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Hydrogen peroxide-induced epithelial injury: the protective role of intracellular nonprotein thiols (NPSH)

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Hydrogen peroxide-induced epithelial injury: the protective role of intracellular nonprotein thiols (NPSH). B. Mulier, I. Rahman, T. Watchorn, K. Donaldson, W. MacNee, P.K. Jeffery. ©ERS Journals Ltd 1998.

ABSTRACT: Injury to the alveolar region is a hallmark of the adult respiratory distress syndrome (ARDS) whereas injury to the epithelium of the conducting airways is a characteristic of asthma. Reactive oxygen species have been implicated as mediators of lung injury in both of these conditions.

We have investigated the relationship between intracellular nonprotein thiols (NPSH), and the release of the cytosolic enzyme lactate dehydrogenase (LDH), as an index of cell injury, following treatment of the human alveolar type II-like epithelial cell line (A549 cells) or the human bronchial epithelial cell line (16HBE140-) with hydrogen peroxide (H_2O_2). We have also assessed the protective effects of pre-incubation of both of these cells lines with H_2O_2 or enhancement of intracellular NPSH against H_2O_2-induced cell injury.

Exposure of A549 and 16HBE140- cells to H_2O_2 (0.1 mM and 1 mM respectively for 16 h) produced the release of 40% of the total cellular LDH. H_2O_2 exposure produced an initial dose-dependent decrease in NPSH in A549 cells, with a subsequent increase to above control values. 16HBE140- cells also showed a dose-dependent decrease in NPSH following exposure to H_2O_2. Pretreatment of A549 cells with 0.1 mM H_2O_2 followed by subsequent exposure to H_2O_2 did not protect against H_2O_2-induced LDH release in this epithelial cell line. Pre-incubation with 2 mM N-acetylcysteine (NAC) increased NPSH but not intracellular reduced glutathione and resulted in total inhibition of H_2O_2-induced LDH release in both cell types. Pretreatment with reduced glutathione protected both cell types against the injurious effects of H_2O_2, whereas glutathione monethyl ester (GSHMEE) only partially protected A549 cells and had no effect in 16HBE140- cells. Intracellular cysteine levels were increased in both cell lines following NAC exposure but not sufficiently to account for the increase in NPSH levels.

These observations raise the possibility that a critical concentration of nonprotein thiols may be necessary to protect pulmonary epithelial cells against hydrogen peroxide-induced injury.

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The epithelium lining the airways and alveoli acts a protective barrier against inhaled toxins. In response to injury the epithelium loses its selective permeability and becomes more permeable to movement of water, ions and macromolecules. The lower respiratory tract is particularly sensitive to injury from inhaled and locally produced oxidants. Alveolar cells are normally covered in a thin protective layer of epithelial fluid which is rich in antioxidants such as glutathione [1]. Following acute oxidant stress the epithelial lining fluid may become depleted of antioxidants, increasing the potential for damage to the underlying epithelial cells. Damage to the alveolar epithelium is associated with the development of adult respiratory distress syndrome (ARDS) [2]. Damage and shedding of the bronchial epithelium is a characteristic finding in asthma and is associated with increased airway responsiveness [3].

Thiols are potentially powerful antioxidants since they contain a sulphydryl (-SH) group in their structure and are readily oxidised by giving up their hydrogen atom to form stable disulphide bonds. Reduced glutathione, a tripeptide consisting of cysteine, glutamic acid and glycine, is a low molecular weight thiol. In addition to its antioxidant properties, it can conjugate xenobiotics to aid their elimination [4–6]. Reduced glutathione reacts to form glutathione disulphide, either nonenzymatically by direct reaction with the xenobiotic, or *via* glutathione peroxidase (GPx). Nonprotein thiols (henceforth referred to as NPSH) is a term used to encompass all low molecular weight thiolcomponds. Reduced glutathione contributes around 90% of the intracellular NPSH. The remaining 10% is made up of other small thiol compounds such as cysteine and methionine [7].

Previous work has shown that animals exposed to 100% oxygen suffer lung injury and subsequently die with signs of progressive respiratory distress [8]. However, pre-exposure to sublethal doses of oxygen (85%) imparted resistance to a subsequent usually lethal dose. Enhanced NPSH

and reduced glutathione in lung homogenates [9] and increased antioxidant enzyme activities in alveolar epithelial and endothelial cells [8] have been associated with this adaptive response.

The aim of the present study was to assess the sulphydryl antioxidant response in alveolar and bronchial epithelial cell lines, cultured *in vitro*, and exposed to the oxidant hydrogen peroxide (H_2O_2). H_2O_2 was chosen to induce injury since it is released in relatively large amounts by activated leucocytes during the process of the lung inflammation which is known to occur in both asthma and ARDS. We wished to determine if pre-exposure to H_2O_2 could protect against the effects of exposure to H_2O_2 and in addition to assess the protective effect of a range of thiol compounds which have the potential to increase intracellular reduced glutathione.

Material and methods

Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM); all substrates, standards and chemicals necessary for the lactate dehydrogenase (LDH), NPSH and reduced glutathione assays were obtained from the Sigma Chemical Co., Poole, UK. Cell culture supplements (foetal calf serum (FCS), penicillin (P)/streptomycin (S), L-glutamine (L-G) and the mixture of nonessential amino acids (AA), and trypsin-ethylendiamine tetraacetic acid (EDTA)) were obtained from Gibco/Life technologies, Glasgow, UK. The protein assay kit was purchased from Pierce and Warriner (UK) limited, Chester, UK.

Cell culture

The human alveolar type II-like epithelial cell line (A549) [10] was obtained from the American Type Culture collection and grown in complete medium (DMEM; supplemented with 10% FCS, 1,000 U·mL^{-1} P, 1 mg·mL^{-1} S (P/S) and 2 mM L-G). Cells were used between passages 42 and 76 when the average intracellular reduced glutathione levels were shown to be stable (data not shown). The human bronchial epithelial cell line (16HBE140-) [11] was kindly donated by D. Gruenert (University of California), and grown in complete medium (MEM; supplemented with 10% FCS, PS, L-G and 1% solution of AA). Cells were used between passages 5 and 29 when intracellular reduced glutathione levels were stable (data not shown).

Both cell lines were grown as monolayers in Costar tissue culture flasks. at 37°C, 5% CO_2 until 95% confluent. The cells were then removed with trypsin, spun and then seeded at 3×10^6 cells·100 mm diameter culture dish^{-1}. Cell numbers were determined using a standard haemocytometer (Weber Scientific Inter. Ltd., Middlesex, UK). Cell viability was checked by trypan blue exclusion. The cells were left for 24 h to reach 75% confluency before treatment.

Treatments

The medium was removed, the cells washed twice with 5 mL sterile phosphate-buffered saline (PBS) and 5 mL of serum-free (SF) medium. H_2O_2, appropriately diluted, was added at time 0 and the cells incubated for up to 16 h without further addition of H_2O_2. The H_2O_2 concentration for absorbance was checked at 240 nm prior to each addition. In some experiments the cells were pretreated for 2 h by adding 5 mL of 2 mM solutions of N-acetylcysteine (NAC) to each culture dish, reduced glutathione and glutathione monethyl ester (GSHMEE), made up in complete medium to a pH of 7.

LDH assay. After each treatment LDH activity was measured in the extracellular medium and in the supernatant obtained after rupturing the cells with 0.01% Triton-X-100 in phosphate buffer pH = 7.4 for 1 h on ice. The cells were than spun at 2,500 revolutions per minute (rpm) and the LDH measured by the method of Wroblewski and Ladue [12]. In brief, the rate of decrease of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm during the formation of sodium lactate from sodium pyruvate was assessed. LDH release from cells was expressed as percentage of the total (intracellular and extracellular) LDH. Correction was made for LDH activity in FCS and the inhibitory effect of H_2O_2 by setting up standard curves containing similar concentrations of FCS and H_2O_2 to those used in the experiments.

NPSH assay. NPSH were measured by a modified Ellman's assay [13]. In brief, the cells were rinsed with PBS, scraped into 1 mL of ice-cold 0.6% sulphosalicylic acid (SSA), treated with Triton X-100 at 0.01% for 1 h on ice, spun and the supernatant assayed. Dithionitrobenzene (DTNB) (2.25 mL of a 0.5 mM solution) was added to 0.5 mL of each sample which was kept on ice. The absolute absorbance at 412 nm was measured 6 min later. Different concentrations of reduced glutathione were used to obtain a standard curve. NPSH levels were then expressed per milligram of protein.

Total reduced glutathione assay. Total reduced glutathione levels were measured, in the same samples used for NPSH measurements to minimize variations due to differences in cell densities, confluency and cell batches, using the reduced glutathione redox cycling method of Tietze [14]. Glutathione reductase and DTNB were added to 50 µL of each sample in PBS (pH = 7.5) and the rate of change of absorbance at 412 nm was measured following the addition of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The results were determined by extrapolation from a reduced glutathione standard curve. Reduced glutathione standards contained Triton-X-100 and SSA in quantities identical to the samples. The results were expressed per milligram protein.

Intracellular cysteine levels. Cysteine levels were kindly measured by J.R. Bonham and S. Moat, Dept of Paediatric Chemistry, Sheffield Hospital for Children, using an adapted method of Araki and Sako [15]. In brief, cysteine concentrations were analysed in deproteinized cell homogenates by a simple but sensitive and selective high performance liquid chromatography (HPLC) method using precolumn derivatization with 7-fluoro-2-oxa-1,3-diazole-4- sulphonate(SBD-F). These levels were then expressed as per milligram of protein.

γ-glutamycysteine synthetase (γ-GCS) activity. The activity of γ-GCS, the rate limiting enzyme in the synthesis of reduced glutathione, was measured in supernatant from cell homogenates. Two hundred microlitres of the supernatant were added to 800 µL of reaction buffer containing 10 mM 1-aminobutyric acid, 5 mM adenosine triphosphate disodium salt (Na_2ATP), 2 mM phosphoenol phosphate, 0.2mM NADH, 20 mM magnesium chloride ($MgCl_2$), 150 mM potassium chloride (KCl), 10 mM L-G, 2 mM ethylene diamine tetra acetic acid disodium salt (Na_2EDTA), 17 µg of pyruvate kinase and 17 µg of LDH. The rate of decrease of absorbance at 340 nm was followed for 1 min. The concentration of γ-GCS activity was calculated using the absorbance coefficient 6.6 [16]. Buthionine sulphoximine (BSO), an inhibitor of γ-GCS activity [17] was used as the control to eliminate the possible role of other metabolic pathways. Samples were divided into two and the assay was performed with or without the presence of 0.1 mM of BSO.

Protein assay. Protein levels were measured using the bicinchionic acid kit from Pierce and Warriner (UK) Limited with bovine serum albumin (BSA) as the standard [18].

Statistical analysis. All data were expressed as mean±SEM of between 4 and 12 samples. Mean values were compared by analysis of variance (ANOVA) and a two way unpaired t-test.

Results

Exposure to H_2O_2 induced a dose and time-dependent increase in LDH release in both cell lines. (figs. la and 1b). After 16 h exposure of A549 cells to 0.1 mM H_2O_2 and 16HBE140- cells to 1 mM H_2O_2, LDH release was 40% of the total and was associated with a 40% decrease in cell numbers compared to the control cells. The viability of the remaining cells was unchanged, at least as assessed by trypan blue exclusion.

Removal of serum from the culture medium of the A549 cells caused a 1.8–2 fold ($p<0.01$) decrease in NPSH which was maintained throughout 24 h. The viability of the remaining cells was unchanged, but cell numbers decreased from $4.5\pm0.17\times10^6$ to $3.74\pm0.14\times10^6$ ($p<0.05$) after 16 h which was associated with 18% LDH release. 16HBE140- cells were less sensitive to the removal of serum from the medium. There was no change in cell numbers ($4.8\pm0.22\times10^6$ to $5.18\pm0.27\times10^6$), or in LDH release but NPSH levels showed an initial dose-dependent decrease at 2 h, ($p<0.05$) which returned to control levels by 6 h.

Treatment with H_2O_2 caused an initial dose-dependent decrease in NPSH at 2 h ($p<0.05$) in both cell lines. For the A549 cells the initial decrease was followed by a subsequent recovery by 8 h and an increase, to above serum-free control levels at 16 and 24 h ($p<0.05$). However, the 16HBE140- cells did not recover their initial fall and the decrease in NPSH was maintained throughout the 24 h period (fig. 2). Similar changes to those with NPSH occurred for reduced glutathione following treatment with H_2O_2 in both cell lines (data not shown).

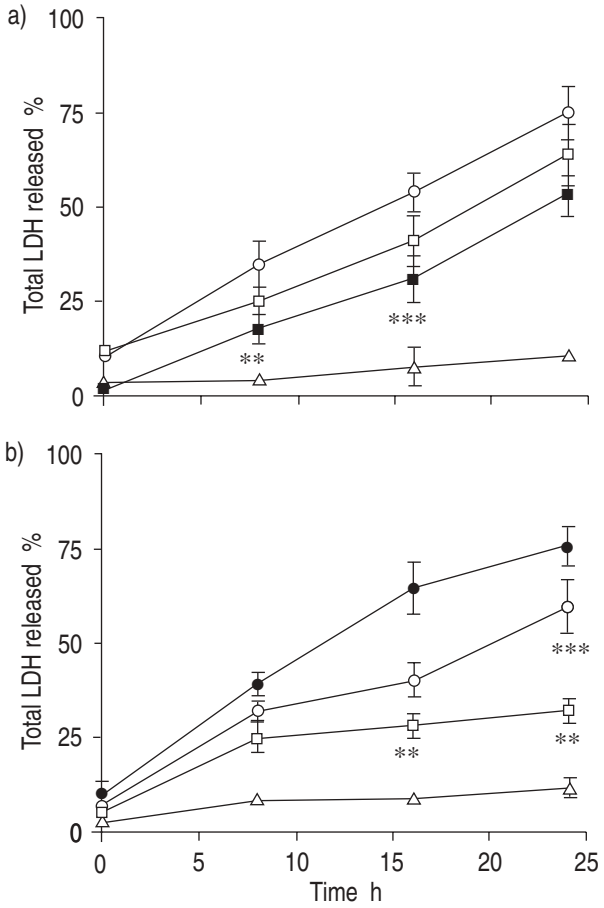

Fig. 1. – The dose and time-independent effects of hydrogen peroxide (H_2O_2) on lactate dehydrogenase (LDH) release in: a) human alveolar type II-like cell line (A549 cells) (0.1 mM H_2O_2) and; b) human bronchial epithelial cell line (16HBE140- cells) (1 mM H_2O_2). –■–: 50 µM H_2O_2; –□–: 100 µM H_2O_2; –●–: 5 mM H_2O_2; –○–: 1 mM H_2O_2; –△–: 10% serum control. The symbols represent mean values and the bars the SEM of 10 experiments. **, ***: $p<0.01$, $p<0.001$ compared to 10% serum control.

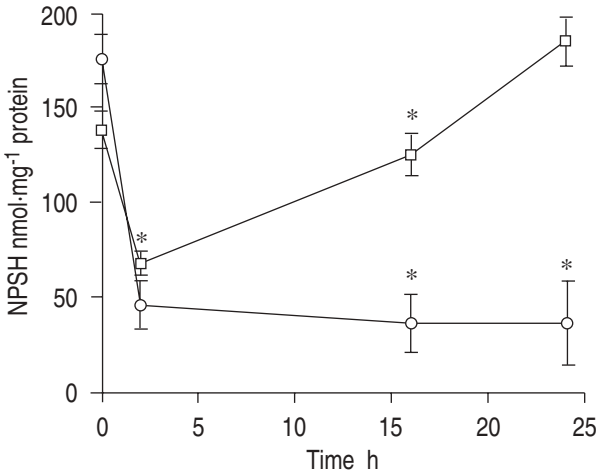

Fig. 2. – The effect of hydrogen peroxide (H_2O_2) on nonprotein thiol (NPSH) levels in human alveolar type II-like cell line (A549 cells) and cell line (16HBE140- cells). –□–: 0.1 mM H_2O_2 on A549 cells; –○–: 1 mM H_2O_2 on 16HBE140- cells. The symbols represent mean values and the bars the SEM of 10 experiments. *: $p<0.05$ compared to serum-free controls (not shown).

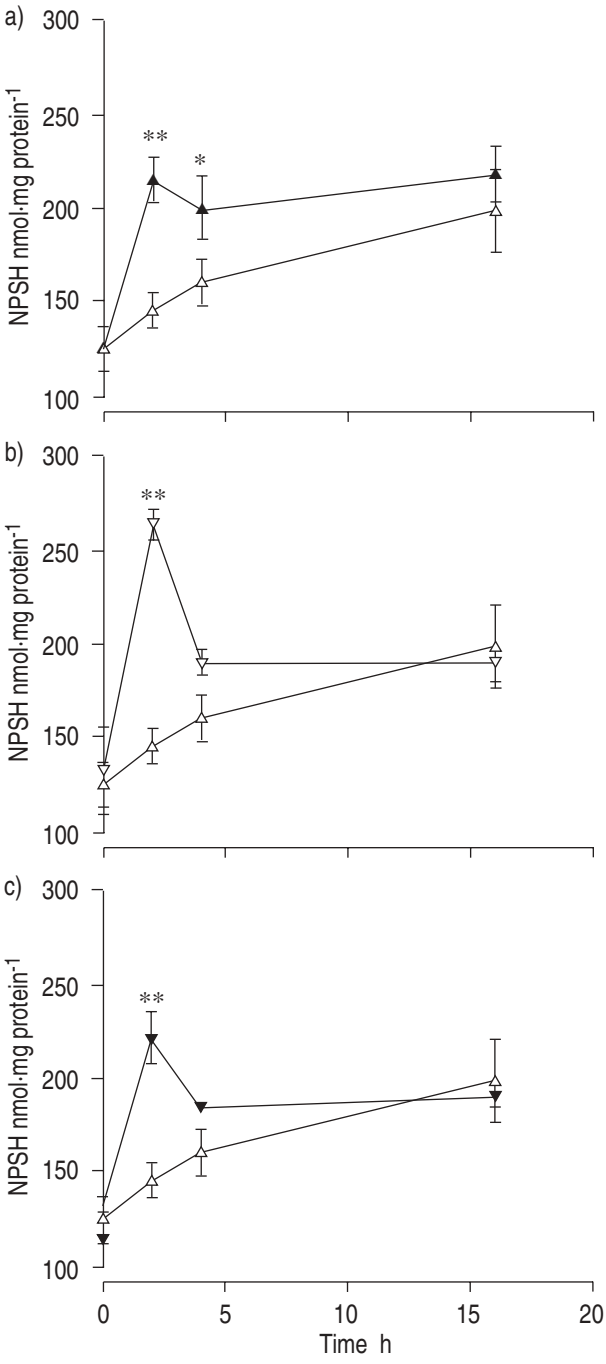

Fig. 3. – The effect of: a) N-acetylcysteine (NAC); b) reduced glutathione (GSH); and c) glutathione monethyl ester (GSHMEE) on nonprotein thiol (NPSH) levels in human alveolar type II-like cell line (A549 cells). –△– : 10% serum control; –▲– : 2 mM NAC; –▽– : 2 mM GSH; –▼– : 2 mM GSHMEE. The symbols represent mean values and the bars the SEM of 10 experiments. *, **: p<0.05, p<0.01 compared to 10% serum controls.

Treatment of both cell lines with exogenous NAC, reduced glutathione and GSHMEE (2 mM) over 24 h caused a dose-dependent increase in NPSH. The three sulphydryl compounds at this concentration did not affect the viability or the proliferative potential of the cells (data not shown). For both cell lines the maximal increase in NPSH occurred 2 h after treatment. The increases were greater for the 16HBE140- cells than for A549 cells following NAC (p<0.01) and reduced glutathione (p<0.001) whereas for GSHMEE there was no difference between the two cell lines. For the A549 cells NAC caused a twofold (p< 0.01), 2 mM reduced glutathione caused a 2.3 fold (p< 0.01) and GSHMEE a 1.8 fold (p<0.01) increase in NPSH respectively at 2 h compared to control values. NAC treatment maintained NPSH levels above control values at 16 h, whereas NPSH levels following treatment with exogenous reduced glutathione and GSHMEE had returned to control levels by 16 h (fig. 3). For the 16HBE140- cells NAC and reduced glutathione caused a fourfold (p<0.01) and threefold (p<0.01) increase respectively, in NPSH levels at 2 h, whereas GSHMEE caused a 2.2 fold (p<0.05) increase compared to control. With all treatments the increase in NPSH returned to control values by 16 h.

The increase in NPSH following treatment with exogenous reduced glutathione and GSHMEE was associated with a similar increase in intracellular reduced glutathione. This was not the case for NAC treatment, in both cell types (table 1). Intracellular cysteine was also increased around 1.5 fold at 2 h following 2 mM NAC treatment compared to control cells (fig. 4).

Based on the results described, a 2 h pretreatment time for exogenous reduced glutathione, GSHMEE and NAC was chosen, which corresponded to the maximal increase in NPSH. A 16 h pretreatment time with H_2O_2 was chosen which also corresponded to the time of maximal increase in NPSH. A 16 h treatment time with 0.1 mM and 1 mM

Table 1. – The effects of 2 h incubation with various thiol treatments to increase intracellular nonprotein thiol (NPSH) and reduced glutathione (GSH) in airspace epithelial cells

Treatment	A549 cells		16HBE140- cells	
	NPSH	GSH	NPSH	GSH
Control	145±8	120±10	171±44	152±9
NAC	225±12***	138±9	548±35***	192±15
GSH	265±1***	245±18***	464±55***	433±37***
GSHMEE	217±22*	199±17*	335±42*	315±23***

Values are presented as nanomole per milligram of protein. A549 cells: human alveolar type II-like cell line; 16HBE140- cells: human bronchial epithelial line. *, ***: p<0.05, p<0.001, compared to control values.

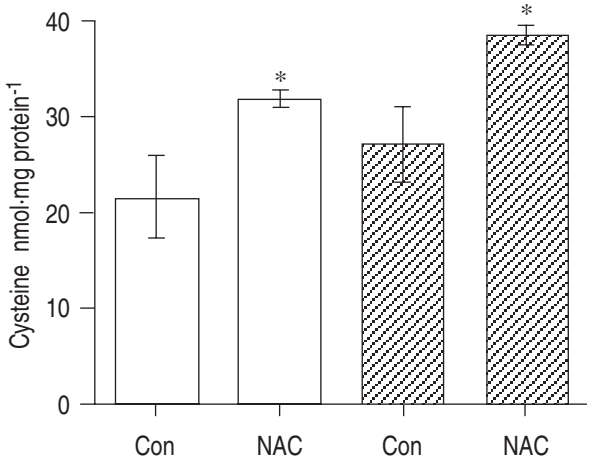

Fig. 4. – The effect of 2 h of N-acetylcysteine (NAC) (2 mM) on intracellular cysteine levels in human alveolar type II-like cell line (A549 cells) and human bronchial epithelial line (16HBE140- cells). □: A549 cells; ▨: 16HBE140- cells. Con: control. The histograms represent mean values and the bars the SEM of three experiments. *: p<0.05 compared to controls.

H_2O_2 was chosen for A549 cells and 16HBE140- cells, respectively. These concentrations caused 40% LDH release from both cell lines.

In both cell types pretreatment with NAC and reduced glutathione reduced the H_2O_2-induced increase in LDH release. NAC also protected alveolar epithelial cells from the injurious effect of the removal of serum (fig. 5a). Pretreatment with GSHMEE protected A549 cells against H_2O_2 to the same extent as reduced glutathione but had no effect in the 16HBE140- cells (figs. 5a and b).

Pretreatment with 2 mM NAC, reduced glutathione and GSHMEE in 2% serum-medium, rather than 10% serum-medium, produced a lesser increase in NPSH, which did not protect against H_2O_2-induced injury in either cell lines (data not shown) [19].

In order to assess the development of resistance, we pretreated A549 cells with 0.1 mM H_2O_2 for 16 h, which we have shown, caused an increase in NPSH/reduced glutathione levels. However, this did not protect against a subsequent dose of 0.1 mM H_2O_2 (fig. 6). Pretreatment of the A549 cells with 50 μM H_2O_2, and 16HBE140- cells with 0.1 mM, both which caused only 25% LDH release from the cells respectively at 16 h treatment (figs 1a and b) but a similar increase in NPSH/reduced glutathione compared to treatment with 0.1 mM H_2O_2, also had no protective effect on subsequent LDH release following 0.1 mM H_2O_2 treatment (data not shown).

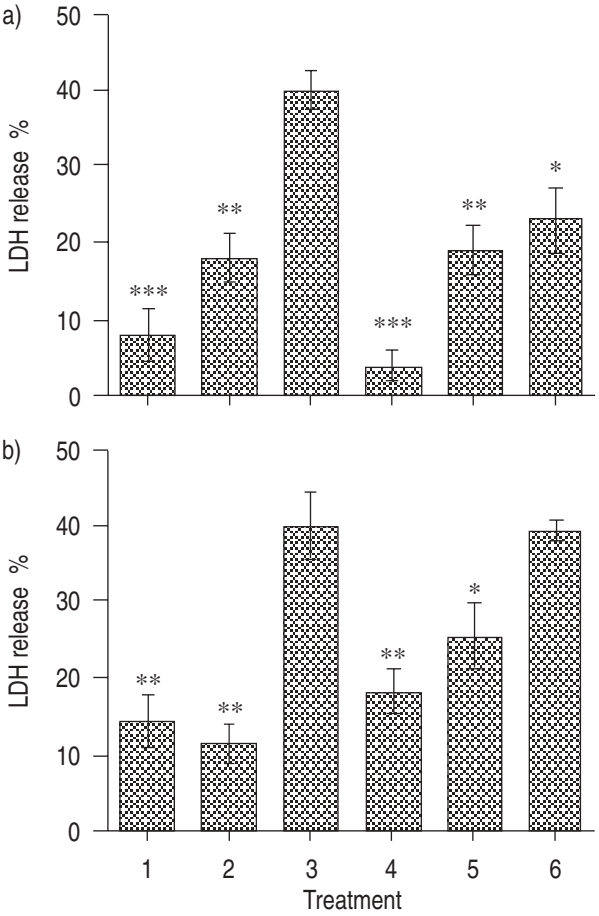

Fig. 5. – The protective effect of N-acetylcysteine (NAC), reduced glutathione (GSH) and glutathione monethyl ester (GSHMEE) (2 mM) pretreatment on hydrogen peroxide (H_2O_2)-induced lactate dehydrogenase (LDH) release in: a) human alveolar type II-like cell line (A549 cells); and b) human bronchial epithelial line (16HBE140- cells). Treatments: 1) 10% serum control; 2) serum-free control; 3) 40% lethal H_2O_2; 4) pretreatment with NAC followed by treatment with 40% lethal H_2O_2; 5) pretreatment with GSH followed by treatment with 40% lethal H_2O_2; 6) pretreatment with GSHMEE followed by treatment with 40% lethal H_2O_2. The histograms represent mean values and the bars the SEM of 10 experiments. *, **, ***: $p<0.05$, $p<0.01$, $p<0.001$ compared to 40% lethal H_2O_2 alone.

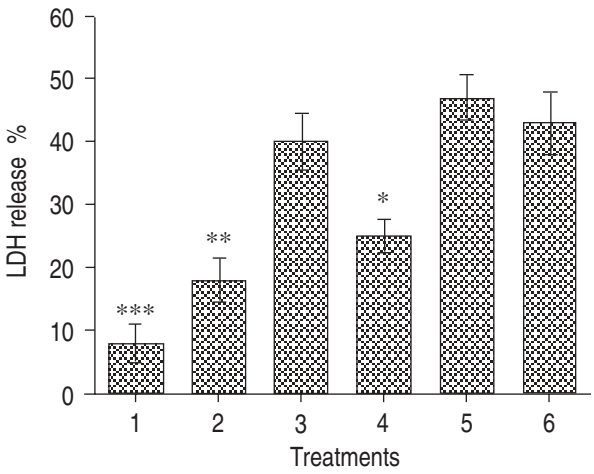

Fig. 6. – The protective effect of 0.1 mM and 0.05 mM hydrogen peroxide (H_2O_2) on 0.1 mM H_2O_2-induced lactate dehydrogenase (LDH) release in human alveolar type II-like cell line (A549 cells). Treatments: 1) 10% serum control; 2) serum-free control; 3) 0.1 mM H_2O_2 for 16 h in serum-free medium; 4) 50 μM H_2O_2 for 16 h in serum-free medium; 5) pretreatment with 0.1 mM H_2O_2 for 16 h followed by treatment with 0.1 mM H_2O_2 for 16 h both in serum-free medium; 6) pretreatment with 50 μM H_2O_2 for 16 h followed by 0.1 mM H_2O_2 for 16 h both in serum-free medium. The histograms represent mean values and the bars the SEM of 10 experiments. *, **, ***: $p<0.05$, $p<0.01$, $p<0.001$ compared to 0.1 mM H_2O_2 alone, *i.e.* treatment.

The activity of γ-GCS increased 2.5 fold and threefold in A549 and 16HBE140- cells, respectively, following 2 h removal of serum from the medium. The activity also increased to a similar level in A549 cells during 0.1 mM H_2O_2 treatment. However, 16HBE140- cells showed a 1.5 fold decrease in γ-GCS activity following the H_2O_2 treatment. In both cell types, NAC had no effect on γ-GCS activity

Table 2. – Effects of various treatments on gamma-glutamycysteine synthetase (γ-GCS) activity in airspace epithelial cells

	A549 cells	16HBE140- cells
Control (4 h)	18.4±0.8	17.5±3.8
Control (16 h)	21.4±2.0	9.2±2.3
Serum-free control (4 h)	31.7±4.9*	24.4±0.6*
Serum-free control (16 h)	29.3±0.9*	40.2±4.7
H_2O_2 (4 h)	37.5±5.2*	5.5±1.4*
H_2O_2 (16 h)	30.9±1.5*	8.5±0.7
NAC 2 mM (4 h)	17.2±2.4	15.3±0.8
NAC 2 mM (16 h)	19.6±1.5	9.2±0.2
GSH 2 mM (4 h)	38.5±2.4*	29.3±3.3*
GSH 2 mM (16 h)	17.6±2.9	8.1±0.4
GSHMEE 2 mM (4 h)	26.2±0.8*	27.8±0.8*
GSHMEE 2 mM (16 h)	15.0±0.7*	10.2±0.2

Values are presented as milliunits of γ-GCS per milligram of protein. Incubation times in parenthesis. A549 cells: human alveolar type II-like cell line; 16HBE140- cells: human bronchial epithelial cell line. H_2O_2: hydrogen peroxide; NAC: N-acetylcysteine; GSH: reduced glutathione; GSHMEE: glutathione monethyl ester. *: $p<0.05$ for 4 and 16 h incubations compared to control 4 and 16 h incubations, respectively.

whereas both exogenous reduced glutathione and GSHMEE produced a 1.5 fold increase in γ-GCS activity after 4 h exposure, with a subsequent return to control levels after 16 h exposure (table 2).

Discussion

This study has shown that a critical level of intracellular NPSH or reduced glutathione can provide protection against injury by H_2O_2. We have compared the inherent capacity of two cell lines and their response to exogenously administered thiols to increase the intracellular NPSH pool and have shown that there are differences which may affect their capacity to mount a protective response to oxidant stress.

Whereas the A549 cells responded to H_2O_2-induced depletion of NPSH/reduced glutathione by a subsequent increase of NPSH levels to a value above that of the control, the 16HBE140- cells lacked the capacity to recover their normal level of NPSH following this insult. This may result from a greater injury to these cells as shown by the decrease in γ-GCS activity which did not recover to allow further reduced glutathione synthesis. The inability to increase γ-GCS activity could be due to loss of enzyme activity or failure of γ-GCS gene transcription or alteration at the post-transcriptional level. Recent studies in rat type II alveolar cells have shown both γ-GCS and γ-glutamyl transpeptidase (γ-GT) activities and messenger ribonucleic acid (mRNA) levels are increased following an oxidant stress [20] by a mechanism involving transcriptional regulation [21]. Further investigations are necessary to verify this in hypothesis.

Cysteine is usually the rate-limiting amino acid in reduced glutathione synthesis. Under culture conditions, most of the cysteine is present as the oxidised dimer cystine [22] and removal of serum from the extracellular medium, which is rich in cysteine [23], may indirectly influence the intracellular cysteine pool. This may explain the lower NPSH and reduced glutathione levels under serum-free culture conditions in A549 cells [24]. Many studies have shown species and cell variations in cysteine and cystine transport [22, 23]. It may be that 16HBE140- cells are capable of utilizing cystine more efficiently than A549 cells for the synthesis of reduced glutathione and are therefore less sensitive to the removal of serum from their growth medium.

It is interesting to note that the bronchial cells were more resistant to H_2O_2 than the alveolar cells since 10 times more H_2O_2 was necessary to produce the same degree of LDH release although both cell lines had similar intracellular reduced glutathione levels. The capacity of 16HBE140- cells to withstand higher doses of H_2O_2 suggests that they may have an alternative major source of antioxidant activity which protects them or their cell membrane against H_2O_2. The most likely candidate is catalase. A549 cells are reported to have low catalase activity [25] which may explain their reliance on reduced glutathione for protection against H_2O_2. Studies in rat primary type II alveolar cells have shown that catalase is important in protecting against H_2O_2-induced injury and that reduced glutathione-dependent reactions also have a protective effect probably by a mechanism other than direct reaction with H_2O_2 [26]. Intracellular cysteine levels were higher in 16HBE140- cells than in A549 cells. This may in part account for their enhanced resistance to H_2O_2.

Our results show that in contrast to pretreatment with hyperoxia, which resulted in acquired protection to a subsequent dose of oxygen in previous studies [22], pretreatment with either of two doses of H_2O_2, which produced two levels of toxicity (40% and 25% LDH release respectively), but increased NPSH and reduced glutathione level to the same extent, did not protect against a subsequent toxic dose of H_2O_2.

The three sulphydryl compounds NAC, reduced glutathione and GSHMEE increased intracellular reduced glutathione to levels higher than those obtained by pretreatment with H_2O_2. NAC has been used to increase pulmonary reduced glutathione levels and many reports have indicated NAC as an effective antioxidant [27, 28]. The mechanism by which NAC enhances intracellular NPSH is unclear. It has been suggested that NAC may become deacylated in the extracellular medium yielding cysteine, which is taken up by a specific transport system and subsequently used to synthesise intracellular reduced glutathione [29]. Another possibility is that NAC reduces extracellular cystine, which is plentiful in the culture medium, to cysteine which is then taken up into the cells [30]. It seems that in general cysteine is taken up at a faster rate than cystine, by two distinct transport systems, although there may be differences between cell types [22, 23]. These cellular transport systems may be influenced by treatment with thiols or oxidants, leading to enhanced uptake [4, 23, 31]. Our results show clearly that NAC increased intracellular NPSH levels, but had no effect on intracellular reduced glutathione. Although intracellular cysteine in-creased within the cells, this increase did not result in increased reduced glutathione synthesis. Our data show that although intracellular cysteine levels were increased slightly following NAC treatment, this was not enough to account for the increase in intracellular NPSH. Therefore, another mechanism of action in increasing NPSH must be involved. It is possible that NAC is taken up into the cells, by an as yet unknown mechanism. and that deacylation occurs slowly to release cysteine. This explanation would account for the increase in intracellular cysteine and NPSH levels. This would also explain why intracellular NPSH levels were still elevated at up to 16 hours.

Removing serum from the medium increased γ-GCS activity in both cell lines, which may therefore allow cysteine, from the deacylation of NAC, to be utilized to synthesise reduced glutathione. It seems that in our experimental model NAC will only increase intracellular reduced glutathione if the cell is deficient in reduced glutathione [20].

Exogenous reduced glutathione has also been shown to increase intracellular reduced glutathione *in vitro*. There are several theories on how this may occur. Some epithelial cells can take up intact reduced glutathione by as yet an unknown mechanism [31]. However, one study has suggested that A549 cells do not have this capacity [32]. Reduced glutathione may also be broken down in the extracellular medium by γ-GT in the presence of an acceptor AA to γ-glutamylamino acid and the dipeptide cysteinylglycine. These two moieties can be subsequently taken up by the cell and reduced glutathione is subsequently resynthesised by the actions of γ-GCS and glutathione synthetase (GS) [33]. It has been recently suggested that

exogenous reduced glutathione may behave in a manner similar to NAC and reduce extracellular cystine to cysteine which is then taken up and resynthesised to reduced glutathione intracellularly [34]. It seems likely that different cell types have distinct mechanisms of action and that a combination of these mechanisms may operate. Our results show that exogenous reduced glutathione increases intracellular NPSH and reduced glutathione to the same extent. γ-GCS activity increased with reduced glutathione treatment in A549 cells. Therefore, regardless of the mechanism by which exogenous reduced glutathione enters the cell, its components are subsequently used to synthesise reduced glutathione. This occurs in medium with or without serum and therefore reduced glutathione appears to switch on γ-GCS activity directly and not as a result of an indirect mechanism due to lack of serum as is the case with NAC.

GSHMEE has been used to increase intracellular reduced glutathione *in vitro* and has been shown in some studies to be more effective than reduced glutathione itself [6, 35, 36]. This is thought to be due to its lipid solubility which enables it to pass easily through plasma membranes into the cell where it is de-esterized to release reduced glutathione. Our results show that GSHMEE increases intracellular NPSH and reduced glutathione to the same extent. However, in both cell lines reduced glutathione was more effective in elevating intracellular reduced glutathione than GSHMEE.

Interestingly, the two cell types increased their NPSH levels to different degrees by the same treatments. The reason for these differences is as yet unknown but could involve different rates for γ-GT, γ-GCS activity or distinct cysteine/cystine transport systems.

In A549 cells, both pre-incubation of NAC and reduced glutathione inhibited the toxic effects of H_2O_2. Pretreatment with NAC had a more effective antioxidant action as it also inhibited the effect of the removal of serum on LDH release.

These studies demonstrate protection against H_2O_2 by increasing NPSH or reduced glutathione levels with exogenous compounds, but not by inducing reduced glutathione by prestimulation of the cell with an oxidant. We believe this is most probably due to differences in the level of increase in NPSH. We have previously shown that NAC pretreatment in 2% serum-medium, which increased intracellular NPSH/reduced glutathione levels to a lesser degree, did not protect as effectively against treatment with H_2O_2 [19]. This supports our contention that a critical level of NPSH or reduced glutathione is required to afford protection against oxidants. We were unable to stimulate the cells to increase NPSH (using endogenous precursors) to similar levels as those obtained using exogenous sources of reduced glutathione. Pretreatment with sublethal doses of oxygen has previously been shown to increase reduced glutathione level in rat type II alveolar cells sufficient to protect against lethal hyperoxia [37]. It may be that hyperoxia is less damaging than H_2O_2 and therefore relatively lower levels of NPSH are needed or it may be that hyperoxia pre-treatment is more effective than H_2O_2 in elevating NPSH. It seems therefore that to obtain protection against H_2O_2-induced injury intracellular NPSH or reduced glutathione levels must be substantially raised.

It is important to note that the increase in intracellular thiols produced by exogenous reduced glutathione and GSHMEE was short lived returning to control levels before 16 h. However, the increase due to NAC treatment decreased at a slower rate and levels were still above those of the control at 16 h. We have shown that this increase was not entirely due to increased intracellular cysteine but may be due to NAC itself which, on entering the cells, may act as a reductant.

It is important at this stage to highlight that this experimental model involves the use of cell lines and therefore cannot be considered as having the exact properties of cells *in vivo*.

These studies show the importance of maintaining intracellular nonprotein thiol/reduced glutathione at a critical level during oxidant stress. N-acetylcysteine, with its reactive sulphydryl group and high antioxidant potential, may be a relatively more potent means of obtaining protection against oxidant injury than reduced glutathione itself. This may be due to N-acetylcysteine not being tightly regulated, as is reduced glutathione and hence its antioxidant capacity is prolonged. The slow release of cysteine from N-acetylcysteine may also be used to synthesise reduced glutathione during an oxidant stress.

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