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PROTECTION AGAINST PARAQUAT-INDUCED INJURY BY EXOGENOUS GSH IN PULMONARY ALVEOLAR TYPE II CELLS

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Abstract—Exogenous GSH provided rat alveolar type II cells with significant protection against injury induced by paraquat. This protection was also observed in cells treated with acivicin to inhibit GSH degradation and buthionine sulfoximine to inhibit GSH synthesis. Exogenous GSH was transported into cells by a Na^+-dependent system. Addition of inhibitors of this transport system, γ-glutamyl-glutamate and probenecid, prevented the protection against injury afforded by GSH. Thus, the results indicate that alveolar type II cells can supplement endogenous synthesis of GSH with uptake of exogenous GSH to protect against paraquat-induced injury.

Oxidative injury in the lung occurs from inhaled air pollutants, oxygen therapy, antitumor compounds, and other redox cycling agents such as paraquat [1–3]. Considerable effort has been made to understand and minimize oxidative injury in lung. However, because of the large number of different cell types, understanding the critical susceptibility of the individual cell types has been difficult. Over the past several years, preparations of some of these cell types have become available. We have focused on the alveolar type II cells because they function in the production and release of surfactant into the alveolar space and also are progenitors of type I cells which form most of the epithelial surface of the alveolus [4, 5]. While type II cells are susceptible to oxidative injury, they are more resistant than are type I cells, but the reasons for this apparent resistance are as yet unclear [6].

Glutathione (GSH) constitutes the major source of low molecular weight thiol in mammalian tissue and functions in detoxification of electrophilic compounds and protection against oxidative injury. Numerous reports show that GSH depletion occurs prior to cell injury for several types of toxic substances [7–9]. Much of the effort has focused on hepatic injury, and there is now substantial understanding of the toxicological consequences involving thiol oxidation, such as inhibition of Ca^{2+} ATPases [10], disruption of cytoskeletal elements [11], and alteration of metabolic regulation [12].

Moldéus and co-workers [13] found that isolated perfused lung can synthesize GSH from the precursor amino acids and that the lung can also use exogenous GSH for this purpose. However, inhibitor studies indicated that the utilization of exogenous GSH was largely due to extracellular breakdown and subsequent resynthesis.

We have found recently that renal epithelial cells and small intestinal epithelial cells contain a sodium-dependent uptake system for intact GSH [14, 15]. In the small intestinal cells, exogenous GSH provides effective protection against oxidative injury due to either *t*-butylhydroperoxide or menadione [15].

In the present study, we investigated whether intact GSH can provide protection in type II cells against the strong oxidant, paraquat, and, if so, the mechanism by which GSH may offer this protection. The results show that intact exogenous GSH was taken up by isolated type II cells and that it provided substantial protection against paraquat-induced injury.

MATERIALS AND METHODS

GSH, Percoll, paraquat, (1,1-dimethyl-4,4′-bypyridinium dichloride), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), probenecid, γ-L-glutamyl-L-glutamate and heparin were purchased from Sigma. [Glycine-2-^{3}H]GSH (specific activity 1.1 Ci/mmole) was purchased from New England Nuclear. Penicillin G potassium was obtained from the Emory University Hospital Pharmacy. Ophthalmic acid was purchased from Bachem Inc., Torrence, CA. Acivicin [L-(α*S*,5s)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; AT-125] was a gift from Dr. J. P. McGovren (Upjohn Co., Kalamazoo, MI). Buthionine-*SR*-sulfoximine was purchased from the Chemical Dynamics Corp.

Alveolar type II cells were obtained from male Sprague–Dawley rats (King outbred albino), weighing approximately 200–250 g, that were kept in air-filtered chambers to reduce the risk of lung infections. Type II cells were isolated by trypsinization and purified by discontinuous albumin density gradient centrifugation as previously described [16]. When examined by polychrome staining [17], 80–85% of the cells contained lamellar bodies, indicating that the preparation is largely type II cells. Primary cultures established with this preparation yield approximately 95% type II cells which produce surfactant

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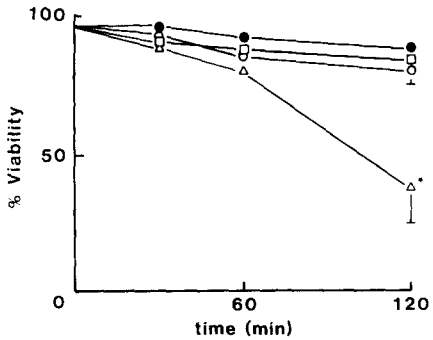

Fig. 1. Protection against paraquat-induced injury by amino acid precursors of GSH (glutamate, cysteine, and glycine) in suspensions of isolated rat pulmonary alveolar type II cells. Cells (0.8×10^6/ml) were incubated in a gyrotory shaker water bath at 37° and 1 mM amino acid precursors of GSH were added 30 min prior to the start of the experiment. Cell viability was determined using trypan blue. Key: control cells (●); cells + 1 mM glutamate, cysteine, and glycine (□); cells + 1 mM glutamate, cysteine, and glycine + 250 μM paraquat (○); and cells + 250 μM paraquat (△). Values are the average of three (±SE) cell preparations. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) between paired experiments without and with amino acid mixture.

[16]. Isolated cells were suspended and incubated in Dulbecco's Modified Minimal Essential Medium for 1 hr at 37° in 10% CO_2 to allow for macrophage attachment. Cells were placed in Nalgene centrifuge tubes and pelleted by centrifugation in a table top centrifuge at the minimum speed sufficient to pellet the cells in 5 min. The supernatant fraction was aspirated and the cell pellet was resuspended in supplemented Krebs–Henseleit medium (pH 7.4) that had been preequilibrated with 95% O_2/5% CO_2. Cells were counted in a hemacytometer, and viability was assessed by the exclusion of 0.2% trypan blue. Typical cell viability was 90–95%.

Incubations. Incubations were performed at 37° in a gyrotory water bath with approximately 0.8×10^6 cells/ml Krebs–Henseleit medium. γ-Glutamyl transferase and γ-glutamylcysteine synthetase were inhibited in some experiments by pretreatment for 30 min at room temperature with 0.25 mM acivicin and 1 mM buthionine sulfoximine. For studies testing the ability of GSH or amino acids to protect against paraquat-induced injury, cells were preincubated for 30 min prior to addition of paraquat. For viability studies using physiological GSH concentrations, 20 μM GSH was added every 30 min over the 2-hr time course. Aliquots (50 μl) were taken at the appropriate times for cell viability measurements. When added, probenecid and γ-glutamyl-glutamate or ophthalmic acid were added at the same time as GSH.

Glutathione uptake studies. Cells were pelleted and resuspended in supplemented Krebs–Henseleit medium to bring the cell number to 10^6 cells/ml. The suspension was pretreated for 30 min with 0.25 mM acivicin and 1.0 mM buthionine sulfoximine and the experiment initiated by addition of 1 mM [^{3}H]GSH (0.1 mCi/mmole). Immediately, duplicate 500-μl aliquots were layered onto 10% Percoll in Krebs–Henseleit medium (pH 7.5) in an Eppendorf microfuge tube (designated time zero). Additional aliquots were taken at times indicated. The tubes were centrifuged for 2 min in a microfuge, the supernatant fraction was aspirated, and the pellets were washed once in Krebs–Henseleit medium and recentrifuged for 1 min. The supernatant fractions were again aspirated and the cells extracted with 150 μl of 30% trichloroacetic acid (TCA). After centrifugation, radioactivity in the supernatant fraction was determined by liquid scintillation counting.

For HPLC analysis of GSH, cell extracts were derivatized with 40 mM iodoacetic acid and 1-fluoro-2,4-dinitrobenzene [1.5% (v/v) in absolute ethanol] as described by Reed *et al.* [17]. Derivatives were separated on a 10 μm Ultrasil amine column (Beckman Instruments, Norcross, GA) and detected at 365 nm. GSH in samples was quantitiated relative to standards by integration (Hewlett–Packard model 3390A).

Na^+ *dependence studies.* Sodium dependence of GSH uptake was studied using a Na^+-free Krebs–Henseleit medium in which choline chloride was substituted for NaCl and the calcium salt of heparin was substituted for the Na^+ salt.

RESULTS

Moldéus and co-workers [13] showed that precursor amino acids are effectively utilized for GSH synthesis in isolated perfused lung. To test whether such synthesis in alveolar type II cells could protect against chemical injury, we examined the effects of cysteine, glutamate and glycine addition on paraquat-induced injury. Initial studies showed that cells treated with 250 μM paraquat resulted in substantial cell death over the 2-hr time course (Fig. 1). Preincubation of cells with a 1 mM concentration of each of cysteine, glycine, and glutamate for 30 min prior to addition of 250 μM paraquat largely prevented this loss of cell viability. Thus, the amino acid precursors of GSH can give substantial protection against paraquat-induced injury in type II cells. Although we have not studied the mechanism for this, Berggren *et al.* [13] and Saito, Warshaw and Prough (personal communication) have shown that GSH synthesis occurs in the presence of cysteine.

To determine whether intact, exogenous GSH can also protect against injury, GSH was added to cells pretreated with inhibitors of GSH degradation and GSH synthesis. γ-Glutamyltransferase was inhibited by addition of a concentration of acivicin sufficient to give >99% inhibition [14]. Buthionine sulfoximine was added at a concentration of (1 mM) sufficient to give >95% inhibition of GSH synthesis [15]. Loss of cell viability in the fraction with only paraquat added (Fig. 2A) was comparable to that for cells which were not treated with acivicin or buthionine sulfoximine (see Fig. 1). Addition of 1 mM GSH provided marked protection; cells had the same degree of viability over the experimental time course as the control incubations. Similar results were also obtained without pretreatment with acivicin or BSO. Thus, the results clearly show that exogenous GSH can protect type II cells from paraquat injury.

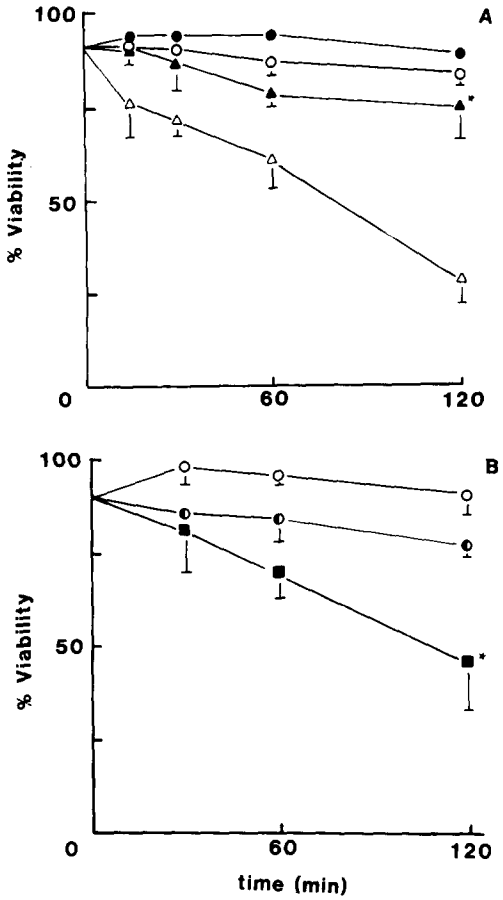

Fig. 2. (A) Effect of exogenous GSH on paraquat-induced injury in isolated pulmonary alveolar type II cells. Acivicin-treated cells (0.8×10^6/ml) were incubated at 37° in a gyrotory shaker bath. Where indicated, cells were pre-incubated with 1 mM GSH for 30 min prior to the start of the experiment. Cell viability was assessed at indicated times in the presence of 0.2% trypan blue. Key: control cells (●); cells + 1 mM GSH (○); cells + 1 mM GSH + 250 μM paraquat (▲); and cells + 250 μM paraquat (△). An asterisk (*) indicates a statistically significant difference ($P < 0.05$) between paired experiments without and with GSH. (B) Effects of inhibitors of GSH uptake on the protection by exogenous GSH against paraquat-induced toxicity. Acivicin-treated cells (0.8×10^6/ml) were pre-incubated with 1 mM GSH. Key: cells + 1 mM γ-glutamyl-glutamate + 250 μM probenecid (○); cells + 1 mM γ-glutamyl-glutamate + 250 μM probenecid + 1 mM GSH (◐); and cells + 1 mM γ-glutamyl-glutamate + 250 μM probenecid + 1 mM GSH + 250 μM paraquat (■). Values are given as averages (±SE) for four cell preparations. Experiments with 1 mM ophthalmic acid instead of γ-glutamyl-glutamate gave the same results. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) between paired experiments without (closed triangles in A) and with γ-glutamyl-glutamate + probenecid.

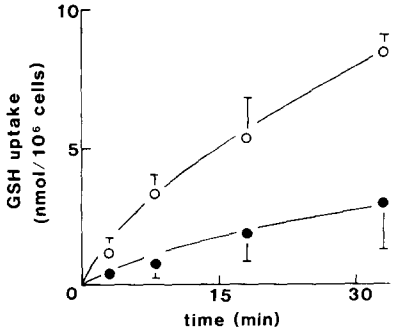

Fig. 3. Uptake of GSH by alveolar type II cells. Cells (1.0×10^6/ml), suspended in modified Krebs–Henseleit medium and pretreated with acivicin and buthionine sulfoximine, were incubated for 30 min prior to addition of 1 mM GSH containing [^{3}H]GSH. At indicated times, 0.5-ml aliquots were centrifuged through 10% Percoll (v/v), the supernatant fraction was aspirated, and the pellet was washed with 1 ml Krebs–Henseleit medium and recentrifuged. Cell pellets were extracted with 150 μl of 30% TCA and 100-μl aliquots were counted. Key: uptake in Na^+-containing Krebs–Henseleit medium (○), and uptake in Na^+-free Krebs–Henseleit medium (●). Resuts are given as the average of four experiments ± SE. At 18 and 33 min, the difference between Na^+-containing and Na^+-free suspensions was statistically significant at $P < 0.05$. No loss in cell viability during the course of these experiments occurred in suspensions with Krebs–Henseleit medium, but 6% loss in viability occurred in the Na^+-free medium following 30 min.

Kidney and small intestinal epithelial cells have a transport system which allows uptake of intact GSH [14, 15]. Studies with plasma membrane vesicles showed that uptake depends on Na^+ cotransport and that GSH can be taken up against its concentration gradient [14]. Since exogenous GSH protected against paraquat toxicity, we examined whether type II cells also have a transport system for GSH. To minimize any artifact due to GSH breakdown and resynthesis, cells were pretreated with acivicin and buthionine sulfoximine as before. The amount of GSH uptake was measured with [^{3}H]GSH over a 2-hr time course. Exogenous radiolabel was separated from cells by centrifuging them into Krebs–Henseleit medium containing 10% Percoll. The initial rate of [^{3}H]GSH was 1.2 nmoles/min/10^6 cells (Fig. 3). Na^+ dependence was examined by performing the same experiment using choline chloride instead of NaCl in the modified Krebs–Henseleit medium. The initial rate of uptake in the choline chloride-containing medium was only 33% of the rate of uptake in the Na^+-containing medium (Fig. 3). Analysis of GSH content by HPLC (Fig. 4, A and B) showed that initial GSH concentration was very low and that cellular GSH increased approximately 4-fold (from 0.3 μmole initially to 1.2 μmoles after 2 hr) in cells incubated in Krebs–Henseleit medium. Saito, Warshaw and Prough (personal communication) have also found very low GSH content in freshly isolated neonatal type II cells. They showed that GSH is released from these cells and, therefore, the low GSH could be due to loss during the lengthy cell preparation procedure. Incubations with [^{3}H]GSH followed by analysis of cell contents by HPLC showed that 90% of the radiolabel was recovered as GSH (Fig. 4C). Thus, the results clearly show that intact GSH is transported into alveolar type II cells.

While the above experiments show that extracellular GSH protects against oxidative injury and intact GSH is taken up by these cells, they do not establish whether GSH added to the medium pro-

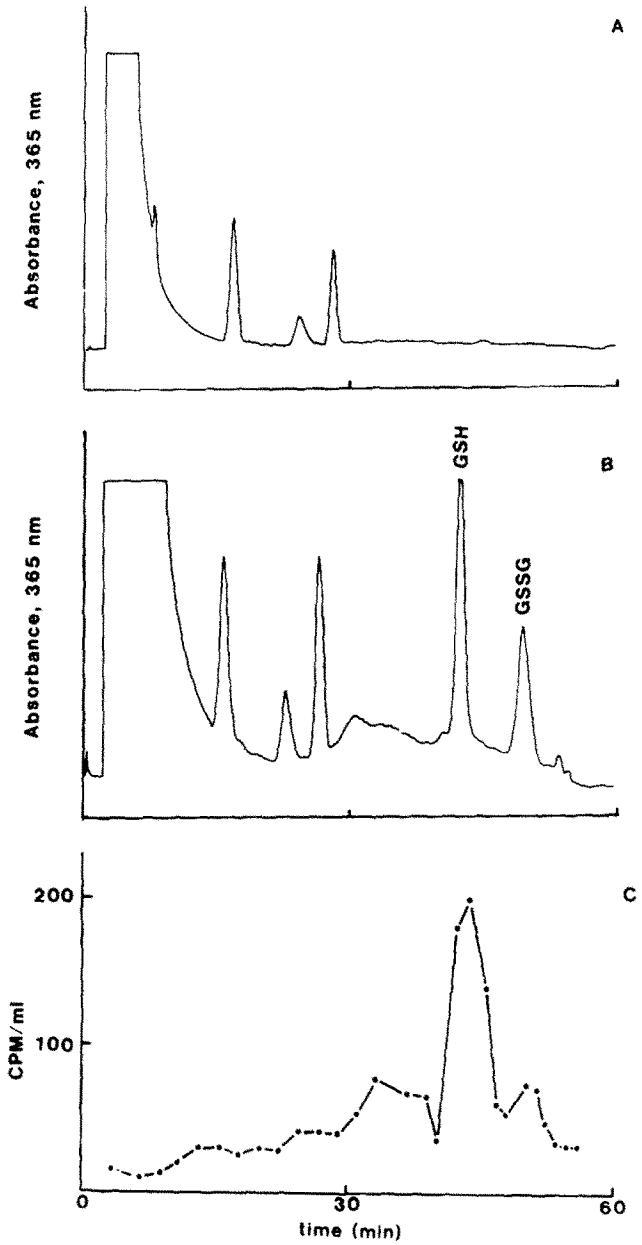

Fig. 4. High performance liquid chromatographic separation at 0 and 30 min of GSH in cell extracts of isolated alveolar type II cells. Trichloroacetic acid extracts were derivatized with iodoacetic acid and Sanger's reagent [17], and 100-μl samples were injected on a 10 μm amine column. Solvent A was 80% v/v) methanol in water. Solvent B was 64% (v/v) methanol in 0.8 M sodium acetate (final concentration), pH 4.6. Operating conditions were as follows. Initial solvent conditions were 75% A for 10 min after injection. A linear gradient was then run to 95% B over 50 min. Flow rate was 1.5 ml/min. (A) HPLC chromatogram of cell extracts at 0 min. (B) Chromatogram after 30 min. GSH retention time, 42.2 min; GSSG = 49.4 min. (C) Elution profile of [^{3}H]GSH on HPLC. Samples (1 ml) of the column eluate were collected, and the radioactivity was counted.

tected cells by extracellular reaction with reactive metabolites or whether GSH transported into the cell provided the protection. The GSH analogs γ-glutamyl-glutamate and ophthalmic acid (γ-glutamyl isobutyric acid) have been shown to competitively inhibit GSH uptake in renal basal-lateral membrane vesicles [14]. Probenecid is a therapeutic agent that inhibits organic anion transport and also inhibits GSH uptake in renal basal lateral membrane vesicles [14]. We used these compounds to determine whether GSH uptake was necessary for protection against paraquat-induced cell death. Cell viability was not altered significantly by treatment with 1 mM γ-glutamyl-glutamate and 250 μM probenecid (Fig. 2B); however, addition of γ-glutamyl-glutamate and probenecid to cells treated with GSH and paraquat resulted in substantial cell death (Fig. 2B). Since the protection by GSH against paraquat-

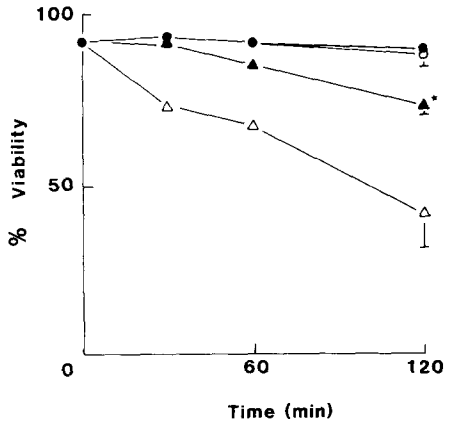

Fig. 5. Effect of a physiological concentration of GSH on paraquat-induced injury in isolated alveolar type II cells. Acivicin-treated cells (0.8×10^6/ml) were incubated at 37° in a gyratory shaker bath. Where indicated, cells were preincubated with 20 μM GSH for 30 min. GSH (20 μM) was added every 30 min to these cells to prevent loss of GSH due to oxidation. Cell viability was assessed as in Fig. 1. Key: control cells (●); cells + 20 μM GSH (○); cells + 20 μM GSH + 250 μM paraquat (▲); and cells + 250 μM paraquat (△). An asterisk (*) indicates a statistically significant difference ($P < 0.05$) between paired experiments without and with GSH.

induced cytotoxicity (Fig. 2A) was lost in the presence of γ-glutamyl-glutamate and probenecid, these results indicate that uptake of GSH is required for protection.

Protection by a physiological concentration of GSH. Since the GSH plasma concentration is approximately 20 μM in rats, the protection of type II cells seen at 1 mM GSH may not accurately reflect the contribution by GSH in protecting these cells at physiological conditions. To address this, the same type of experiments were performed using 20 μM GSH instead of 1 mM GSH. Results showed that type II cells incubated with 20 μM GSH were protected against paraquat injury as compared to the paraquat containing control (Fig. 5). Cells with 20 μM GSH and 250 μM paraquat added remained approximately 67% viable after 2 hr, whereas cells with 250 μM paraquat alone were only 43% viable. These studies show that GSH can protect type II cells not only at 1 mM but also at physiological GSH concentrations as well.

DISCUSSION

The role of GSH in protection against chemical injury has been well documented. It is synthesized in almost every tissue, including lung, and functions in detoxication of peroxides, free radicals, and reactive electrophiles. When the GSH supply becomes limiting, cell injury due to these types of compounds is increased. Smith and Bend [18] incubated benzo[*a*]pyrene-4,5-oxide and 5 mM GSH with isolated lung cells from rabbit and showed only 30% GSH-*S*-transferase activity compared to that of sonicated cells. Conclusions were that cellular GSH synthesis was not able to support maximal transferase activity and GSH availability could be a limiting factor. The presence of an uptake mechanism for GSH provides a way for GSH-dependent processes to continue to detoxify oxidants and xenobiotics at rates faster than allowed by GSH synthesis alone.

Intact GSH has been found to be taken up by certain epithelial cells which provide a defensive barrier against exogenous toxins. Previous work has shown that intestinal epithelial cells have a system that allows GSH to be transported from the blood [15]. Exogenously supplied GSH substantially protected isolated enterocytes against cytoxicity caused by *t*-butyl hydroperoxide or menadione and may aid in the protection of these cells from injury by oxidative compounds encountered in the diet. The kidney epithelium also has an uptake system for GSH [14], and recent studies in this laboratory demonstrated that exogenous GSH protected against oxidative injury in these cells (T. M. Hagen and D. P. Jones, unpublished results). In both of these tissues, the uptake mechanism is Na^+ dependent and electrogenic, allowing uptake of GSH against a substantial concentration gradient [14].

In the present study, we found that pulmonary alveolar type II cells can transport GSH and this uptake provided protection against paraquat-induced injury. Studies by both radiotracer and HPLC techniques established that uptake of intact GSH occurred and was Na^+ dependent. HPLC analysis with [^{3}H]GSH showed that the radiolabel transported into the cells was largely in the form of GSH under conditions where GSH hydrolysis and synthesis were inhibited. Ascorbate, a known antioxidant, is also accumulated by cultured type II cells via a transport system for ascorbate which is sodium dependent [19]. The ability of type II cells to take up exogenous antioxidants such as GSH or ascorbate may explain why these cells are more resistant to oxidative injury than other pulmonary cell types.

As judged by light microscopy, cell morphology with added GSH and paraquat was similar to control whereas significant blebbing occurred in the cells incubated with paraquat (data not shown). In incubations with paraquat, not only was cell viability reduced, but the total number of cells decreased by ~50% over 2 hr, whereas there was negligible loss of control cells. Therefore, the results given in the viability studies are probably an underestimate of the true protection given by GSH.

Addition of γ-glutamyl-glutamate and probenecid, inhibitors of GSH transport, with GSH and paraquat resulted in the loss of GSH-dependent protection. The loss of protection indicates that GSH must be taken up to give protection and is not simply reacting with paraquat-derived toxic species in the extracellular medium. Moldéus and co-workers showed that, in isolated lung cells and perfused lung, added GSH could stimulate the rate of GSH conjugation with 1-chloro-2,4-dinitrobenzene. Their conclusions were that the lung was able to utilize extracellular GSH and that extracellular GSH may be able to be taken up by lung tissue [20]. Our results confirm this earlier study and extend it to show that type II cells in particular can take up intact GSH and use it to protect against paraquat-induced damage. Whether other lung cell types may also have this ability is unknown at present.

The effect of paraquat on thiol/disulfide homeostasis in alveolar cells preincubated with GSH has not yet been studied. However, previous studies of paraquat effects in rabbit lung indicate that GSSG is increased, but GSH is not depleted [21]. Thus, a detailed study of the effects of paraquat on type II cell thiol/disulfide pools and related reductase systems is needed to clarify the role of GSH in protection against paraquat-induced injury.

For the current studies, type II cells were specifically chosen due to the availability of methods to prepare a nearly homogeneous population of viable cells and considerable information about oxidative injury in these cells. The type II cells are significant because they are constantly exposed to an oxidative environment. They are involved in regeneration of the alveolar epithelium as progenitors of type I cells, the major cell type lining the alveolar sac. Prior studies demonstrated that type I cells are very susceptible to oxidative injury while type II cells are relatively resistant [21]. Type II cells obtained from oxygen-treated neonatal rats demonstrated increased glutathione levels after their isolation compared to type II cells obtained from animals maintained in room air [22]. In conjunction with increased GSH levels, increased glucose-6-phosphate dehydrogenase activity and glutathione reductase activity were also observed in oxygen-treated lung explants. NADPH generated by glucose-6-phosphate dehydrogenase activity provides reducing equivalents for the reduction of GSSG to GSH by glutathione reductase. Therefore, type II cell resistance to oxidative injury may be due to increase in enzyme levels, concentration of reducing equivalents, and transport of GSH.

The plasma is constantly supplied with GSH from the liver in a process under hormonal control [23]. This process may maintain plasma GSH to allow cells with an uptake system to utilize the GSH synthesized in liver to aid in protection against cellular injury. The current study shows that, like epithelial cells of the intestine and kidney, alveolar type II cells can potentially utilize plasma GSH for detoxication processes.

These studies provide a basis to suggest that GSH may be useful as a direct therapeutic agent to protect lung tissue against certain types of chemical and oxidative injury. Such injury occurs as a consequence of oxygen therapy to premature infants, administration of antitumour agents such as *bis*-chloronitrosourea [24] and bleomycin [25], and environmental agents such as the defoliant paraquat [4]. *In vivo* studies are required to determine potentially effective treatment regimens and to test the clinical utility of such therapy.

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