Paraquat Increases Cyanide-insensitive Respiration in Murine Lung Epithelial Cells by Activating an NAD(P)H:Paraquat Oxidoreductase

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Pulmonary fibrosis is one of the most severe consequences of exposure to paraquat, an herbicide that causes rapid alveolar inflammation and epithelial cell damage. Paraquat is known to induce toxicity in cells by stimulating oxygen utilization via redox cycling and the generation of reactive oxygen intermediates. However, the enzymatic activity mediating this reaction in lung cells is not completely understood. Using self-referencing microsensors, we measured the effects of paraquat on oxygen flux into murine lung epithelial cells. Paraquat (10–100 μM) was found to cause a 2–4-fold increase in cellular oxygen flux. The mitochondrial poisons cyanide, rotenone, and antimycin A prevented mitochondrial- but not paraquat-mediated oxygen flux into cells. In contrast, diphenyleneiodonium (10 μM), an NADPH oxidase inhibitor, blocked the effects of paraquat without altering mitochondrial respiration. NADPH oxidases, enzymes that are highly expressed in lung epithelial cells, utilize molecular oxygen to generate superoxide anion. We discovered that lung epithelial cells possess a distinct cytoplasmic diphenyleneiodonium-sensitive NAD(P)H:paraquat oxidoreductase. This enzyme utilizes oxygen, requires NADH or NADPH, and readily generates the reduced paraquat radical. Purification and sequence analysis identified this enzyme activity as thioredoxin reductase. Purified paraquat reductase from the cells contained thioredoxin reductase activity, and purified rat liver thioredoxin reductase or recombinant enzyme possessed paraquat reductase activity. Reactive oxygen intermediates and subsequent oxidative stress generated from this enzyme are likely to contribute to paraquat-induced lung toxicity.

Exposure of humans and animals to toxic doses of paraquat (1,1’-dimethyl-4,4’-bipyridyl) is known to damage the lung.

1 This work was supported in part by National Institutes of Health Grants U54AR055073, ES006897, CA100994, CA093798, ES003647, ES010791, ES004738, GM034310, RR001395, and ES005022. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 The abbreviations used are: ROI, reactive oxygen intermediates; DPI, diphenyleneiodonium; NAME, nitroarginine methyl ester; MLE-15, murine lung epithelial cells; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); ATM, sodium aurothiomalate; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography.
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\[
\begin{align*}
\text{NAD(P)H} & \xrightarrow{\text{oxidase}} \text{NAD(P)}^+ \text{H}^+ \quad \text{REACTION 1} \\
\text{NAD(P)}^+ \text{H}^+ & \xrightarrow{\text{paraquat}} \text{NAD(P)}^+ \text{H}_2 \quad \text{paraquat radical}
\end{align*}
\]

enzyme known to reduce thioredoxin as well as a number of other oxidants, was identified as the active enzyme (23–28). Paraquat has been reported to accumulate in type II alveolar epithelial cells via an active transport system (29). Selective lung toxicity may be the result of coupling of this transport system with the redox cycling of paraquat by thioredoxin reductase.

MATERIALS AND METHODS

Chemicals and Reagents—10-Acetyl-3,7-dihydroxyphenoxazine (AMPLEX-RED) and 3H-phenoxyazine (resorufin) were obtained from Molecular Probes (Eugene, OR). Rat cytochrome P450 reductase (Supersomes™, catalog no. 456514) was from BD Biosciences. Polyclonal antibodies to rat cytochrome P450 reductase (whole rabbit serum, catalog no. OSA-300) were from Stressgen (Victoria, BC). Paraquat, NADPH, NADH, horseradish peroxidase, 2’,5’-ADP-agarose, and all other reagents were purchased from Sigma-Aldrich. The Superose 12 HR 10/30 size exclusion column was obtained from Amersham Biosciences. Rat liver thioredoxin reductase was obtained from Cayman Chemical (Ann Arbor, MI).

Cells and Treatments—MLE-15 murine lung epithelial cells were kindly provided by Dr. Jacob Finkelstein (University of Rochester) (30). Parental CHO cells were obtained from the American Type Culture Collection (Manassas, VA). The preparation of CHO cells expressing cytochrome P450 reductase or control empty vector has been described previously (31). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in 5% CO2 in a humidified incubator. Tissue culture reagents were from Invitrogen. For recordings, cells were plated at varying densities (0.05–0.5 × 10⁵ cells) onto 35-mm plastic Petri dishes in phenol-red-free Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. At low density cells grow individually, whereas at higher densities cells grow as confluent monolayers. Recordings were performed in phosphate-buffered saline. Paraquat and other reagents were added sequentially directly to the medium. Recordings were suspended during the 10–15 s required to apply the drugs. Time was recorded continuously during the addition of reagents.

Self-referencing Electrodes—The preparation and characterization of the oxygen microsensors has been described previously (32–35). Microsensors were used in a self-referencing format to obtain a directional flux of oxygen by individual cells and monolayers, which is detected as a difference in current (Δfemtoamperes). The advantage of this method is that it minimizes noise and random drift in the measurements, factors that limit the use of standard oxygen electrodes (33).

Enzyme Assays—NAD(P)H:paraquat oxidoreductase activity was assayed in cell lysates by quantifying ROI generated via redox cycling of paraquat (36–38), by the paraquat-dependent consumption of oxygen or NADPH in the enzyme assay (39), and by the NADPH-dependent formation of paraquat radical (36, 37, 40). To prepare cell lysates for the assays, cells were gently scraped from the culture dishes, resuspended in phosphate-buffered saline, and sonicated on ice with three 15-s bursts and 1 min of cooling on ice between each sonication burst. Disrupted cells were centrifuged (3000 × g, 10 min) to remove cellular debris, and the supernatants were either assayed immediately for enzyme activity or further used to purify and characterize the paraquat reductase (see further below). Unless otherwise specified, standard reaction mixes contained 50 mm phosphate buffer, pH 7.4, 0.5 mm NADPH or NADH, 10–100 μg of cell lysate protein, and 1–1000 μm paraquat in 0.1 ml. All enzyme assays were run at 37 °C.

Hydrogen peroxide production was assayed in 100-μl reaction mixes supplemented with 25 μm 10-acetyl-3,7-di-hydroxyphenoxazine and 0.1 units/sample of horseradish peroxidase as previously described (41). The fluorescent product resorufin was quantified using an HTS 7000 Plus Bio Assay Reader (PerkinElmer Life Sciences) fitted with a 540-nm excitation filter and a 595-nm emission filter. Kinetic assays were performed in the presence of increasing concentrations of paraquat, and fluorescence of resorufin was measured every 2.5 min for 30 min. The rate of hydrogen peroxide formation was calculated based on a standard curve generated with hydrogen peroxide in a concentration range of 0.5–30 μM. Initial velocity studies of the NAD(P)H oxidase activity were performed as described previously (42). In some experiments purified rat liver thioredoxin reductase (5 units/ml) or recombinant enzyme (9.8 units/ml) was added in place of cell lysates.

Superoxide anion production was assayed by its ability to oxidize hydroethidine to 2-hydroxyethidium cation in the reaction mix; the product was detected using HPLC with fluorescence detection as described by Mishin and Thomas (44).

The generation of paraquat radicals and depletion of NADPH in reaction mixtures were quantified in 0.5-ml cuvettes using a Lambda 20 UV/visible spectrophotometer...
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of post-microsomal supernatant), and the suspension was rocked in a cold room for 1 h and then centrifuged at 2000 × g for 5 min. The supernatant was discarded, and the resin was washed five times with phosphate-buffered saline containing 0.1% Nonidet P-40. The resin was then washed with phosphate-buffered saline containing 0.1% Nonidet P-40 and 5 mM NADPH. The NADPH eluate was concentrated using an Amicon Ultra centrifugal filter (Millipore, Billerica, MA) and then fractionated by size exclusion chromatography on a Superose 12 HR 10/30 column (GE Healthcare) in phosphate-buffered saline containing 0.1% Nonidet P-40, pH 7.4, at a flow rate of 0.3 ml/min. Absorbance of effluent fractions was monitored at 280 nm. The column was previously calibrated with Bio-Rad gel filtration standard proteins (thyroglobulin, Mr, 60,000; bovine globulin, Mr, 158,000; chicken ovalbumin, Mr, 44,000). Fractions containing enzyme activity were analyzed on 10% SDS-polyacrylamide gels. Sequence analysis of protein bands in the gels were performed as previously described (48) and analyzed using NCBI Blast algorithms.

Thioredoxin Reductase Assay—Thioredoxin reductase activity of the purified paraquat reductase or the recombinant enzyme was assayed by the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of NADPH as described by Kang et al. (49). The assay mixture contained 0.2 mM phosphate buffer, pH 7.4, 1 mM EDTA, 0.25 mM NADPH, 1 mM DTNB, and appropriate concentrations of purified recombinant thioredoxin reductase or lung cell extract. Enzyme activity was followed by increases in absorbance at 412 nm. In some experiments, 100 μM 1-chloro-2,4-dinitrobenzene or sodium aurothiomalate (ATM), inhibitors of mammalian thioredoxin reductase, were added to the reaction mix.

Purification of Thioredoxin Reductase—A construct containing human cytosolic thioredoxin reductase (hTR1) in which the TGA (Sec) codon was replaced with the TGC (Cys) codon (Sec498Cys) cloned into the PET 28a+ vector (Novagen) was kindly provided by Anton Turanov (University of Nebraska). The thioredoxin reductase was purified from E. coli (BL21(DE3), Novagen) transformed with the construct and induced to express the enzyme as previously described (50). Purity of the enzyme was confirmed by fractionation of the purified protein on a 10% SDS-polyacrylamide gel followed by Coomassie staining. Enzymatic activity was confirmed by measuring the enzymatic reduction of

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FIGURE 1. Oxygen flux near surface membranes of lung epithelial cells. Panel A, micrograph showing self-referencing oxygen microsensor placed near an MLE-15 cell. Panel B, oxygen flux measured at increasing distances from the cell membrane. Note that oxygen gradients were detected at distances of up to 25–50 μm from the cell. Panel C, effects of antimycin A on oxygen flux in lung epithelial cells. Initially recordings were obtained by placing the oxygen microsensor in the culture medium 1 mm above an MLE-15 cell. After obtaining a stable background (Bkgd) the probe was placed within 5 μm of the cell to obtain a recording of oxygen flux. After an additional 3 min, antimycin A (1 μg/ml, final concentration) was added to the culture medium. Panel D, effects of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μM) on oxygen flux into lung epithelial cells.

(PerkinElmer Life Sciences) scanning at 1 nm/s, recording at 1-nm intervals, and repeating the scan at 2.5-min intervals for 30 min. Paraquat radicals were quantified by increases in absorbance at 603 nm (ε₆₀₃ = 1.20 × 10⁴) (45). Because paraquat radicals are sensitive to oxygen (46), experiments were run in filled cuvettes covered tightly with Parafilm. NADPH depletion was measured by decreases in absorbance at 340 nm (39). A polarographic system fitted with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) was used to characterize oxygen utilization during enzymatic redox cycling of paraquat.

Purification Studies—Lysates containing enzyme activity were centrifuged in an Eppendorf 5417R high speed centrifuge at 12,000 × g for 10 min to sediment membrane and mitochondrial fractions. The resulting supernatant was centrifuged in a Beckman L7–55 ultracentrifuge at 100,000 × g for 1 h, and the microsomal and post-microsomal supernatant fractions were collected. Approximately 40% of the paraquat oxidoreductase activity was contained in the post-microsomal supernatant fractions, whereas the remaining activity was present in membrane and microsomal fractions. The post-mitochondrial supernatant fractions were further purified by NAPDH affinity chromatography as described by Wolff et al. (47). Briefly, 2',5'-ADP-agarose was added (0.2 volume of settled resin per volume of post-microsomal supernatant), and the suspension was rocked in a cold room for 1 h and then centrifuged at 2000 × g for 5 min. The supernatant was discarded, and the resin was washed five times with phosphate-buffered saline containing 0.1% Nonidet P-40. The resin was then washed with phosphate-buffered saline containing 0.1% Nonidet P-40 and 5 mM NADPH. The NADPH eluate was concentrated using an Amicon Ultra centrifugal filter (Millipore, Billerica, MA) and then fractionated by size exclusion chromatography on a Superose 12 HR 10/30 column (GE Healthcare) in phosphate-buffered saline containing 0.1% Nonidet P-40, pH 7.4, at a flow rate of 0.3 ml/min. Absorbance of effluent fractions was monitored at 280 nm. The column was previously calibrated with Bio-Rad gel filtration standard proteins (thyroglobulin, Mr, 60,000; bovine globulin, Mr, 158,000; chicken ovalbumin, Mr, 44,000). Fractions containing enzyme activity were analyzed on 10% SDS-polyacrylamide gels. Sequence analysis of protein bands in the gels were performed as previously described (48) and analyzed using NCBI Blast algorithms.

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FIGURE 2. Paraquat stimulates cellular oxygen flux. Panel A, oxygen flux into lung cells was measured after treatment with paraquat (100 μM) followed by KCN (5 mM). Panel B, cells were treated with the proton ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP; 1 μM) followed by KCN. Panel C, oxygen flux was measured in paraquat-treated cells pretreated with KCN. Panel D, the sensitivity of paraquat-stimulated oxygen flux to NAME (5 mM) and antimycin A (1 μg/ml). Bkgd, background.

DTNB and inhibition of enzyme activity with 1-chloro-2,4-dinitrobenzene or ATM (51, 52).

RESULTS

Characterization of Lung Epithelial Cell Oxygen Flux—In initial experiments we used the polarographic self-referencing microsensor to characterize oxygen utilization by lung epithelial cells. When the sensor was placed 1–5 μm from the cells, an oxygen gradient could readily be detected (Figs. 1, A and B). By moving the probe away from the cells, the gradient was mapped. Oxygen flux was detectable at distances up to 50 μm from individual cells and 400–500 μm from confluent monolayers (Fig. 1B and not shown). The fact that oxygen gradients could be detected in areas surrounding the cells indicates that oxygen consumption by the cells is greater than the amount of oxygen available by its diffusion through the medium to the cells.

Treatment of the cells with mitochondrial electron transport chain inhibitors rotenone (complex I), antimycin A (complex III), or potassium cyanide (complex IV) markedly reduced the oxygen gradients surrounding the cells, indicating that the oxygen consumption was mainly mediated by mitochondrial respiration (Figs. 1, panel C, and 2 and not shown). This conclusion is supported by our findings that the mitochondrial protonophore uncouplers, carbonyl cyanide p-trifluoromethoxyphenylhydrazone and carbonyl cyanide m-chlorophenylhydrazone, enhanced oxygen uptake by the cells (Figs. 1D and 2B).

In further experiments we examined the effects of paraquat on oxygen flux into lung cells. The addition of 100 μM paraquat to the cells resulted in a marked increase (2–4-fold) in oxygen flux that was evident within 15–30 s and persisted for at least 30 min (Fig. 2A); smaller increases in oxygen flux (~1.5-fold) were observed with 10 μM paraquat (not shown). Neither antimycin A nor potassium cyanide, which block mitochondrial respiration, altered paraquat-induced oxygen flux (Figs. 2, panels A and D, and 3). These data indicate that the effects of paraquat are independent of mitochondrial respiration. Pretreatment of lung cells with inhibitors of mitochondrial respiration also had no effect on paraquat-induced oxygen flux (Fig. 2C and not shown). In contrast, carbonyl cyanide p-trifluoromethoxyphenylhydrazone and carbonyl cyanide m-chlorophenylhydrazone-induced increases in oxygen flux were completely blocked by potassium cyanide, demonstrating that the actions of the mitochondrial protonophores and paraquat are distinct (Fig. 2B and not shown).

Paraquat has also been reported to stimulate the diaphorase activity of nitric-oxide synthase, a process inhibited by the nitric-oxide synthase inhibitor nitroarginine methyl ester (NAME) (2). We found that treatment of the lung epithelial cells with NAME had no effect on mitochondrial or paraquat-induced oxygen flux (Fig. 2D and not shown). These data suggest that nitric-oxide synthase does not affect paraquat-induced oxygen uptake.

It is well recognized that several NADPH oxidases, including cytochrome P450 reductase, contribute to extra-mitochondrial oxygen utilization (53, 54). Paraquat is known to redox cycle via cytochrome P450 reductase; whether it is a substrate for NADPH oxidase is unknown (6). We found that diphenyleneiodonium (DPI), a non-selective inhibitor of FAD-dependent enzymes, including NADPH oxidase, readily blocked paraquat-induced oxygen flux into the cells without affecting mitochondrial respiration (Fig. 2A). This effect was independent of the order of addition of paraquat and DPI to the cultures (compare Figs. 4, A and B). Taken together, these data indicate that paraquat-induced alterations in oxygen uptake are dependent on an FAD-containing oxidoreductase activity in the cells.

To eliminate the possibility that cytochrome P450 reductase was responsible for paraquat-induced increases in oxygen flux, we analyzed the effects of the herbicide in cells overexpressing the enzyme. For these studies we used CHO cells stably transfected with mouse CYP450 reductase that have 35-fold more
NADPH oxidase enzyme activity can readily be quantified by the NADPH-dependent consumption of oxygen, utilization of NADPH, and the production of ROI due to redox cycling of paraquat (36–39). Mitochondria-free lysates prepared from lung epithelial cells contained very low levels of NADPH oxidase enzyme activity, as measured by oxygen consumption using a Clark oxygen electrode (Fig. 5). The addition of paraquat to the reaction mix caused a marked increase in enzyme activity that was dependent on NADPH. Oxygen consumption by this enzyme was inhibited by DPI (Fig. 5). These findings are consistent with our microsensor studies in intact cells and support the concept that the actions of paraquat are mediated by an FAD-containing NADPH oxidase.

In further studies we characterized the paraquat-stimulated NADPH oxidase activity in lung cell lysates. A one-electron reduction of paraquat by this enzyme would be expected to generate the paraquat radical (see Reaction 1). This radical is stable at low oxygen tension and can be quantified spectrophotometrically by its absorption maximum at 603 nm (45). Using sealed cuvettes to facilitate depletion of oxygen by the enzyme, reduced paraquat was found to readily accumulate with time in the reaction mixture in an NADPH-dependent reaction (Fig. 6A). When the cuvettes were re-oxygenated, the paraquat radical was oxidized back to paraquat, thus confirming the redox cycling reaction (data not shown). Paraquat also stimulates NADPH consumption in lung cell lysates. In the absence of paraquat cell lysates were found to metabolize relatively low basal levels of NADPH (Fig. 6C). The addition of paraquat to the reaction mix caused a marked increase in NADPH metabolism (Fig. 6D). Both paraquat radical formation and NADPH metabolism were inhibited by DPI (Fig. 6, panels B and E).

We next determined if the NADPH oxidase activity in lung cell lysates could generate ROI in response to paraquat. For these studies the formation of hydrogen peroxide, superoxide anion and hydroxyl radicals was analyzed. In the absence of paraquat, only low basal levels of hydrogen peroxide were observed; a small increase in activity was noted when either NADPH or NADH was added to the assay mix (Fig. 7, panels A and B). The addition of paraquat to the reaction resulted in a marked increase in enzymatic activity with both cofactors. Approximately four times more activity was evident with NADPH than with NADH (Fig. 7, panels A and B). The effects of paraquat on hydrogen peroxide production were concentration- and time-dependent; maximal activity was observed with 100 μM paraquat (Fig. 7C). The accumulation of hydrogen peroxide was abolished by catalase (10 units/ml) but not superoxide dismutase (400 units/ml), confirming that hydrogen peroxide was formed in the assay (data not shown).

Increased activity of paraquat in the presence of NADPH when compared with NADH was due to a lower half-maximal activating concentration (K_{act}) and increased V_{max} for paraquat. Thus, the K_{act} for paraquat was 41.5 μM with NADPH and 680.5 μM for NADH. The V_{max} for enzyme activity with paraquat was 108 nmol of H_2O_2/min/mg of protein and 226 nmol of H_2O_2/ min/mg of protein with NADH and NADPH, respectively (Table 1). In the presence of 100 μM paraquat, there was a marked increase in the V_{max} value for NADH and NADPH, with only small changes in the K_{m} values for these cofactors.
Paraquat was also found to stimulate production of superoxide anion and hydroxyl radicals (Fig. 7, panels D–F). The accumulation of these ROI was dependent on NADPH; superoxide anion detection was inhibitable by superoxide dismutase but not by catalase (data not shown). Hydroxyl radical production was supported by the presence of redox-active iron. In the absence of iron only very low levels of hydroxyl radicals were formed (Fig. 7E). These findings indicate that hydroxyl radicals are derived from hydrogen peroxide via the Fenton reaction (55). Hydroxyl radical production was prevented by catalase and the hydroxyl radical scavenger dimethyl sulfoxide (Ref. 44 and data not shown). The fact that low levels of hydroxyl radical were detected in the absence of added iron may be due to the presence of traces of redox active transition metals in the cell lysates.

We also found that paraquat-induced NADPH oxidase activity was suppressed by DPI; the IC₅₀ for this inhibitor was 1 μM (Fig. 7G). In contrast, enzyme activity was unaffected by the nitric-oxide synthase inhibitor, NAME or the NADPH-quinone reductase inhibitors, dicoumarol or chrysin (5,7-dihydroxyflavone), indicating that these enzymes do not mediate paraquat NAD(P)H oxidoreductase activity (Fig. 7G and not shown). Although cDNA-expressed rat cytochrome P450 reductase was inhibited by a polyclonal antibody to this enzyme, this antibody did not block lung epithelial cell paraquat NAD(P)H oxidoreductase activity (Fig. 7H). These data provide additional evidence that the effects of paraquat are not dependent on cytochrome P450 reductase.

We next characterized the lung epithelial cell paraquat NAD(P)H oxidoreductase activity. As indicated under ‘Materials and Methods,’ 40% of the activity was identified in post-microsomal supernatant fractions of the cells. Enzyme activity in this fraction was purified by 2',5'-ADP-agarose affinity and Superose size exclusion chromatography. Two peaks with approximate molecular weights of 100 and 50 were identified. When analyzed by SDS-polyacrylamide gel electrophoresis, the first peak contained two proteins (Mᵣ 58,000 and 39,000), whereas the second peak contained a single Mᵣ 58,000 protein (not shown). Based on sequence analysis, the Mᵣ 58,000 protein was identified as thioredoxin (56). The identity of the lower molecular weight band was not determined. To confirm the identity of the paraquat reductase in the cytosolic fraction of the lung epithelial cells, we next assayed the 50-kDa fraction for...
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**DISCUSSION**

Of fundamental importance to the survival of mammalian cells is the availability of molecular oxygen. Although largely used in mitochondrial respiration, cells also consume oxygen in numerous extra-mitochondrial enzymatic and non-enzymatic processes (53, 54). Indeed it has been estimated that in healthy respiring tissues non-mitochondrial respiration accounts for ~6–14% of oxygen consumption (54). Redox cycling has been recognized as one important pathway by which cells can utilize oxygen to generate reactive oxygen species (46). Many endogenous cellular components have the capacity to participate in redox reactions, most notably, transition metals and a variety of single electron donating enzymes (36–38). Redox cycling is also thought to mediate the action of many toxicants including paraquat (20).

Paraquat is unusually toxic to the lung, presumably due to its selective uptake by lung cells and localized redox cycling in the alveolar epithelium (57, 58). The present studies demonstrate that micromolar concentrations of paraquat increase cellular oxygen consumption by lung epithelial cells. This activity was not inhibited by cyanide or antimycin A, indicating that the actions of paraquat were not dependent on mitochondrial respiration. These data are consistent with redox cycling of paraquat (14, 17). Increases in oxygen utilization by paraquat in lung epithelial cells were inhibited by DPI, indicating that redox cycling occurs via an FAD-dependent enzyme. However, DPI did not alter cellular respiration, supporting the idea that the effects of paraquat are independent of mitochondria functioning.

In our studies a highly sensitive oxygen microsensor was used to quantify the effects of paraquat on oxygen uptake by lung epithelial cells (33–35, 59). Of interest was our finding that a significant gradient of oxygen is generated around actively respiring cells. Thus, the availability of oxygen in the cell culture medium may limit metabolic activity. In paraquat-treated cells, a steeper gradient was observed surrounding the cells. This is consistent with the idea that additional oxygen is utilized during redox cycling, potentially limiting the amount of oxygen available for respiration. This could result in increased oxidative stress, leading to dysregulation of gene expression, altered energy reserves, and toxicity (60, 61). Similar actions may also occur in bacteria where thioredoxin reductase activity. We found that this fraction readily reduced DTNB, which was inhibited by DPI and the thioredoxin reductase inhibitors 1-chloro-2,4-dinitrobenzene and sodium aurothiomalate (51, 52) (Fig. 8A and not shown). These data indicate that the paraquat redox active material purified from lung epithelial cells was thioredoxin reductase.

To confirm the reactivity of thioredoxin reductase with paraquat, we next analyzed its activity using recombinant thioredoxin reductase or purified rat liver enzyme. In the presence of NADPH, these enzymes generated low levels of hydrogen peroxide; this reaction was markedly increased by paraquat (Fig. 8B). Both recombinant thioredoxin reductase and the rat liver enzyme also generated paraquat radical in the absence of molecular oxygen in an NADPH-dependent reaction (Fig. 8C and not shown). Furthermore, using a Clark oxygen electrode, paraquat was shown to stimulate oxygen utilization by the recombinant thioredoxin reductase (Fig. 8D). Oxygen utilization was inhibited by DPI. Taken together, these data demonstrate that thioredoxin reductase is an important component of the cellular redox cycling of paraquat.
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FIGURE 7. Paraquat redox cycling stimulates ROI production in lung epithelial cell lysates. Panels A and B, effects of NADPH or NADH, respectively, on the formation of hydrogen peroxide as assayed using 10-acetyl-3,7-dihydroxyphenoxazine (AMPLEX-RED) (82). PQ, paraquat. Panel C, increasing concentrations of paraquat stimulate hydrogen peroxide production. Reaction mixes contained 500 μM NADPH. Panel D, redox cycling of paraquat generates superoxide anion. Superoxide anion was assayed in reaction mixes after 45 min by the formation of 2-hydroethidium cation (indicated by the arrow) in the absence and presence of paraquat (100 μM). 2-Hydroethidium was separated from hydroethidine (second peak) by HPLC using fluorescence detection. Panels E and F, redox active iron is required for the formation of hydroxyl radicals by paraquat. Hydroxyl radicals were assayed in reaction mixes after 3 h of incubation by the formation of 2-hydroxyterephthalate from terephthalate using HPLC with fluorescence detection. In panel E reactions containing 100 μM paraquat were run in the absence and presence of 100 μM Fe²⁺/EDTA. In panel F reactions containing Fe²⁺/EDTA were run in the absence and presence of 1 mM paraquat. Panel G, effects of inhibitors on paraquat-stimulated NADPH oxidase activity. Paraquat-stimulated hydrogen peroxide formation was assayed after 30 min in reaction mixes containing NADPH and increasing concentrations of DPI, dicoumarol, or NAME. Panel H, effects of P450 reductase antibodies on redox cycling by paraquat. Reaction mixes containing cell lysates or recombinant P450 reductase were incubated without and with antibody to P450 reductase and then assayed for paraquat-stimulated hydrogen peroxide production.
Hassan and Fridovich (18) reported that paraquat-induced oxygen utilization in *E. coli* occurs directly at the expense of normal respiration.

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Previous studies have suggested that paraquat-induced redox cycling occurs via cytochrome P450 reductase (3, 14, 62). In contrast, our findings demonstrate that cells overexpressing this enzyme do not display increased oxygen uptake when compared with non-overexpressing parental cells, indicating that cytochrome P450 reductase is not a major contributor to paraquat-induced changes in cellular oxygen flux. Thus, increased oxygen utilization in response to paraquat must be mediated by a distinct one electron reductase. In this regard the present studies demonstrate that murine lung epithelial cells contain a paraquat reductase activity that utilizes molecular oxygen to initiate paraquat redox cycling with resultant generation of ROI. This enzymatic activity, which is FAD- and NADH- or NADPH-dependent, readily catalyzes the generation of the characteristic blue-violet paraquat radical (see Reaction 1) and is distinct from cytochrome P450 reductase. Thus, in contrast to P450 enzyme, the paraquat reductase is present in cytosolic fractions of cells and is capable of utilizing NADH as a source of reducing equivalents. Moreover, the paraquat reductase is not inhibitable by antibodies to cytochrome P450 reductase. The molecular weight of purified paraquat reductase is significantly lower than cytochrome P450 reductase.

Under aerobic conditions the paraquat reductase activity in lung epithelial cells was directly associated with the NADPH-dependent utilization of dioxygen as paraquat radical recycled back to paraquat. Both superoxide anion and hydrogen peroxide were generated during the process. Interestingly, in the absence of redox active iron, only low amounts of hydroxyl radicals were formed. This was not unexpected since in mammalian cells hydroxyl radical is largely derived from hydrogen peroxide by the Fenton reaction (55). This reaction requires redox active transition metals which are in low abundance in mammalian cells. Thus, damage to lung epithelial cells by paraquat-generated hydroxyl radicals may be limited.

Identification of the NAD(P)H:paraquat oxidoreductase activity as thioredoxin reductase was accomplished after purification and sequence analysis. This was confirmed by demonstrating that the enzyme activity purified from lung epithelial cells contains thioredoxin reductase activity and that highly purified rat liver thioredoxin reductase and recombinant human thioredoxin reductase were able to redox cycle paraquat and to reduce paraquat to paraquat radical under anaerobic conditions. It should be

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**TABLE 1**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>( K_m )</th>
<th>( V_{max} )</th>
</tr>
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<tbody>
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<td>NADH</td>
<td>680.5</td>
<td>108</td>
</tr>
<tr>
<td>NADPH</td>
<td>41.5</td>
<td>226</td>
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*Initial reaction velocities for the production of hydrogen peroxide using increasing concentrations of NADH or NADPH were analyzed in standard reaction mixes in the absence and presence of paraquat (100 \( \mu M \)).

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>( K_m )</th>
<th>( V_{max} )</th>
</tr>
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<tbody>
<tr>
<td>+NADH</td>
<td>9.7</td>
<td>23.1</td>
</tr>
<tr>
<td>+NADH + paraquat</td>
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<td>57.4</td>
</tr>
<tr>
<td>+NADPH</td>
<td>5.9</td>
<td>12.7</td>
</tr>
<tr>
<td>+NADPH + paraquat</td>
<td>8.9</td>
<td>245</td>
</tr>
</tbody>
</table>

*Initial reaction velocities for the production of hydrogen peroxide using increasing concentrations of NADH or NADPH were analyzed in standard reaction mixes in the absence and presence of paraquat (100 \( \mu M \)).

**FIGURE 8.** Thioredoxin reductase activity of purified material from lung epithelial cells and characterization of paraquat redox cycling using recombinant thioredoxin reductase. Panel A, thioredoxin reductase activity in material purified from lung epithelial cells was analyzed using DTNB as the substrate. The thioredoxin reductase inhibitor ATM (100 \( \mu M \)) inhibited DTNB reduction (inset). Panel B, ability of paraquat (PQ) to redox cycle using recombinant thioredoxin reductase or rat liver thioredoxin reductase (inset). Redox cycling was quantified using the AMPLEX-RED reaction. Reaction mixes contained 0.5 mM NADPH and increasing concentrations of paraquat (0 –1000 \( \mu M \); 1000 \( \mu M \) inset). Panel C, recombinant thioredoxin reductase catalyzes the formation of paraquat radical. Reaction mixes contained NADPH and paraquat (100 \( \mu M \)) and were run in sealed cuvettes as in the legend to Fig. 6. Panel D, paraquat stimulates oxygen utilization by recombinant thioredoxin reductase. Oxygen utilization was measured as indicated in the legend to Fig. 5. The arrows show the addition of 0.5 mM NADPH, 100 \( \mu M \) paraquat, and 10 \( \mu M \) DPI.
Paraquat-induced Activation of an Oxidoreductase

noted that, besides P450 reductase, additional paraquat reductases have been identified in both mammalian cells and in bacteria (18, 19, 36–38). Thus, Day et al. (2) reported that rat nitric-oxide synthase can reduce paraquat in a process inhibitable by nitric-oxide synthase inhibitors that prevent NADPH oxidation. In contrast, we found that the nitric-oxide synthase inhibitor NAME had no effect on paraquat redox cycling in our system, suggesting that the enzyme is not nitric-oxide synthase. The molecular weights for nitric-oxide synthase and paraquat reductase are also clearly distinct (63). In E. coli several paraquat reductases have been identified including NADPH:thioredoxin reductase, NADPH: ferredoxin reductase, and the ferredoxin-containing NADPH:sulfite reductase (64, 65). It is important to note that the thioredoxin reductases from mammals and E. coli are mechanistically and structurally distinct, making it difficult to compare activity between these two proteins (56).

In addition to increasing oxygen consumption, paraquat dramatically reduces cellular NADPH concentrations (66). Because NADPH is required in multiple biological pathways, including the reduction of oxidized glutathione, the function of cytochrome P450 enzymes, and many biosynthetic reactions, depletion of this pyridine nucleotide can inhibit many essential cellular functions and contribute to toxicity (67). Overstimulation of the glucose 6-phosphate shunt by accumulated NADP+ may also contribute to toxicity by diverting glucose from ATP production. This hypothesis is supported by findings that overexpression of glucose 6-phosphate dehydrogenase enhances paraquat toxicity in NIH-3T3 cells (68).

Attempts to ameliorate paraquat-induced lung toxicity have met with mixed success. Although anti-inflammatory corticosteroids are commonly administered, there is limited evidence that these compounds provide therapeutic benefits (69). Many compounds that reduce production of ROI, including antioxidants (N-acetylcysteine and glutathione), catechols and flavonoids (epigallocatechin gallate and apocynin), and metal chelators (desferoxamine), protect against paraquat toxicity in cell culture systems (70–75). However, these treatments have been less successful in various in vivo models, particularly when administered after, rather than before, paraquat exposure (76–80). Our data suggest that inhibition of thioredoxin reductase in animals and humans may ameliorate toxicity. Further studies are needed to identify therapeutic inhibitors of this enzyme in lung tissues.

In summary, the present studies show that paraquat increases cyanide-insensitive respiration in murine lung epithelial cells. Paraquat is known to function via redox cycling, and our findings that paraquat-stimulated respiration in lung cells was inhibited by DPI suggest that its actions are mediated by an FAD-dependent cellular oxidoreductase. Based on these findings we purified an NAD(P)H-dependent oxidoreductase from the cytoplasm of lung epithelial cells and demonstrated that it readily utilized oxygen and generated highly toxic reactive oxygen species. This activity was independent of cytochrome P450 reductase and identified as the FAD-containing enzyme thioredoxin reductase. Thioredoxin reductase is an essential enzyme for protection against oxidative stress (51, 79–81). Thus, it is surprising that it can also promote oxidative stress by redox cycling of paraquat. Further studies are required to characterize the mechanism by which paraquat activates thioredoxin reductase and to more precisely define its role in paraquat pneumotoxicity.

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REFERENCES

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