Differential regulation of glutathione by oxidants and dexamethasone in alveolar epithelial cells

IRFAN RAHMAN,1 AGNES BEL,1 BRIGITTE MULIER,1 KENNETH DONALDSON,2 AND WILLIAM MACNEE1

1The Rayne Laboratory, Respiratory Medicine Unit, Department of Medicine, University of Edinburgh, Edinburgh EH8 9AG; and 2Department of Biological Sciences, Napier University, Edinburgh EH10 5DT, United Kingdom

Rahman, Irfan, Agnes Bel, Brigitte Mulier, Kenneth Donaldson, and William MacNee. Differential regulation of glutathione by oxidants and dexamethasone in alveolar epithelial cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L80–L86, 1998.—We studied the regulation of GSH and the enzymes involved in GSH regulation, γ-glutamylcysteine synthetase (γ-GCS) and γ-glutamyl transpeptidase (γ-GT), in response to the oxidants menadione, xanthine/xanthine oxidase, hyperoxia, and cigarette smoke condensate in human alveolar epithelial cells (A549). Menadione (100 µM), xanthine/xanthine oxidase (50 µM/10 µU), and cigarette smoke condensate (10%) exposure produced increased GSH levels (240 ± 6, 202 ± 12, and 191 ± 2 nmol/mg protein, respectively; P < 0.001) compared with the control level (132 ± 8 nmol/mg protein), which were associated with a significant increase in γ-GCS activity (0.18 ± 0.006, 0.16 ± 0.01, and 0.17 ± 0.008 U/mg protein, respectively; P < 0.001) compared with the control level (0.08 ± 0.001 U/mg protein) at 24 h. Exposure to hyperoxia (95% O2) resulted in a time-dependent increase in GSH levels. γ-GCS activity increased significantly at 4 h (P < 0.001), returning to control values after 12 h of exposure. Dexamethasone (3 µM) exposure produced a significant time-dependent decrease in the levels of GSH and γ-GCS activity at 24–96 h. The activity of γ-GT did not change after oxidant treatment; however, it was decreased significantly by dexamethasone at 24–96 h. Thus oxidants and dexamethasone modulate GSH levels and activities of γ-GT and γ-GCS by different mechanisms. We suggest that the increase in γ-GCS activity but not in γ-GT activity may be required for the increase in intracellular GSH under oxidative stress in alveolar epithelial cells.

γ-glutamylcysteine synthetase; γ-glutamyl transpeptidase; A549 cells

THE RESPIRATORY EPITHELIUM is often exposed to oxidants, whether inhaled, such as cigarette smoke and ozone, or from reactive oxygen intermediates released from neutrophils recruited to the lungs during airway inflammation. GSH, a ubiquitous cellular nonprotein sulfhydryl, is an important antioxidant in the maintenance of intracellular redox balance and is involved in the detoxification of oxidants, free radicals, electrophiles, and organic peroxides either through direct thiol conjugation or by enzyme-catalyzed reactions (26, 28).

The synthesis of intracellular GSH is controlled by two rate-limiting factors. One is the enzyme γ-glutamylcysteine synthetase (γ-GCS), which catalyzes the first reaction of de novo GSH synthesis. The other is the availability of cysteine (18, 30), which is produced as a breakdown product of extracellular GSH by the membrane-bound enzyme γ-glutamyl transpeptidase (γ-GT) (7). The activities of both of these enzymes are important in maintaining the intracellular GSH pool and thus preventing oxidative injury to cells. Intracellular GSH can also be increased by cysteine transport (9, 17) in cells under oxidative stress, which can subsequently be used for the synthesis of GSH by γ-GCS. Hence the maintenance of intracellular GSH is mediated either by modification of cellular uptake of its precursors (cysteine) or by an increase in the activity of γ-GCS. Rahman and colleagues (24, 27) recently showed that GSH synthesis is induced in lung cells during oxidative stress as an adaptive response. However, different forms of oxidant stress may have differential effects on GSH regulation through the enzymes γ-GCS and γ-GT. Therefore, in this study, we used a range of different oxidative stresses including cigarette smoke condensate (CSC) to assess their effects on GSH homeostasis in lung type II epithelial cells. The oxidants that were studied were hyperoxia (>95% O2); xanthine/xanthine oxidase (X/XO), an intracellular generator of superoxide anion (O2·−) (11); 2-methyl-1,4-naphthoquinone [menadione (MQ)], a quinone that generates O2•− and hydrogen peroxide (H2O2) by redox cycling (2); and CSC, a complex oxidant that contains 1014 to 1016 free radicals/puff and electrophilic compounds capable of generating H2O2 (21).

Corticosteroids such as dexamethasone are widely used as for their anti-inflammatory properties in various inflammatory lung diseases (1). The anti-inflammatory action of dexamethasone appears to be mediated through an effect on transcription factors such as nuclear factor-xB and activator protein-1 (AP-1) for inflammatory mediator gene expression (1). The AP-1 binding site also appears to be critical for γ-GCS gene regulation and hence GSH synthesis (27). The effects of corticosteroids on lung GSH metabolism have not been studied so far. Therefore, we studied the effects of the oxidants described above and dexamethasone on GSH and its enzymes γ-GCS and γ-GT, which are critical for GSH synthesis in human alveolar epithelial cells (A549).

MATERIALS AND METHODS

Unless otherwise stated, all of the biochemical reagents used in this study were purchased from Sigma (Poole, UK); cell culture media were purchased from Gibco-BRL (Paisley, UK).

A549 epithelial cells. The type II alveolar epithelial cell line A549 (European Culture Collection no. 86012804), which was mycoplasma free, was maintained in continuous culture at...
37°C with 5% CO2 in DMEM, sodium bicarbonate, l-glutamate, and 10% fetal bovine serum.

Preparation of CSC. CSC (100%) was produced from standard cigarettes, each containing 23 mg of tar and 2.2 mg of nicotine (University of Kentucky UK2R1). CSC was produced by blowing smoke generated by a smoking machine with a vacuum syringe system from three cigarettes over 3 ml of PBS in a siliconized glass tonometer (Vitalograph, Buckingham, UK). The smoke-generating machine delivers a 37-ml puff of whole cigarette smoke, including particulates, every minute to the tonometer, which was rotated gently (13). The solution was made up fresh on the morning of each experiment, and CSC (100%) was filtered with a 0.22-mm filter (Millipore, Molsheim, France) to remove large particles and bacteria (27). Ten percent CSC was prepared from this solution by dilution with PBS.

To define a convenient surrogate marker for the amount of cigarette smoke entering into the solution, we measured the products of nitric oxide, nitrite, and nitrate in the filtered solution by dilution with PBS.

- The concentration was 12.2 ± 1.2 µM, which is in agreement with previous reports (5).

Epithelial cell exposure to CSC, X/XO, MQ, and dexamethasone. Monolayers of confluent epithelial cells were prepared by seeding 3 × 106 cells/well in a six-well plate and reculturing in DMEM with 10% fetal bovine serum at 37°C with 5% CO2 for 24 h. Confluent monolayers were rinsed twice with DMEM and exposed to CSC (10%), X/XO (50 µM/10 mU/mL), MQ (100 and 200 µM), or dexamethasone (3 µM) for time intervals between 1, 6, and 24 h in 2 ml of full medium incubated at 37°C with 5% CO2. In some experiments to study the recovery of intracellular GSH and enzyme activities after various exposures, monolayers were washed with fresh medium after exposure to the oxidants for 1 h and reincubated for 24 h. Thereafter, the monolayers were washed twice with cold PBS (pH 7.4), scraped into PBS, and centrifuged at 250 g for 5 min at 4°C. Cell viability was determined by staining with trypan blue.

Exposure of epithelial cells to hyperoxia. Cell monolayers were placed in anaerobic chambers humidified with wet tissue paper at 37°C. The chambers were sealed, and 95% O2-5% air was flushed through for 3 min (until the reading on the O2 monitor was 95%). The level of O2 was then maintained for 24 h. Confluent monolayers were seeded with 100% confluent monolayers of epithelial cells and cultured in this manner for 4 h. The cells were then harvested by trypsinization, and the whole cell suspension was immediately analyzed for GSH and enzyme activities.

- The results are expressed as means ± SE. Differences between values were compared by Duncan’s multiple range test.

RESULTS

Effects of MQ, CSC, X/XO, hyperoxia, and dexamethasone on GSH levels in alveolar epithelial cells. Rahman and colleagues (24, 27) previously demonstrated that MQ (100 µM) and CSC (10%) deplete GSH levels significantly at 1 h followed by a significant increase in GSH levels 24 h after exposure. In this study, A549 cells were exposed to various generators of oxidant stress to investigate whether these agents exert their effects on GSH levels in a time-dependent manner. MQ at concentrations of 100 and 200 µM significantly decreased GSH levels at 1 h (P < 0.01) compared with the control values. A return to the control levels by 6 h and a significant increase in intracellular GSH concentration after a 24-h exposure were observed with both concentrations of MQ (Fig. 1). X/XO did not produce any change in GSH after 1 and 6 h of exposure. However, GSH levels significantly increased at 24 h compared with the control values (Fig. 2).

Protein and DNA assays. The protein concentration was determined with the bicinchoninic acid reagent (Pierce, Rockford, IL) (35). The DNA concentration was estimated with the method of Richards (29) using the diphenylamine reagent.

- The results are expressed as means ± SE. Differences between values were compared by Duncan’s multiple range test.
treatment; however, the activity was significantly decreased at 24, 48, 72, and 96 h compared with the control values (Fig. 8).

Effects of MQ, CSC, X/XO, hyperoxia, and dexamethasone on γ-GT activity in alveolar epithelial cells. The activity of γ-GT was not affected by any of the oxidants after 1, 6, and 24 h of treatment (Table 1). By contrast, dexamethasone produced a significant decrease in γ-GT activity at 24, 48, 72, and 96 h (Table 2) compared with the control values. There were no changes in enzyme activity after 4 and 10 h of treatment with dexamethasone.

Fig. 1. Effect of 100 and 200 µM menadione (MQ) at different time intervals on GSH levels in A549 type II alveolar epithelial cells. Each symbol is mean ± SE of 6 experiments. Significant difference compared with control value: **P < 0.01; ***P < 0.001.

Fig. 2. Effect of xanthine/xanthine oxidase (X/XO; 50 µM/10 mU) at 1, 6, and 24 h on GSH levels in A549 cells. Each symbol is mean ± SE of 6 experiments. ** Significant difference compared with control value, P < 0.01.

Fig. 3. Effect of hyperoxic (95% O2) exposure at 2, 8, 12, and 24 h on GSH levels in A549 cells. Each symbol is mean ± SE of 4 experiments. Significant difference compared with control value: **P < 0.01; ***P < 0.001.

Fig. 4. Effect of 3 µM dexamethasone at different time points on GSH levels in A549 epithelial cells. Each symbol is mean ± SE of 4 experiments. Significant difference compared with control value: *P < 0.05; **P < 0.01.
Effects of MQ, X/XO, hyperoxia, CSC, and dexamethasone on protein and DNA concentrations in A549 cells. We determined the concentration of protein per cell or per milligram of DNA after treatment with oxidants and dexamethasone in A549 cells. The concentrations of cellular protein after MQ (100 µM), X/XO, hyperoxia, and CSC exposures at 24 h were not significantly different (0.84 ± 0.1, 0.86 ± 0.07, 0.84 ± 0.1, and 0.79 ± 0.08 mg protein/10⁶ cells, respectively) compared with the control value (0.82 ± 0.1 mg protein/10⁶ cells; n = 3 experiments). Similarly, the concentration of protein per milligram of DNA remained constant after MQ, X/XO, hyperoxia, and CSC treatments in A549 cells at 24 h (6.7 ± 0.6, 6.8 ± 0.9, 7.2 ± 1.1, and 6.8 ± 0.4 mg protein/mg DNA, respectively) compared with the control value (6.8 ± 0.8 mg protein/mg DNA; n = 3 experiments). By contrast, dexamethasone produced a slight but insignificant time-dependent decrease in cellular protein concentration at 24, 48, 72, and 96 h.
Several mechanisms can account for the de novo synthesis of GSH in response to sublethal oxidative stress. It has been reported that γ-GCS normally functions submaximally due to feedback inhibition by GSH (30). A transient decrease in cellular GSH content could release this feedback inhibition of γ-GCS activity, leading to higher GSH levels. This could partially explain the increased intracellular GSH levels produced by MQ and CSC. As the GSH levels increase, further feedback inhibition may result, causing a subsequent decrease in γ-GCS activity. However, an initial depletion of GSH cannot explain the later increase in GSH synthesis after X/XO or hyperoxia exposure. The lack of an initial depletion of GSH with hyperoxia in contrast to MQ and CSC may be explained by the fact that MQ and CSC result in GSH depletion by a mechanism that produces GSH conjugates (26), which does not occur with hyperoxia or X/XO, or that a transient fall in GSH was missed because measurements were made at limited time points.

A second mechanism that can result in increased GSH synthesis is an increase in γ-GCS activity through increased synthesis of the enzyme. Recently, Rahman and colleagues (24, 27) showed that cigarette smoke and MQ can induce γ-GCS-heavy subunit (HS) mRNA expression in human alveolar type II cells that possess the catalytic activity of this enzyme.

Other investigators (15, 22, 33) have also shown that oxidants can increase GSH synthesis in various cell lines. We suggested that reactive oxygen species, particularly O$_2^*$ and H$_2$O$_2$, released from MQ (2) and H$_2$O$_2$ generated by CSC-derived free radicals and electrophilic compounds (21) could result in transcriptional upregulation of γ-GCS-HS. Our data showing an increase in GSH levels and γ-GCS activity by X/XO therefore suggest upregulation of GSH synthesis by the direct action of O$_2^*$ within the cell. This observation is supported by previous studies showing increased γ-GCS-HS mRNA expression after MQ (24) and CSC (27).

Hyperoxia is known to increase GSH levels by increasing cystine uptake in endothelial cells (17). Our data showing increased levels of GSH 2–24 h after hyperoxia exposure in A549 cells suggest that an increased synthesis of GSH occurred in response to hyperoxia. However, the activity of γ-GCS was only increased at 4 h and not at 12 and 24 h. This suggested that the increased GSH levels produced by hyperoxia may not be related to increased γ-GCS activity in alveolar epithelial cells.

Activity of γ-GT has been suggested to be important in maintaining intracellular GSH (28). GSH levels have been shown to be twofold higher in the epithelial lining fluid of chronic cigarette smokers (3, 19). In this study, we hypothesized that cigarette smoke may increase the level of epithelial lining fluid GSH, which might result from the inhibition of γ-GT in epithelial cells, which would prevent the degradation of extracellular GSH. However, we showed that CSC had no effect on γ-GT activity in epithelial cells. Thus our data suggest that γ-GT may not be involved in the regulation of GSH synthesis in alveolar epithelial cells in response to oxidants and dexamethasone.

**Table 1. Effects of MQ, X/XO, CSC, and hyperoxia on γ-GT activity in alveolar epithelial cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>γ-GT Activity, U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>MQ</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>X/XO</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>CSC</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>0.17 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 experiments except n = 2 experiments for hyperoxia. MQ, menadione; X/XO, xanthine/xanthine oxidase; CSC, cigarette smoke condensate; γ-GT, γ-glutamyl transpeptidase. There are no significant differences compared with control values.

(0.72 ± 0.1, 0.69 ± 0.08, 0.65 ± 0.1, and 0.63 ± 0.11 mg protein/10$^6$ cells, respectively) compared with the control values (0.82 ± 0.1, 0.78 ± 0.07, 0.80 ± 0.1 and 0.74 ± 0.12 mg protein/10$^6$ cells; P > 0.05; n = 3 experiments). However, dexamethasone produced a time-dependent increase in the concentration of protein per milligram of DNA (0.6 mg protein/mg DNA; P < 0.05; n = 3 experiments).

**DISCUSSION**

The capacity of mammalian cells to maintain homeostasis of cellular functions during oxidative stress depends on the rapid induction of protective antioxidant enzymes (25, 34). In the present study, we demonstrated that exposure to sublethal concentrations of MQ and CSC caused a transient depletion of GSH followed by an elevation in intracellular GSH levels in A549 epithelial cells. X/XO also produced an elevation in GSH levels at 24 h without an initial depletion, at least at the time points at which measurements were made. One mechanism we considered for this elevation in GSH was an increase in γ-GCS activity, the rate-limiting enzyme of GSH biosynthesis. The results of this study demonstrated that the increase in GSH levels was associated with an increase in γ-GCS activity that occurred in response to exposure of cells to MQ and X/XO. A previous study (27) showed a similar effect with CSC.

**Table 2. Effect of dexamethasone on γ-GT activity in alveolar epithelial cells**

<table>
<thead>
<tr>
<th>Time, h</th>
<th>γ-GT Activity, U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>24</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>48</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>72</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>96</td>
<td>0.24 ± 0.07</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 experiments. *P < 0.01 compared with control values.
to CSC. Indeed the previous work by Rahman and colleagues (24, 27) suggests that the increase in GSH after CSC is mainly due to transcriptional upregulation of γ-GCS-HS mRNA. In contrast, γ-GT has been shown to be induced in rat lung epithelial L2 cells by MQ and tert-butylhydroquinone (12, 14). However, our data failed to show any change in the γ-GT activity in human alveolar epithelial cells in response to MQ, X/OX, or hyperoxia. The possible explanation for differential regulation of γ-GT activity in response to oxidative stress may be due to differential expression of the γ-GT gene in different cell lines and organs (4, 6, 23). A similar discrepancy was also observed recently in a study (39) on the regulation of γ-GT gene in different cell lines and organs (4, 6, 23). A similar discrepancy was also observed recently in a study (39) on the regulation of γ-GT gene in different cell lines and organs (4, 6, 23). A similar discrepancy was also observed recently in a study (39) on the regulation of γ-GT gene in different cell lines and organs (4, 6, 23).

Corticosteroids such as dexamethasone are thought to suppress inflammation by interacting with various transcription factors (1). The role of redox status, particularly GSH redox status, in the regulation of transcription factors is of considerable interest currently (1). However, the effect of dexamethasone on GSH synthesis has not been extensively studied so far. We show for the first time a time-dependent depletion of intracellular GSH by dexamethasone in A549 alveolar epithelial cells. The decrease in GSH is associated with inhibition of the activity of γ-GCS and γ-GT. Similar hemorrhodal inhibition of GSH synthesis has been observed in a rat hepatic cell line (16). Dexamethasone treatment also produced a time-dependent small decrease in the concentration of protein per cell and the milligrams of protein per milligram of DNA. However, the extent of the decrease in protein or DNA levels did not account for the dramatic decrease in the levels of GSH, γ-GCS, and γ-GT activity (30–77%) in response to dexamethasone in A549 cells because the decrease in the level of milligrams of protein per cell and per milligrams of DNA was only 12–15 and 15–27%, respectively. The reason for the inhibition of γ-GCS by dexamethasone is not known; however, it is of interest that the γ-GCS gene is regulated by an AP-1 transcription factor (27), which may be inhibited by dexamethasone. Further studies are required to understand the molecular mechanism(s) of the action of dexamethasone on the GSH synthesis.

In conclusion, this study has shown that sublethal concentrations of MQ, X/OX, and hyperoxia produce elevations in intracellular GSH content in alveolar epithelial cells. This elevation appears to be dependent on an increase in γ-GCS enzymatic activity but not on the increase in γ-GT activity. Dexamethasone decreases GSH levels by inhibiting γ-GT and γ-GCS activity and hence GSH synthesis. We suggest that γ-GT activity may not be necessary for the regulation of GSH under oxidative stress in human alveolar epithelial cells.

This work was supported by the British Lung Foundation and the Chest Heart and Stroke Association (Scotland).

Address for reprint requests: W. MacNee, Respiratory Medicine Unit, Dept. of Medicine, Univ. of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh EH3 9WT, UK.

Received 21 November 1997; accepted in final form 3 April 1998.

REFERENCES


