Chapter 4

GASEOUS-PHASE CIGARETTE SMOKE IRREVERSIBLY MODIFIES

GLUTATHIONE IN AIRWAY EPITHELIAL CELLS

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ABSTRACT

In patients with COPD an imbalance between oxidants and anti-oxidants is acknowledged to result in disease development and progression. Cigarette smoke (CS) is known to deplete total glutathione (GSH+GSSG) in the airways. We hypothesized that components in the gaseous-phase of CS may irreversibly react with GSH to GSX, thereby causing this depletion. To understand this phenomenon, we investigated the GSH metabolism in response to CS, and identified the actual GSX compounds. Both CS and H_2O_2-control depletes reduced GSH in solution (Δ -54.1±1.7 μM, $P < 0.01$ and Δ - 39.8±0.9 μM; $P < 0.01$). However, a significant decrease of total glutathione was observed after CS exposure (Δ -75.1±7.6 μM, $P < 0.01$) but not after H_2O_2 exposure. Exposure of A549 cells and primary bronchial epithelial cells to CS decreased free sulfhydryl (–SH) groups (Δ -64.2±14.6 μM/mg protein; $P < 0.05$) and irreversibly modified total glutathione (Δ -17.7±1.9 μM; $P < 0.01$) compared to non-exposed cells or H_2O_2 control. Mass spectrometry (MS) showed that GSH was modified into glutathione-aldehyde derivatives. Further MS identification showed that GSH was bound to acrolein and crotonaldehyde, and another, yet unidentified structure. Our data shows that CS did not oxidize GSH to GSSG, but reacts to non-reducible glutathione-aldehyde derivatives, thereby depleting the total available GSH-pool.

INTRODUCTION

Smoking and genetic susceptibility are the main risk factors for the development of Chronic Obstructive Pulmonary Disease (COPD) (2, 22). One important hypothesis with respect to the pathophysiology of COPD is an imbalance between oxidants and antioxidants in the airways. This imbalance results in disease, when the anti-oxidant capacity of the lung is unable to sufficiently neutralize reactive compounds present in cigarette smoke (CS) or generated during the persistent airway inflammation present in COPD (18).

Reactive compounds in the gaseous-phase of CS are thought to constitute the main component of the oxidative stress present in COPD (15). In patients with COPD, increased levels of oxidative stress parameters have been documented in exhaled breath condensate, sputum and blood (e.g. higher H_2O_2, 8-isoprostane, malondialdehyde (MDA), and lower reduced glutathione (GSH) levels) (3, 11). Changes induced by these reactive components may result in inactivation of anti-proteases, epithelial cell injury, apoptotic and necrotic cell death, mitochondrial dysfunctions, disturbance of extracellular matrix repair, and maintenance of airway inflammation, all potentially of importance in the development of COPD (25, 32).

Since the airways of smokers are exposed to highly reactive components, the lung is always at risk of oxidative injury (26). To ensure an appropriate defense against this injury, the respiratory tract is equipped with the epithelial lining fluid (ELF) and the airway epithelium, which both contain large amounts of GSH (9). GSH plays a key role in the cellular redox balance, and is thought to be one of the most important anti-oxidant defenses against CS inhaled reactive components (4).

Under non-stress conditions, most of the intracellular GSH is 'stored' in its reduced form. However, the balance between GSH and oxidized glutathione (GSSG) can change significantly under conditions of oxidative stress, and their ratio provides information on the redox status of cells and tissues. During states of increased oxidative stress, the free sulfhydryl (-SH) groups become oxidized (33). It has been described that exposure to the gaseous-phase of CS in vitro and in vivo generally results in a loss of GSH, whereas the amount of GSSG does not increase significantly (21, 26). This was confirmed by preliminary experiments performed in our laboratory, where an irreversible loss of total glutathione in epithelial cells was observed when exposed to gaseous-phase CS.

We therefore hypothesized that components in the gaseous-phase of CS may irreversibly react with GSH to GSX. As a consequence of this, GSH derivatives that can not be reduced (GSX) may induce a chronic lack of protection against additional CS-exposure under conditions where *de novo* synthesis of GSH is rate-limiting. This may occur when there is a genetic deficiency in *de novo* synthesis of GSH, which predisposes smokers to the development of COPD (8, 14).

MATERIALS AND METHODS

Chemicals

L-cystein, bovine serum albumin (BSA), reduced glutathione (GSH), oxidized glutathione (GSSG), hydrogen peroxide (H_2O_2), 5,5'-dithiobis-(2)-nitrobenzoic acid

(DTNB), trichloroacetic acid (TCA), acrolein and crotonaldehyde were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

Cell cultures
The human alveolar type II epithelium-like adherent cell line, A549 was purchased from American Type Culture Collection (ATCC, Manassas, VA). Human primary bronchial epithelial cells were collected and cultured by the methods described previously (32). All cells were grown in RPMI 1640 with 25 mM HEPES, L-Glutamine (BioWitthaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (BioWitthaker, Verviers, Belgium) and 20 µg/ml Gentamycin (Centafarm Services, Etten-Leur, The Netherlands). All cells were grown in 25 cm_2 plastic culture flasks (Costar, Cambridge, MA) at 37°C in an atmosphere of 5% CO_2 until 80-90% confluency was reached. Before the experiments both the A549 cells and the human primary bronchial epithelial cells were incubated for 16 h in serum free RPMI 1640 media.

Exposure of a GSH solution to different oxidants
A solution of GSH (150 µM in pure H_2O, pH 4.2) was exposed to air, the gaseous-phase of one cigarette (CS) or two cigarettes (CS (2x)), or a solution of H_2O_2 (1 mM). Briefly, 25 ml solution was placed in a 50 ml Falcon tube (BD Biosciences, Alphen aan den Rijn, The Netherlands) at 37°C. Kentucky 2R4F research-reference cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) were smoked using a peristaltic pump. Just before the experiments, filters were cut from the cigarettes. Each cigarette was smoked in exactly 5 min at a flowrate of 8 L/h and bubbled through the GSH solution. This solution was used immediately for the experimental procedures. Air, produced with the same peristaltic pump, but without the use of a cigarette, was used as a negative control under the same conditions. The oxidative agent H_2O_2 (final concentration 1 mM) was used as a positive control.

Exposure of airway epithelial cells to CS
A549 cells were exposed to air, CS or a solution of 5 mM H_2O_2. Briefly, A549 cells were grown in 25 cm2 plastic culture flasks as described above. Just before the experiments, medium was removed and the culture flask was positioned up-side down, allowing a direct contact of smoke with epithelial cells. Kentucky 2R4F research reference cigarettes were smoked using a peristaltic pump. Just before the experiments, filters were cut from the cigarettes. Each cigarette was smoked in exactly 5 min at a rate of 8 L/hr. Gaseous-phase CS was directly distributed inside the culture flasks, by blowing the smoke inside through a small plastic tube. After the exposure, cells were washed with PBS, lysed with water and analyzed. Air was used as negative control under the same conditions as CS. As positive control, H_2O_2 (final concentration 5 mM) was incubated at room temperature for 5 min, after which the cells were washed with PBS, lysed with water and analyzed.

Quantitative determination of total -SH groups
A549 cells were washed with PBS and lysed by one freeze-thaw cycle in 2.5 ml pure H_2O. Total protein concentration was determined by the Bradford method (7), using BSA as standard (Bio-Rad Laboratories, The Netherlands). Ellman's reagent (12) was used for the determination of free -SH groups in cell culture and a cell-free solution of GSH. Ellman's reagent (12 mM DTNB) was added to the lysed cells or GSH solution to a final concentration of 6 mM DTNB followed by 10 min incubation. Thereafter, samples were centrifuged at 1000 g for 5 min. The supernatant was used in the assay and measured at 405 nm in a Biotek EL808 microplate reader (Bio-Tek Instruments, Abcoude, The Netherlands). The amount of free thiol groups was calibrated against a standard curve of L-cystein.

Quantitative determination of total glutathione using the Tietze assay
The sum of reduced and oxidized glutathione in different samples was determined by the enzymatic method of Tietze (31). Briefly, cells were washed with PBS and treated with 5% TCA. The samples of the GSH solution were also treated with 5% TCA. All samples were centrifuged at 10.000 g for 5 min, at 4°C. 150 µL of each standard and sample were pipetted into a 96-wells Elisa plate. 25 µL Ellman reagents (DTNB) and 5 µL glutathione reductase (GR) were added. This supernatant was enzymatically reduced by the added GR and immediately before reading, NADPH was added and the increase absorbance at 405 nm was recorded for 10 min at room temperature on a Biotek EL808 microplate reader. The results were compared with a standard curve of GSH.

Mass spectrometry
Before analyzing A549 cell lysates, primary bronchial epithelial cell lysates, and solutions of GSH and GSSG by mass spectrometry (MS), samples were filtrated using an Amicon Ultra 5-kDa cut-off centrifugal filter device (Millipore Co., Cork, Ireland). The collected ultrafiltrate was diluted in a 1:1 ratio with 0.1% (v/v) formic acid (Merck, Haarlem, The Netherlands) in acetonitrile (Merck, Haarlem, The Netherlands). The samples were analyzed by direct infusion at 5 µL/min into an SL ion trap mass spectrometer (Agilent, Santa Clara, USA) equipped with an electrospray ionization source operated in positive mode, using the following conditions: capillary voltage (3000V), nebulizer gas (N_2, 10 psi), drying gas (N_2, 4 L/min) at 275ºC. MS data were acquired over a scan range of 50-650 m/z (mass/charge) and with a scan rate of 5,500 m/z per second. The target m/z was 615, the compound stability was set at 70%. Data were collected for 10 min prior to mass spectrum averaging and analyzed using data analysis software for LC/MSD Trap version 3.2 (Bruker Daltonics, Bremen, Germany). Peaks of interest were fragmented using manual MS/MS (MS2) settings (width of precursor ion selection: 1.0-1.5 Da; amplitude of 0.8-1.0 V). MS2 data were collected for 5 min.

Statistical Analysis
Data were analyzed using Prism 4 for Windows (GraphPad Software, inc., San Diego, USA). Comparisons between different experimental groups were performed with Dunnet's multiple comparison test (figure 1 A, B and 3 A, B). $P < 0.05$ was

considered significant. Results are presented as mean values (±SEM) unless otherwise mentioned.

RESULTS

Cigarette smoke decreases total glutathione in solution

GSH is one of the most important anti-oxidants present in the ELF and participates in the defense of pulmonary epithelial cells against inhaled reactive components of CS. To test whether these CS components are able to modify the free thiol groups of GSH, a solution of GSH was exposed to CS. While air, used as negative control, did not affect the level of free -SH groups of GSH, exposure to gaseous-phase CS (CS (2x), Δ -54.1±1.7 μM, $P < 0.01$; Fig. 1A) or the addition of 1 mM H_2O_2 (Δ -39.8±0.9 μM, $P < 0.01$; Fig. 1A) resulted in a dose-dependent decrease of free -SH groups. To determine whether CS oxidizes GSH to GSSG, the enzymatic reducing cycle of Tietze was used. A significant reduction of total glutathione (GSH+GSSG) was observed after exposure to gaseous-phase CS (CS (2x): Δ -75.1±7.6 μM, $P < 0.01$; Fig. 1B) whereas H_2O_2, which has been proven to oxidize GSH (Fig. 1A), did not affect the total glutathione concentration compared to control (Fig. 1B).

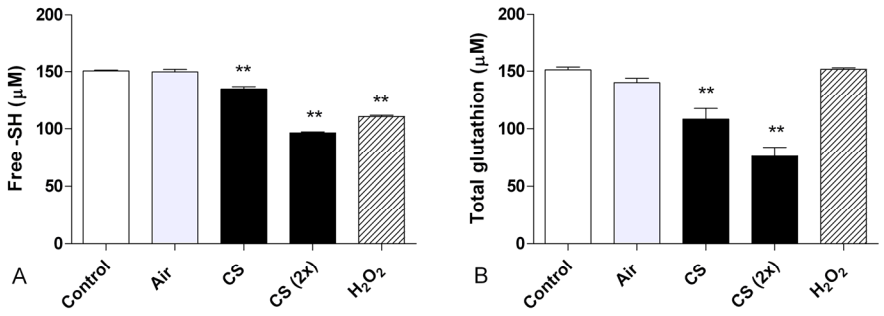


Figure 1. The effect of cigarette smoke, control air and H_2O_2 on the level of free –SH groups of GSH in solution. Free –SH groups were studied using Ellman's reagent (A). Total glutathione was studied using the enzymatic reducing cycle system of Tietze (B). CS = exposure to 1 cigarette, CS (2x) = exposure to 2 cigarettes. Data are expressed as mean values ± SEM and are referred to 4 experiments. **, $p<0.01$ vs control by Dunnet's multiple comparison test.

MS analysis of CS-exposed GSH in solution

To understand why CS decreases free –SH groups in solution, samples were analyzed by mass spectrometry using direct infusion. Figure 2A and B show the MS background of pure water and water exposed to the gaseous-phase of two cigarettes. Water exposed to CS shows a substantial background between m/z 80-300. Because the background was less prominent above m/z 300 we were able to study glutathione modification (m/z 308.1, singly charged protonated GSH, Fig. 2C). In figure 2D two peaks corresponding to GSSG can be seen. The peak with the m/z value of 307.1 indicates double charged GSSG, and the peak of m/z 613.2 indicates single charged GSSG. MS analysis of GSH in solution exposed to the gaseous-phase of two cigarettes did not result in the formation of oxidized GSSG. However, two

additional components (component 1 and 2) were observed with m/z values of 364.1 and 378.1 respectively (Fig. 2E). Addition of H_2O_2 to GSH in solution, as control experiment, resulted in two peaks of m/z 307.2 and 613.2, proving the direct oxidation of GSH into GSSG (Fig. 2F).

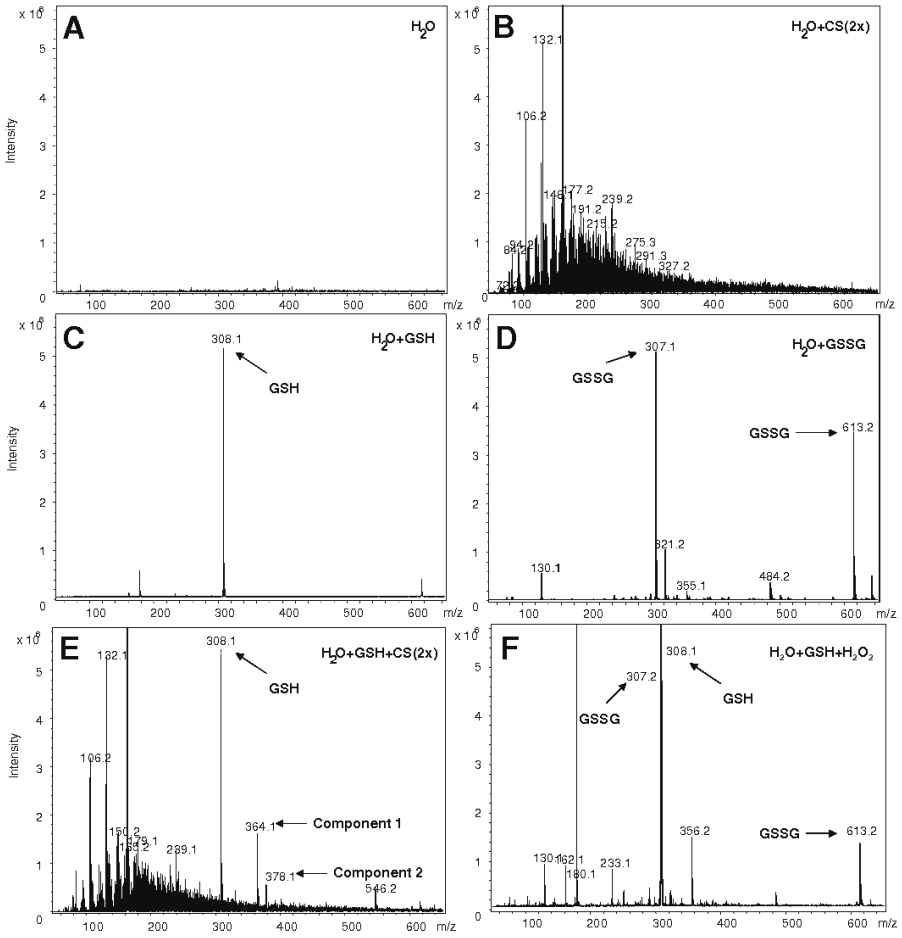


Figure 2. The effect of cigarette smoke and H_2O_2 on the modification of a cell-free GSH solution by mass spectrometry. A) sample solution (pure H_2O); B) exposure of H_2O to 2 cigarettes (H_2O+CS(2x)); C) solution of GSH (final concentration 150 μM) (H_2O+GSH); D) exposure of GSH to 2 cigarettes (H_2O+GSH+CS(2x)); E) solution of GSSG (final concentration 150 μM) (H_2O+GSSG); F) exposure of GSH to H_2O_2 (final concentration 1 mM) (H_2O+GSH+H_2O_2). The mass spectra are representative of one experiment out of three.

Cigarette smoke decreases total –SH groups in airway epithelial cells

To investigate the effect of CS on the redox status of free -SH groups, airway epithelial cells were directly exposed to CS. Air exposure used as a negative control did not affect free -SH groups in the cells, whereas the gaseous-phase of CS significantly decreased this level by -64.2±14.6 μM/mg protein, $P < 0.05$ (Fig. 3A). H_2O_2 used as positive control, did not show an effect, which might be due to the short time of incubation (30).

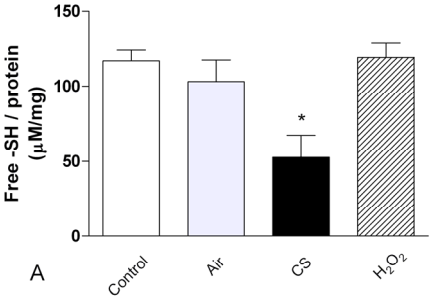
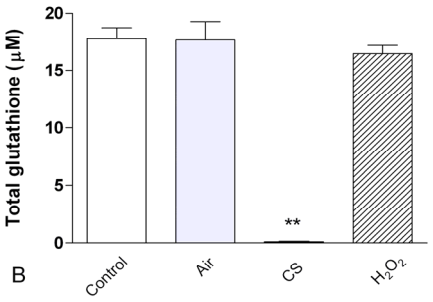


Figure 3A. The effect of cigarette smoke on free –SH groups in A549 cells. Free –SH groups in A549 cells were measured using Ellman's reagent. Data are expressed as mean values ± SEM and are referred to 4 experiments. *, $P < 0.05$ vs control by Dunnet's multiple comparison test.

Cigarette smoke decreases total glutathione in airway epithelial cells

To examine whether glutathione was irreversibly modified after CS exposure, total glutathione was determined using the enzymatic GSSG-reducing cycle system described by Tietze (31). A significant reduction of total glutathione was observed after the A549 cells were exposed to gaseous-phase CS (CS, Δ -17.7±1.9 μM, $P < 0.01$; Fig. 3B). Exposure to air and H_2O_2 did not affect the total glutathione concentration in the epithelial cells compared with control cells.

Figure 3B. To examine irreversible modification of glutathione after exposure of A549 cells to cigarette smoke, total glutathione was measured using the enzymatic reducing cycle system of Tietze. Data are expressed as mean values ± SEM and are referred to 4 experiments. **, $P < 0.01$ vs control by Dunnet's multiple comparison test.



MS analysis of airway epithelial cells

A549 cells were exposed directly to the gaseous-phase of CS and thereafter lysed. This lysate was directly analyzed by MS by direct infusion. Non-exposed A549 cells (Fig. 4A) and cells exposed to air (Fig. 4B) show a peak at m/z 308.1, corresponding to the reduced form of GSH. We observed a decreased signal for GSH in the CS-exposed cells (Fig. 4C) compared to the untreated- or air exposed cells and observed the appearance of three different components (Component 1, 2 and 3). The first two components were also observed in the CS-exposed GSH solution: the peaks at m/z 364.1 (Component 1) and 378.1 (Component 2). Furthermore, a new peak appeared

corresponding with an m/z value of 391.2 (Component 3). Short exposure of A549 cells to H_2O_2 did not alter GSH, compared to the non-exposed cells (Fig. 4D). Interestingly GSSG (peaks at m/z of 307.1 and 613.2) was not detectable in any of the A549 cultures.

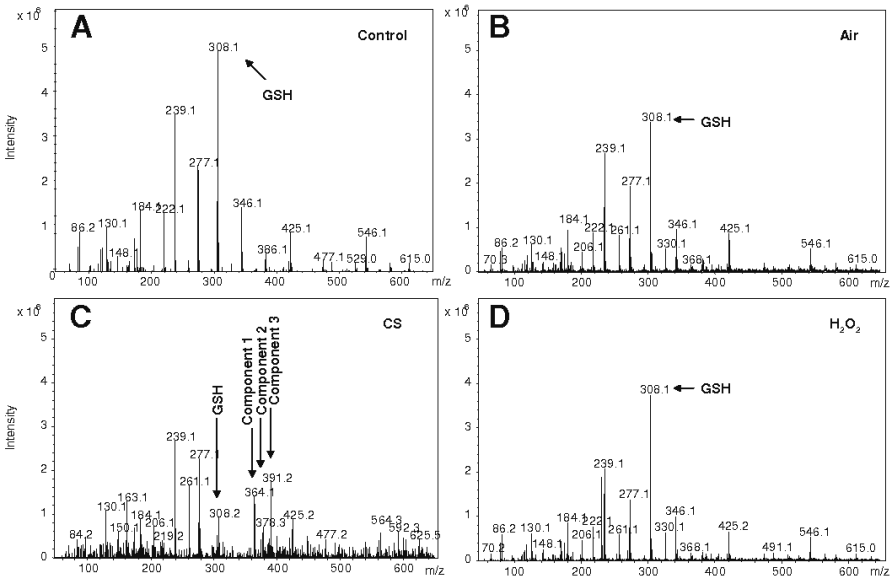


Figure 4. The effect of cigarette smoke and H_2O_2 on modification of GSH in A549 cells by mass spectrometry. A) control cells (no exposure); B) exposure to air; C) exposure to cigarette smoke (CS); D) exposure to H_2O_2 (final concentration 5 mM). Characterization of the peaks at m/z 364.1 (Component 1), 378.1 (Component 2) and 391.2 (Component 3) was done by MS2 spectrometry (Fig. 5). The mass spectra are representative of one experiment out of three.

Mass spectrometry showed GSH modification into glutathione-aldehyde derivatives

MS analysis of CS-exposed A549 cells resulted in a decreased peak at m/z 308.1, reduced GSH, and additional peaks at m/z values of 364.1 (Component 1), 378.1 (Component 2) and 391.2 (Component 3). The isotope distribution confirmed that these m/z values corresponded to singly-charged molecules. By subtracting the mass of these components from the mass of GSH we were able to select a few candidate molecules that would fit the observed mass difference including acrolein or crotonaldehyde. After incubation of GSH with acrolein and crotonaldehyde we were able to detect peaks at m/z values of 364.1 and 378.1 respectively (Fig. 5A and B). Identification of these peaks was done by MS2 fragmentation. The fragments obtained from the A549 cells exposed to gaseous-phase CS were compared to GSH incubated with acrolein (GSH+acrolein) or crotonaldehyde (GSH+crotonaldehyde). These findings were also confirmed by MS2 fragmentation of primary bronchial epithelial cell lysates under the same experimental conditions as the A549 cells (Fig. 5C and D). Fragmentation confirmed the identity of the peaks to be GSH-acrolein (Component 1) and GSH-crotonaldehyde (Component 2) (Fig. 5C and D). The same

cell lysate also showed a peak at m/z 391.2 (Component 3). So far we have not been able to identify this peak, apart from the fact that it did not derive from a phthalate contamination. Phthalates are well known contaminants in MS, coming from plastic bottles, flasks and packing materials (13). MS2 fragmentation of peak m/z 391.2 (Component 3) obtained from traces of phthalate contamination differs from the MS2 fragmentation of peak m/z 391.2 obtained from the cell lysate (Fig. 5E and F).

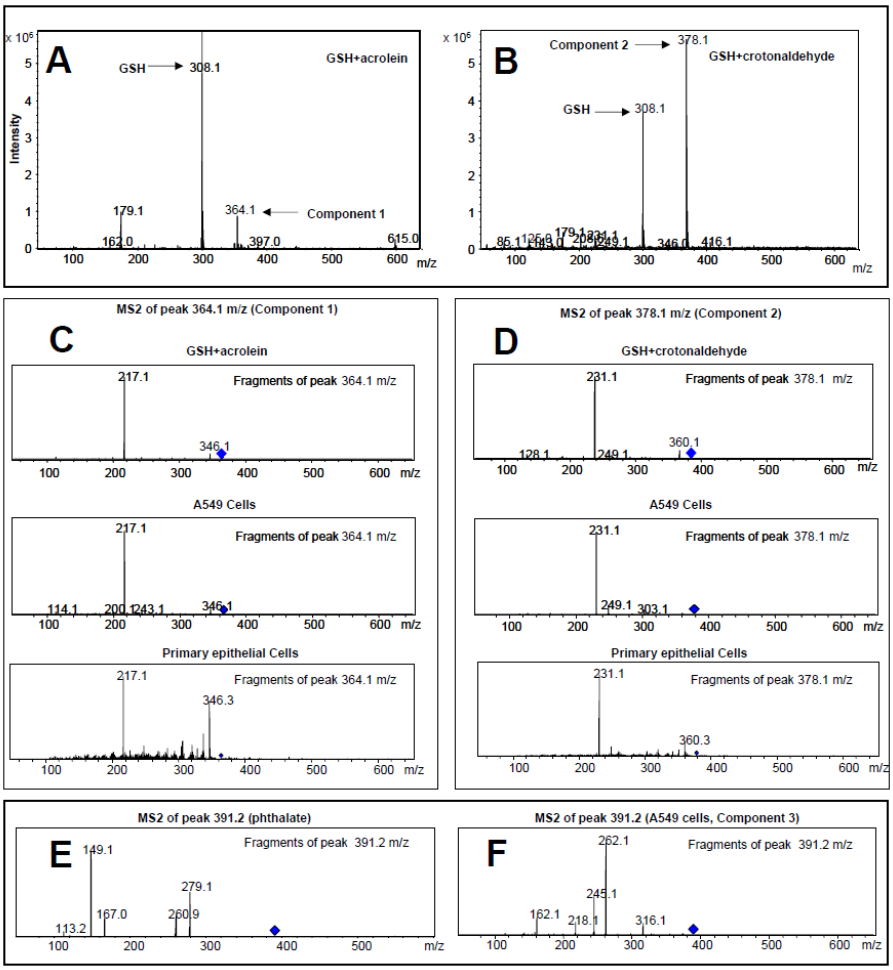


Figure 5. Identification of GSH-acrolein (m/z 364.1, component 1) and GSH-crotonaldehyde (m/z 378.1, component 2) derivatives by MS2. The mass spectra of GSH + acrolein (A) and GSH + crotonaldehyde are shown (B). First daughter ion spectra of GSH+acrolein, A549 cells and primary bronchial epithelial cells are shown (C) and the first daughter ion spectra of GSH+crotonaldehyde, A549 cells and primary bronchial epithelial cells are shown (D). First daughter spectra of phthalate (E) and the unknown component from A549 cells (Component 3) are shown (F). The mass spectra are representative of one experiment out of three.

DISCUSSION

In this study we investigated the modification of glutathione (GSH) and more general the total reduced thiol content of airway epithelial cells by the gaseous-phase of CS. We hypothesized that CS can irreversibly modify GSH, rendering it unavailable for the enzymatic reducing cycle system that is thought to play an important role in protection of airway epithelial cells against oxidative stress. Our results clearly showed that the gaseous-phase of CS decreases free -SH groups of GSH in solution and in airway epithelial cells. We observed that the reactive components present in the gaseous-phase of CS did not oxidize GSH to the disulfide-containing GSSG. Instead of this physiological reaction, GSH was irreversibly modified by unsaturated aldehydes that are being generated during the combustion of tobacco. In vitro experiments showed that exposure of CS changed almost the entire pool of GSH into GSH-aldehyde components.

These findings shed new light on the GSH redox cycle in airway epithelial cells. The enzymatic redox cycle, which is normally activated after oxidative stress and the formation of GSSG, could not be activated because of the depletion of GSH into nonreducible glutathione components, with loss of the GSH pool. This exhaustion of the pool of reduced GSH may induce a chronic lack of anti-oxidant protection. Persistent smokers may in that case inhale more ROS than can be scavenged by the residual anti-oxidants, resulting in increased vulnerability to oxidative stress. This makes the synthesis of GSH essential for cellular survival and protection of the lung.

The development of COPD is associated with increased oxidative stress and reduced antioxidant resources (5; 6; 18). Smoking cigarettes is the most important factor for the development of COPD, which is currently the 5th leading cause of death worldwide (19). Cellular stress induced by CS is critically dependent on the intracellular reduced GSH concentration. For instance, intracellular GSH depletion significantly facilitates stress signal transduction pathways, cell proliferation, apoptosis and inflammation (1). Studies performed by Rahman *et al.* showed that a GSH/GSSG ratio of less than 90% influences a variety of cellular signaling processes, such as phosphorylation of stress kinases JNK, p38, MAPK and PI-3K as well as activation of the transcription factors AP-1 and NFκB (23, 27). In these publications it has been demonstrated that increasing the intracellular levels of GSH can provide protection. In other respiratory diseases like cystic fibrosis a significant decrease of GSH efflux from cells, which leads to deficiency of GSH in the ELF of the lung, as well as in other compartments, including cells of the immune system and the gastrointestinal tract, was observed (16).

Although reduced GSH has been shown to be elevated in the ELF of chronic smokers, CS acutely lowers intracellular levels of GSH (17, 24). An animal study by Cotgreave *et al.* showed that acute effects of CS inhalation by rats caused significant depletion of GSH in the whole lung, lavage cells and lavage fluid. The depleted GSH could not be reduced by a reducing agent like dithiothreitol. They suggested that GSH was irreversible conjugated with electrophilic components of the CS (10). In line with these results, intratracheal instillation of CS condensate in the rat resulted in depletion of intracellular GSH, concomitant formation of GSH-conjugates without significant elevation of oxidized GSSG nor any GSH efflux from

the cells (24). These studies are in agreement with our current findings. We showed in a GSH solution that GSH exposed to gaseous-phase CS was not oxidized into GSSG but became irreversibly modified into glutathione derivatives (Fig. 1 and 2). Where addition of the enzyme GR to a solution of GSH exposed to H_2O_2 resulted in a restoration of the initial GSH concentration, this enzyme was not able to reduce the components in the GSH solution exposed to CS, indicating that GSH had been irreversibly modified. However, this enzyme was not able to reduce the components in the GSH solution exposed to CS, indicating that GSH had been irreversibly modified. Exposure of cultured alveolar A549 cells to the gaseous-phase of CS also showed that GSH was irreversibly modified into the same GSH derivatives, whereas incubation with H_2O_2 for 5 minutes did not alter the intracellular pool of GSH.

MS showed that the depletion of GSH could be attributed to the formation of glutathione-aldehyde derivatives. Direct infusion of cell lysate into the mass spectrometer showed three peaks (m/z 364.1, 378.1 and 391.2), while the peak of GSH (m/z 308.1) was diminished. We confirmed by MS2 that two of the three peaks were GSH aldehyde derivatives, respectively GSH-acrolein (m/z 364.1) and GSH-crotonaldehyde (m/z 378.1). In line, a recent study by Reddy *et al.* demonstrated that exposure of neutrophils to the gaseous-phase of CS also resulted in the formation of GSH-acrolein (29). In contrast to our observations, Reddy *et al.* were not able to show GSH-aldehyde derivatives in airway epithelial cells. This may be due either to a lower exposure to CS, to a longer incubation time of the cells allowing the release of the toxic components into the culture medium or to the use of cell culture medium resulting in no direct contact of smoke with the cultured epithelial cells. In our system, medium was removed from the cells with only a very thin layer of medium covering the cells, allowing a more direct contact of the gaseous phase of CS with the epithelial cells. After the exposure, cells were rapidly lysed and analyzed by MS by direct infusion. Reported EC50 values of a variety of aldehydes that are able to deplete GSH, showed that acrolein was by far the most effective one to bind to GSH followed by formaldehyde and crotonaldehyde (20). In contrast to our data from airway epithelial cells, the third peak (m/z 391.2) was not seen within the CS-exposed GSH solution. This peak is similar to a well known contaminant in MS, coming from phthalate compounds in plastic, however, fragmentation of the peak by MS2 showed that it was not a phthalate compound (13). It may be proposed that this component is derived from membrane degradation by radicals in CS, but the mass of this component could not be ascribed to known membrane degradation products.

The presence of oxidative stress and decreased anti-oxidant capacity has important consequences for the pathogenesis of COPD. Activation of redox-sensitive transcription factors enhances pro-inflammatory mediators and protective anti-oxidant gene expression. In biochemistry, *de novo* synthesis of GSH from glutamate, cysteine and glycine is catalyzed sequentially by the two cytosolic enzymes, γ-GCS and GS. It is already known that epithelial cells of the lung respond rapidly and sensitively to oxidative stress, and that this adaptive response is mediated by an increase of γ-GCS mRNA and enzyme activity (28). Although CS is the main environmental risk factor for developing COPD, only about 15-20% of smokers develop COPD. Genetic factors are likely to modify the risk of developing COPD. It can therefore be hypothesized that restricted ability to synthesize sufficient GSH may be due to polymorphisms in genes linked to *de novo* synthesis of GSH

leading to COPD. A few studies are available involving polymorphisms in genes related to GSH synthesis and lung disease (8; 14). Therefore, further research on the variability among genes encoding for *de novo* GSH synthesis in smokers would be of great interest in human genetics of COPD.

For the first time, we have demonstrated by direct infusion MS combined with enzymatic assays, that a substantial amount of GSH in epithelial cells is irreversibly modified into GSH-acrolein and GSH-crotonaldehyde derivatives and a yet unknown component. Under these circumstances, a chronic lack of protection against oxidative stress might be induced, especially when a genetic predisposition of rate-limiting *de novo* synthesis of GSH is present. These findings might be a possible biochemical mechanism of CS induced toxicity which has been found in patients with COPD.

GRANTS

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