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Oxidant-mediated lung epithelial cell tolerance: the role of intracellular glutathione and nuclear factor-kappaB

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Abstract

The airway epithelium is injured by oxidants inhaled as atmospheric pollutants or produced during inflammatory responses. We studied the effect of modulating the antioxidant intracellular glutathione, both using thiol compounds and by the adaptive effect of hyperoxia, on oxidant-induced injury and activation of the nuclear factor-*kappa*B (NF-κB) in two cell lines: the human bronchial (16HBE) and type II alveolar epithelial cells (A549). The thiol antioxidants glutathione (GSH) and glutathione monoethyl ester (GSH-MEE) [2 mM] increased GSH levels (nmol/mg protein) in A549 cells (GSH 383 ± 26 and GSH-MEE 336 ± 23 vs control 171 ± 13, $P < 0.001$) and in 16HBE cells (GSH 405 ± 33, GSH-MEE 362 ± 37 vs control 198 ± 12, $P < 0.001$, N = 3). Treatment of hyperoxia (95% oxygen) also increased GSH levels between 4 and 24 hr exposure compared with control ($P < 0.01$). Hydrogen peroxide (H_2O_2) (0.01 mM) induced NF-κB activation, whereas hyperoxia exposure did not affect NF-κB activation in either cell line. Pretreatment with DL-buthionine (*SR*)-sulfoximine, which decreased intracellular glutathione, increased NF-κB binding induced by H_2O_2 and increased lactate dehydrogenase (LDH) release ($P < 0.001$). Pretreatment with the thiol compounds and hyperoxia totally inhibited H_2O_2-induced NF-κB binding and cell injury as measured by LDH release. These data indicate the importance of intracellular glutathione and inhibition of NF-κB in both protection/tolerance against oxidant-induced epithelial cell injury, and NF-κB activation in response to oxidative stress which may be important in lung inflammation. Thus, increasing intracellular glutathione may be of therapeutic relevance if able to modulate NF-κB activation and hence attenuate inflammation.

Keywords: GSH; NF-κB; Hyperoxia; Tolerance; 16HBE; A549

1. Introduction

The airway epithelium is a target for ROS either inhaled from the external environment or released during lung inflammation during tissue recruitment and accumulation of leukocytes. In order to protect host tissues during an acute inflammatory response, the alveolar epithelial cells and epithelial lining fluid possess protective antioxidant molecules and enzymes [1]. One such protective molecule is GSH, which is the most abundant, ubiquitous non-protein thiol found in cells in its reduced state [2]. Its reactive sulphydryl group confers GSH with direct antioxidant properties, giving it a pivotal role in cellular defense against oxidative stress. The lung is unique in that the epithelial lining fluid is extremely rich in glutathione (about 10 times higher than plasma) [3]. Inflammatory conditions such as adult respiratory distress syndrome, COPD, smoking, fibrosing alveolitis, cystic fibrosis, HIV seropositive patients, and asthma are all associated with changes in the reduced GSH content of the epithelial lining and possibly also in the pulmonary epithelium [reviewed in Ref. 2]. The levels of intracellular GSH have been shown to be related to the vulnerability of

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Abbreviations: A549 cells, human alveolar epithelial type II cell line; AP-1, activator protein-1; BSO; DL-buthionine (*SR*)-sulfoximine; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GSHMEE, glutathione monoethyl ester; H_2O_2, hydrogen peroxide; IkB, inhibitory binding protein kB; LDH, lactic dehydrogenase; NAC, *N*-acetyl-L-cysteine; NF-kB, nuclear factor-kappa b; NPSH, non-protein sulphydryls; PKC, protein kinase C; and ROS, reactive oxygen species.

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a lung cell to oxidative injury [4–6]. Therefore, maintaining intra-cellular GSH may be an important mechanism in resistance to oxidant toxicity.

Oxidative stress, resulting from excessive release of ROS or depletion of antioxidants, plays an important role in the pathogenesis of many inflammatory lung diseases [2]. ROS also play a critical role in the activation of cells as messengers in the signal transduction pathways leading to gene expression and proliferation [7,8]. NF-κB is a dimeric transcription factor that is present in the cytosol in an inactive form bound to its inhibitory protein, I-κB. When activated by a variety of stimuli, I-κB is phosphorylated, ubiquitinated, and generally degraded in the proteasome releasing active NF-κB which then translocates to the nucleus, where it binds to its consensus site present within the promoters of many genes, such as those of proinflammatory cytokines [9]. Increased activation of NF-κB is thought to occur in several inflammatory lung conditions, for example asthma [10] and chronic obstructive pulmonary disease [11]. NF-κB activation also occurs following an array of different stimuli, such as oxidants, tumor necrosis factor-α and phorbol ester [9]. It has been suggested that each of these different stimuli generate intracellular ROS, which converge into a common signaling pathway responsible for the activation of NF-κB [12,13]. A recent study by Haddad and co-workers has shown the importance of antioxidant/pro-oxidant equilibrium in the redox regulation of NF-κB in rat fetal alveolar type II epithelial cells [14].

Airway epithelial cells are able to act as immune effector cells by secreting proinflammatory mediators [15,16]. Excessive production of proinflammatory mediators by the airway epithelium is proposed to have a key role in the development of tissue injury during acute and chronic inflammatory conditions, implicating the airway epithelium in the pathogenesis of inflammatory lung diseases. Since activation of NF-κB is a crucial event in the up-regulation of these inflammatory mediators, inhibition of the activation of this redox-sensitive transcription factor may therefore have therapeutic implications [17,18]. We have previously shown that increasing intracellular NPSH/GSH can protect against H_2O_2-induced cell death [5]. In view of the importance of glutathione in protecting cells against oxidant-induced injury and the growing interest in the central role of intracellular redox status in cell signal transduction and metabolism [6,8,19], we have assessed herein the effects of oxidants in the presence of augmented and depleted intracellular glutathione on cell injury and NF-κB activation in two human pulmonary epithelial cell lines. The effects of adaptation to the effects of oxidant stress are also presented.

2. Materials and methods

2.1. Materials

GSH, GSSG, 5,5′-dithiobis-(2-nitrobenzoic acid), sulfosalicyclic acid, glutathione reductase, NADPH, triethanolamine, pyruvate kinase, lactate dehydrogenase, BSO, ATP, NAC, GSHMEE, H_2O_2, EDTA, NaCl, and HEPES were obtained from the Sigma and 2-vinylpyridine from Aldrich Chemical Co.

2.2. Cell culture

Two cell lines were used in these studies: the human type II alveolar cell line-A549, obtained from the American Type Cell Collection (ATCC), and 16HBE, a human bronchial epithelial cell line kindly donated by Dr. Gruenert of the University of California, San Francisco, USA [20]. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine at 37°, 5% CO_2. At 95% confluency, the cells were trypsinized and seeded into 100-mm^3 Costar Petri dishes at a density of 3 million cells and grown as above until they had reached 70% confluency (2 days). At this point they were treated as described below.

2.3. Cell treatments

To increase intracellular NPSH and/or GSH levels, cells were treated for 2 and 4 hr with either 2 mM NAC, GSH, or GSHMEE made up in normal growth medium (pH 7.4). The cells were exposed in serum-free medium to two oxidants, either hydrogen peroxide (0.1/1 mM) or to 95% oxygen/5% air (hyperoxia). Hyperoxia is known to increase intracellular GSH levels [21]. To investigate this effect on oxidant-induced cell injury, cells were pretreated with hyperoxia for 12 hr, rinsed twice with warm phosphate-buffered saline, and then treated with 0.1 mM (A549) or 1 mM (16HBE) of H_2O_2 for 16 hr. To investigate the effect of increasing intracellular NPSH/GSH levels on oxidant-induced cell injury, cells were pretreated with either 12 hr of hyperoxia as mentioned above or with 2 mM NAC, GSH, or GSHMEE for 2 and 4 hr followed by treatment with H_2O_2 in the concentrations described above for 30 min. To investigate the effect of depleting intracellular GSH, cells were exposed with 0.1/0.25 mM BSO for 24 hr, rinsed with warm PBS and then exposed to either lower concentration of 0.01 mM H_2O_2 or 95% O_2 for either 16 hr or 30 min, respectively. We used high concentrations of H_2O_2 (0.1 mM for A549 cells and 1 mM for 16HBE cells) to demonstrate the role of glutathione in cellular tolerance. However, a low concentration of H_2O_2 (0.01 mM) was used to study the cellular tolerance with respect to the regulation of NF-κB, without affecting the cell toxicity (LDH release).

2.4. GSH and GSSG assays

Intracellular GSH levels were measured using the method of Tietze [22] with slight modifications [23]. In brief, cells were rinsed twice with warm PBS, scraped off the Petri dish, using Costar cell scrapers, into 1 mL of ice-cold PBS supplemented with 0.01% Triton, homoge-

Table 1
Intracellular GSH and NPSH levels in A549 and 16HBE cells following 2-hr incubation with 2 mM NAC, GSH, or GSHMEE

Treatment	A549		16HBE	
	GSH	NPSH	GSH	NPSH
Control	171 ± 13	185 ± 17	198 ± 12	202 ± 20
2 mM NAC	204 ± 15	325 ± 22***	212 ± 19	350 ± 23***
2 mM GSH	383 ± 26***	377 ± 39***	405 ± 33***	427 ± 23***
2 mM GSHMEE	336 ± 23***	362 ± 22***	362 ± 37***	356 ± 29***

GSH and NPSH levels are expressed as (nmol/mg protein). Data are means ± SEM of N = 3.
*** $P < 0.001$ compared to control values.

nized briefly using a hand-held Teflon homogeniser and spun at 4° for 5 min at 2500 rpm. Twenty microliters of the supernatant was added to 120 μL of 0.1 M phosphate buffer, 5 mM EDTA, pH 7.5, containing 100 μL of 5 mM DTNB and 0.5 units of glutathione reductase. Sixty microliters of 2.4 mM NADPH was added and the rate of change in absorbance measured for 1 min at 410 nm. A standard curve using GSH in the range of 0.33 to 1.35 nmol was prepared prior to making measurements in the samples. The results were expressed in nmoles of GSH per milligram of total protein. For the GSSG assay, supernatant was treated with 2-vinylpyridine and triethanolamine as described previously [24] and thereafter was used in the assay for GSH as described above.

2.5. NPSH assay

Intracellular NPSH were measured by a standard technique using Ellman's reagent [25]. In brief, 500 μL of the sample supernatant was added to 2.5 mL of 0.1 M potassium phosphate−5 mM EDTA buffer containing 5 mM DTNB, (pH 8), and the absorbance at 412 nm was measured over 6 min. A standard curve was generated using GSH in the range of 0.33 to 1.35 nmol. The results were expressed per milligram of protein.

2.6. LDH assay

Extracellular LDH as a percentage of total extra and intracellular was measured as an index of cell injury. Following treatments, the extracellular medium was collected. The cells were scraped into 1 mL of ice-cold PBS containing 0.01% Triton and placed on ice for 15 min. All the samples were then spun at 1000 rpm, 4°C for 5 min and the supernatants were then used to measure LDH using the method of Wrobleski and Ladue [26]. The amount of LDH released into the extracellular medium was expressed as the percentage of the total intra- and extracellular content.

2.7. Total protein assay

Total protein was measured from the supernatants in all samples using the bicinchoninic acid assay [27].

2.8. Electrophoretic mobility shift assay

Nuclear protein was extracted from the cells as described previously [28]. Labeling reaction was performed using NF-κB-specific double-stranded oligonucleotide (Promega) with [γ-^{32}P]ATP. Binding experiments were performed with 5 μg of nuclear protein, 2 μL of 5X binding buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 25% glycerol, 5 mM EDTA, 0.25 mg/mL of poly(dl-dC).poly(dl-dC) and 1 μL of the radiolabelled NF-κB-specific oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ (Promega) for 20 min at room temperature. Non-denaturing 8% polyacrylamide gel electrophoresis was performed with 0.5X Tris borate–EDTA buffer, pH 8.0 for 4 hr at 100 V. The gel was dried for 30 min after which the autoradiography was carried out. To monitor the specificity of the binding reaction, the assay was performed in the presence of 1000-fold excess of the non-labelled oligonucleotide [28].

2.9. Statistical analysis

All data were expressed as the mean and standard error of the mean (SEM) of 3 experiments. Means were compared by analysis of variance and a two-way unpaired *t*-test (Skewness analysis showed data to be normally distributed). $P < 0.05$ was considered as significant value. The densitometric intensities of the NF-κB-specific bands were measured relative to the control samples. These experiments were repeated three times and the mean and SEM of these relative intensities plotted.

3. Results

Treatment of A549 and 16HBE cells with 2 mM NAC, 2 mM GSH, and 2 mM GSHMEE increased intracellular NPSH levels ($P < 0.001$) at 2 hr (Table 1) with a return to control levels by 4 hr and maintained at this level for up to 24 hr (data not shown). The increases in intracellular NPSH following treatment with GSH and GSHMEE were associated with increases in intracellular GSH in both cell lines ($P < 0.001$), whereas treatment with NAC increased

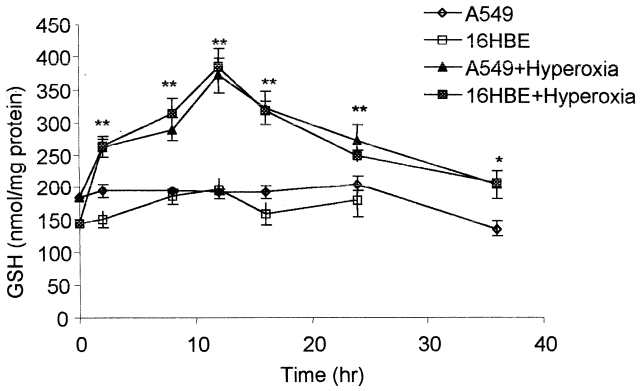

Fig. 1. Intracellular GSH levels in A549 and 16HBE cells following incubation in hyperoxia (95% O_2/5% air) for up to 36 hr. Treatments are as follows: (1) control A549 cells, (2) control 16HBE cells, (3) A549 cells incubated in hyperoxia, and (4) 16HBE cells incubated in hyperoxia. Data are expressed as the means ± SEM (N = 3). *$P < 0.05$, **$P < 0.01$, compared to control values.

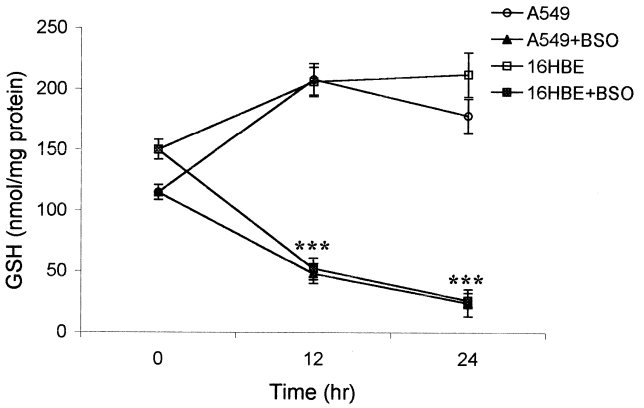

Fig. 2. Intracellular GSH levels in A549 and 16HBE cells following incubation for 24 hr with 0.1 and 0.25 mM of BSO, respectively. Treatments are as follows: (1) control A549 cells, (2) A549 cells incubated with 0.1 mM BSO, (3) control 16HBE cells, and (4) 16HBE cells incubated with 0.25 mM BSO. Data are expressed as the means ± SEM (N = 3). ***$P < 0.001$ compared to control values.

NPSH in both cells lines but without an increase in intracellular GSH over this time point (Table 1). However, 2 mM NAC produced an increase in intracellular GSH at 4-hr treatment in both cells (A549, 305 ± 11 vs control 171 ± 13; 16HBE 321 ± 18 vs 198 ± 12, N = 3, $P < 0.001$). The increased GSH levels returned to control levels by 6 hr and maintained at this level for up to 24 hr (data not shown). The levels of GSSG (nmol/mg protein) did not change at 2 hr by these thiol treatments in either A549 cells (NAC 10.4 ± 2.2, GSH 12.8 ± 4, GSHMEE 13.2 ± 3.8 vs control 11.2 ± 1.8, N = 3), or in 16HBE cells (NAC 14.9 ± 3.2, GSH 16.1 ± 4.2, GSHMEE 14.8 ± 2.9 vs control 12.8 ± 2.6, N = 3).

Exposure of A549 and 16HBE cells to hyperoxia (95% O_2/5% air), in serum-free medium increased intracellular GSH levels (and associated NPSH levels, data not shown) after 4, 6, 12, and 24 hr exposure compared to cells grown in 5% CO_2/95% air ($P < 0.01$) (Fig. 1). GSSG levels were unchanged in response to hyperoxia exposure either in A549 or in 16HBE cells (data not shown). The level to which intracellular GSH was increased was similar to that following the treatments with GSH and GSHMEE (Table 1). Treatment of A549 cells and 16HBE cells with 0.1 and 0.25 mM BSO respectively, for 24 hr decreased intracellular GSH levels ($P < 0.001$) (Fig. 2). There was no significant difference in GSSG levels in response to BSO treatment at 24 hr (A549, 8.9 ± 3.4 and 16HBE, 11.8 ± 3.0 vs controls A549, 10.8 ± 1.2 and 16HBE, 12.8 ± 2.6 nmol/mg protein).

Pretreatment of A549 and 16HBE cells with hyperoxia for 12 hr significantly decreased H_2O_2 (0.1/1 mM)-induced LDH release compared to H_2O_2 treatment alone ($P < 0.05$) (Fig. 3). However, pretreatment of A549 cells or 16HBE cells with 0.1 or 0.25 mM BSO respectively for 24 hr increased LDH release following 24 hr of hyperoxia in both cell lines ($P < 0.001$) and also increased LDH release in A549 cells following 24 hr exposure to 0.01 mM H_2O_2 ($P < 0.05$) (Fig. 4). BSO alone did not affect significant LDH release (%) in either cell type.

Treatment with H_2O_2 (0.01 mM) for 2 hr increased NF-κB nuclear binding, as seen in the electrophoretic mobility shift assay in both A549 (Fig. 5, a and b) and 16HBE cells (Fig. 6, a and b). This was associated with the increased GSSG levels (nmol/mg protein) in A549 (H_2O_2 21.8 ± 2.2 vs 11.2 ± 2.0 controls, N = 3, $P < 0.01$), and in 16HBE (H_2O_2 24.3 ± 4.2 vs 10.8 ± 1.9 controls, N = 3, $P < 0.01$). H_2O_2 (0.1 mM) treatment caused NF-κB activation in both cell lines (data not shown), which was associated with increased LDH release (Fig. 3). In contrast, A549 and 16HBE cells treated with hyperoxia for up to 2 hr did not show any increase in the NF-κB nuclear binding when compared to control cells (Fig. 5, a and b and Fig. 6,

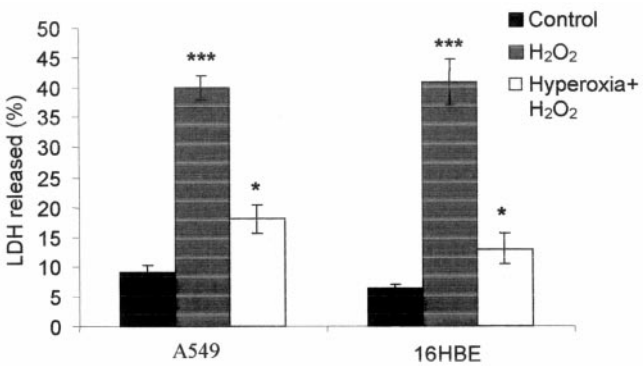

Fig. 3. The effect of pretreatment with hyperoxia (95% O_2/5% air) for 12 hr on the concentration of LDH in the extracellular medium as a percentage of the total intra- and extracellular LDH following treatment of A549 and 16HBE cells with 0.1 and 1 mM H_2O_2, respectively, for 16 hr. Treatments are as follows: (1) control, (2) 0.1/1 mM H_2O_2, and (3) pretreatment for 12 hr with hyperoxia followed by exposure to 0.1/1 mM H_2O_2 for 16 hr. Each histogram represents the mean and the error bars the SEM of three experiments. *$P < 0.05$, ***$P < 0.001$, compared to control values.

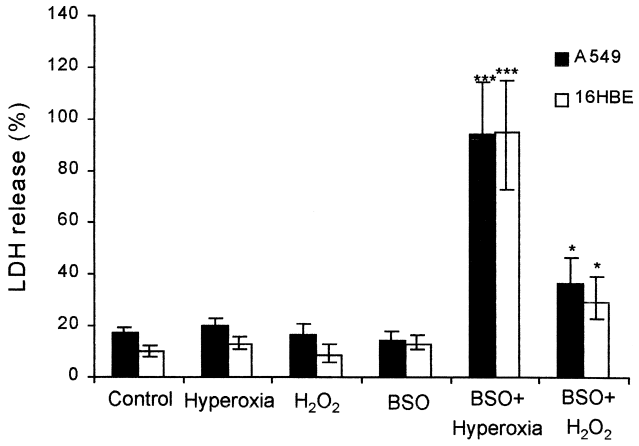

Fig. 4. The effect of pretreatment of A549 and 16HBE cells with 0.1 or 0.25 mM BSO respectively for 24 hr on the percentage of LDH in the extracellular medium as a percentage of the total intra- and extracellular LDH following exposure for 24 hr to either hyperoxia or 0.01 mM H_2O_2. Treatments are as follows: (1) control, (2) 24-hr hyperoxia exposure, (3) 24-hr exposure with 0.01 mM H_2O_2, (4) pretreatment with BSO for 24 hr, 0.1 mM for A549 and 0.25 mM for 16HBE cells, (5) pretreatment with BSO for 24 hr, rinsed with PBS followed by 24-hr exposure to hyperoxia, and (6) pretreatment with BSO for 24 hr, rinsed with PBS followed by 24-hr exposure to 0.01 mM H_2O_2. Each histogram represents the mean and the error bars the SEM of three experiments. $^*P < 0.05$, $^{***}P < 0.001$, compared to control values.

a and b). Two and four hours incubation with 2 mM NAC, GSH, or GSHMEE had no effect on NF-κB nuclear binding in either cell line (data not shown). Whereas pretreatment of A549 and 16HBE cells with BSO (0.1 and 0.25 mM, respectively) for 24 hr increased NF-κB nuclear binding induced by 0.01 mM H_2O_2 ($P < 0.001$) compared to the marginal BSO alone treatment effect (Fig. 5, a and b and Fig. 6, a and b). Similarly, pretreatment with BSO (0.1 mM) for 24 hr also increased NF-κB nuclear binding following hyperoxia treatment in the A549 cell line ($P < 0.001$) (Fig. 5, a and b). Pretreatment of A549 and 16HBE cells with hyperoxia for 12 hr or with 2 mM NAC, GSH, or GSHMEE for 2 hr totally inhibited 0.01 mM H_2O_2-increased NF-κB nuclear binding in both cell lines (Fig. 6, a and b). Similar results were obtained when the cells were treated with 2 mM NAC at 4 hr (data not shown).

4. Discussion

We and other investigators have previously shown that increasing intracellular GSH can provide protection against hydrogen peroxide-induced cell injury [5,6,29]. In this study, we show that prior exposure to a non-lethal concentration of another oxidant, hyperoxia (95% O_2), can lead to the development of protective response/tolerance against subsequent hydrogen peroxide-induced cell injury. This adaptive response was concomitant with an increase in intracellular GSH levels. These data are in agreement with

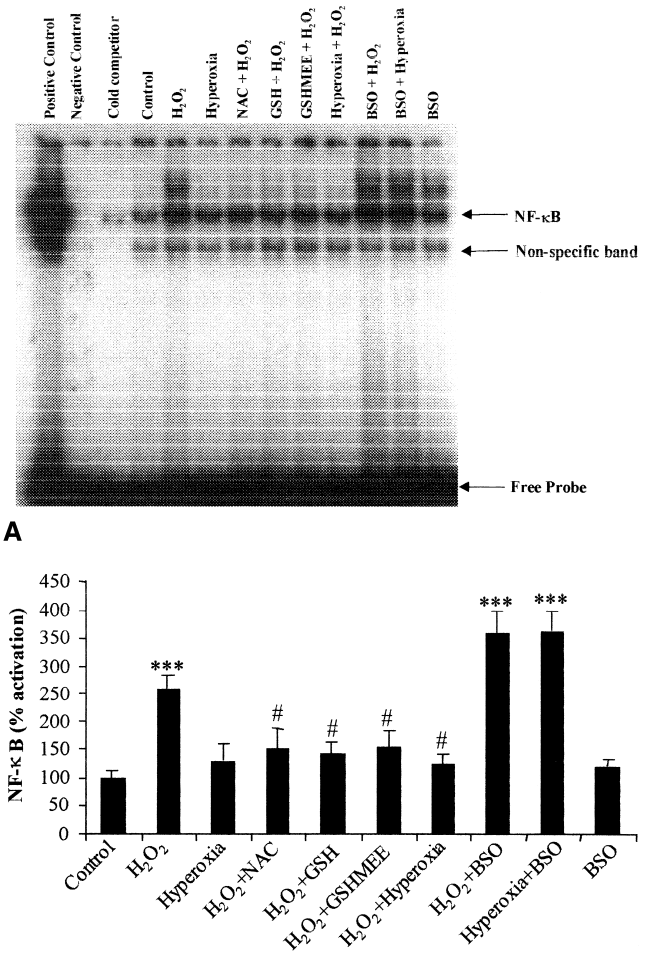

Fig. 5. (a) Electrophoretic mobility shift assay of nuclear extracts isolated from A549 cells showing nuclear binding of NF-κB following various treatments. Nuclear extracts were prepared and analysed by electrophoretic mobility shift assay using (γ^{32}P)-labeled NF-κB oligonucleotide. The DNA–protein complexes formed are indicated (arrow) of NF-κB-specific band following exposure of A549 cells to hyperoxia or 0.01 mM H_2O_2 and the protective effects of pretreatment with the thiol compounds [2 mM] NAC, GSH, or GSHMEE; pretreatment with hyperoxia (95% O_2/5% air) or pretreatment with BSO for 24 hr. Treatments are as follows: control; 2-hr incubation with 0.01 mM H_2O_2; 2-hr incubation with hyperoxia; 2-hr pretreatment with exogenous 2 mM NAC followed by 2-hr exposure to 0.01 mM H_2O_2; 2-hr pretreatment with exogenous 2 mM GSH followed by 2-hr exposure to 0.01 mM H_2O_2; 2-hr pretreatment with exogenous 2 mM GSHMEE followed by 2-hr exposure to 0.01 mM H_2O_2; 12-hr pretreatment with hyperoxia followed by 2-hr exposure to 0.01 mM H_2O_2; 24-hr pretreatment with 0.1 mM BSO followed by 2-hr exposure to 0.01 mM H_2O_2; 24-hr pretreatment with 0.1 mM BSO followed by 2-hr exposure to hyperoxia and 24-hr treatment with 0.1 mM BSO. Negative water control; positive HeLa nuclear extract control and cold competitor using 100-fold molar excess of unlabelled oligonucleotides. Autoradiographs shown are representative of at least three separate experiments. (b) Densitometric quantitation of specific NF-κB binding was compared with the control values set at 100%. The histograms represent the mean values and the error bars the SEM of the relative intensity of the bands of three experiments. $^{***}P < 0.001$, compared to control values, and $^{\#}P < 0.001$ compared to H_2O_2 alone.

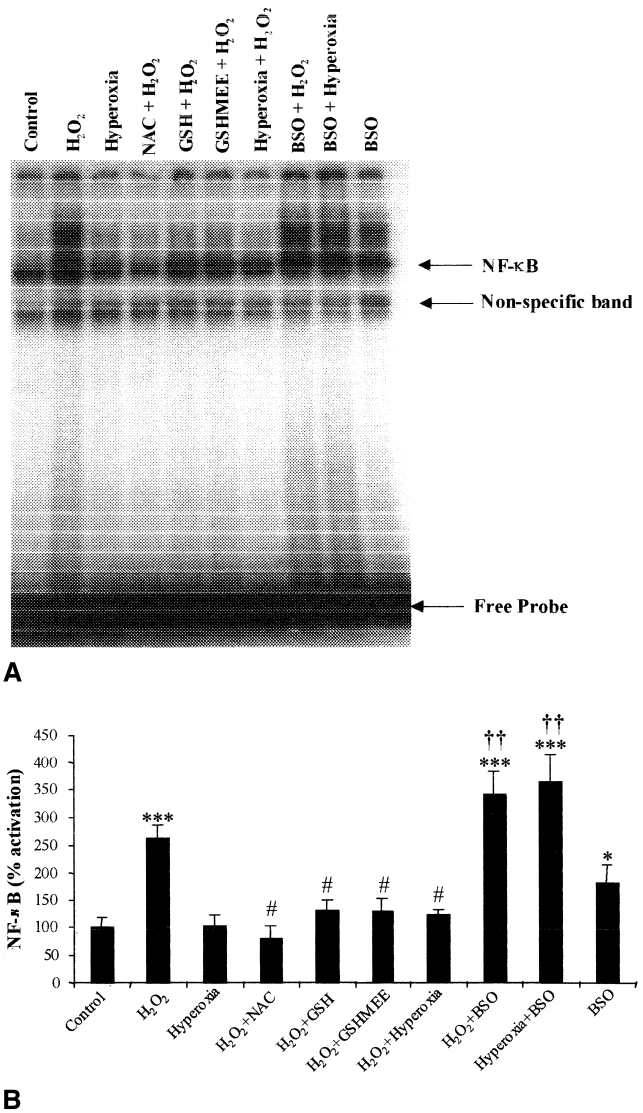

Fig. 6. (a) Electrophoretic mobility shift assay of nuclear extracts isolated from 16HBE cells showing nuclear binding of NF-κB following various treatments. Nuclear extracts were prepared and analysed by electrophoretic mobility shift assay using (γ^{32}P)-labeled NF-κB oligonucleotide. The DNA–protein complexes formed are indicated (arrow) of NF-κB-specific band following exposure of 16HBE cells to hyperoxia or 0.01 mM H_2O_2 and the protective effects of pretreatment with the thiol compounds [2 mM] NAC, GSH, or GSHMEE; pretreatment with hyperoxia (95% O_2/5% air) or pretreatment with BSO for 24 hr. Treatments are as follows: control; 2-hr incubation with 0.01 mM H_2O_2; 2-hr incubation with hyperoxia; 2-hr pretreatment with exogenous 2 mM NAC followed by 2-hr exposure to 0.01 mM H_2O_2; 2-hr pretreatment with exogenous 2 mM GSH followed by 2-hr exposure to 0.01 mM H_2O_2; 2-hr pretreatment with exogenous 2 mM GSHMEE followed by 2-hr exposure to 0.01 mM H_2O_2; 12-hr pretreatment with hyperoxia followed by 2-hr exposure to 0.01 mM H_2O_2; 24-hr pretreatment with 0.25 mM BSO followed by 2-hr exposure to 0.01 mM H_2O_2; 24-hr pretreatment with 0.25 mM BSO followed by 2-hr exposure to hyperoxia and 24-hr treatment with 0.25 mM BSO. Autoradiographs shown are representative of at least three separate experiments. (b) Densitometric quantitation of specific NF-κB binding was compared with the control values set at 100%. The histograms represent the mean values and the error bars the SEM of the relative intensity of the bands of three experiments. $^{*}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{***}P < 0.001$, compared to control values, and $^{\#}P < 0.001$ compared to H_2O_2 alone.

the earlier reports of the role of GSH in the development of tolerance against oxidative stresses [30,31]. Our data are also supported by the recent studies of Franek and co-workers showing that oxidant-induced apoptosis is inhibited in lung epithelial cells pre-exposed to hyperoxia [32]. We show that depletion of intracellular GSH enhanced the effect of H_2O_2 on cell lysis/toxicity as measured by LDH: this was even more pronounced when GSH was depleted and then followed by hyperoxia exposure. Thus, it is likely that hyperoxia causes more oxidant-induced cell injury in GSH depleted cells than does H_2O_2 treatment.

GSH has a high affinity for hydrogen peroxide through the enzyme glutathione peroxidase. Due to its highly nucleophilic sulphydryl group, it can react with electrophilic agents such as the superoxide radical, which is the major free radical generated in hyperoxic conditions. Several lines of evidence suggest that ROS may contribute to cell activation including the induced expression of several genes encoding proinflammatory cytokines [2,9]. In this study we show that depletion of intracellular GSH (increased GSSG levels or increased GSH/GSSG ratio) regulates the activation of NF-κB. NF-κB is an oxidant-sensitive transcription factor that plays a central role in inflammatory and immune regulation [3,13]. It is believed that hydrogen peroxide acts as a second messenger in NF-κB activation in various cell lines, which in turn is critical for maximal expression of many cytokines [9,12]. It is also believed that excessive cytokine-mediated inflammation is likely to be pathogenic in several inflammatory pulmonary conditions such as asthma, adult respiratory distress syndrome, and idiopathic pulmonary fibrosis [9,11,33]. The present study shows that increasing intracellular GSH or non-protein thiols by use of thiol compounds decreases oxidant-induced NF-κB nuclear binding in lung epithelial cells. Similar results were obtained in rat fetal alveolar epithelial cells, showing that NF-κB is redox-sensitive and is tightly regulated by the GSH/GSSG equilibrium, and that NAC, a glutathione precursor, ameliorated NF-κB activation [14]. In addition, NAC has previously been reported to inhibit cytokine-stimulated NF-κB activation, suggesting that this thiol compound might be effective where oxidant stress is driving inflammation [34]. Interestingly, our results show that the development of an adaptive response by mild pretreatment with hyperoxia also decreases the level of oxidant-induced NF-κB activation in both cell lines. This may be due to the fact that exposure of A549 and 16HBE cells to hyperoxia increases GSH synthesis [5,21]. Hyperoxia exposure alone did not result in induction of NF-κB nuclear translocation in both cell types. Similarly, Allen and co-workers also did not find any induction of NF-κB activation by hyperoxia exposure in A549 cells [35]. Further confirmation of the potential role of GSH in NF-κB regulation also comes from our data, which show that BSO pretreatment (which depletes GSH by inhibition of γ-glutamylcysteine synthetase) in both cell lines enhances NF-κB when the cells are exposed to either hyperoxia or H_2O_2. This suggests that (a) GSH plays a key

role in the regulation of NF-κB and oxidant-induced cellular tolerance and (b) the development of tolerance is associated with inhibition of NF-κB activation in epithelial cells. There are other reports which support our observation: for example (i) pretreatment with low doses of endotoxin, which also acts via generation of oxidants, has been associated with inhibition of subsequent endotoxin-induced NF-κB activation in rat lungs [34] and (ii) the development of a stress response to TNF-α treatment in L929 fibroblast cells is also associated with decreased NF-κB activation [36]. However, neither of these studies implicated the role of intracellular GSH in NF-κB related tolerance.

There is increasing evidence which links the redox status of a cell with inhibitory/stimulatory effects on signalling pathways [6,12,37]. Phosphorylation and dephosphorylation of proteins have been shown to depend on enzymes sensitive to the redox status of the environment in which they act [7,8]. It is, therefore, likely that intracellular GSH redox levels are also important in such processes.

We suggest that part of the protective effect of increased intracellular glutathione, either by exogenous means or during the development of an adaptive response, is to inhibit oxidant-induced NF-κB activation. Prolonged activation of NF-κB may result in the expression of several proinflammatory cytokines and other mediators of inflammation which may, in excess, have detrimental effects on the host tissues [9,14,36]. Thus, increasing intracellular glutathione (reduced thiols) may be of clinical therapeutic relevance if able to modulate NF-κB activation and hence attenuate inflammation. Hyperoxia pre-exposure may provide tolerance to lung epithelial cells by buffering the glutathione antioxidant capacity. This may be important in protection against a inflammatory/oxidative stress response that occurs in inflammatory lung diseases [38].

In summary, this study shows the importance of intracellular GSH in protecting the pulmonary epithelium against oxygen and hydrogen peroxide-induced injury and suggests that changes in intracellular GSH levels are associated with adaptive responses to oxidants and may modulate NF-κB activation. Furthermore, the development of tolerance may be directly associated with inhibition of NF-κB activation in epithelial cells.

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