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# POTENTIATION OF THE CELL SPECIFIC TOXICITY OF PARAQUAT BY 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU)

## IMPLICATIONS FOR THE HETEROGENEOUS DISTRIBUTION OF GLUTATHIONE (GSH) IN RAT LUNG*

SIMON J. HARDWICK, AISHAH ADAM, LEWIS L. SMITH† and GERALD M. COHEN

Toxicology Unit, Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX and †Central Toxicology Laboratory, Imperial Chemical Industries plc, Alderley Park, Macclesfield, Cheshire SK10 4TJ, U.K.

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**Abstract**—In order to study oxidative stress in the lung, we have developed a rat lung slice model with compromised oxidative defences. Lung slices with markedly inhibited glutathione reductase activity (~80% inhibition) were prepared by incubating slices, with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (100 μM) in an amino acid-rich medium for 45 min at 37°. These lung slices had similar levels of GSH and ATP and polyamine uptake (a marker of alveolar epithelial type I and II cell function) to control rat lung slices. We have utilized these BCNU pretreated slices to study the effects of the herbicide, paraquat, in comparison to those of 2,3-dimethoxy-1,4-naphthoquinone, a potent redox cycler. Paraquat (10–100 μM) caused only minimal changes in the levels of GSH or ATP in control or compromised slices. In contrast, 2,3-dimethoxy-1,4-naphthoquinone caused a decrease in GSH in control slices but a markedly enhanced decrease in both GSH and ATP in compromised slices. Both compounds had only limited effects on putrescine and spermidine uptake in control slices. However, they caused a marked inhibition in compromised slices. Paraquat had little effect on 5-hydroxytryptamine uptake (a marker of endothelial cell function) in either control or compromised slices whereas the quinone inhibited uptake in the compromised slices. Thus, the lack of effect of paraquat on GSH and ATP does not support the involvement of oxidative stress in its toxicity. In contrast, using polyamine uptake, as a functional marker of alveolar epithelial cell damage, suggests a role for redox cycling. As paraquat is known to be accumulated primarily in alveolar type I and II cells (a small fraction of the lung cell population), our data suggest that only a small proportion of pulmonary GSH and ATP is present in alveolar epithelial type I and II cells but that much larger amounts may be present in endothelial cells. These studies highlight the problem of gross tissue measurements in heterogeneous tissues such as the lung.

Oxidative stress has been implicated in the pulmonary toxicity of a number of chemicals including the herbicide, paraquat (1,1′-dimethyl-4,4′-bipyridilium) and the antimicrobial agent, nitrofurantoin [2]. A key feature of paraquat toxicity is lung damage. The mechanism of paraquat toxicity is not unequivocally established but is generally agreed to involve its selective accumulation in lung followed by oxidative damage to those cells in which it is accumulated [3].

Paraquat is accumulated into lung tissue by an energy-dependent process [4] which obeys saturation kinetics and appears to be identical to an uptake system present for the accumulation of the oligoamines, putrescine, spermidine and spermine [5] and the disulphide cystamine [6]. The uptake system for paraquat and the polyamines appears to be present in alveolar epithelial type I and type II cells and in Clara cells [7, 8]. This accumulation process differs from the uptake system present for the accumulation of the monoamine 5-hydroxytryptamine which is associated with pulmonary endothelial cells [9]. Following its pulmonary accumulation, paraquat undergoes a one electron reduction most probably catalysed by the microsomal enzyme NADPH-cytochrome P-450 reductase forming the paraquat radical cation [2, 10]. In the presence of molecular oxygen, the radical is rapidly reoxidized, generating superoxide anion radical ($O_2^-$) and reforming the parent molecule, which may be re-reduced thereby establishing a futile redox cycle [2, 3]. Superoxide anion so formed by redox cycling may directly or indirectly induce toxicity by a number of different mechanisms including DNA-strand breaks, lipid peroxidation and enzyme inactivation [11]. The toxic effects of paraquat may be due to this sustained redox cycling generating active oxygen species resulting in NADPH depletion and/or lipid peroxidation [3, 12, 13].

The susceptibility of the lung to oxidant stress depends on the balance of pro-oxidant and anti-oxidant activities. The latter include glutathione (GSH), α-tocopherol (Vitamin E), superoxide dismutase (SOD), catalase and the glutathione peroxidase–reductase system [14, 15]. Glutathione peroxidase catalyses the decomposition of hydrogen

* A preliminary report of the method for preparation of lung slices with compromised oxidative defences has been published [1].

peroxide with the concomitant oxidation of GSH. Glutathione reductase maintains intracellular GSH by reducing GSSG at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) [16].

We have been particularly interested in the role of GSH in the pulmonary toxicity of paraquat. Early work by Bus and co-workers revealed a depletion of GSH in liver but not in the lungs of mice dosed with paraquat. Keeling *et al.* observed no thiol depletion in rat lung following a range of doses of paraquat [18]. These results are surprising, in view of the proposed mechanism of paraquat toxicity involving redox cycling and the generation of $O_2^{\cdot-}$ and hydrogen peroxide. Whilst increases in glutathione mixed protein disulphides have been reported, the levels of GSSG in the lung following paraquat administration are unchanged [18], suggesting minimal involvement of glutathione in the mechanism of toxicity.

In order to clarify the role of GSH in paraquat toxicity, we have developed a novel system using rat lung slices with impaired oxidative defences, specifically with a markedly inhibited glutathione reductase activity. This was achieved by incubating slices with the anti-tumour agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a relatively specific inhibitor of glutathione reductase [19]. Previous studies have utilized BCNU in order to study oxidative stress in hepatocytes [20].

We have studied the effects of paraquat on control and BCNU pretreated lung slices and compared them to a potent redox cycling compound 2,3-dimethoxy-1,4-naphthoquinone [21]. In addition to biochemical measurements, we have employed polyamine uptake as a functional marker of the integrity of the alveolar epithelium [8]. Our studies highlight the problems associated with gross tissue measurements in heterogeneous tissues, such as the lung, when chemicals may exert their effects in specific cell types. Our results suggest that much of the pulmonary GSH is present in cell types other than alveolar epithelial type I and II cells possibly in pulmonary endothelial cells.

## MATERIALS AND METHODS

*Chemicals.* 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) was obtained from Bristol-Myers Pharmaceuticals (Uxbridge, U.K.). Paraquat dichloride was a generous gift of ICI plc (Macclesfield, U.K.). 2,3-Dimethoxy-1,4-naphthoquinone was prepared as previously described [21]. Minimal essential medium (MEM) with amino acids (without L-glutamine) was obtained from Gibco Ltd (Paisley, U.K.). All other chemicals were obtained from the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated and were of the highest purity grade available.

[1,4-$^{14}$C]Putrescine dihydrochloride (99%) was obtained from NEN Research Products. [$^{14}$C]Spermidine trihydrochloride (99.7% pure) and 5-hydroxy[side-chain-2-$^{14}$C]tryptamine creatinine sulphate (99% pure) were purchased from Amersham International plc (Bucks, U.K.).

*Preparation of control lung slices and slices with compromised oxidative defences.* Male Wistar rats (160–250 g) were from Olac Ltd (Southampton, U.K.). The lungs were perfused *in situ* with Krebs–Ringer phosphate buffer, pH 7.4 (KRP) with glucose using a recirculating system employing a Watson–Marlow 502-S peristaltic pump. Slices were prepared using a McIlwain tissue chopper as described previously [9]. In order to obtain rat lung slices with inhibited glutathione reductase, slices (35–45 mg) were preincubated with BCNU (100 $\mu$M) in 3 mL Krebs–Ringer phosphate buffer, pH 7.4, supplemented with L-cysteine (0.2 mM), L-glycine (0.67 mM), L-methionine (0.2 mM), L-serine (0.2 mM) and Gibco's MEM amino acid solution (without L-glutamine) as employed in hepatocyte studies [22], for 45 min at 37° in 25 mL conical flasks shaken at 60 cycles/min. BCNU was added in DMSO (final concentration 0.3%). Control slices were treated similarly except BCNU and the amino acids were omitted. Lung slices were then washed in amino acid-free Krebs–Ringer phosphate buffer, pH 7.4 (at 37°) and reincubated in similar medium for periods up to 4 hr.

*Measurement of lung nonprotein sulphydryls (NPSH) and ATP.* Lung slices were homogenized at 4° using a Polytron homogenizer for 30 sec and assayed for NPSH and ATP for 30 sec in 6.5% TCA/5 mM EDTA. NPSH was assayed using the fluorimetric method of Hissin and Hilf [23] using *o*-phthaldialdehyde. Fluorescence was detected with a Perkin–Elmer Fluorescence Spectrophotometer. NPSH levels are reported as GSH levels since previous work has shown that the cytoplasm of rat lung cells contains only small amounts of acid-soluble thiols other than GSH [24].

ATP was assayed using a luciferase-based bioluminescence method as previously described [25], employing a Thorn–EMI Photon Detection System coupled to Amstrad PC1512 software.

*Accumulation of putrescine, spermidine and 5-hydroxytryptamine.* Accumulation of putrescine, spermidine and 5-hydroxytryptamine was assessed by incubation of lung tissue (20–40 mg) with 0.1 $\mu$Ci of radiolabel with an appropriate concentration of unlabelled compound. In experiments with 5-hydroxytryptamine, iproniazid (500 $\mu$M) was included to inhibit monoamine oxidase activity [9]. Lung slices were washed, blotted and dissolved in 0.5 M KOH (400 $\mu$L), neutralized with 0.5 M HCl (400 $\mu$L) and radioactivity determined as previously described [5].

In some experiments, slices were preincubated with either paraquat (10–100 $\mu$M) or 2,3-dimethoxy-1,4-naphthoquinone (50 $\mu$M) before determining pulmonary uptake. These slices were removed and washed to remove any extraneous compound, which may inhibit the uptake of putrescine, spermidine or 5-hydroxytryptamine.

*Enzyme activities.* Lung slices were homogenized at 4° in phosphate/EDTA buffer (100 mM $KH_2PO_4$, 5 mM EDTA; pH 7.4) with a Polytron for 20 sec. Lung homogenates were centrifuged in a Denley BR 401 refrigerated centrifuge at 800 *g* for 15 min and the supernatant fractions sampled for enzyme assays.

Glutathione reductase activity was determined by monitoring the oxidation of NADPH at 340 nm at 37° essentially by the method of Carlberg and Mannervik

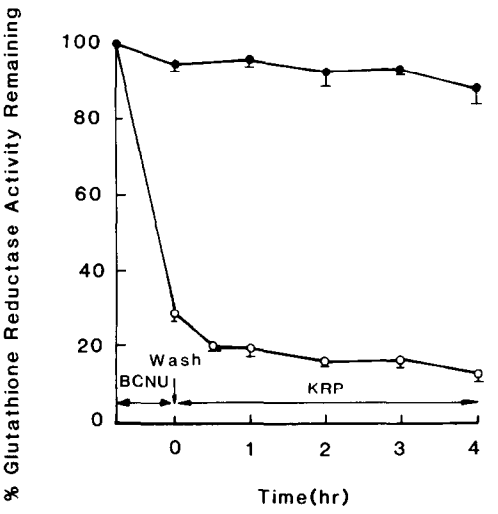


Fig. 1. Inhibition of pulmonary glutathione reductase by BCNU. Rat lung slices were incubated for 45 min either as controls (0.3% DMSO) (●—●) or with BCNU (100 μM) in the presence of amino acid supplemented KRP (○—○). Slices were then washed and incubated for 0–4 hr in fresh KRP medium and slices were removed and glutathione reductase assayed at appropriate times. Results shown are the means ± SE of three experiments.

[26]. The assay solution contained 1 mM GSSG in phosphate/EDTA buffer, pH 7.4, and the reaction was initiated by the addition of 100 μM NADPH.

The activity of catalase was determined by following the reduction of $H_2O_2$ at 240 nm at 25° [27]. The amount of reduced $H_2O_2$ was calculated using an extinction coefficient of 43.6/M/cm.

NADPH-cytochrome *c* reductase activity was determined by following the reduction of cytochrome *c* by NADPH at 37° at 550 nm. The reaction mixture contained cytochrome *c* (0.5 mg/mL) in phosphate/EDTA buffer, pH 7.4 and was initiated by 100 μM NADPH. The specific activity of the enzyme was calculated using an extinction coefficient of 19.5/mM/cm [28].

## RESULTS

### *Preparation and characterization of lung slices with compromised oxidative defences*

The inhibition of glutathione reductase activity in lung slices by BCNU was both time and concentration dependent (40–1500 μM). At the highest concentration of BCNU, in agreement with previous results with hepatocytes, near maximal inhibition (~80%) was observed after 45 min incubation at a BCNU concentration of 100 μM without marked depletion of pulmonary GSH (see later). After 45 min incubation, the lung slices were transferred to a BCNU-free medium when the marked inhibition was maintained for up to 4 hr (Fig. 1).

We wished to examine whether the incubation of the lung slices with BCNU impaired other key biochemical functions of the lung in addition to glutathione reductase. Viability of lung slices as assessed by $^{14}CO_2$ evolution from D-[$^{14}$C(U)]glucose was unaffected by BCNU pretreatment [1]. Pretreatment with BCNU did not affect the activity of NADPH-cytochrome *c* reductase, catalase, ATP levels and caused only a small decrease in GSH levels (Table 1). However, preincubation with BCNU in an amino acid-rich medium was essential for maintenance of pulmonary GSH as lung slices preincubated with BCNU in an amino acid-free medium had significantly lower GSH levels than controls (results not shown).

Thus, following incubation with BCNU (100 μM) for 45 min, lung slices were obtained with compromised oxidative defences as defined by a markedly inhibited glutathione reductase (~80%) together with relatively normal levels of GSH, ATP, NADPH-cytochrome *c* reductase and catalase.

### *Effects of paraquat on GSH and ATP levels in control and BCNU pretreated lung slices*

Lung slices were incubated with paraquat (1–100 μM) for up to 4 hr. Control slices exhibited little or no thiol depletion (Fig. 2a). In BCNU pretreated slices, a small reduction in GSH was observed (Fig. 2b). No marked differences were observed in GSH levels in BCNU exposed slices with paraquat (1–

Table 1. Comparison of biochemical parameters in control and BCNU pretreated lung slices

| Parameter | Time (hr) | Control | BCNU |
|---|---|---|---|
| GSH* | 0 | 0.60 ± 0.01 | 0.56 ± 0.01 |
| GSH* | 4 | 0.51 ± 0.02 | 0.43 ± 0.03 |
| ATP* | 0 | 1.14 ± 0.05 | 1.09 ± 0.04 |
| ATP* | 4 | 1.19 ± 0.10 | 1.07 ± 0.07 |
| NADPH-cytochrome *c* reductase† | 0 | 0.53 ± 0.01 | 0.50 ± 0.03 |
| NADPH-cytochrome *c* reductase† | 4 | 0.40 ± 0.02 | 0.40 ± 0.02 |
| Catalase‡ | 0 | 0.59 | 0.60 |
| Catalase‡ | 4 | 0.64 | 0.57 |

Lung slices were incubated at 37° in buffer or with BCNU (100 μM) for 45 min and then transferred to Krebs–Ringer phosphate buffer, pH 7.4, and incubated for a further 4 hr. GSH, ATP, NADPH-cytochrome *c* reductase and catalase were measured immediately after the initial incubation, i.e. 45 min ($t = 0$) or after 4 hr incubation as described in Materials and Methods.

* μmol/g wet wt lung.

† nmoles cytochrome *c* reduced/min/mg wet wt lung.

‡ μmoles $H_2O_2$ reduced/min/mg wet wt lung.

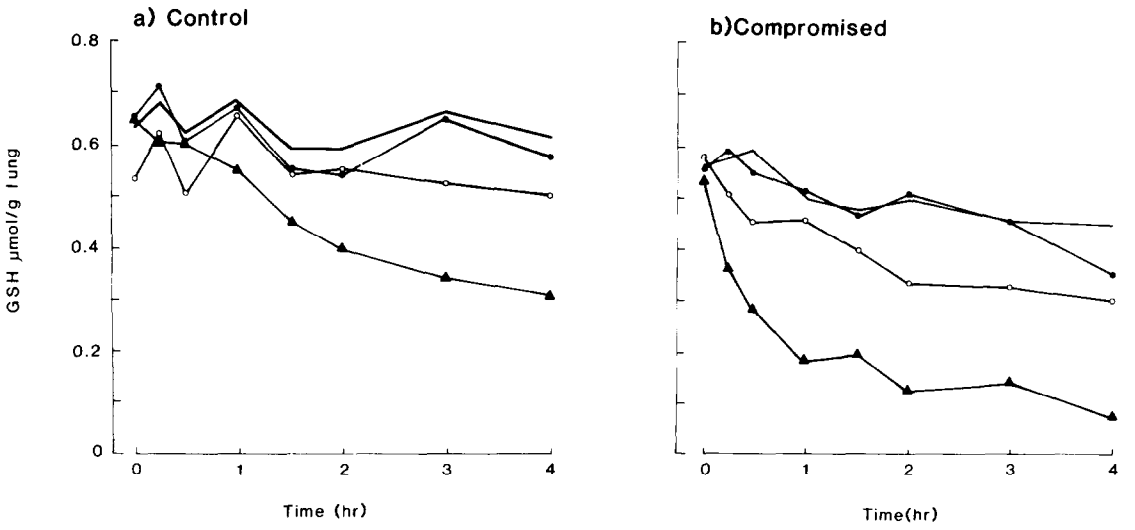


Fig. 2. GSH levels in control and compromised lung slices incubated with paraquat. Control lung slices (a) or BCNU pretreated lung slices (b) were incubated either alone (———) or with paraquat, 10 and 100 µM, (●—● and ○—○ respectively), or with 2,3-dimethoxy-1,4-naphthoquinone, 50 µM (▲—▲). At the indicated times, slices were removed and GSH measured as described in Materials and Methods. Results shown are the means of three experiments. Standard errors were small and have been omitted for clarity.

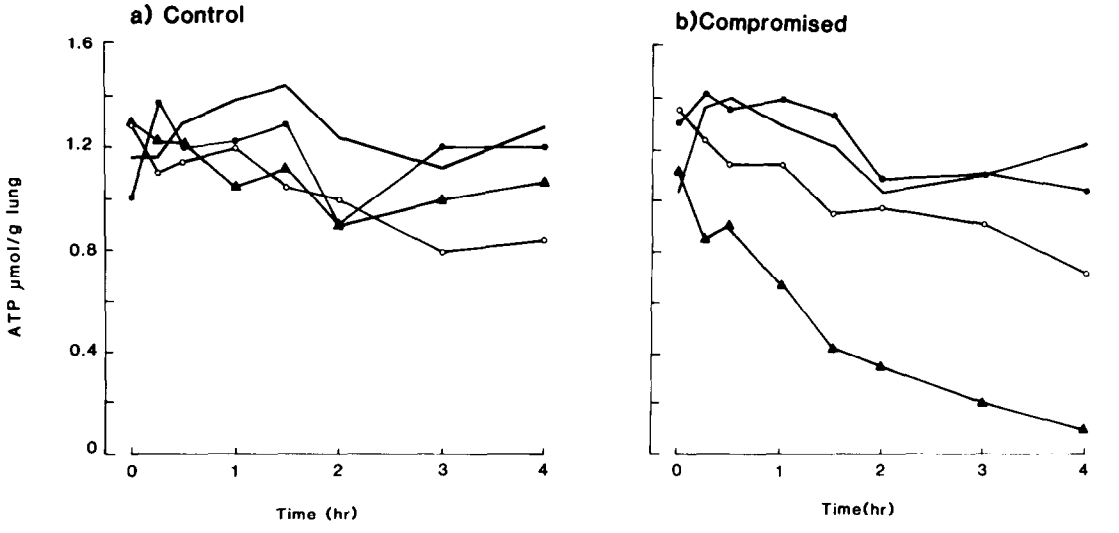


Fig. 3. ATP levels in control and compromised lung slices incubated with paraquat. Control lung slices (a) or BCNU pretreated lung slices (b) were incubated either alone or with paraquat, 10 and 100 µM, (●—● and ○—○ respectively), or with 2,3-dimethoxy-1,4-naphthoquinone, 50 µM (▲—▲). At the indicated times, slices were removed and ATP measured as described in Materials and Methods. Results shown are the means of three experiments. Standard errors were small and have been omitted for clarity.

100 µM) (Fig. 2). The lack of a marked potentiation of GSH depletion by paraquat, particularly in the compromised slices, was surprising as it is believed to exert its toxicity by redox cycling. To elucidate this apparent paradox, we utilized 2,3-dimethoxy-1,4-naphthoquinone, a potent redox cycler [21] as a positive control.

2,3-Dimethoxy-1,4-naphthoquinone (50 µM) produced a time dependent depletion of GSH in control slices (Fig. 2a). With compromised slices, this effect was markedly potentiated, in particular, during the first 90 min of the incubation (Fig. 2b).

Incubation of lung slices from control or BCNU pretreated slices with paraquat (10 µM) did not affect pulmonary ATP levels (Fig. 3). Incubation with a higher concentration of paraquat (100 µM) caused a depletion of ATP but no difference was observed between control and BCNU pretreated slices (Fig.

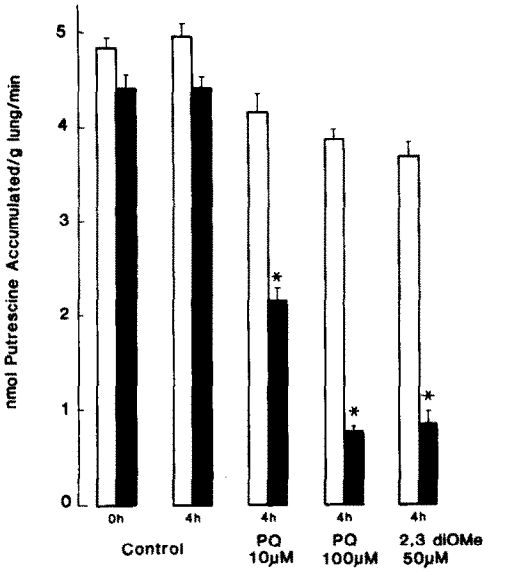


Fig. 4. Putrescine uptake in control and BCNU treated slices following incubation with paraquat. Putrescine accumulation was assessed following incubation of slices with paraquat (PQ) and 2,3-dimethoxy-1,4-naphthoquinone (2,3-diOMe). Open and closed bars denote control and BCNU pretreated slices respectively. Slices were incubated with 0.1 μCi [$^{14}$C]putrescine (10 μM) for 30 min at 37° in a shaking water bath. Results shown represent mean ± SE (N = 4). (*) Significant at $P < 0.05$ using a paired $t$-test comparing BCNU pretreated slices to their corresponding controls.

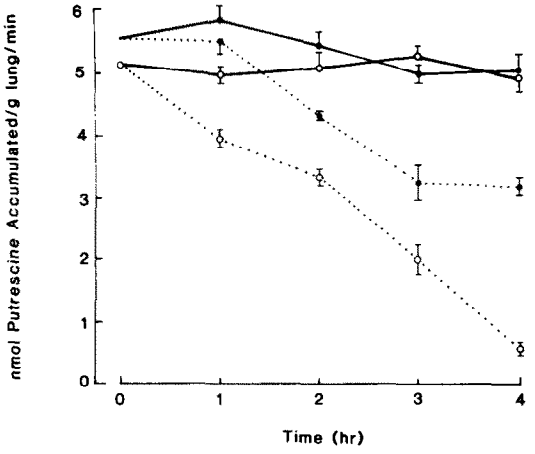


Fig. 5. Time course of loss of putrescine accumulation in BCNU compromised lung slices incubated with paraquat. Control lung slices (●) or BCNU pretreated lung slices (○) were incubated either in the absence (———) or the presence (· · · · · ·) of paraquat (100 μM). At the indicated times, slices were removed and the accumulation of [$^{14}$C]putrescine (10 μM) assessed as described in Materials and Methods. Results are mean ± SE (N = 4).

Table 2. Kinetic parameters of putrescine accumulation in control and BCNU pretreated lung slices

| Treatment | Apparent $K_m$ (μM) | $V_{max}$ (nmol/g lung/hr) |
|---|---|---|
| Control | 13.5 ± 0.4 | 718 ± 30.2 |
| Compromised | 15.6 ± 0.1 | 720 ± 23.7 |

Control or compromised lung slices were incubated with putrescine (1, 5, 10, 50 and 100 μM) for up to 60 min and uptake determined as described in Materials and Methods. Results are expressed as mean ± SE (N = 3). $K_m$ and $V_{max}$ values were derived from Hanes–Woolf plots.

3). Of particular interest was the very marked potentiation of ATP depletion observed with 2,3-dimethoxy-1,4-naphthoquinone in BCNU pretreated slices (Fig. 3b).

As these results demonstrated no potentiation of the effects of paraquat on GSH or ATP (Figs 2 and 3) with BCNU pretreated slices, we wished to investigate any possible effects on a marker of type I and II cell integrity i.e. pulmonary uptake of putrescine.

*Putrescine uptake after incubation for 4 hr with paraquat in control and compromised rat lung slices*

Incubation of lung slices in control medium for 4 hr did not impair their ability to accumulate putrescine (10 μM). PQ (10 and 100 μM) caused a reduction in putrescine uptake which was potentiated in BCNU pretreated slices compared to control (Fig. 4).

2,3-Dimethoxy,1,4-naphthoquinone (50 μM) alone caused a slight decrease in the pulmonary uptake of putrescine and this was also markedly potentiated in BCNU pretreated slices (Fig. 4).

In order to characterize the potentiation of the loss of putrescine uptake in BCNU pretreated slices in more detail, we determined the time course of the inhibition and the kinetic parameters of the uptake system in both control and compromised lung slices.

In the absence of paraquat, BCNU pretreated and control slices exhibited virtually identical putrescine accumulation, over a 4 hr incubation period (Fig. 5). In control slices, paraquat (100 μM) produced a gradual but significant decrease in ability to accumulate the diamine whereas in compromised slices a rapid and much more extensive loss of uptake was observed (Fig. 5). In this experiment, a somewhat greater inhibition of putrescine uptake by paraquat (100 μM) was observed than in previous experiments (compare Figs 4 and 5). However, in all experiments, paraquat caused a much greater inhibition of putrescine uptake in compromised than in control slices.

*Determination of kinetic parameters of putrescine uptake in BCNU pretreated slices*

BCNU pretreatment caused a small inhibition of putrescine uptake but this was unaltered after 4 hr incubation (Fig. 4). However the relevance of this is unclear as the kinetic parameters in control and compromised slices were virtually identical (Table 2). These values were similar to those observed by others [5].

Table 3. Uptake of spermidine into control and BCNU pretreated lung slices following paraquat incubation (4 hr)

| Paraquat (μM) | Time (hr) | Spermidine uptake (nmol/g lung/min) Control | Compromised |
|---|---|---|---|
| — | 0 | 4.95 ± 0.29 | 4.91 ± 0.07 |
| — | 4 | 4.70 ± 0.23 | 4.48 ± 0.27 |
| 10 | 4 | 4.09 ± 0.09 | 2.95 ± 0.08* |
| 100 | 4 | 2.73 ± 0.14 | 1.00 ± 0.11* |

Control or BCNU compromised lung slices were incubated for 4 hr either alone or in the presence of paraquat. Spermidine (10 μM) uptake was then measured as described in Materials and Methods. In addition spermidine uptake was measured after the initial incubation of 45 min ($t = 0$ hr). Results are expressed as mean ± SE (N = 3).

* $P < 0.05$ using a paired $t$-test.

*Uptake of spermidine in control and compromised rat lung slices*

The uptake of spermidine was assessed in BCNU compromised slices and compared to control slices. Paraquat (10 and 100 μM) caused a reduction in spermidine accumulation which was potentiated in BCNU treated slices similarly incubated. However the magnitude of the reduction in spermidine uptake was less than that observed with putrescine (compare Table 3 and Fig. 4).

*Effects of paraquat on uptake of 5-hydroxytryptamine*

Pulmonary accumulation of 5-hydroxytryptamine has been suggested as a marker of endothelial cell integrity [9, 29]. Incubation of control slices for 4 hr did not affect the accumulation of 5-hydroxytryptamine (Fig. 6). Lung slices pretreated with BCNU exhibited a small but significant reduction in uptake both initially and after 4 hr (Fig. 6), which may have been due to a toxic effect of BCNU on endothelial cells. Paraquat slightly increased 5-hydroxytryptamine uptake into control slices. In compromised slices, accumulation was reduced but was not significantly lower than the lung slices exposed to BCNU alone (Fig. 6).

In control slices, 2,3-dimethoxy-1,4-naphthoquinone (50 μM) did not affect the pulmonary accumulation of 5-hydroxytryptamine. However, in slices pretreated with BCNU, a significant inhibition of uptake was observed (Fig. 6).

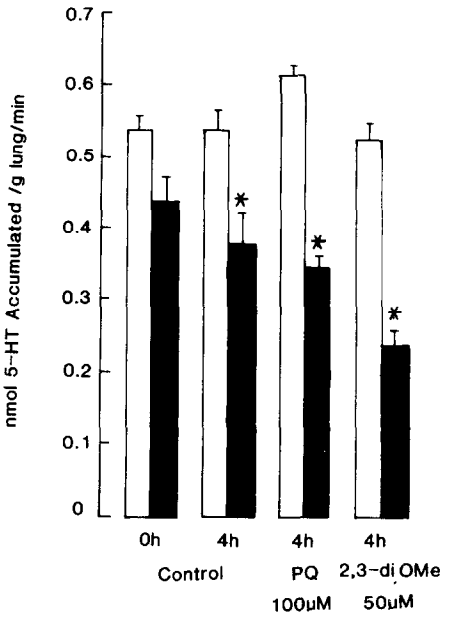


Fig. 6. Uptake of 5-hydroxytryptamine (5-HT) in control and BCNU compromised lung slices. Lung slices were incubated with 0.1 μCi [$^{14}$C]5-HT (1 μM) for 20 min, following 4 hr incubation with either paraquat (PQ, 100 μM) or 2,3-dimethoxy-1,4-naphthoquinone (2,3-diOMe, 50 μM). Open and closed bars indicate control and BCNU pretreated slices respectively. Results are the means ± SE (N = 3). (*) Significant at $P < 0.05$ using a paired $t$-test comparing BCNU pretreated slices to their corresponding controls.

## DISCUSSION

The generally accepted mechanism of the pulmonary toxicity of paraquat is that it is initially accumulated in alveolar epithelial type I and II cells, where it then exerts its toxicity by redox cycling [2, 3]. However, if redox cycling is intimately associated with paraquat toxicity it is difficult to reconcile the lack of pulmonary thiol depletion observed in mice [17] and rats [18] dosed with paraquat. In the latter study, no increase in glutathione disulphide was observed, however an increase in pulmonary mixed protein disulphides was noted. Brigelius *et al.* [30] noted a decrease in GSH/GSSG ratio in isolated perfused rabbit lung treated with paraquat.

In an attempt to clarify the role of GSH in the pulmonary toxicity of paraquat, we have used BCNU in a rat lung slice model with compromised oxidative defences. BCNU is used in the chemotherapy of a variety of human tumours including gliomas, small cell carcinoma of the lung, lymphomas and carcinoma of the gastrointestinal tract [31]. Its effects are believed to be due to carbamylation and/or alkylation effects of degradation intermediates of the parent compound [32]. In clinical studies, BCNU produced a specific and irreversible inhibition of erythrocytic glutathione reductase activity without affecting 19 additional enzymes [19]. *In vivo* BCNU has been shown to inhibit pulmonary glutathione reductase [33]. BCNU has been utilized as an experimental tool to inhibit glutathione reductase in hepatocytes in order to study the role of oxidative stress in the cytotoxicity of a number of compounds including Adriamycin® [20], menadione [34], paraquat [35] and diquat [35, 36]. With the hepatocyte model, following BCNU exposure, it was necessary to incubate the hepatocytes for up to 90 min in an amino acid rich medium in order to restore normal GSH levels [22]. This was not essential in the lung slice model we have described, when initial incubation with amino acids was sufficient to maintain approximately normal levels of GSH in the presence of

BCNU (Table 1 and Fig. 2) and these levels were then maintained during a further 4 hr incubation (Fig. 2). A possible reason for this difference between hepatocytes and lung slices is a more rapid utilization of GSH by liver tissue [37].

BCNU pretreatment of lung slices did not potentiate the effects of paraquat on pulmonary GSH (Fig. 2) or ATP (Fig. 3) but did potentiate the inhibitory effects on the pulmonary uptake of the polyamines putrescine (Fig. 4), spermidine (Table 3) and spermine (Walther, Hardwick and Cohen, unpublished results). The lack of effect of paraquat on GSH and ATP was most interesting and was in marked contrast to the marked potentiation by 2,3-dimethoxy-1,4-naphthoquinone of the loss of both GSH (Fig. 2) and ATP (Fig. 3) observed in lung slices pretreated with BCNU. With hepatocytes and rat liver microsomes, we have shown that 2,3-dimethoxy-1,4-naphthoquinone is a potent redox cycling compound causing a concentration dependent depletion of GSH and increase in GSSG, which precede cytotoxicity [21]. The most likely explanations for the differential effects of paraquat and 2,3-dimethoxy-1,4-naphthoquinone on GSH levels in BCNU pretreated slices are: (i) paraquat redox cycles much more slowly than 2,3-dimethoxy-1,4-naphthoquinone, therefore the small amount of residual glutathione reductase activity may be capable of reducing any GSSG formed in the presence of paraquat and (ii) paraquat is selectively accumulated by alveolar type I and II cells and possibly Clara cells [7, 8], whereas 2,3-dimethoxy-1,4-naphthoquinone, because of its lipid solubility would be expected to diffuse into all cell types in the lung, thus the much more extensive GSH depletion with the quinone is compatible with the hypothesis that only a small fraction of pulmonary GSH is present in alveolar type I and II cells. This hypothesis is further substantiated by the observations that BCNU pretreatment of rat lung slices potentiates the toxicity of paraquat, based upon the use of a functional assessment of viability of alveolar epithelium i.e. putrescine and spermidine uptake (Fig. 4 and Table 3) in contrast to the lack of effects on GSH and ATP (Figs 2 and 3). Thus, consideration of the GSH levels in compromised lung slices in the presence of paraquat argues against potentiaion of an oxidative stress-induced toxicity, whereas using a functional assessment of alveolar epithelial damage indicates that paraquat may be toxic due to redox cycling. These findings highlight the problems encountered when investigating biochemical mechanisms of toxicity in heterogeneous tissues such as the lung, which contains at least 40 different cell types [38]. If critical biochemical changes are occurring in a target cell population, which constitutes only a small percentage of the total lung cell population, then these changes could be masked by the other cells present. In particular, changes in levels or activities of enzymes or metabolic intermediates present in all cell types may not be detected, whereas changes in intermediates present primarily in the target cell population should be readily detected. Therefore as paraquat is accumulated primarily into alveolar type I and II cells [7, 8, 39] which comprise 8 and 14% respectively of the parenchymal lung cell population [40], we conclude that much of the pulmonary glutathione is present in other cell types. This conclusion is further substantiated by studies with isolated rabbit lung cells which showed a wide variation in GSH concentrations, macrophages > Clara cells ≫ type II cells [41], although in this study some of the observed differences may have been caused by losses during isolation of the cells. In the lung slice model we have used, a significant proportion of the cells should compromise pulmonary endothelial cells, which we would hypothesize contain a large proportion of the pulmonary GSH. The function of this GSH in endothelial cells is unknown but may be related to leukotriene formation [42]. If pulmonary GSH has an uneven cellular distribution, as suggested by our studies, then this may have important implications in the susceptibility of individual cell types to toxicity. A similar consideration of the data with paraquat and 2,3-dimethoxy-1,4-naphthoquinone on ATP in control and BCNU pretreated lung slices suggests that much of the pulmonary ATP is also present in the endothelial cells.

The data with 5-hydroxytryptamine, suggests that paraquat does not have a major effect on the endothelium. Paraquat alone caused a small increase in uptake (Fig. 6) in lung slices similar to that reported by others following *in vivo* dosing with paraquat although the mechanism remains unknown [8]. However, the redox active 2,3-dimethoxy-1,4-naphthoquinone did not alter uptake of the amine in control lung slices but in compromised slices caused a reduction in accumulation (Fig. 6) suggestive of toxicity to the endothelial cells. Interestingly, the BCNU control groups show reduced accumulation at both 0 and 4 hr compared with control lung slices, suggesting a toxic effect of BCNU on the endothelium. Endothelial damage in lung tissue following BCNU administration *in vivo* has been reported [43].

In summary, we have described a new model of lung slices with compromised oxidative defences (i.e. inhibited glutathione reductase activity) for the study of oxidative stress in the lung. The compromised lung slices possessed apparently normal levels of both key metabolic intermediates such as ATP and GSH as well as antioxidant and prooxidant enzymes (catalase and NADPH-cytochrome *c* reductase respectively) and normal type I and II cell function (as assessed by polyamine uptake). We have utilized these compromised slices to further study the mechanism of toxicity of paraquat. Inactivation of glutathione reductase potentiates the effects of paraquat in the lung as assessed by functional markers of type I and II cell function but not by biochemical parameters such as GSH and ATP. From these results we infer that much of the pulmonary GSH is distributed in cells other than alveolar type I and II cells, most probably in endothelial cells. The system we have described in addition to being a valuable model for the study of oxidative stress should aid in increasing our knowledge of basic lung biochemistry. These studies also highlight the problem of gross tissue measurements in a heterogenous tissue such as lung.

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