Ozone Interaction with Rodent Lung

III. OXIDATION OF REDUCED GLUTATHIONE AND FORMATION OF MIXED DISULFIDES BETWEEN PROTEIN AND NONPROTEIN SULFHYDRYLS

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ABSTRACT Nonprotein sulfhydryls (NPSH), a major source of cellular reducing substances, were examined in lung tissue after short-term exposure of rats to O_3. While the NPSH level was unaffected by low-level exposures (e.g., 0.8 ppm for up to 24 h or 1.5 ppm for up to 8 h), it was significantly lowered by higher exposure regimens (e.g., 25% after 2 ppm for 8 h and 49% after 4 ppm for 6 h). After exposure to 4 ppm O_3 for 6 h the level of reduced glutathione (GSH), which accounted for approximately 90% of NPSH in the lung, decreased 40% but without a rise in the level of oxidized glutathione (GSSG).

Treatment of lung homogenate with borohydride led to recovery of NPSH in exposed lungs to control values, suggesting that NPSH or GSH oxidation during in vivo O_3 exposure resulted in formation of mixed disulfides with other sulfhydryl (SH) groups of lung tissue. Extracts of borohydride-treated particulate and supernatant fractions of lung homogenate were analyzed for NPSH by paper chromatography. From this analysis GSH appeared to be the only NPSH bound to lung tissue proteins via mixed disulfide linkage.

The formation of mixed disulfides appeared to be a transient phenomenon. Immediately after a 4-h exposure to 3 ppm O_3 the level of mixed disulfides was small (15% of the total NPSH) but attained a peak (equivalent to 0.6 μmol NPSH/lung) after a recovery for 24 h. However, the level diminished considerably within 48 h of recovery.

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INTRODUCTION

A number of authors reviewing the biochemical mechanisms of oxidant toxicity have stressed that thiol oxidation is an important factor in cellular injury (1–7). In this regard, the effects of O_3, the predominant oxidant of photochemical smog, on oxidation of sulfhydryls (SH) [1] and activities of SH-dependent enzymes have been of particular concern. Studies of Mountain (8) demonstrated that in vivo O_3 exposure (8 ppm, 3 h) caused an oxidation of reduced glutathione (GSH) and depression of succinate dehydrogenase activity in mouse lung. King (9) showed a loss of SH content as well as enzymatic function of partially purified glyceraldehyde-3-phosphate dehydrogenase in rat lung subsequent to O_3 exposure (1.2 ppm, 4 wk). Recent studies of DeLucia, Hoque, Mustafa, and Cross (10) demonstrated that the levels of protein sulfhydryls (PSH) and nonprotein sulfhydryls (NPSH) in rat lung were depressed, and that, concomitant with the loss of thiols, the activities of a number of enzymes in the lung (viz., glucose-6-phosphate dehydrogenase, glutathione reductase, and NADH and succinate-cytochrome *c* reductases) were inhibited by in vivo O_3 exposure (2 ppm, 4–8 h). The foregoing observations, and also those of Fairchild, Murphy, and Stokinger (11), which indicate that exogenous SH compounds can protect against mortality and pulmonary edema re-

[1] *Abbreviations used in this paper:* DTNB, 10 mM 5,5'-dithiobis(2-nitrobenzoic acid); DTNP, 2,2'-dithiobis(5-nitropyridine); GSH, glutathione; GSSG, oxidized gluthathione; NPSH, nonprotein sulfhydryls; PSH, protein sulfhydryls; SH, sulfhydryls.

sulting from O_3 exposure of rats, testify that this oxidant causes oxidation of thiols and inhibition of SH-dependent enzymes in the lung.

Although the occurrence of thiol oxidation in the lungs of O_3-exposed animals is well documented, no attempts have been made to characterize the thiol oxidation products or their reversibility to original reduced forms. As has been shown by Mudd, Leavitt, Ongun, and McManus (12) and Menzel (13) using in vitro O_3 exposure, and by Little and O'Brien (14) using lipid peroxides, oxidation of GSH resulted mainly in the formation of oxidized glutathione (GSSG), which like other disulfides is relatively stable to further oxidation (15). In vivo measurements of oxidized glutathione levels in various tissues, however, have shown that only about 1–3% of the total glutathione occurs in the free oxidized form (16, 17). Other disulfide forms of glutathione, namely mixed disulfides consisting of PSH and GSH, appear to be more common, and have been shown to occur in mammalian erythrocytes (18), ascites tumor cells (19), human lens (20), and several rat tissues (21) as a significant fraction of the total thiols present. Although O_3 exposure has been shown to cause diminution of NPSH level in lung tissue (10), it has not been determined whether in vivo oxidation of GSH in the lungs of O_3-exposed animals results in an increase of GSSG above the normal level or whether it results in an increased level of mixed disulfides.

In this study the products of thiol oxidation in the lungs of O_3-exposed rats have been analyzed. The results demonstrate that mixed disulfides are formed as the product of NPSH oxidation, and that glutathione is the major identifiable component which can be liberated from the mixed disulfides.

METHODS

Exposure protocol

Male Sprague-Dawley rats 60–90 days old, initially free of chronic respiratory disease and weighing 300–400 g, were used in the experiments. The exposures were conducted at either 1.5–4 ppm O_3 for up to 8 h or 0.8 ppm O_3 for up to 24 h under conditions similar to those described previously (10).

Tissue preparation

Animals were killed by decapitation immediately after the exposure. The lungs were perfused with saline (0.15 M NaCl) through the pulmonary artery to remove as much blood as possible, and then were excised, trimmed of extraparenchymal bronchovascular connective tissue, and homogenized in a glass Teflon homogenizer using an ice-cold medium containing 0.15 M NaCl, 5 mM Tris-chloride, and 1 mM Tris-EDTA at pH 7.5. The homogenate was filtered through a two-layer cheese cloth and the filtrate adjusted to a 12-ml final volume with ice-cold medium so as to allow calculation of data on a per lung basis.

To prepare the particulate and soluble fractions of lung homogenate the original homogenate was diluted 1:1 with cold distilled water and then centrifuged at 40,000 *g* 30 min. The supernate was saved for thiol identification. The resulting pellet was washed once by resuspending in a 50 mM Tris-chloride buffer (pH 7.5) and centrifuged as before. The protein components of the original 40,000 *g* supernate were precipitated by addition of 0.3 ml of 50% (wt/vol) trichloroacetic acid (TCA). The precipitate was collected by centrifuging at 2,500 rpm for 5 min in a clinical centrifuge, resuspended in 1 ml of 50 mM Tris-chloride (pH 7.5), and then dissolved by adding a drop of 10% NaOH.

To prepare a mitochondria-rich fraction the filtered lung homogenate was centrifuged first at 700 *g* for 10 min (Sorvall RC-2B, rotor SS-34, Ivan Sorvall, Inc., Newton, Conn.) to remove the nuclei and broken cell debris, and then at 9,000 *g* for 10 min to sediment the mitochondrial fraction (22). The mitochondria thus obtained were washed three times with fresh medium, and the final pellet was resuspended in a 2-ml volume.

Assays

NPSH. The NPSH levels of lung homogenate were determined according to Sedlak and Lindsay (23) with the following modifications. A 2-ml aliquot of lung homogenate was added to 2 ml of distilled water and 50 μl of 0.2 M EDTA. After a thorough mixing, a 2.5-ml aliquot was deproteininzed with 2.5 ml of 10% TCA and centrifuged at 3,000 rpm for 10 min. 1-ml aliquots of the clear supernate (in duplicate) were transferred to test tubes containing 1.8 ml of 0.4 M Tris-chloride (pH 8.9) and 0.2 ml of 0.2 M EDTA. To each tube 50 μl of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (in absolute methanol) was added and the contents mixed and allowed to stand for 1 min. The optical density at 412 nm was read in a spectrophotometer (Beckman DB-GT, Beckman Instruments, Inc., Fullerton, Calif.) against an appropriate blank. The concentration of NPSH was calcuated using ϵ_{mM} of 13.1 for reduced DTNB.

Glutathione. Glutathione (GSH and GSSG) was determined by a modification of the method of Klotsch and Bergmeyer (24). 2-ml aliquots of lung homogenate were first deproteinized by immersion in a beaker of boiling water for 5 min, then cooled immediately in an ice-bath and treated with a drop of 10% TCA to obtain a clear supernate. Samples were centrifuged for 10 min at 3,000 rpm to remove particulate matter. To measure GSH concentration, 0.3 ml of the supernate was added to a 1-ml quartz cuvette containing 0.6 ml of 0.25 M potassium phosphate buffer (pH 6.8), 50 μl of 1% egg albumin, and 5 μl of glyoxalase I (5 μg protein). The contents were mixed and after a stable base line was obtained, 10 μl of 0.1 M methylglyoxal was added. The increase in absorbance at 240 nm was monitored until a new, stable absorbance was attained (within 5–8 min). The concentration of GSH present was calculated from the net increase in absorbance observed, using the ϵ_{mM} of 3.37 for *S*-lactyl glutathione, which was produced stoichiometrically from the reaction of GSH with methylglyoxal. The wave length was then changed to 340 nm and 5 μl of 12-mM NADPH (in 1% $NaHCO_3$) was added to the cuvette. After stabilization, 5 μl of glutathione reductase (10 μg protein) was introduced. The decrease in absorbance at 340 nm subsequent to addition of glutathione reductase was used to calculate GSSG concentrations, using the ϵ_{mM} of 6.22 for NADPH, since NADPH oxidation was stoichiometric with GSSG reduction. The precision of the method

for GSSG assay was checked using standard GSSG solutions and the recovery was 92–97%.

NPSH after borohydride reduction. The NPSH involved in forming disulfide bonds was estimated as the difference in NPSH levels with and without borohydride reduction. A 2-ml aliquot of lung homogenate was added to 2 ml of a freshly prepared solution of 1.25% (wt/vol) $NaBH_4$ plus 50 μl of 0.2 M EDTA, and the mixture was incubated at 37°C for 30 min. A 2.5-ml aliquot of this mixture transferred to a test tube containing 2.5 ml of 10% TCA to deproteinize the treated homogenate as well as to destroy excess borohydride. A complete elimination of $NaBH_4$ was important. Residual evolution of bubbles after deproteinization was indicative of incomplete destruction of the reagent, which in the subsequent steps of the NPSH assay would reduce DTNB and produce spuriously high values. NPSH was estimated by the method of Sedlak and Lindsay (23) as described above.

To determine the level of total NPSH (i.e., NPSH plus its soluble disulfide forms) in lung homogenate, the homogenate was deproteinized before borohydride treatment as described below. A 2.5-ml aliquot of lung homogenate was immersed in a boiling water bath for 5 min and then combined with 0.5 ml of 10% TCA. The mixture was thoroughly mixed and centrifuged for 10 min at 12,000 rpm. The clear supernate was neutralized by dropwise addition of 1 N NaOH, and a 1-ml aliquot was treated with 50 μl of 0.2 M EDTA and 2 ml of 1% $NaBH_4$. After 30 min of incubation at 37°C excess borohydride was destroyed by adding 0.1 ml of 100% TCA, and the sample was assayed for NPSH content as above.

Chromatographic identification of protein-bound NPSH. To a 1-ml suspension of particulate fraction or to a 1-ml supernatant fraction of lung homogenate were added 50 μl of 0.2 M EDTA and 20 mg of $NaBH_4$. After incubation at 37°C for 30 min, 1 ml of 50% TCA was added dropwise to destroy the excess $NaBH_4$ and to precipitate proteins. After centrifugation for 10 min at 10,000 rpm the supernate was filtered through a Whatman no. 50 filter paper. 1 ml of the filtrate was applied to a column (1 × 30 cm) packed with Bio-Gel P2 beads Bio-Rad Laboratories, Richmond, Calif., 100–200 mesh) in 50 mM Tris-chloride (pH 7.5). The column was eluted with the same buffer. After a void volume of 7 ml, 15 fractions (each 1 ml) were collected. Each fraction was evaporated to dryness at 50° C in a vacuum evaporator and redissolved in 50 μl of water. A 10-μl aliquot of the solution was spotted on Whatman no. 1 paper (23 × 57 cm) and subjected to descending chromatography. Solvent systems used were proprionic acid: butanol: water (10:5:4, vol/vol) (25) or phenol: isopropyl alcohol: water (70:25:5, vol/vol) (26). Standard solutions of GSH, GSSG, cysteine, and cystine were run simultaneously to serve as references. Color development for localization of separated compounds was achieved by dipping the chromatograms in 1% ninhydrin in acetone containing 0.5 mM sodium ascorbate and drying overnight in a hood. In parallel experiments the chromatograms were also sprayed with a 0.03% solution of 2,2′-dithiobis(5-nitropyridine) (DTNP) in acetone for sulfydryl color reaction.

Presentation of data

In an earlier study (10) involving 2 ppm O_3, SH levels of control and exposed rat lungs were compared on the basis of nanomole NPSH per milligram of protein. For exposures involving 4 ppm O_3, however, such expressions of data would be erroneous because of pulmonary edema causing influx of plasma proteins into the lung. In this report, data related to lung homogenate are presented as nanmoles NPSH per lung, a convention that has proven to be satisfactory in discerning the changes in lung NPSH content.

Triplicate samples of the mitochondrial fraction were assayed for protein content by the Hartree modification of the Lowry method (27) for quantitative expression of NPSH released from lung mitochondria.

For statistical computations the Student's *t* test for unpaired samples was used.

TABLE I

*Effect of O_3 Exposure on NPSH Levels in Lung Tissue**

Exposure conditions	Column 1: Level of NPSH before reduction	Column 1: Loss relative to control		Column 2: Level of NPSH after reduction‡	Column 3: Amount of NPSH involved in disulfide formation§	Column 3: Increase relative to control		Column 4: Fraction of NPSH as disulfides‖	
	μmol/lung	*%*	*P value*	*μmol/lung*	*μmol/lung*	*%*	*P value*	*%*	*P value*
2 ppm O_3									
0 h	1.51±0.32 (24)	—	—	1.96±0.48 (16)	0.45±0.12 (16)	—	— (16)	23.0	—
4 h	1.36±0.40 (24)	10.0	NS	1.91±0.51 (12)	0.55±0.24 (14)	22.2	NS (11)	28.8	NS
6 h	1.19±0.34 (23)	21.2	<0.01	1.78±0.51 (13)	0.59±0.27 (14)	31.1	<0.01 (13)	33.2	<0.05
8 h	1.13±0.32 (24)	25.2	<0.001	1.87±0.43 (16)	0.74±0.23 (15)	64.4	<0.001 (16)	39.6	<0.05
4 ppm O_3									
0 h	1.77±0.53 (26)	—	—	2.02±0.40 (32)	0.25±0.20 (14)	—	— (14)	1.25	—
2 h	1.39±0.29 (5)	21.5	<0.05	1.94±0.36 (6)	0.55±0.17 (5)	120.0	<0.01 (5)	28.4	<0.02
4 h	1.17±0.50 (26)	34.9	<0.001	1.80±0.37 (33)	0.63±0.22 (14)	152.0	<0.001 (14)	35.0	<0.001
6 h	0.93±0.45 (14)	48.5	<0.001	1.74±0.12 (5)	0.81±0.12 (4)	224.0	<0.001 (4)	46.5	<0.001

* Conditions for assay of NPSH and disulfides are given in the Methods section. Values given represent mean±SD, where *n* for each group is given in parentheses. Significance of results was determined using unpaired Student's *t* test, and *P* value >0.05 was considered nonsignificant NS.

‡ After $NaBH_4$ reduction the differences in values relative to control were nonsignificant.

§ Amount of NPSH was obtained as the difference between the levels of NPSH in lung homogenate before and after treatment with $NaBH_4$.

‖ Fraction of NPSH as disulfides was computed from the differential amount of NPSH (as in column 3) divided by the total amount of NPSH resulting from $NaBH_4$ reduction (as in column 2).

Chemicals

Chemicals used were of reagent grades commercially available. Particularly, glutathione reductase, glyoxalase I, NADPH, $NaBH_4$, methylglyoxal, GSH, GSSG, and DTNB were obtained from Sigma Chemical Co., St. Louis, Mo., and DTNP from Newcell Biochemicals, Berkeley, Calif.

RESULTS

NPSH and GSH. The average content of NPSH in the homogenate of a control rat lung (weighing approximately 1 g) was found to be 1.6 μmol per lung. Independent determinations of reduced GSH gave values which accounted for 85–95% of the total NPSH present in whole lung homogenate.

As shown in Table I (Column 1), O_3 exposure depressed the level of NPSH in rat lung. The magnitude of this depression was dependent upon the concentration of O_3 and the duration of exposure. For example, exposure of animals to 0.8 ppm O_3 for up to 24 h, to 1.5 ppm O_3 for up to 8 h, or to 2 ppm O_3 for up to 4 h did not cause a significant decrease of NPSH level. On the other hand, exposure to 2 ppm O_3 for 6 and 8 h depressed the NPSH level to 21 and 25%, respectively. Likewise, exposure to 4 ppm O_3 for 4 and 6 h decreased the NPSH level by 35 and 49%, respectively. In terms of the absolute value, the drop in the NPSH level for a 6-h exposure to 4 ppm O_3 amounted to a loss of 0.8 μmol NPSH per lung.

Under these exposure conditions the level of GSH was determined to compare it with that of total NPSH. As shown in Fig. 1, O_3 exposure diminished the level of GSH in the lung similar to that of NPSH. A 40% ($P < 0.001$) decrease of GSH was noted for a 6-h exposure of rats to 4 ppm O_3. Although the GSH level dropped significantly, there was no corresponding rise in the level of GSSG. As shown in Fig. 1, the GSSG level remained constant throughout the exposure period. Since the disappearance of GSH was not balanced by an increase of GSSG, there was a continuous decrease in the level of total assayable glutathione. A 33% ($P < 0.001$) decrease in the total GSH level occurred after a 6-h exposure to 4 ppm O_3. It should be pointed out that the measured levels of GSSG in control lung homogenates may be high, accounting for up to 10% of the levels of total glutathione (i.e., GSH plus GSH equivalent of GSSG). In liver and kidney tissues, GSSG levels are reported to be 3–5% of the total tissue glutathione levels (21). Several authors (24–28), working with different tissues, have indicated that high GSSG levels may be observed unless samples are immediately assayed or special precautions are taken to prevent oxidation of GSH before assay. In our assay of the GSSG levels, as shown in Fig. 1, if any such factors were involved they would have influenced control and exposed samples alike.

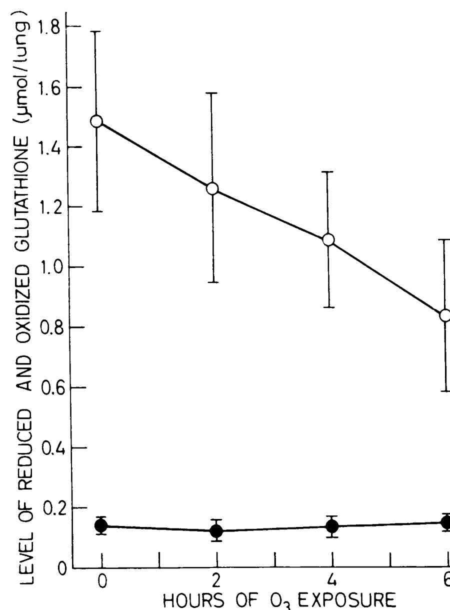

FIGURE 1 Effect of O_3 exposure on the levels of GSH and GSSG in lung. Conditions for assay of GSH (○) and GSSG (●) are given in the Methods section. 5, 30, and 18 rats were exposed to 4 ppm O_3 for 2, 4, and 6 h, respectively. 31 rats treated under identical conditions with room air served as controls. Vertical lines represent SD.

NPSH and mixed disulfides. As described in the previous section, GSH (or NPSH) oxidation in O_3-exposed lungs was not accompanied by an increase in GSSG. As assays were carried out in the supernatant fractions of deproteinized lung homogenates using a specific enzymatic method (24), the GSSG determinations did not include any bound (acid-insoluble) form of disulfides involving GSH, or any unbound (acid-soluble) form of disulfides other than GSSG. To determine if mixed disulfides other than GSSG were formed in the lung subsequent to O_3 exposure, sodium borohydride, which quantitatively reduces –SS– bonds to SH groups, was employed for quantitative analysis of disulfides involving NPSH. In contrast to the procedure for GSSG assay, determination of the disulfide forms of NPSH involved sequentially (*a*) reduction of –SS– bonds of both protein-bound and all acid-soluble disulfides to SH groups by borohydride treatment of lung homogenate, (*b*) deproteinization, and (*c*) assay for NPSH.

As shown in Table I (columns 1 and 2), NPSH levels were higher in borohydride-treated homogenate compared to untreated homogenate of the same lung. In control lungs the elevations were relatively small (15–30%). In exposed lungs the elevations were relatively large and quantitatively related to the magnitude of O_3 exposures. Lung homogenates from rats

Table II
*Effect of $NaBH_4$ Treatment on NPSH Levels in Lung Homogenate and Supernate of Deproteinized Lung Homogenate**

Exposure condition	Level of NPSH		
	Untreated homogenate‡	$NaBH_4$-treated homogenate	$NaBH_4$-treated supernate of deproteinized homogenate‡
		μmol/lung	
4 ppm O_3			
0 h (control)	1.98±0.19 (20)	2.12±0.22 (21)	1.87±0.16 (21)
4 h (exposed)	1.56±0.28 (20)	2.13±0.27 (22)	1.54±0.20 (20)
% of control	78.8	100.6	82.1
P value	<0.001	NS	<0.001

* Conditions for assay of NPSH are given in the Methods section. Statistical conventions are the same as in Table I.
‡ This column also represents NPSH levels in untreated supernate of deproteinized homogenate.

exposed to 4 ppm O_3 for 4 and 6 h showed 54 and 115% increases in the NPSH level, respectively. In terms of the absolute values (Table I, column 3), these increases amounted to 0.63 and 0.81 μmol NPSH per lung, respectively. The results suggest that the amount of NPSH which disappeared during O_3 exposure was recoverable in the form of disulfides not represented by GSSG. Furthermore, as shown in Table I, column 3, the relative amount of NPSH involved in disulfide formation increased with increasing dosage of O_3 (cf. increases due to higher O_3 level, 4 vs. 2 ppm, and longer exposure time, 6–8 vs. 2–4 h). Of the total NPSH in the lung, the fraction that formed disulfides was estimated as shown in Table I, column 4. Although formation of a certain amount of disulfides occurred in control lungs, a significantly larger fraction of NPSH was involved in disulfide formation in each series of exposed lungs.

In view of the observations that increased GSH or NPSH oxidation did not yield GSSG in the lung (cf. Fig. 1), the data presented in Table I suggested that the formation of disulfides from NPSH might have involved protein thiols as the counterparts. To test this hypothesis we compared the effect of borohydride reduction on whole lung homogenate and on deproteinized supernate of whole lung homogenate. Borohydride treatment of whole lung homogenate resulted in a recovery of NPSH lost due to O_3 exposure, whereas the same treatment of deproteinized supernate (which still contained free NPSH and soluble disulfides) did not lead to any such recovery (Table II). These results demonstrate that NPSH was bound to protein via a mixed disulfide linkage, as interpreted by Modig (19) for similar findings with radiation exposure.

In another set of experiments isolated lung mitochondria, a particulate fraction which showed a 20% decrease of assayable SH groups relative to unexposed control (22) were treated with $NaBH_4$ and assayed for the release of NPSH. The liberation of NPSH was 32% ($P < 0.001$) higher in exposed lung mitochondria (8.58±1.76 nmol NPSH per mg protein) compared to control lung mitochondria (6.52±2.93 nmol NPSH per lung). These findings, showing association of NPSH with mitochondrial SH via –SS– linkage, further demonstrate that O_3 exposure resulted in mixed disulfide formation between protein and nonprotein thiols in the lung.

Identification of protein-bound NPSH. It is clear from the above results that O_3 exposures elicited the formation of mixed disulfides in the lung with a concomitant loss in NPSH or GSH level. Since the mixed disulfides were protein-bound (acid-insoluble), a question arose as to the nature of NPSH moiety involved. To identify this moiety the acid-insoluble mixed disulfides in the supernatant fraction (as well as the particulate fraction) of lung homogenate were treated with $NaBH_4$, and the acid-soluble portion of these samples were analyzed for the free sulfhydryl compounds by paper chromatography.

As shown in Fig. 2 and Table III, the major sulfhydryl compound liberated after borohydride reduction was GSH, as judged from its mobility in the two solvent systems against the standard GSH. This was true for both control and exposed lung samples. A minor ninhydrin-positive spot with a lower R_f (0.04, 0.06) was also detected in both types of samples, which corresponded with the standard GSSG. This compound might have been formed from the oxidation

of liberated GSH during the course of chromatographic operation.

It should be pointed out that GSH spot was obtained for both control and exposed lung samples, although a relatively greater quantity of GSH would be expected to result from exposed lung samples (cf. Table I). In this study a rigorous quantitation of the liberated GSH was not undertaken; however, simple comparison of the ninhydrin color spots on the chromatogram revealed that such was the case. The color intensity of the major spot in both the solvent systems was greater for the extracts of exposed lungs relative to those of control lungs for the same amount of supernate or particulate homogenate, suggesting the occurrence of a greater quantity of protein-bound GSH in exposed lung. No such difference was observable for the color intensity of the minor spot (GSSG).

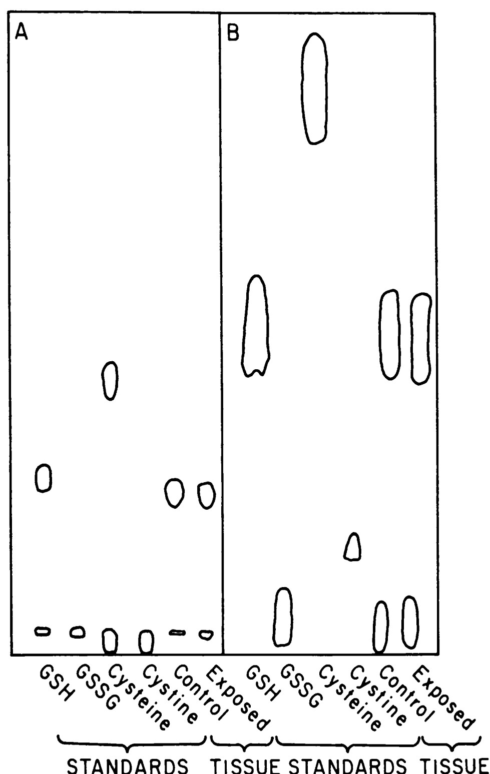

FIGURE 2 Chromatographic analysis of NPSH derived from $NaBH_4$-treated lung proteins. The figure is drawn from a representative chromatograms of standards and borohydride-treated lung cytosol from control and exposed rats. The eighth fraction (1 ml) of column eluate mentioned in the text was selected for spotting after it was identified, using DTNB, to be the major NPSH-containing fraction. Conditions for chromatographic analysis of extracts prepared from acid-precipitated proteins are described in Methods. All spots were developed with ninhydrin with the exception of cysteine for which DTNP was used for color reaction. A, solvent system: proprionic acid: butanol: water (10:5:4) and B, solvent system: phenol isopropyl alcohol water (70:25:5).

TABLE III

*Chromatographic Characteristics of NPSH Derived from $NaBH_4$-Treated Lung Proteins**

Samples	R_f values‡ for solvent systems: Propionic acid: butanol: water	R_f values‡ for solvent systems: Phenol: isopropyl alcohol: water
Control tissue extract§		
Major spot	0.18	0.44
Minor spot	0.03	0.05
Exposed tissue extract§		
Major spot	0.17	0.46
Minor spot	0.04	0.06
Standard compounds		
GSH	0.18	0.45
GSSG	0.04	0.06
Cysteine‖	0.28	0.59
Cystine	0.04	0.12

* Conditions for chromatographic analysis are given in the Methods section.

‡ R_f values were calculated from chromatographic runs as shown in Fig. 2. Each number represents an average of at least five runs.

§ Extracts were prepared from the acid-precipitated proteins of the supernatant fraction (cf. Methods).

‖ Cysteine on chromatogram gave no color reaction with ninydrin. Its R_f was determined with the aid of DTNP color reaction.

Reversal of mixed disulfide formation. In view of the formation of mixed disulfides in the lung during O_3 exposure, a question arose as to the time-course for their disappearance after the exposure is withdrawn. Animals exposed to 3 ppm O_3 for 4 h were allowed to recover in room air for up to 7 days. (This exposure regimen was chosen to allow for greater survival of animals compared to those surviving a 4 ppm O_3 exposure for 6 h.) Immediately after the exposure there was a 15% decrease of NPSH level in lung tissue relative to control (Fig. 3). Since $NaBH_4$ treatment of exposed lung homogenate brought the NPSH level back to control value, the NPSH lost could be accounted for by the formation of mixed disulfides. The recovery seemed to manifest two kinds of phenomena. First, the level of NPSH in exposed lung attained the control value within 6 h of recovery, and then increased 42 and 100%, respectively, after 1 and 2 days of recovery. Thereafter, the NPSH level in exposed lung dropped but remained significantly elevated (approximately 40% higher than the control value) for up to 7 days of recovery. Secondly, as judged from the $NaBH_4$ treatment of lung homogenate, 0.25 μmol or 15% of the total NPSH in exposed lung appeared as mixed disulfides immediately after the exposure. After 1 day of recovery the amount of mixed disulfides increased in exposed lung (representing 0.6 μmol or 25% of the total NPSH). However, after 2 days of re-

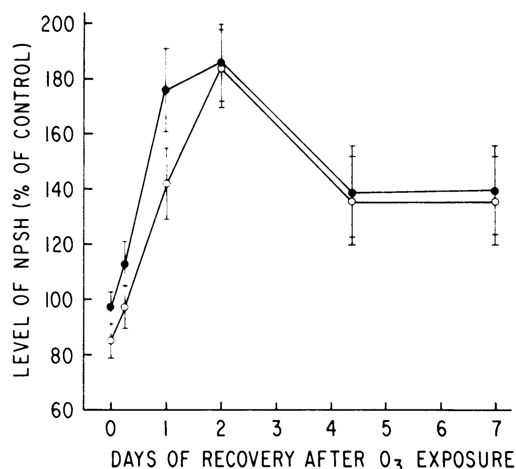

FIGURE 3 Effect of recovery on the levels of NPSH and mixed disulfides. Conditions for assay of NPSH (○) and NPSH after borohydride reduction (●) are given in the Methods section. The average NPSH content in control rat lung homogenate was 1.6±0.1 μmol/lung. A total of 89 rats, exposed to 3 ppm O_3 or room air for 4 h, were used in these experiments. For all points except day 2, 8–10 rats were used for controls and 6–8 in the experimental groups. For day 2, four rats were used in each group. Vertical lines represent SD.

covery the level of mixed disulfides diminished considerably.

DISCUSSION

The results demonstrate that thiol levels are depressed in the lungs of O_3-exposed animals and that O_3-mediated in vivo thiol oxidation is reversible, since essentially all of the oxidation products (i.e., disulfides) can be recovered in the reduced forms (i.e., SH groups) by borohydride treatment or by allowing animals to recover. In the following, various products of thiol oxidation in the lung resulting from O_3 exposure are discussed with respect to their possible physiologic significance.

Simple disulfides. Although a 48% decrease of the NPSH level and a 40% decrease of the GSH level was observed in the lung after O_3 exposure, the level of GSSG was only 3% above the control, suggesting that GSSG was not a major product of O_3-mediated NPSH oxidation in the lung. However, it is not certain whether any excess GSSG formed in exposed lungs enters the circulation, since cell membranes may be permeable to GSSG, as has been shown to occur in erythrocytes (28) and perfused rat liver under peroxidative stress (29). The present findings for in vivo exposures are in contrast to those for in vitro condition in which O_3 reaction with GSH favors GSSG formation (12, 13). These observations are also in contrast to these reported for short-term exposure of rats to hyperbaric O_2 (e.g., 3.5 atm for 4 h) (30) where the GSH level in the lung remained constant but the GSSG level increased 25%.

Mixed disulfides. Mixed disulfide formation between PSH and NPSH seems to be an important phenomenon in O_3-exposed animal lungs, although it occurs only when edema producing doses of O_3 were used. Essentially all of the NPSH lost during O_3 exposure appears to form mixed disulfides. The liberation of NPSH in an amount equal to that lost during O_3 exposure that takes place after borohydride reduction of exposed lung homogenate, but not of deproteinized supernate from exposed lung homogenate, indicates the formation of mixed disulfides between PSH and NPSH.

Further comments on mixed disulfides. NPSH are thought to play important roles in counteracting free radical chain propagation and preventing oxidation of functional SH groups of enzymes (cf. references 1 and 2 which discuss these functions with regard to O_3-mediated oxidations). For example, in erythrocytes the intracellular oxidation of cysteine residues in hemoglobin, oxidative hemolysis and, under some circumstances, the formation of Heinz bodies has been related to changes in erythrocyte GSH levels (31). However, under oxidative conditions where mixed disulfides between GSH and hemoglobin are formed, the process is presumably harmful and may involve binding of denatured precipitated hemoglobin to the inside of the erythrocyte membrane, constituting the Heinz body (32–34).

In the lung, mixed disulfide formation in oxidative circumstances (viz., under O_3 stress) could represent a protective mechanism rather than a harmful event. For example, mixed disulfide –SS– linkages would be resistant to further oxidation by O_3 (15), thus preventing irreversible oxidation of SH groups of enzymes and proteins. However, the binding of PSH with NPSH in forming a disulfide bond may interfere with cellular enzymatic activities requiring SH groups (35, 36) or maintenance of structural integrity in subcellular membranes dependent on SH groups (37–39). Likewise, by being bound to proteins, the potential functions of NPSH as mobile reducing compounds and free radical scavengers are presumably impaired (1, 2). Although mixed disulfide formation in the lung under oxidant stress is a phenomenon that causes a transient disappearance of a fraction of NPSH, enzymatic (as well as nonenzymatic) mechanisms for disulfide reduction may exist in the lung that will lead to regeneration of the respective thiols as conditions become favorable.

Comments on dose-effect relationships. The relatively high levels of ozone employed in this study (3–4 ppm) as opposed to low-level exposures (0.05–0.5 ppm) which are encountered in some photochemical

smog-polluted atmospheres warrant further comment. In low-level exposures oxidation products of SH do not appear to accumulate. It seems possible that SH oxidation caused by low-level exposure occurs in focal areas and that the resultant disulfides remain undetected due to the averaging of healthy and injured tissues during homogenization. It is also possible that normal (or augmented) enzymatic pathways that favor disulfide reduction are able to prevent significant mixed disulfide increases from occurring under conditions of low-level exposures. In high-level exposures the damage to the lung is widespread, resulting in demonstrable alterations of thiol levels despite averaging of ozone-damaged and healthy lung tissues. We conclude that high-level ozone exposures (although not usually encountered under ambient conditions) provide evidence that oxidant stress results in SH oxidation and formation of mixed disulfides in the lung. The significance of these findings may be of physiological and clinical importance in that they serve as a model for ozone and other oxidant damage to the lung, and indicate potential biochemical changes that may occur focally with low-level exposures and more extensively with higher exposure levels.

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