inc., Rockville, Md.) with additives 0.33 nM retinoic acid, 2.75 mM epinephrine, and the antibiotic combination 1% penicillin/streptomycin on plates precoated with coating media containing 29 μg/ml collagen (Vitrogen: Collagen, Palo Alto, Calif.), 10 μg/ml bovine serum albumin (Biofluids), and 10 μg/ml fibronectin (Calbiochem) for 5 min (24). Human airway epithelial cells (HAEC) obtained by bronchial brushing were cultured in serum-free Lechner and LaVeck media (LHC8) on plates precoated with coating media. Primary HAEC cultures of passages 0–2 were used in experiments. To evaluate the response to ROS, the cells were stimulated at 70% confluence with the intracellular superoxide-producing agent pyrogallol (25) (J. T. Baker Inc., Phillipsburg, N.J.), hydrogen peroxide (Sigma), and/or reduced and oxidized glutathione in a dose- and time-dependent manner.

GSH/GSSG levels

GSH levels in cell lysate and media were measured by standard methods as described previously (26). In brief, total glutathione levels were determined by mixing equal amounts of cell lysate or media with 10 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mM potassium phosphate, pH 7.5, which contained 17.5 μM EDTA. An aliquot (50 μl) of the solution was added to a cuvette containing 0.5 U of glutathione disulfide reductase (Sigma type III) in 100 mM potassium phosphate and 5 mM EDTA, pH 7.5. After 1 min, the reaction was initiated with 220 nmol of NADPH in a final reaction volume of 1 ml. The rate of reduction of DTNB was recorded continuously at 412 nm by a spectrophotometer with a Kinetics/Time feature (Beckman DU-640, Beckman Instruments, Fullerton, Calif.).

To assay GSSG, equal volumes of cell lysate or media and N-ethylmaleimide (NEM) were added. An aliquot (50 μl) of the solution was passed at one drop/sec through a C15 Sep-Pak cartridge (Walters Associates, Framingham, Mass.) that had been washed with methanol, followed by water. The cartridge was then washed with 1 ml of 100 mM potassium phosphate and 5 mM EDTA, pH 7.5. A 750 μl aliquot of the eluate was added to a cuvette with 250 nmol of DTNB and 0.5 U of GSSG reductase. The assay then proceeded as in the measurement of total GSH. The levels of GSH and GSSG were based on standard curves.

GPx activity

Total glutathione peroxidase activity was determined spectrophotometrically in BET1A cell lysate (intracellular) and overlying media (extracellular) after exposure to 100 μM pyrogallol and/or 10 mM GSH for 24 and 48 h. The cell lysate or

media were incubated in the presence of 0.1 mM sodium azide, 1 U/ml glutathione reductase, 0.1 mM glutathione, and 0.12 mM reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH), 0.016 mM dithiothreitol, 0.38 mM EDTA, and 50 mM sodium phosphate (pH 7.0) for 2 min at 25°C. The reaction was initiated by the addition of 0.2 mM hydrogen peroxide. The decrease in absorbance at 340 nm over 3 min as NADPH is converted to NADP is proportional to the GPx activity. One unit of activity is defined as the activity that catalyzed the oxidation of 1 nmol NADPH/min using an extinction molar coefficient of $6.22 \times 10^6\ M^{-1}\ cm^{-1}$ for NADPH (1).

Northern analysis of eGPx expression

Total RNA from BET1A cells or epithelial cells freshly obtained by bronchoscopic brushing of control and asthmatic airways 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 analysis using a ^{32}P-labeled eGPx probe (pCCF33) or as control GAPDH cDNA probe (27), and then subjected to autoradiography. Expression of eGPx mRNA relative to GAPDH mRNA was accomplished using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) to quantitate relative units.

Infection of BET1A cells with adenoviral vectors containing human Cu/ZnSOD (AdSOD) or catalase cDNA (AdCL)

BET1A cells at 50% confluence were infected with adenovirus-expressing SOD (AdSOD), adenovirus expressing catalase (AdCL), or AdNull at 10 multiplicity of infection (MOI) per cell, as described previously (28), and exposed to 100 μM pyrogallol and 10 mM GSH for 24 h.

eGPx mRNA half-life

To determine the half-life of eGPx mRNA, BET1A cells stimulated with GSH/pyrogallol for 24 h or unstimulated were exposed to actinomycin D to inhibit new RNA synthesis. The cells were subsequently harvested at different time points to evaluate eGPx mRNA.

Characterization of the 5′ flanking region of the human eGPx gene

A 1003 base pair eGPx promoter fragment was isolated from human lung DNA by using polymerase chain reaction (PCR) with primers based on the known sequence of eGPx (29), cloned into enhancer pGL3 vector (Promega, Madison, Wis.), and sequenced using Sequenase 2.0 (U.S. Biochemical, Cleveland) and/or 373 DNA sequencing system (Applied Biosystems, Foster City, Calif.). To identify more of the 5′ flanking region of the gene, a rapid PCR-based human GenomeWalker method (Clontech, Palo Alto, Calif.) was used. PCR was performed using the following two nested reactions (F, forward primer; R, reverse primer): R- adaptor primer (AP1) (Clontech) and F-eGPx1 (5′ ACTGAGTGGGAAACCCAGCAAGGC 3′) for the first PCR, and R-AP2 (Clontech) and F-eGPx2 (5′ CTATCTGTGGCCAAACCACCTGGC 3′) for the nested reaction. The 2402 bp product was ligated to the known 1.2 kb eGPx promoter fragment using an internal *Apa*I site. The resulting 3202 bp fragment was cloned into the *Sma*I and the Mlu I site of the enhancer pGL3 vector (Promega). Deletion constructs p-52, p-252, p-443, and p-652 were made by PCR, using the same downstream primer R-eGPx-12 (5′ ATTGCAAGCTTCCGCGGCCAAGCCGAGACC 3′) with a *Hin*dIII site incorporated (underlined). A *Xho*I site (underlined) was included in each of the 5′ primers.

p-52: 5′ ATTCCTCGAGCCTTGCCCTGGCTGTAATGG 3′
p-252: 5′ ATTCCTCGAGCCCAGGACACCCACTCTTTG 3′
p-443: 5′ ATTCCTCGAGGTTTTCCTTGAGTCTTTGGG 3′
p-652: 5′ ATTCCTCGAGGAGGCCCAGAGAGTAACTGC 3′.

Each truncated segment was cloned into the pGL3 enhancer reporter vector. This vector contains an SV40 minimal promoter that drives the expression of the luciferase reporter gene. Sequencing confirmed identity of all constructs.

DNA transfection

Transfections in BET1A cells were accomplished using Liposome (DOTTY, from Boehringer Mannheim, Mannheim, Germany) and 2.5 μg plasmid. To normalize transfection efficiencies, a plasmid expressing renilla luciferase (Promega) was cotransfected with the test plasmid in each experiment. The cell extracts were prepared and firefly luciferase activity was measured. The luciferase assay was performed with Dual-Luciferase Reporter Assay protocol provided by Promega and the activity was normalized to renilla luficerase.

RESULTS

Clinical characteristics

Healthy control and asthmatic individuals were similar in terms of age (control 41±4 year; asthma 39±3 year: $P>0.05$). Pulmonary function testing showed no difference in airflow limitation between groups, although asthmatics had positive methacholine challenge and/or evidence of spontaneous airway reactivity [forced vital capacity (FVC % predicted) control 103 ± 6, asthma 97 ± 4; forced expiratory volume in 1 s (FEV_1 % predicted) control 99 ± 6, asthma 93 ± 4; FEV_1/FVC control 79 ± 2, asthma 78 ± 1; all $P>0.05$]. Bronchoscopy was performed in all individuals without complications.

Recovery of BAL fluid and cells from asthmatic individuals were similar to controls ($P>0.05$). The predominant cells obtained by BAL were >96% macrophages, with the cell differentials and viability (>95%) similar for the two groups [control: macrophages, 96 ± 1; lymphocytes, 3 ± 1; neutrophils, 0.8 ± 0.5; eosinophils, 0.2 ± 0.2; and asthmatics: macrophages, 96 ± 1; lymphocytes, 3.3 ± 1; neutrophils, 0.7 ± 0.5; eosinophils, 0.1 ± 0.2; all comparisons $P>0.05$].

Increased eGPx in BAL fluid

The eGPx protein was increased in ELF of asthmatic individuals [eGPx μg/ml ELF: controls, 6 ± 1 (n=6); asthma, 11 ± 1 (n=7); P=0.02; (**Fig. 1*A***)], although total protein levels were similar between the groups (protein μg/ml ELF: controls, 69 ± 22; asthma, 76 ± 18; P=0.81).

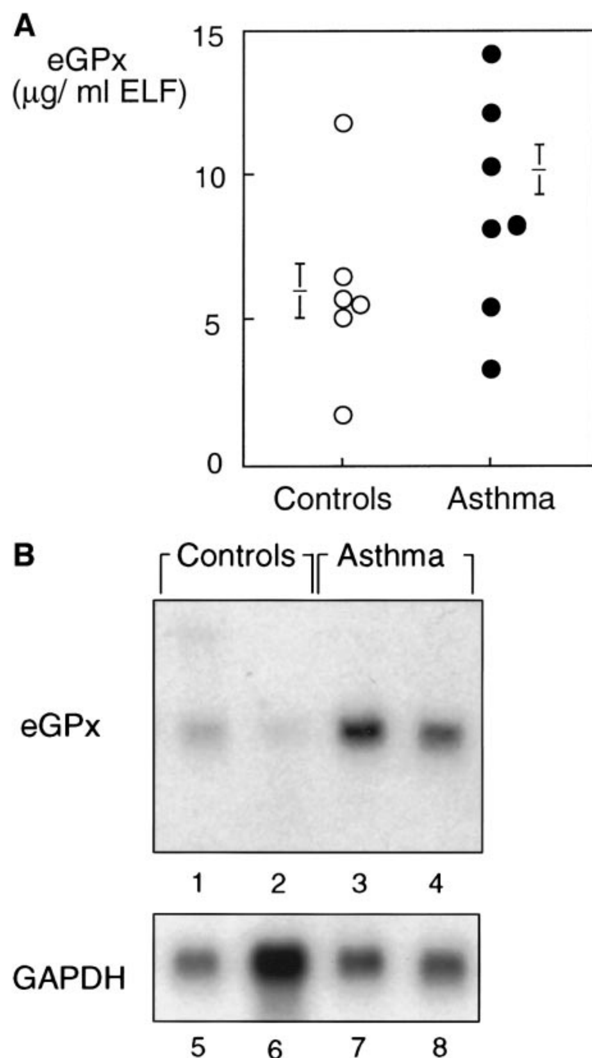


Figure 1. *A)* Increased eGPx in ELF of asthmatic individuals. eGPx is present in healthy control ELF, but is present at higher levels in ELF from asthmatic lungs (P=0.02). Each circle represents eGPx level of a single individual; mean and SE are also shown. Each value is derived from average of duplicate determinations. *B)* Increased eGPx mRNA expression in human airway epithelial cells of asthmatic individuals. Representative Northern analysis of total RNA (10 µg RNA/lane) from airway epithelial cells obtained by bronchial brushing from two healthy controls (lanes 1, 2) and two asthmatic individuals (lanes 3, 4) using a ^{32}P-labeled eGPx cDNA reveals higher eGPx mRNA in asthmatic airway epithelium than controls. GAPDH is shown as control for RNA integrity and loading (lanes 5–8).

Up-regulation of eGPx mRNA

To investigate the mechanisms leading to increased eGPx protein, eGPx mRNA expression was evaluated by Northern analysis of total RNA from airway epithelial cells freshly obtained by bronchoscopic brushing of healthy controls and asthmatic individuals. The predominant cells obtained by bronchial brushing were epithelial cells, with ciliated cells comprising the majority of the epithelial cell type. eGPx mRNA was present at the predicted size of 1.9 kb in all samples using a ^{32}P-labeled eGPx probe (pCCF31). Asthmatic individuals had levels of eGPx mRNA 2.4-fold higher than those of controls [eGPx mRNA/GAPDH mRNA: controls, 6 ± 1 (n=17); asthmatic individuals, 14 ± 3 (n=6); P=0.009] (Fig. 1*B*). Based on these results, we reasoned that elevated eGPx protein in BAL fluid was due to oxidant induction of eGPx gene expression.

Reactive oxygen species induce the eGPx expression

To investigate whether induction of eGPx was related to ROS, BET1A cells or HAEC were exposed to various ROS *in vitro*. Northern analysis showed that both BET1A and HAEC express the eGPx gene in culture with higher eGPx mRNA levels in HAEC (eGPx mRNA/GAPDH mRNA: cultured HAEC, 17 ± 4; BET1A, 1.3 ± 0.4). Furthermore, eGPx mRNA transcripts increased after a minimum of 24 h exposure (P<0.05) to the oxidative stress of pyrogallol (100 µM, P<0.001) (**Fig. 2**). Hydrogen peroxide modestly increased eGPx expression twofold at 48 h [eGPx mRNA/GAPDH mRNA relative to baseline; 10 µM H_2O_2, 1 ± 0.08; 25 µM, 1.02 ± 0.09; 50 µM, 1.3 ± 0.2; 100 µM, 1.9 ± 0.2; (P=0.001)]. Similarly, primary HAEC from healthy controls exposed to pyrogallol (24 h) have increased eGPx mRNA [eGPx mRNA/GAPDH mRNA relative to basal expression: 0.1 µM, 1.2 ± 0.05; 1 µM, 2.6 ± 0.8; 10 µM, 1.7 ± 0.5; (P=0.01)]. Thus, eGPx gene expression is increased in human bronchial epithelial cells in response to ROS.

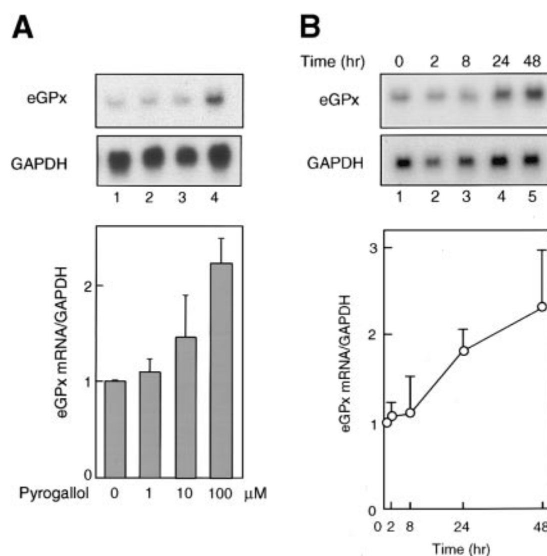


Figure 2. Induction of eGPx mRNA in transformed bronchial epithelial cells (BET1A) by ROS. *A)* BET1A cells were exposed to the superoxide-generating compound pyrogallol (0–100 µM), for 48 h. Representative Northern analysis of total RNA (10 µg/lane) demonstrates increase of eGPx mRNA with 100 µM pyrogallol (lane 4). *B)* Time course of pyrogallol in BET1A cells. The representative Northern analysis of total RNA (10 µg/lane) demonstrates increase of eGPx mRNA with 100 µM pyrogallol at 24 and 48 h (lanes 4, 5). GAPDH is shown as control. Relative units of eGPx mRNA/GAPDH (means±SD) are summarized for a minimum of 3 experiments

Effect of GSH and pyrogallol on the eGPx mRNA

Glutathione is an abundant antioxidant in the intracellular and extracellular lung compartments and an essential cofactor for eGPx reactions (5, 6). The redox state of glutathione is one indicator of the oxidizing state intracellularly and may play a role in the regulation of eGPx expression. Pyrogallol (100 μM) rapidly decreased intracellular GSH in BET1A, followed by a significant increase in GSH at later time points (P=0.002) (**Fig. 3**). Intracellular GSSG increased at 30 min, followed by a decrease to baseline over 48 h (Fig. 3). In contrast, a significant increase in GSSG occurred in the overlying culture media after 8 h of pyrogallol, whereas GSH decreased in the overlying culture media after 8 h (P=0.02) (Fig. 3).

Levels of GSH in culture (μM) are several orders of magnitude lower in comparison to physiological levels (mM) (4, 5); thus, we supplemented GSH in tissue culture media to investigate effects of ROS in more physiological conditions. The 10 mM levels of GSH alone had no effect on the eGPx gene expression. However, a combination of 100 μM pyrogallol with GSH for 24 h strikingly augmented gene induction (P<0.001) (**Fig. 4**), although 10 mM of GSSG did not affect pyrogallol induction of eGPx.

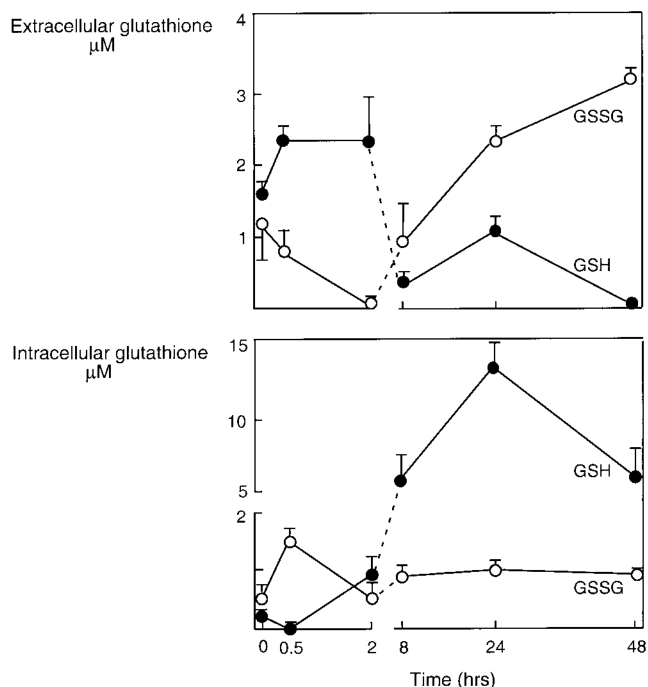


Figure 3. Time course of changes in GSH and GSSG induced by pyrogallol in cultures of BET1A cells. BET1A cells were exposed to 100 μM of pyrogallol for varying times. Supernatant (upper panel) and cells (lower panel) were harvested simultaneously for determination of GSH and GSSG in the extracellular and intracellular compartments. Values are means and SD of a minimum of 3 experiments.

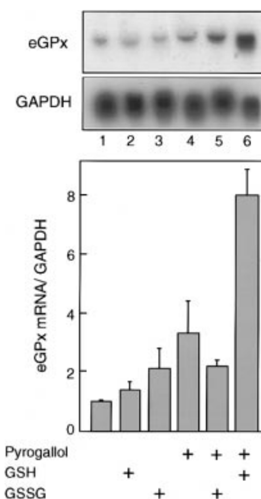


Figure 4. Potentiation of ROS induction of eGPx by GSH. BET1A cells were cultured in the presence of combinations of pyrogallol, GSH, and GSSG for 24 h. Representative Northern analysis of total RNA (10 μg/lane) demonstrates that the combination GSH/pyrogallol significantly increased the eGPx mRNA expression (lane 6). Relative units of eGPx mRNA/GAPDH (means±SD) for 3 experiments are shown.

Increase in extracellular GPx protein and activity

To investigate whether the induction of eGPx mRNA leads to increased eGPx protein and activity, BET1A cells were exposed to pyrogallol, GSH, and a combination of pyrogallol and GSH for 24 and 48 h. The eGPx protein was not detectable intracellularly. However, the overlying media had a significant increase of the eGPx protein by pyrogallol or combination of GSH and pyrogallol [eGPx protein (ng/10^6 cells) mean ± SD: baseline <0.125; pyrogallol, 0.4 ± 0.17; pyrogallol and GSH, 0.5 ± 0.2; P<0.05]. Furthermore, the extracellular GPx activity increased after stimulation of pyrogallol, and in combination with GSH led even to higher levels of activity. However, the intracellular GPx activity was not induced by ROS (**Fig. 5**). Thus, the induction of the eGPx mRNA resulted in increased extracellular activity and protein.

Overexpression of SOD abrogates eGPx mRNA induction

To determine the primary ROS (i.e., superoxide or hydrogen peroxide involved in the induction of the eGPx gene), we infected the BET1A cells with AdSOD and/or catalase (AdCL) (28), which are known to induce the expression and activity of SOD more than 14-fold, and catalase more than 16-fold (28). Null virus (AdNull) or AdCL did not prevent induction of eGPx in BET1A cells. In contrast, the eGPx mRNA induction in cells was significantly inhibited by AdSOD (P=0.02) (**Fig. 6**). These data suggest that eGPx gene induction is dependent in part on intracellular superoxide levels.

eGPx mRNA half-life

Increases in mRNA may occur at the level of transcription or RNA stability, thus eGPx mRNA stability was evaluated in BET1A cells. An inhibitor of RNA synthesis, actinomycin D (AD) prevented pyrogallol induction of eGPx mRNA supporting a transcriptional mechanism [eGPx mRNA/GAPDH relative to baseline eGPx

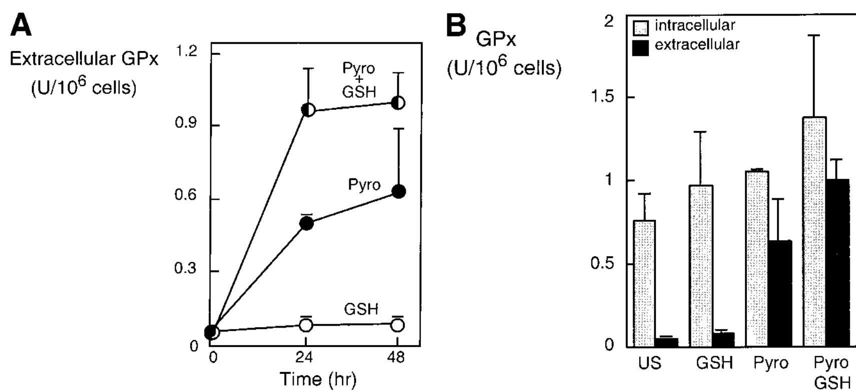


Figure 5. : Increase of extracellular GPx activity by ROS. BET1A cells were exposed to 100 μM pyrogallol or combination of pyrogallol and GSH (10 mM) for various times. Supernatant and the cells were harvested simultaneously for determination of GPx activity in the extracellular and intracellular compartments. *A)* Increased GPx activity in the extracellular compartment is noted at 24 h ($P<0.001$) and 48 h ($P=0.001$). *B)* In contrast to extracellular Gpx, intracellular GPx activity at 48 h did not increase ($P>0.2$). Values are means and SD of 3 experiments.

expression (mean±SD): pyrogallol, 2.7 ± 0.02; pyrogallol and AD, 0.8 ± 0.07; $P=0.001$ (**Fig. 7*A***)]. The eGPx mRNA half-life determined in BET1A cells was prolonged but not affected by ROS [half-life: unstimulated, 34 ± 8 h; GSH/pyrogallol stimulation, 32 ± 9 h (Fig. 7*B*)]. Based on these results, we investigated the regulation of eGPx transcription.

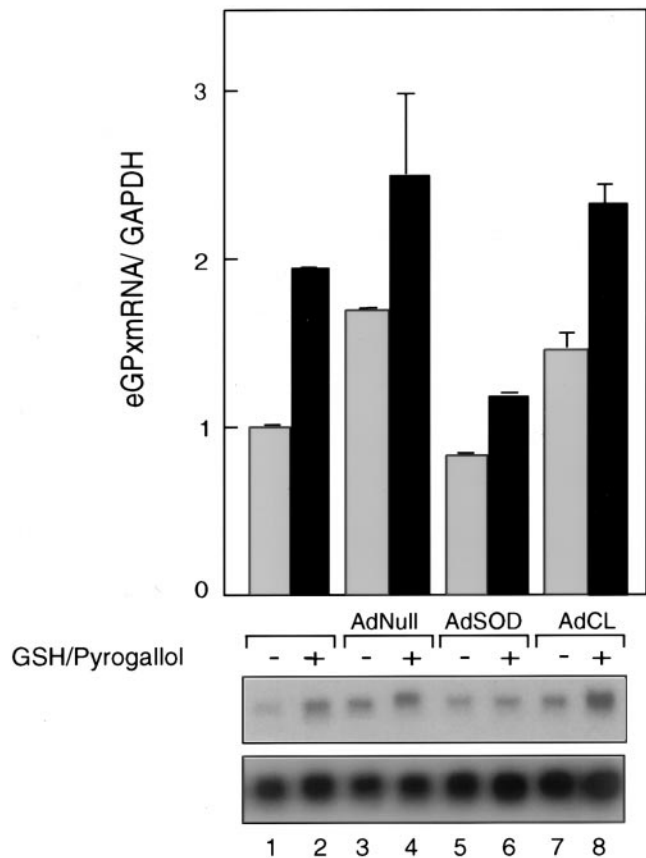


Figure 6. Inhibition of eGPx mRNA induction by adenovirus-mediated transfer of Cu, ZnSOD. Cells were incubated with the control adenovirus (AdNull), vector encoding the human catalase cDNA (AdCL), or vector encoding the human Cu,Zn-SOD cDNA (AdSOD) at MOI of 10/cell in the presence or absence of GSH and pyrogallol for 24 h. eGPx expression was evaluated by Northern analysis (10 μg/lane). The eGPx mRNA normalized to GAPDH is summarized relative to maximal induction by GSH and pyrogallol. Means and SD of a minimum of 3 experiments are shown.

Analysis of 5′ flanking sequence of the human eGPx gene defines region of promoter responsible for gene activation

Comparison of the 5′ flanking region of eGPx promoter with the known sequence (10) revealed 15 insertions, 7 deletions, and 4 mismatches (Genbank accession #AF285633). To test whether the eGPx promoter was responsible for ROS inducibility, a genomic fragment of 3.2 kb, including 5′ upstream sequences and part of the cDNA sequence, was placed 5′ to a firefly luciferase reporter gene, and its capacity to direct firefly luciferase synthesis was compared to a

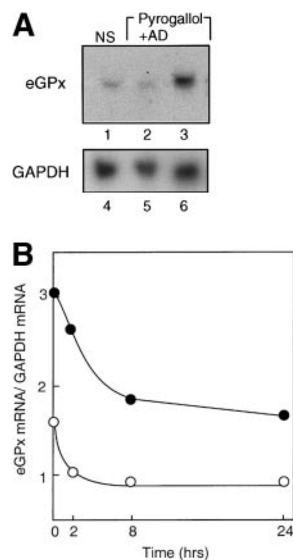


Figure 7. Half-life of eGPx mRNA in BET1A cells. *A)* BET1A cells were cultured in the presence and absence of actinomycin D (3 μg/ml) and stimulated with 100 μM pyrogallol for 24 h. Total RNA (10 μg/lane) was subjected to Northern analysis with ^{32}P-labeled eGPx cDNA and GAPDH cDNA as control for RNA loading (lanes 4–6). Exposure to pyrogallol with AD (lane 2) shows no induction of the eGPx gene, whereas pyrogallol (lane 3) alone induces eGPx mRNA. *B)* BET1A cells cultured in the absence (open circles) and in the presence of GSH (10 mM) and pyrogallol (100 μM) (filled circles) for 24 h. Cells were collected at the times (h) indicated after addition of actinomycin D (3 μg/ml) to block new mRNA synthesis and determine mRNA half-life. Values are means and SD of a minimum of 3 experiments.

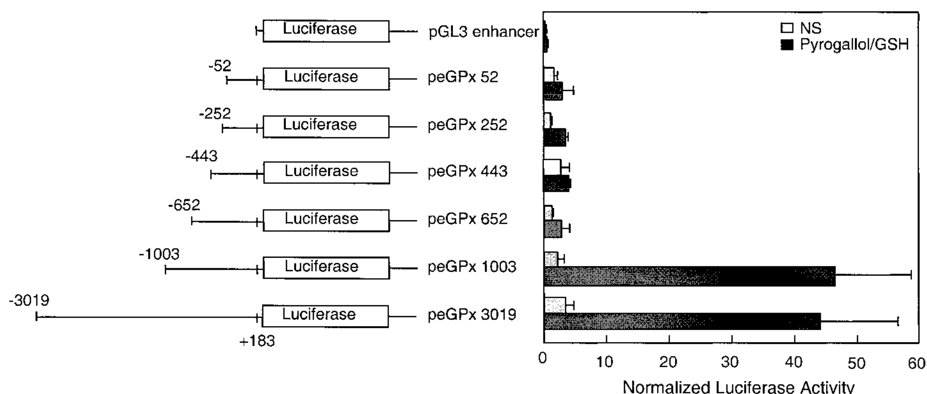


Figure 8. Promoter activity of the eGPx gene 5′ flanking region. Levels of firefly luciferase expression by fusion gene constructs of eGPx 5′ flanking region and a luciferase reporter gene are shown relative to the expression of the renilla luciferase reporter gene. Each point is the average of 3 independent transfection experiments. Promoter function analyses were carried out with BET1A cells incubated with and without GSH/pyrogallol stimulus. Normalized luciferase activity is summarized in the graph, with means and SD of a minimum of 3 experiments shown.

series of deletion mutant constructs in BET1A cells. Cells were transfected and exposed to oxidant stress of GSH and pyrogallol for 24 h. All constructs had low levels of promoter activity in the absence of ROS. The region between −1003 bp and −652 bp led to 45-fold increase in the eGPx promoter activity with ROS (**Fig. 8**).

DISCUSSION

The airway epithelium is an important cellular barrier between the lung parenchyma and the surface epithelial lining fluid. Therefore, these cells are immediately and directly exposed to any change in the redox environment on the airway surface, which makes them especially susceptible to environmental oxidative damage. The presence of eGPx and other extracellular antioxidants on the airway surface undoubtedly protects the lung from external oxidizing damage. Similarly, eGPx serves an important antioxidant role in many other extracellular surfaces and spaces (9, 30–36). Specifically, eGPx transcripts have been demonstrated in epithelial cells with well-developed brush borders, e.g., visceral yolk sac, the intestine, and the S1 and S2 segments of the renal tubules (9, 10, 31). The human airway epithelium expresses and secretes eGPx into the apical surface lining fluid (9). We have recently shown that eGPx in ELF obtained from lungs of cigarette smoking individuals is higher than in nonsmoking controls, which suggests that the airway has the capacity to increase eGPx in response to inhalation of exogenous ROS (4). Here, increases of eGPx are demonstrated in asthmatic airways that have increased endogenous generation of ROS.

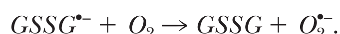
Asthma is a condition characterized by chronic airway inflammation (15–21). Inflammatory and epithelial cells in asthmatic lungs generate increased amounts of ROS that correlate with disease severity (15–21, 35). Asthmatics also have increased nitric oxide (NO) and RNS in the airway due to increased NO synthesis by epithelial cells. NO and superoxide rapidly react to form RNS, which modify tyrosine in proteins by numerous complex mechanisms to create nitrotyrosine, allowing nitrotyrosine to be used as a collective marker of RNS and ROS (15, 22, 36, 37). Despite a clear increase of oxidative and nitrosative stress in the asthmatic airway, intracellular antioxidant enzymes are not increased (1, 2, 20). In fact, asthmatic lung cells have catalase and GPx levels similar to controls and decreased levels of SOD (1, 2). Although eGPx protein is increased in asthmatic ELF in this study, total glutathione peroxidase activity in asthmatic ELF previously has not been different from controls (20). This may reflect that eGPx accounts for only 57% of the total glutathione peroxidase activity in ELF, with the remainder of activity derived from cellular GPx (9). Furthermore, NO, which is increased in asthma, is capable of inactivating GPx (38, 39).

Expression of eGPx mRNA in bronchial epithelial cells in healthy controls indicates that eGPx synthesis and secretion into ELF occur in part by the bronchial epithelial cells. The eGPx protein in ELF may also be due in part to eGPx expression by alveolar macrophages (9). For example, eGPx is expressed in macrophages and is increased with oxidant stress of cigarette smoke (40). However, the striking increase of eGPx mRNA in asthmatic bronchial epithelial cells provides clear evidence that these cells are also the source of the increased eGPx in ELF. Parallel to *in vivo* findings, bronchial epithelial cells significantly increase eGPx mRNA expression in response to increased intracellular or extracellular ROS *in vitro*. Rapid changes in GSH and GSSG in cells and in the overlying supernatant occur after ROS exposure, verifying alterations in the redox environment. Similarly, alterations of GSH and GSSG in asthmatic airways have been reported by us and others in previous studies (1–3, 20, 41). Rapid induction of intracellular GSH is a known response to oxidative stress (42, 43) and a critical determinant of cellular tolerance to oxidizing environments (43). In the present study, exposure to pyrogallol caused a transient depletion of GSH, followed later by a prolonged elevation in intracellular GSH levels. Other protective responses to oxidative stress include uptake of GSH into cells (45, 46) and export of the oxidized form to overcome an accumulation of GSSG within the cytosol. Studies have shown that a 24 h exposure to ROS increases GSH through induction of γ-glutamylcysteine synthetase (γ-GCS) (43, 44).

Since glutathione is a critical cofactor for eGPx activity, coordinate induction of this coupled system is likely necessary for efficient antioxidant defense. For example, previous studies have shown a positive correlation between the eGPx protein and GSH levels in ELF from cigarette smokers (4). Based on this, and because glutathione in cell culture media is more than 100-fold less than in ELF, we tested whether augmentation of glutathione levels would influence the ROS induction of eGPx. Physiological levels of GSH potentiated the effect of ROS on eGPx expression. Overexpression of SOD prevented the induction of eGPx, suggesting the importance of superoxide in eGPx induction. Moreover, the lack of effect on eGPx induction by catalase overexpression indicates that hydrogen peroxide or RNS are less likely key mediators of eGPx induction.

Usually considered an antioxidant, physiological levels of GSH potentiated the effect of ROS on eGPx expression, which may be due to GSH participation in oxidative processes (47–50). In the generation of ROS through autocatalytic processes (i.e., pyrogallol), GSH does not inhibit production of superoxide, but rather promotes the formation of oxidizing species by facilitating autoxidation. At low levels of GSH, autoxidation of compounds is less rapid and initiated by molecular oxygen, which leads to superoxide. However, GSH in the range of 100-1000 μM leads to a dose-dependent increase in oxygen uptake during autoxidation of alloxan, a pyrimidine compound (48). GSH is oxidized to glutathionyl during autocatalytic reactions, which subsequently generate superoxide:

$$GS^{\bullet} + GS^{-} \rightarrow GSSG^{\bullet -}$$



$$GSSG^{\bullet -} + O_2 \rightarrow GSSG + O_2^{\bullet -}.$$

In support of this reaction mechanism, SOD inhibits oxygen uptake and GSH oxidation by alloxan (48). Similarly, GSH may interact in the cyclic process that leads to pyrogallol autoxidation, accelerates free radical production, and subsequently potentiates the oxidant induction of eGPx. Alternatively, recent work suggests that GSH is essential to efficiently transduce oxidative stress into a functional response at the transcriptional level by S-glutathiolation of redox-sensitive transcription factors (51). For example, the oxidant-sensitive cysteine in the DNA binding site of c-Jun, a component of the activator protein 1 (AP-1) transcription factor, undergoes reversible S-glutathiolation during oxidative stress in the presence of physiological levels of GSH. Thus, GSH may potentiate the transcriptional response to oxidative processes inside the cell through several mechanisms.

The regulation of genes in response to ROS may occur via transcription and/or stabilization of mRNA (11–14, 51, 52). Our results show that eGPx mRNA stability is not affected by ROS. In contrast, the 5′ flanking region of the human eGPx gene, which contains the consensus element for AP-1, is exquisitely ROS inducible. Studies from a number of laboratories have demonstrated that ROS induce transcription by the activation of redox-regulated transcription factors, e.g., AP-1 (11–14). For example, the transcriptional induction of γ-GCS by ROS is related to activation of AP-1 (44).

Together with the finding of increased ROS and eGPx expression in asthmatic lungs, the eGPx induction in respiratory epithelial cells by ROS *in vitro* provides strong support for an oxidative mechanism of eGPx gene induction in the asthmatic airway epithelium. Based on this, we propose the following molecular mechanism of eGPx gene induction in asthma. Increased ROS formation by inflammatory and epithelial cells in the lung leads to alterations in the intracellular and extracellular reducing/oxidizing environment, i.e., GSH/GSSG levels. Loss of SOD antioxidant activity is present in asthma and favors increased superoxide and RNS formation. The high level of oxidative and nitrosative stress leads subsequently to induction of eGPx mRNA transcription, protein expression, and secretion into ELF. Since susceptibility of cells to ROS depends largely on the ability to up-regulate protective antioxidant systems (51), increased eGPx is undoubtedly an important defense against oxidative injury to the airway surface of asthmatic individuals. FJ

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