

Oxidant stress induces gamma-glutamylcysteine synthetase and glutathione synthesis in human bronchial epithelial NCI-H292 cells

S. Ray, D. N. Watkins, N. L. A. Misso and P. J. Thompson

Asthma and Allergy Research Institute, and Department of Medicine, The University of Western Australia, Perth, Australia

Summary

Background The bronchial epithelium is exposed to reactive oxygen species (ROS) derived from cigarette smoke, air pollutants and activated leucocytes. Glutathione (GSH) prevents ROS-mediated loss of cell function, tissue injury and inflammation, and its synthesis is regulated by γ -glutamylcysteine synthetase (γ -GCS). However, the capacity of bronchial epithelial cells to adapt to oxidative stress and the mechanisms involved are not known.

Objective To investigate the effects of oxidative stress on the regulation of GSH synthesis in human bronchial epithelial (NCI-H292) cells.

Methods NCI-H292 cells were exposed to menadione and intracellular GSH concentrations were measured by spectrophotometry. γ -GCS activity was measured by HPLC assay and changes in γ -GCS mRNA by Northern blotting.

Results Exposure to menadione (MQ, 10–200 μ M, 30–120 min) decreased total cellular GSH content, measured immediately after exposure to MQ. However, GSH content measured 6–12 h after withdrawal of the oxidant stress (MQ, 50 μ M, 30 min), increased *c.* two fold over baseline levels ($P < 0.001$). γ -GCS activity measured 6 h (21.7 ± 3.4 nmol/min/mg, SD, $n = 5$, $P < 0.01$) or 12 h (23.2 ± 4.6 , $P < 0.001$) after MQ treatment was also significantly increased compared with untreated cells (12.8 ± 1.0). Similarly, γ -GCS mRNA expression increased 1.3–1.6-fold relative to GAPDH mRNA, 3–6 h after MQ treatment. The MQ-induced increase in γ -GCS mRNA expression was completely inhibited by actinomycin D.

Conclusions Bronchial epithelial (NCI-H292) cells respond rapidly and sensitively to oxidant stress, and this adaptive response is mediated by increased γ -GCS mRNA transcription and enzyme activity.

Keywords bronchial epithelial cells, γ -glutamylcysteine synthetase, glutathione, menadione, oxidative stress

Submitted 14 April 2001; revised 2 July 2001; accepted 29 August 2001

Introduction

Reactive oxygen species (ROS) have the potential to interfere with signal transduction, amplify and perpetuate inflammation and cause tissue injury [1]. The airway epithelium is uniquely vulnerable to oxidant stress arising from inhalation of cigarette smoke and air pollutants (ozone, NO₂, SO₂) as well as from endogenous H₂O₂ and superoxide anion, which are released from activated leucocytes during airway inflammation [2,3].

Glutathione (L- γ -glutamyl-L-cysteine-L-glycine, GSH), is the predominant antioxidant in airway epithelial cells and epithelial lining fluid and serves as the first line of defence against exogenous oxidants [3–5]. GSH reduces H₂O₂ and lipid hydroperoxides via the glutathione peroxidase reaction and detoxifies electrophiles and xenobiotics through direct thiol conjugation or by enzyme catalysed reactions [3,6]. The capacity of cells to

maintain GSH during an oxidative challenge is important in preventing loss of cellular function and integrity.

Most cells rely on *de novo* synthesis of GSH for maintaining or increasing intracellular GSH [6]. The biosynthesis of GSH is a two-step process in which gamma glutamylcysteine synthetase (γ -GCS) catalyses the initial rate limiting step. Therefore, the expression and activity of this enzyme is an important determinant of the GSH concentration within cells [3,6]. γ -GCS is a heterodimer with a heavy catalytic subunit (73 kDa) and a light subunit (30 kDa) that regulates the affinity of the heavy subunit for substrates and inhibitors [3,7].

Induction of antioxidant defences has been proposed as the major mechanism for adaptation to oxidative stress in mammalian cells [8–10]. However, there is wide variability in the cellular response to inhaled reactive oxidant gases at different sites along the respiratory tract that may be due to regional differences in the metabolic potential of epithelial cells to regulate intracellular GSH [11]. In this regard, the capacity of bronchial epithelial cells to adapt to oxidative stress and the mechanisms involved are not known. Therefore, the aim of this study was to define the concentration dependence and time course of

Correspondence: Philip J. Thompson, Asthma and Allergy Research Institute, Ground Floor, E Block, Sir Charles Gairdner Hospital, Nedlands, WA 6009, Australia. E-mail: pjthomps@cyllene.uwa.edu.au

menadione-induced changes in GSH and γ -GCS expression in a human bronchial epithelial (NCI-H292) cell line.

Materials and methods

Materials

RPMI 1640 medium was purchased from GIBCO BRL (Melbourne, Australia) and fetal calf serum (FCS) from CSL (Melbourne, Australia). [α - ^{32} P]UTP and RNazol B were purchased from GeneWorks (Adelaide, Australia) and γ -glutamyl- α -aminobutyric acid from Auspep (Melbourne, Australia). Reagents, enzymes and deoxynucleotide triphosphates for reverse transcription, pGEM-T vector and the Gemini II riboprobe kit were purchased from Promega (Sydney, Australia). Amplitaq DNA polymerase was purchased from Perkin Elmer (Melbourne, Australia). All other reagents and enzymes were purchased from Sigma Chemical Co. (Sydney, Australia).

Cell culture

NCI-H292 cells, that were derived from a human pulmonary mucoepidermoid carcinoma, were obtained from American Type Culture Collection (Rockville, MD, USA). NCI-H292 cells retain their squamous epithelial cell differentiation in culture, and were routinely grown in RPMI 1640 medium supplemented with FCS (10%, v/v), sodium bicarbonate (2.2 g/L), L-glutamine (2 mM) and gentamicin (50 μ g/mL) at 37°C and pH 7.0–7.2 in a humidified atmosphere of 95% air and 5% CO₂ [12]. When confluent, cells were disaggregated in trypsin-versene solution, washed in RPMI-10% FCS, centrifuged at 1200g for 5 min, resuspended and subcultured according to standard protocols.

Exposure of cells to menadione (MQ)-induced oxidative stress

MQ was dissolved in DMSO and subsequently diluted in serum free RPMI. Confluent cells were incubated in serum free medium with or without various concentrations of MQ at 37°C for different periods of time (0–2 h). Control cells were treated with the same concentrations of DMSO used in the MQ treatment groups. The DMSO content in each culture was no more than 0.01% and at this concentration it had no effect on γ -GCS activity or GSH content. In some experiments, after exposure to MQ, cells were washed twice in PBS, incubated in RPMI-10% FCS for varying periods and then lysed for measurement of GSH content, γ -GCS activity or steady state γ -GCS mRNA levels.

Actinomycin D and cycloheximide treatment

Confluent cells were treated with 0.05 μ g/mL actinomycin D or 0.5 μ g/mL cycloheximide in serum free medium with or without 50 μ M MQ for 30 min. Cells were then washed and incubated in RPMI-10% FCS in the presence of actinomycin D (0.05 μ g/mL) or cycloheximide (0.5 μ g/mL) for varying periods (0–12 h).

Assessment of MQ-related cytotoxicity

Cultures were assessed for MQ toxicity by counting non-adherent cells in the culture medium under light microscopy and by measuring lactate dehydrogenase leakage using a standard assay [13]. The viability of cells was also assessed by trypan blue exclusion.

Cellular GSH and GSSG measurement

Confluent cells on six-well plates were washed twice with cold PBS after MQ treatment and lysed in cold 4% sulfosalicylic acid. The lysate was vortexed vigorously and centrifuged (10000g, 10 min, 4°C) to remove precipitated protein. Total cellular GSH in the supernatant was immediately determined using the DTNB-GSSG reductase recycling assay [14]. To determine the GSSG content, the supernatant was treated with 2-vinylpyridine and triethanolamine to mask reduced GSH prior to the DTNB-GSSG assay [15].

HPLC assay for γ -GCS activity

After exposure to MQ, cells were harvested and resuspended in 0.1 M Tris-HCl, pH 8.2 containing 20 mM MgCl₂, 50 mM KCl and 2 mM EDTA. Cells were lysed by sonication on ice, the lysate was centrifuged (13000g, 10 min, 4°C) and γ -GCS activity in the supernatant was assayed by measuring the rate of formation of the dipeptide product of the enzyme reaction as described [16]. L- α -aminobutyrate was used instead of L-cysteine, in order to avoid aerial oxidation of the natural substrate, L-cysteine. Equal rates of reaction are obtained with saturating concentrations of L-cysteine and L- α -aminobutyrate. The assay medium (100 μ L, final volume), containing 0.1 M Tris-HCl (pH 8.2 at 37°C), 20 mM MgCl₂, 50 mM KCl, 2 mM EDTA, 10 mM ATP, 5 mM glutamic acid, 5 mM L- α -aminobutyric acid and cell extract was incubated for 30 min at 37°C. The reaction was terminated with 20 μ L of 50% trichloroacetic acid, and the reaction products were quantified by HPLC using a Physiological AA Lithium column (AA-503, 125 $\times$ 4.6 mm, 5 μ m, Interaction Chemicals, San Jose, CA, USA) at 34°C and a pH gradient (lithium citrate, pH 2.65; lithium borate, pH 9.7; lithium hydroxide, pH 13.2), as described previously [17]. After post-column derivatization with ninhydrin, products were detected at 546 nm on a UV-VIS detector. The amount of γ -glutamyl- α -aminobutyrate produced (retention time 25.6 min) was quantified by comparison with an authentic standard that was subjected to similar HPLC separation and derivatization.

Synthesis and labelling of RNA probe for γ -GCS heavy subunit (γ -GCS_H)

Partial γ -GCS_H cDNA (882 bp) was prepared by reverse transcription-polymerase chain reaction (RT-PCR) of total RNA from airway epithelial (BEAS-2B) cells. Total RNA (10 μ g) and hexameric random primers were heated to 75°C for 5 min, then incubated at 42°C for 60 min in a 20- μ L reaction containing 4 U avian myeloblastosis virus reverse transcriptase, 2 U of RNasin, 2.5 mM each of deoxynucleotide triphosphates (dNTPs), 50 mM KCl, 10 mM Tris-HCl and 2 mM MgCl₂. Subsequent PCR using γ -GCS_H cDNA specific primers [5'-primer (332–350), 5'-GAAGGGGGAAAGGACAAAC-3' and 3'-primer (1213–1195), 5'-AGATGAGCAACATGCTGGG-3'] was performed in a 25- μ L reaction containing 1.5 mM MgCl₂, 50 ng specific primers, 12.5 ng β -actin primers, cDNA equivalent to 1 μ g of total RNA, and Amplitaq DNA polymerase. PCR was performed with 35 cycles of 94°C for 5 min, 55°C for 1 min and 72°C for 2 min and yielded a single 882 bp DNA fragment. This PCR product was subcloned by ligation to the pGEM-T vector in JM 109 *E. coli* competent cells. Positive clones were selected and the correct plasmid DNA was verified by restriction enzyme mapping, automatic DNA sequencing

(Applied Biosystems, Sydney, Australia) and comparison with the published γ -GCS_H cDNA sequence (GenBank accession number M90656).

The γ -GCS_H plasmid construct was linearized with *Sph* 1, and used as a template for the synthesis of an [α -³²P]UTP antisense RNA probe. The reaction mixture (25 μ L), containing 1x transcription buffer, 1U RNasin, 6mM dithiothreitol, 2.5 mM each of ATP, CTP and GTP, 10 μ M UTP, 0.5–1 μ g linearized template, [α -³²P]UTP (50 μ Ci), and 1U of bacterial SP6 RNA polymerase, was incubated at 37°C for 1 h and the synthesized RNA probe was purified by phenol:chloroform extraction and ethanol precipitation.

Northern blot analysis of total RNA

Total cellular RNA (20 μ g) was extracted from NCI-H292 cells with ice cold RNazol B as described [18]. The RNA was heat denatured, electrophoresed overnight in 1% agarose–1.5% formaldehyde and transferred onto Hybond N⁺ membrane (Amersham, Sydney, Australia) in 10 $\times$ standard saline citrate (SSC). RNA was fixed to the membrane (UV light, 30s) which was then pre-hybridized at 55°C in 5 $\times$ saline sodium phosphate EDTA (SSPE), 5 $\times$ Denhardt's solution, 0.5% SDS, 20 μ g/mL heat denatured herring sperm DNA and 50% formamide followed by overnight hybridization with the [³²P]rUTP antisense γ -GCS_H RNA probe at 55°C. Post-hybridization washes in 2 $\times$ SSC, 0.1% SDS and 0.1 $\times$ SSC, 0.1% SDS were performed at 70°C for 20 min each. Autoradiography of the membrane was performed on a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA, USA) using Imager SI v 4.0 and ImageQuaNT v 4.2 software for quantitative densitometry. The membrane was stripped of γ -GCS_H probe and rehybridized with a [³²P]-labelled GAPDH RNA probe, prepared from a plasmid containing rat kidney GAPDH cDNA, to provide an internal control for the amount of RNA loaded in each lane.

Analysis and statistical methods

γ -GCS activity is expressed as nmol/min/mg of soluble protein and GSH content is expressed as nmol/10⁶ cells. Assay data are expressed as means $\pm$ standard deviation. Statistical analyses were performed by one-way ANOVA followed by the Tukey-Kramer multiple comparison test (GraphPad Prism, GraphPad Software, San Diego, CA, USA). A *P*-value <0.05 was considered significant.

Results

Effect of menadione on the GSH content of NCI-H292 cells

When confluent NCI-H292 cells were exposed to increasing concentrations of MQ for 30–120 min, total cellular GSH content measured immediately after MQ exposure decreased in a concentration and time dependent manner (Fig. 1). In contrast, GSH levels measured 24 h after a 30-min exposure to MQ, increased significantly over baseline levels for all concentrations of MQ, with a maximum twofold increase in GSH after 24 h, compared with control cells (Fig. 2, *P* < 0.001).

Total cellular GSH content after a 30-min exposure to 50 μ M MQ was restored to the baseline level of untreated cells within 1 h of withdrawing the oxidant stimulus and then increased to 180% and 205% of control at 6 and 12 h, respectively (Fig. 3). GSH levels in treated cells remained significantly elevated

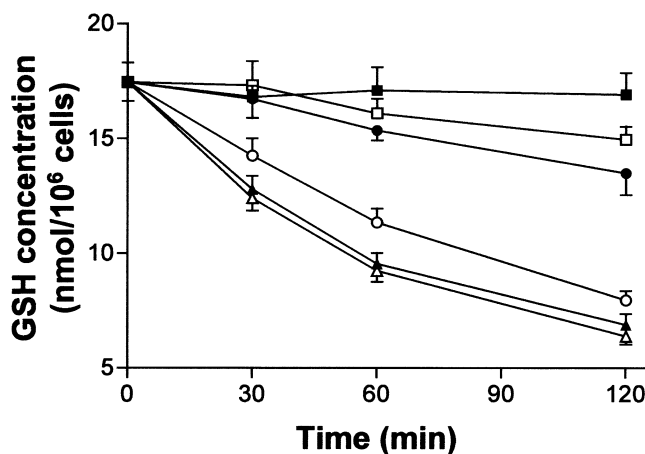


Fig. 1. Effect of menadione (MQ) on cellular glutathione. Confluent human bronchial epithelial (NCI-H292) cells were cultured in medium alone (closed squares), or treated with 10 (open squares), 25 (closed circles), 50 (open circles), 100 (closed triangles) or 200 μ M (open triangles) MQ for 0, 30, 60, 90 or 120 min, and the total GSH content of the cells was determined immediately after treatment. Data are presented as means $\pm$ SD (*n* = 5).

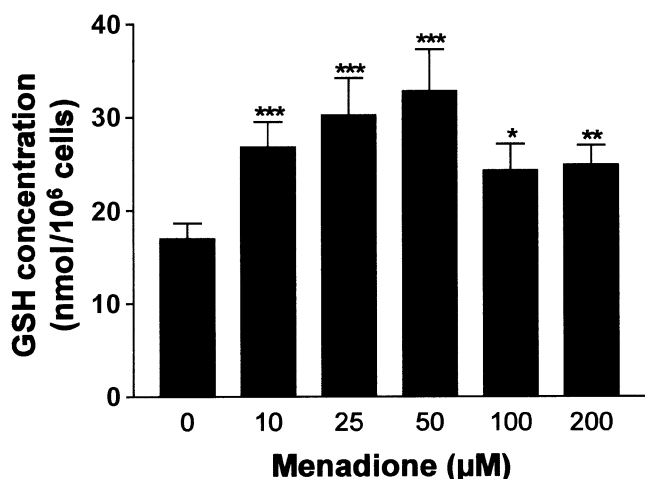


Fig. 2. Concentration-dependent increases in GSH content 24 h after a 30-min exposure to MQ. Confluent cells were cultured in medium alone or treated with 10, 25, 50, 100 or 200 μ M MQ in serum free RPMI for 30 min. The cells were then washed twice with PBS and incubated for 24 h in RPMI containing 10% FCS, after which GSH content was measured. Data are presented as means $\pm$ SD (*n* = 5). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with untreated cells.

compared with control cells for at least 24 h after exposure to MQ. Both immediately after exposure to MQ and 1, 6, 12 or 24 h after withdrawal of MQ, cellular GSH remained almost entirely in the reduced form.

MQ had no effect on the viability of cells as judged by trypan blue exclusion and lactate dehydrogenase release at 0, 1 and 24 h after exposure (data not shown). Furthermore, there were no morphological changes in cells exposed to MQ, indicating that MQ-related toxicity was minimal.

Effect of menadione on γ -GCS activity

Control and MQ-treated cells did not differ in γ -GCS activities measured immediately after exposure to MQ or 1 h after removal of MQ (*P* > 0.05). In contrast, γ -GCS activities in MQ-treated cells at 6 h (21.67 ± 3.38 nmol/min/mg protein) and 12 h

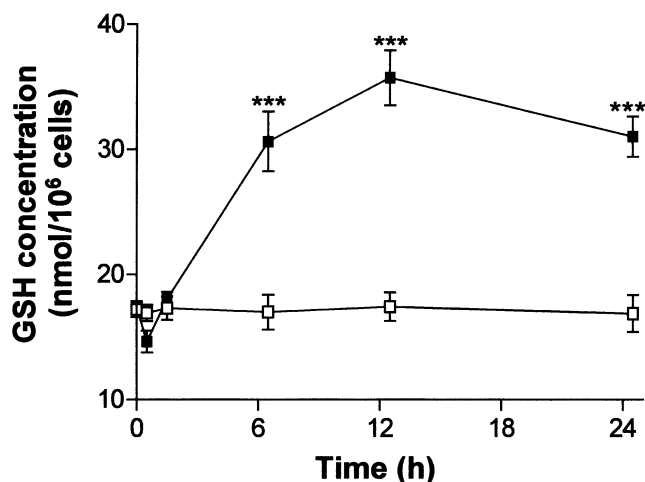


Fig. 3. Time course of increase in GSH content after exposure to MQ (50 μ M, 30 min). Confluent cells were cultured in serum free RPMI with (closed squares) or without (open squares) 50 μ M MQ for 30 min. The cells were then washed twice with PBS and cultured for an additional 0, 0.5, 1, 6, 12 or 24 h in fresh RPMI containing 10% FCS. The GSH content of cells was measured immediately after each period of culture in fresh medium. Data are presented as means $\pm$ SD ($n=5$). *** $P < 0.001$ compared with untreated cells.

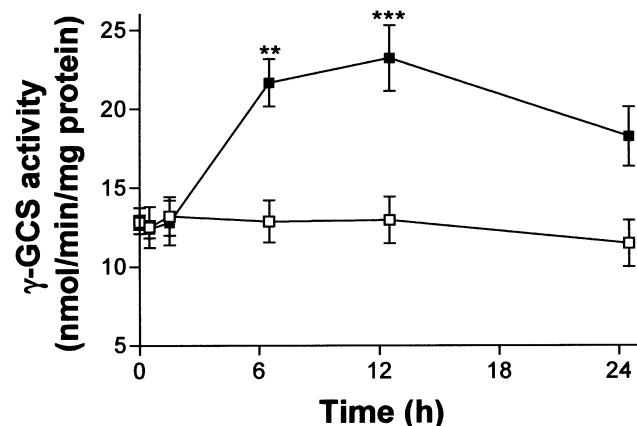


Fig. 4. γ -GCS activity of NCI-H292 cells after exposure to MQ. Confluent cells were cultured in serum free RPMI with (closed squares) or without (open squares) 50 μ M MQ for 30 min. The cells were then washed twice with PBS and cultured for an additional 0, 0.5, 1, 6, 12 or 24 h in fresh RPMI containing 10% FCS. The γ -GCS activity of cells was measured immediately after each period of culture in fresh medium. Data are presented as means $\pm$ SD ($n=5$). ** $P < 0.01$ and *** $P < 0.001$ compared with untreated cells.

(23.22 $\pm$ 4.63) after withdrawal of the oxidant stimulus were significantly elevated as compared with control cells (12.82 $\pm$ 1.02, $P < 0.01$ and $P < 0.001$, respectively, Fig. 4). A decline in γ -GCS activity of MQ-treated cells was observed by 24h.

Effect of menadione on γ -GCS mRNA expression

The [³²P]-labelled γ -GCS_H riboprobe hybridized with two mRNAs (around 3.2 kb and 4.1 kb) isolated from NCI-H292 cells, consistent with the size of the two transcripts reported for γ -GCS_H mRNA from human tissues [19]. For quantification of γ -GCS mRNA, the sum of the densities of these two bands was used. The steady state γ -GCS_H mRNA levels in treated cells increased significantly 3 h after withdrawal of MQ ($P < 0.001$)

and this was sustained for at least 6 h ($P < 0.05$, Fig. 5a). At 12 h, γ -GCS_H mRNA levels had almost returned to control levels, with no further change at 24 h. The autoradiographic densities of γ -GCS_H mRNA relative to GAPDH mRNA were 160%, 145% and 114% of control at 3, 6 and 12 h, respectively (Figs 5a and b). A similar pattern was observed in several experiments ($n=4$), with MQ-treated cells showing a 1.3–1.6-fold maximum relative increase in γ -GCS_H mRNA compared with GAPDH mRNA.

Effects of actinomycin D and cycloheximide on induction of γ -GCS mRNA expression

The MQ-induced increase in γ -GCS_H mRNA expression was completely blocked by the transcription inhibitor, actinomycin D, but only partially blocked by inhibition of protein synthesis with cycloheximide (Fig. 6). Similar results were obtained in two separate experiments.

Discussion

This study has demonstrated that a human bronchial epithelial (NCI-H292) cell line adapts to sublethal oxidative stress by elevating γ -GCS mRNA levels and γ -GCS activity, thereby increasing *de novo* GSH synthesis. After an initial depletion in GSH, the levels returned to baseline at 1 h, followed as early as 6 h after removal of MQ, by an almost twofold increase in GSH and γ -GCS activity above control levels.

Oxidant stress was induced by treatment with menadione, a lipid soluble quinone that is readily taken up by cells, where it generates reactive oxygen species such as superoxide radical and H₂O₂ by redox cycling [20,21]. However, the depletion of cellular GSH, observed immediately after exposure of NCI-H292 cells to MQ, was probably due to the formation of a GSH-MQ conjugate that is rapidly transported out of pulmonary cells [22,23]. During prolonged exposure to MQ, GSH continues to be depleted, probably because loss of GSH as the GSH-MQ conjugate exceeds the rate of GSH synthesis.

The effects of other biologically relevant inducers of oxidant stress may or may not be similar to those seen with menadione, depending on the type of oxidant. Thus cigarette smoke condensate, which contains a high percentage of quinones, depletes cellular GSH in alveolar epithelial cells *in vitro* and rat lungs *in vivo* [22] and appears to increase γ -GCS mRNA expression in the lungs of human subjects [24]. Particulate air pollution and SO₂ also induce oxidant stress and reduce lung GSH levels in rats [25,26]. In contrast, inhalation of ozone or NO₂ by humans increases airway responsiveness and reduces pulmonary function [27], but does not deplete lung GSH levels [28,29].

Feedback inhibition by GSH normally limits γ -GCS activity within cells to substantially less than its maximal rate [3,6,30,31]. The transient decrease in the GSH content of cells, immediately after exposure to MQ, probably released this feedback inhibition of γ -GCS activity such that there was an increase in GSH synthesis and restoration of GSH to control levels within 1 h of MQ withdrawal. However, any changes in γ -GCS activity would not have been observed in our *in vitro* assay in which γ -GCS activity was measured under optimal conditions designed to minimize feedback inhibition by GSH. *De novo* GSH synthesis is also regulated by the availability of the substrate, L-cysteine [3,30]. The enzyme γ -glutamyl transpeptidase (γ -GT) catalyses

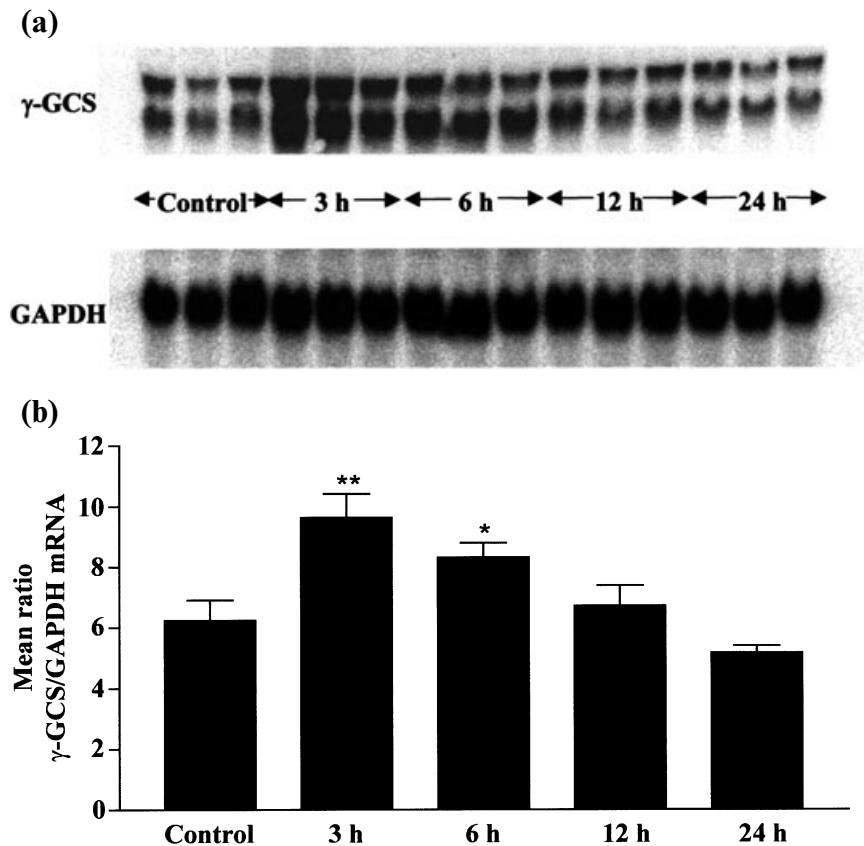


Fig. 5. Effect of MQ on γ -GCS mRNA expression in NCI-H292 cells. Confluent cells were cultured in serum free RPMI with or without 50 μ M MQ for 30 min. The cells were then washed twice with PBS and cultured for an additional 3, 6, 12 or 24 h in fresh RPMI containing 10% FCS. Total cellular RNA was extracted and Northern analysis for γ -GCS_H (a, upper bands) and GAPDH mRNA (a, lower bands) was performed as described in Methods. (b) For densitometric quantification, the ratio of the sum of the densities of the two γ -GCS_H mRNA bands to the density of the GAPDH mRNA band was determined for each sample. Bars show the mean $\pm$ SD values for three samples at each time-point. * P < 0.05, ** P < 0.001 compared with control untreated cells.

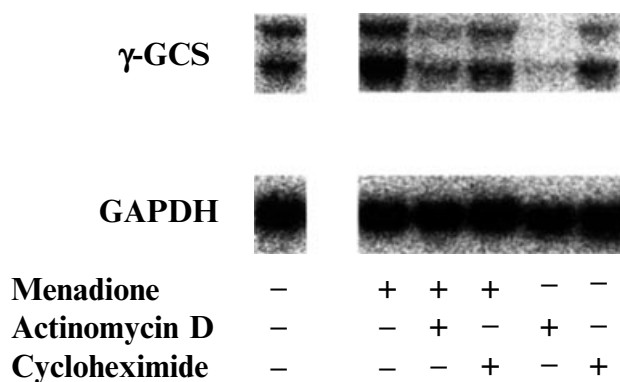


Fig. 6. Effect of actinomycin D and cycloheximide on menadione-induced increase in γ -GCS_H mRNA expression in NCI-H292 cells. Cells were cultured with or without 50 μ M MQ in serum free RPMI for 30 min in the presence or absence of actinomycin D (0.05 μ g/mL) or cycloheximide (0.5 μ g/mL). The cells were then washed twice with PBS and cultured for an additional 6 h in fresh RPMI/10% FCS with or without actinomycin D (0.05 μ g/mL) or cycloheximide (0.5 μ g/mL). Total cellular RNA was extracted and Northern analysis for γ -GCS_H and GAPDH mRNA was performed as described in Methods.

the breakdown of extracellular GSH to cysteinylglycine, which is further degraded by dipeptidase to cysteine and glycine. γ -GT activity is enhanced in rat lung epithelial L2 cells exposed to oxidant stress [32], and extracellular cysteine produced by this

mechanism may be actively transported into the cell [33], ensuring the availability of substrate for GSH synthesis during oxidative stress.

Although the temporary release of feedback inhibition of γ -GCS might explain the restoration of cellular GSH to control levels, a sustained increase in GSH synthesis requires an increase in γ -GCS V_{max} , due to increased transcription and/or translation [34]. This study demonstrated that steady state γ -GCS_H mRNA levels in NCI-H292 cells were increased 3–6 h after withdrawal of MQ and returned to control levels by 12 h. The larger 4.1 kb transcript appeared to persist longer, suggesting possible differences in stability of the two mRNA transcripts. Actinomycin D inhibited the increase in γ -GCS_H mRNA, suggesting that it was probably due to increased transcription. However, the partial inhibitory effect of cycloheximide also suggests that new protein synthesis may be required. Other factors, such as mRNA and/or enzyme stabilization, may also contribute to the observed increase in γ -GCS activity [30].

Although previous studies have shown that oxidants and cigarette smoke induce γ -GCS and glutathione synthesis in human alveolar epithelial type II cells [35–37], the present study adds significantly to those previous observations. In the NCI-H292 bronchial epithelial cell line an increase in GSH levels and γ -GCS activity occurs as early as 6 h after MQ exposure. In contrast, A549 alveolar epithelial cells showed an increase in γ -GCS activity and mRNA expression only 24 h after

MQ treatment [35,36]. In this study we demonstrated a significant increase in bronchial epithelial cell γ -GCS mRNA expression as early as 3 h after MQ treatment. However, the levels of γ -GCS activity in control NCI-H292 bronchial epithelial cells were very similar to the levels we recently measured in A549 alveolar epithelial cells [17]. It should be noted that because the NCI-H292 cells used in this study were derived from a carcinoma, their responses to oxidant stress may differ somewhat from those of normal ciliated bronchial epithelial cells. Furthermore, this model will not allow differentiation of the responses of bronchial epithelial cells of healthy individuals compared with those of patients with respiratory disease [38]. Nevertheless, the results of this study indicate a more rapid and sensitive up-regulation of γ -GCS in a bronchial epithelial cell line in response to oxidant stress compared with A549 alveolar epithelial cells and identify a possible mechanism by which bronchial epithelial cells adapt to oxidant stress *in vivo*.

It is possible that in NCI-H292 cells, menadione increases γ -GCS activity by increasing transcription of both the γ -GCS_H and γ -GCS_L genes, as has been reported in other cell types [3,34], and a MQ-induced increase in H₂O₂ may be the intracellular signal leading to increased transcription of these γ -GCS genes [34]. In addition, the binding of Jun family transcription factors to an AP-1 site in the proximal promoter region, a distal antioxidant response element (ARE) site and NF κ B binding activity have all been implicated in the up-regulation of γ -GCS_H gene expression in response to oxidant stress [3,30]. MQ-induced up-regulation of γ -GCS_H gene transcription in NCI-H292 cells may involve similar signal transduction mechanisms.

In conclusion, the present study demonstrates for the first time that a human bronchial epithelial cell line responds to sublethal oxidative stress by a sustained elevation of GSH content resulting from an increase in steady-state levels of γ -GCS_H mRNA and γ -GCS enzyme activity. Induction of *de novo* GSH synthesis is an important adaptive response mechanism to oxidative stress in bronchial epithelial cells, and further investigation of the regulation of this pathway could potentially reveal important opportunities for disease modification.

Acknowledgements

We thank Mr E. Rasins, Department of Botany, The University of Western Australia, for help with amino acid analyses for the γ -GCS assay.

References

- Cross CE. Reactive oxygen species and the lung. *Lancet* 1994; 344:930–3.
- Dworski R. Oxidant stress in asthma. *Thorax* 2000; 55 (Suppl. 2): S51–3.
- Rahman I, MacNee W. Oxidative stress and regulation of glutathione in lung inflammation. *Eur Respir J* 2000; 16:534–54.
- Smith LJ, Houston M, Anderson J. Increased levels of glutathione in bronchoalveolar lavage fluid from patients with asthma. *Am Rev Respir Dis* 1993; 147:1461–4.
- van Klaveren RJ, Demedts M, Nemery B. Cellular glutathione turnover *in vitro*, with emphasis on type II pneumocytes. *Eur Respir J* 1997; 10:1392–400.
- Deneke SM, Fanburg BL. Regulation of cellular glutathione. *Am J Physiol* 1989; 257:L163–73.
- Seelig GF, Meister A. γ -Glutamylcysteine synthetase. *J Biol Chem* 1984; 259:3534–8.
- Ho Y-S, Dey MS, Crapo JD. Antioxidant enzyme expression in rat lungs during hyperoxia. *Am J Physiol* 1996; 270:L810–18.
- White AC, Maloney EK, Boustani MR, Hassoun PM, Fanburg BL. Nitric oxide increases cellular glutathione levels in rat lung fibroblasts. *Am J Respir Cell Mol Biol* 1995; 13:442–8.
- Shi MM, Kugelman A, Iwamoto T, Tian L, Forman HJ. Quinone-induced oxidative stress elevates glutathione and induces γ -glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J Biol Chem* 1994; 269:26512–7.
- Plopper CG, Hatch GE, Wong V et al. Relationship of inhaled ozone concentration to acute tracheobronchial epithelial injury, site – specific ozone dose, and glutathione depletion in rhesus monkeys. *Am J Respir Cell Mol Biol* 1998; 19:387–99.
- Levine SJ, Benfield T, Shelhamer JH. Corticosteroids induce intracellular interleukin-1 receptor antagonist type I expression by a human airway epithelial cell line. *Am J Respir Cell Mol Biol* 1996; 15:245–51.
- Bergmeyer HU, Bernt E. Lactate dehydrogenase. In: Bergmeyer HU, ed. *Methods of Enzymatic Analysis*. New York: Academic Press, 1974; 574–8.
- Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidised glutathione: applications to mammalian blood and other tissue. *Anal Biochem* 1969; 27:502–22.
- Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980; 106:207–12.
- Forman HJ, Shi MM, Iwamoto T, Liu R-M, Robison TW. Measurement of gamma-glutamyltranspeptidase and gamma-glutamylcysteine synthetase activities in cells. *Meth Enzymol* 1995; 252:66–71.
- Ray S, Misso NLA, Lenzo JC, Robinson C, Thompson PJ. Gamma-glutamylcysteine synthetase activity in human lung epithelial (A549) cells: factors influencing its measurement. *Free Radic Biol Med* 1999; 27:1346–56.
- Watkins DN, Peroni DJ, Basclain KA, Garlepp MJ, Thompson PJ. Expression and activity of nitric oxide synthases in human airway epithelium. *Am J Respir Cell Mol Biol* 1997; 16:629–39.
- Gipp JJ, Bailey HH, Mulcahy RT. Cloning and sequencing of the cDNA for the light subunit of human liver γ -glutamylcysteine synthetase and relative mRNA levels for heavy and light subunits in human normal tissues. *Biochem Biophys Res Commun* 1995; 206:584–9.
- Brunmark A, Cadenas E. Redox and addition chemistry of quinoid compounds and its biological implications. *Free Radic Biol Med* 1989; 7:435–77.
- Thor H, Smith MT, Hartzell P et al. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J Biol Chem* 1982; 257:12419–25.
- Rahman I, Li XY, Donaldson K, Harrison DJ, MacNee W. Glutathione homeostasis in alveolar epithelial cells in vitro and lung in vivo under oxidative stress. *Am J Physiol* 1995; 269:L285–92.
- Chang M, Shi MM, Forman HJ. Exogenous glutathione protects endothelial cells from menadione toxicity. *Am J Physiol* 1992; 262: L637–43.
- Rahman I, van Schadewijk AA, Hiemstra PS et al. Localisation of γ -glutamylcysteine synthetase messenger RNA expression in lungs of smokers and patients with chronic obstructive pulmonary disease. *Free Radic Biol Med* 2000; 28:920–5.
- Li XY, Gilmour PS, Donaldson K, MacNee W. Free radical activity and proinflammatory effects of particulate air pollution (PM₁₀) in vivo and in vitro. *Thorax* 1996; 51:1216–22.

- 26 Langley-Evans SC, Phillips GJ, Jackson AA. Sulphur dioxide: a potent glutathione depleting agent. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1996; 114:89–98.
- 27 Jenkins HS, Devalia JL, Mister RL et al. The effect of exposure to ozone and nitrogen dioxide on the airway response of atopic asthmatics to inhaled allergen: dose- and time-dependent effects. *Am J Respir Crit Care Med* 1999; 160:33–9.
- 28 Blomberg A, Mudway IS, Nordenhall C et al. Ozone-induced lung function decrements do not correlate with early airway inflammatory or antioxidant responses. *Eur Respir J* 1999; 13:1418–28.
- 29 Kelly FJ, Tetley TD. Nitrogen dioxide depletes uric acid and ascorbic acid but not glutathione from lung lining fluid. *Biochem J* 1997; 325:95–9.
- 30 Griffith OW. Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med* 1999; 27:922–35.
- 31 Richman PG, Meister A. Regulation of γ -glutamylcysteine synthetase by nonallosteric feedback inhibition by glutathione. *J Biol Chem* 1975; 250:1422–6.
- 32 Liu R-M, Shi MM, Giulivi C, Forman HJ. Quinones increase gamma-glutamyl transpeptidase expression by multiple mechanisms in rat lung epithelial cells. *Am J Physiol* 1998; 274:L330–6.
- 33 Bannai S. Transport of cystine and cysteine in mammalian cells. *Biochim Biophys Acta* 1984; 779:289–306.
- 34 Tian L, Shi MM, Forman HJ. Increased transcription of the regulatory subunit of γ -glutamylcysteine synthetase in rat lung epithelial L2 cells exposed to oxidative stress or glutathione depletion. *Arch Biochem Biophys* 1997; 342:126–33.
- 35 Rahman I, Bel A, Mulier B, Donaldson K, MacNee W. Differential regulation of glutathione by oxidants and dexamethasone in alveolar epithelial cells. *Am J Physiol* 1998; 275:L80–6.
- 36 Rahman I, Bel A, Mulier B et al. Transcriptional regulation of γ -glutamylcysteine synthetase heavy subunit by oxidants in human alveolar epithelial cells. *Biochem Biophys Res Commun* 1996; 229:832–7.
- 37 Rahman I, Smith CAD, Lawson MF, Harrison DJ, MacNee W. Induction of γ -glutamylcysteine synthetase by cigarette smoke is associated with AP-1 in human alveolar epithelial cells. *FEBS Lett* 1996; 396:21–5.
- 38 Mills PR, Davies RJ, Devalia JL. Airway epithelial cells, cytokines and pollutants. *Am J Respir Crit Care Med* 1999; 160:S38–43.