An Assessment of the Role of Redox Cycling in Mediating the Toxicity of Paraquat and Nitrofurantoin

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The abilities of paraquat, diquat, and nitrofurantoin to undergo cyclic oxidation and reduction with rat microsomal systems have been assessed and compared to that of the potent redox cycle, menadione. Diquat and menadione were found to be potent redox cyclers with comparable abilities to elicit a nonstoichiometric increase in both the consumption of \( \text{O}_2 \) and the oxidation of NADPH, compared to the amounts of substrate added. In contrast, paraquat and nitrofurantoin redox cycled poorly, being an order of magnitude less potent than either diquat or menadione. This was reflected in kinetic studies using lung and liver microsomes, which showed that NADPH-cytochrome P-450 reductase had a lower affinity \( (K_m) \) for paraquat and nitrofurantoin than for menadione and diquat, although values of \( V_{\text{max}} \) were comparable for all the substrates except nitrofurantoin, which was lower.

In order to assess redox cycling of the substrates in an intact lung system, the \( \text{O}_2 \) consumption of rat lung slices was measured in the presence of all four compounds. A small increase in lung slice \( \text{O}_2 \) uptake was observed with paraquat \( (10^{-4} \text{ M}) \) in the first 2.5 hr of incubation, possibly because of redox cycling of a high intracellular concentration of paraquat resulting from active accumulation into target cells. This stimulation in \( \text{O}_2 \) uptake was no longer observed when slices were incubated for a longer period or with higher paraquat concentrations \( (10^{-4} \text{ M}) \), possibly because of toxic effects in target cells. High concentrations of diquat \( (10^{-4} \text{ M}) \) had no effect on \( \text{O}_2 \) consumption of lung slices. These results together with the poor ability to redox cycle with microsomes and the absence of a specific uptake system highlight the problem of associating redox cycling and oxidative stress in the mechanism of nitrofurantoin toxicity.

Introduction

Oxidative stress arises when there is an imbalance in the prooxidant-antioxidant system of a cell, in favor of the former. The condition has been implicated in a diversity of processes including drug action and toxicity, aging, and carcinogenesis. Many compounds, including the herbicides paraquat and diquat, and the antibiotic nitrofurantoin, are capable of inducing oxidative stress. Menadione \( (2\text{-methyl-1,4-naphthoquinone}) \) has been widely used as an investigative tool in assessing the effects of oxidative stress in biological systems.

Many cases of accidental or intentional poisoning with paraquat \( (\text{PQ}: 1,1'\text{-dimethyl-4,4'-bipyridyl}) \) have been described \( (1-3) \). The lung is the primary target organ of toxicity and early effects include edema, inflammation, and pulmonary epithelial cell destruction, followed by a phase of fibroblastic infiltration \( (4) \).

Nitrofurantoin \( (\text{NF}: \text{N-(5-amino-2-furanyliden)-1-amino hydantoin}) \), an antibiotic used in the treatment of urinary tract infections, also causes lung damage \( (5) \), generally in patients who have received the drug from 6 months to 6 years. The major pathological feature is interstitial fibrosis of the lungs, which may be associated with interstitial pneumonitis \( (5,6) \).

While pulmonary fibrosis observed after PQ and NF appears similar, their mechanisms of toxicity have not been fully elucidated. Under aerobic conditions with an appropriate electron donor, both compounds undergo a cyclical reduction and reoxidation reaction; thus, their toxic effects have been postulated to be because of redox cycling \( (7,8) \). Redox cycling describes the one electron reduction of a compound and its subsequent reoxidation in the presence of \( \text{O}_2 \) with the concomitant production of superoxide anion radical \( (\text{O}_2^-) \). The superoxide anion radical may dismutate, either spontaneously or in the presence of superoxide dismutase to form hydrogen...
peroxide (9), which in turn may undergo an iron-catalyzed Haber-Weiss reaction to form the highly reactive hydroxyl radical (OH•) (10). These reactive oxygen species may be scavenged by endogenous antioxidant defenses, such as glutathione peroxidase, which hydrolyses H₂O₂ to water at the expense of reduced glutathione, and vitamin E, which interferes with the membrane-damaging process of lipid peroxidation resulting from the reaction of free radicals on polyunsaturated fatty acids (11).

The lung is a major target organ for PQ, in part, because of its active accumulation (12,13) into susceptible cell types, i.e., Types I and II alveolar epithelial cells and Clara cells of the bronchiolar epithelium (14,15) where it is proposed that the redox cycling of PQ with the formation of free radicals leads to a disturbance in normal lung function. No active uptake system for NF has been described. However, unlike PQ, it has been shown to covalently bind to cellular macromolecules (16).

Another bipyridylum herbicide, diquat (DQ: N,N’-ethylene-1,1’-bipyridyl), is also capable of redox cycling (17). However, DQ causes minimal lung damage in cases of intoxication, probably because of the absence of an uptake system for its accumulation into the lungs (13).

The present studies attempt to elucidate the role of redox cycling in mediating the toxicity of PQ and NF. Menadione, a potent redox cycier in liver microsomal preparations (18), was used as a positive control in experiments designed to assess the abilities of PQ, NF, and DQ to redox cycle. Much of the work presented here was carried out using rat liver and lung microsomal preparations since the microsomal enzyme NADPH-cytochrome P-450 reductase has been shown to mediate the one electron reduction of PQ, NF, DQ, and menadione (17,19,20).

Materials and Methods

NF was purchased from Sigma (Sigma Chemical Company, Poole, Dorset, UK); menadione was purchased from Aldrich (Aldrich Chemical Company, Gillingham, Dorset, UK); PQ and DQ were kindly provided by ICI (Imperial Chemical Industries plc, Cheshire, UK); other chemicals were obtained from either Sigma, or British Drug Houses (Poole, Dorset, UK).

Preparation of Microsomes

Washed hepatic and pulmonary microsomes were prepared from fed, male Wistar-derived Alderley Park rats, essentially by the method of Ernster et al. (21). The organs were perfused with ice-cold 1.15% KCl, minced, and homogenized with a Polytron homogenizer at full speed for 30 and 45 sec for liver and lung, respectively. The washed microsomal pellet was resuspended in Tris-EGTA buffer (100 mM Tris, 5 mM EGTA; pH 7.4). Microsomal protein was determined by the method of Lowry et al. (22).

NADPH Oxidation

The oxidation of NADPH at 37°C was followed at 340 nm on a Shimadzu MPS 2000 spectrophotometer as previously described (23). Liver and lung microsomes were used at concentrations of 0.2 and 0.4 mg of protein/mL, respectively. PQ and DQ were dissolved in buffer (100 mM Tris, 5 mM EGTA, pH 7.4), while NF and menadione were dissolved in 1% dimethylsulfoxide (DMSO).

O₂ Consumption

The microsomal consumption of O₂ was measured on a Yellow Spring Clark oxygen electrode maintained at 37°C, as previously described (24). Microsomes were used at a concentration of 0.2 mg protein/mL.

Respirometry Studies

O₂ consumption of rat lung slices at 37°C was measured using a Gilson respirometer (25). PQ and DQ were dissolved in Krebs-Ringer phosphate buffer, pH 7.4, while NF and menadione were dissolved in 0.1% DMSO.

Results

Microsomal NADPH Oxidation and O₂ Consumption

All four compounds stimulated the initial rates of liver microsomal NADPH oxidation; however, PQ and menadione produced a much greater stimulation than either PQ or NF (Fig. 1). Similarly, all four compounds stimulated the initial rates of rat liver microsomal O₂ consumption. The greatest stimulation was elicited by menadione and diquat (Fig. 2). Similar results were obtained for lung microsomal NADPH oxidation (data not shown).

Kinetic Parameters

Lung and liver microsomal NADPH oxidations were studied in the presence of various concentrations of PQ (20 μM–5 mM), DQ (10 μM–1 mM), NF (20 μM–500 μM), and menadione (2 μM–60 μM). K_m (a measure of the affinity of the enzyme for the substrate) and V_max (maximal rate of reaction) values were calculated from Lineweaver-Burk, Eadie-Hofste, Hanes-Woolf, and direct linear plots using a computer program (ENZPACK) (Table 1).

The microsomal enzyme NADPH-cytochrome P-450 reductase appeared to have a higher affinity (low K_m values) for DQ and menadione than for PQ and NF. V_max values for PQ, DQ, and menadione were comparable in
both liver and lung microsomes, while that for NF was lower in rat liver microsomes.

**O₂ Consumption of Lung Slices**

The O₂ consumption of lung slices was followed for 4 hr of incubation at 37°C. The rate of O₂ uptake of control slices was linear over the period studied (90-120 μL O₂/100 mg wet wt lung/hr).

A small, but significant stimulation (p < 0.05) in the O₂ uptake of the lung slices was observed in the first 2.5 hr of incubation with 10⁻⁵ M PQ (Fig. 3a). NF, DQ, and menadione, used at the same concentration, did not significantly affect the O₂ utilization of the slices.

Treatment of lung slices with a higher concentration (10⁻⁴ M) of PQ did not elicit a significant stimulation in lung slice O₂ uptake (Fig. 3b). However, the same concentration of DQ produced a significant stimulation (p < 0.001) in O₂ uptake of lung slices. In contrast, menadione produced a significant depression (p < 0.001) in O₂ uptake of slices when compared to controls. NF did not produce any significant effects on O₂ consumption (Fig. 3).

| Table 1. Kinetic parameters of microsomal NADPH oxidation with various substrates. |
|-----------------------------------------------|
| Substrate | Rat liver microsomes | Rat lung microsomes |
|          | Kₘ, μM | Vₘₐₓ a | Kₘ, μM | Vₘₐₓ a |
| PQ       | 143 ± 13.7 | 148 ± 3.3 | 198 ± 6.2 | 54 ± 0.9 |
| DQ       | 6.2 ± 0.7 | 124 ± 2.0 | 17 ± 0.4 | 45 ± 0.3 |
| Menadione| 5.1 ± 0.2 | 132 ± 1.5 | 6.9 ± 0.4 | 45 ± 0.9 |
| NF       | 102.6 ± 19.4 | 53.2 ± 6.6 | ND | ND |

*aVₘₐₓ = nmole NADPH oxidized/milligram microsomal protein/min.

*bResults are means ± SEM of four different methods of calculations (Lineweaver-Burk, Eadie-Hofstee, Hanes-Weil, and direct linear) on results from three different experiments per substrate used.

**Discussion**

Redox cycling has been proposed to play a key role in the mechanism of toxicity of PQ and NF (27). We have assessed the redox cycling abilities of these compounds by measuring the stimulated initial rates of NADPH
oxidation and $O_2$ consumption in liver and lung microsomal preparations, and we have compared these to those of two redox-active compounds, DQ and menadione. Although both PQ and NF stimulated microsomal NADPH oxidation and $O_2$ consumption, the stimulation produced was about an order of magnitude lower than that of DQ or menadione (Figs. 1 and 2).

The poor redox cycling of PQ and NF was also reflected in the kinetic parameters measured (Table 1). Apparent $K_m$ values for PQ and NF in liver microsomes were comparable and were about 20-fold greater than those for DQ and menadione. Values of $V_{max}$ were similar for the compounds except NF that was lower (Table 1). Similar results were obtained for PQ, DQ, and menadione in lung microsomes. The specific activity of lung NADPH-cytochrome P-450 reductase is about one-third to one-fifth that reported for liver microsomes (28); this probably accounts for the lower values of $V_{max}$ for PQ, DQ, and menadione in lung microsomes.

In this study, $K_m$, a measure of the affinity of the enzyme for the substrate, is a composite of two reactions: first, the one electron reduction of the substrate utilizing NADPH and catalyzed, most probably, by NADPH-cytochrome P-450 reductase and forming the radical, which, in the second reaction, interacts with oxygen reforming the parent compound. Since the latter reaction is diffusion limited with a rate constant of $7.7 \times 10^9$ M/sec (29), we assume that the $K_m$ value reflects the one electron reduction reaction. Thus, the affinities of NADPH-cytochrome P-450 reductase for PQ and NF are much lower than for DQ or menadione. $V_{max}$ values, which reflect turnover rates of the enzyme, are about the same for PQ, DQ, and menadione in lung and liver microsomes but are lower for NF (Table 1) in liver microsomal preparations.

As both PQ and NF are relatively poor redox cyclers, it is necessary to evaluate the contribution of this cycling in mediating their toxicity. We measured the $O_2$ consumption of rat lung slices treated with low ($10^{-5}$ M) and high ($10^{-4}$ M) concentrations of the four compounds (Fig. 3). Low concentrations of NF, DQ, and menadione did not produce significant effects on $O_2$ uptake, but PQ ($10^{-5}$ M) showed a small but significant increase in the first 2.5 hr of incubation (Fig. 3a). However, at higher concentrations ($10^{-4}$ M), PQ caused no change in $O_2$ uptake, compared to control slices (Fig. 3b).

Rose et al. (30) calculated the rate of PQ accumulation into rat lung slices incubated with $10^{-5}$ M PQ to be between 90 to 40 nmol PQ/g wet wt lung/hr. Thus, after a 3-hr incubation with PQ ($10^{-5}$ M), the intracellular concentration should be 90 to 120 nmole PQ/g wet wt lung. This concentration appeared to depress the increase in lung slice $O_2$ uptake, as shown by the lack of significant stimulation in $O_2$ consumption beyond 3 hr of incubation with $10^{-5}$ M PQ (Fig. 3a) and in slices incubated with $10^{-4}$ M PQ (Fig. 3b), possibly because of a toxic effect on susceptible cells.

In contrast, despite the absence of an active pulmonary uptake system (13), DQ ($10^{-4}$ M) significantly stimulated $O_2$ uptake in lung slices (Fig. 3b) possibly because of passive diffusion of sufficient redox active molecules into susceptible cells. Menadione decreased $O_2$ consumption of lung slices (Fig. 3b) possibly because of toxicity due to either or both its redox cycling and covalent binding to tissue macromolecules (23). The inability of a redox active compound such as menadione to stimulate lung $O_2$ uptake highlights the problem of directly correlating increases in $O_2$ uptake to the redox cycling of a substrate. This may be because of redox

\begin{figure}[h]
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\caption{$O_2$ consumption of rat lung slices. (a) Rat lung slices were incubated with $10^{-3}$ M and (b) $10^{-4}$ M of PQ ($\Delta$, $\triangle$), NF (O--O), DQ (+---+), and menadione (----). Control slices (x--x) were either incubated in buffer only, or in 0.1% DMSO. Values are the means of experiments performed on six rats. Asterisk (*) indicates significantly different from control ($p<0.05$, t test).}
\end{figure}
cycling occurring in a small proportion of total lung cells; therefore, O₂ consumption due to redox cycling represents only a small percentage of total lung O₂ used. NF did not significantly affect lung slice O₂ uptake (Fig. 3).

Summary
In summary, PQ is accumulated in Type I and Type II alveolar epithelial cells by an energy-dependent system (13). Although PQ is a poor redox cycle, its concentration in target cells predisposes these cells to toxicity, dependent on the balance of prooxidant to antioxidant enzymes. However, a similar rationale cannot be applied to NF since it is not specifically accumulated by the lungs.

NF is lipid soluble, and has been shown to inhibit liver mitochondrial respiration (31), it covalently binds to cellular macromolecules (16), and it is a poor redox cycle in microsomal systems. The inhibition of mitochondrial respiration may mask any increase in lung slice O₂ consumption because of the relatively slow redox cycling of NF. The mechanism of toxicity of NF, in particular a role of redox cycling in mediating its pulmonary toxicity, remains unproven.

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