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MECHANISTIC ASPECTS OF PARAQUAT TOXICITY IN *E. COLI*

A SPIN TRAPPING STUDY

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Abstract—Mechanistic aspects of paraquat monocation radical ($PQ^{\cdot+}$) and copper involvement in paraquat toxicity have been examined using *E. coli B* cells. Electron spin resonance (ESR) spectrometry combined with cell survival studies were used to explore the correlation between radical production and biological damage. The line broadening agent oxalato-chromiate (CrOx) was used to characterize the anoxic partition of $PQ^{\cdot+}$ inside and outside the cell. In the presence of CrOx the ESR signal was totally eliminated, indicating that intracellular species were undetectable and that, contrary to previous reports, $PQ^{\cdot+}$ exclusively accumulates outside the cell. The $PQ^{\cdot+}$ radical does not react with H_2O_2 but disappears in the presence of H_2O_2 when catalytic traces of Cu(II) are present. Spin-trapping studies using DMPO showed that in aerobic environment paraquat-induced $O_2^{\cdot-}$ radicals are detectable exclusively in the extracellular compartment. The correlation between $PQ^{\cdot+}$ appearance and the biological damage is not simple. $PQ^{\cdot+}$ non-toxically accumulates, in the absence of oxygen and either Cu(II) or H_2O_2. By contrast, with both H_2O_2 and Cu(II) the cells are rapidly killed but $PQ^{\cdot+}$ was undetectable. These results reconfirm the key catalytic mediatory function of transition metals in paraquat toxicity.

The cycling reaction involving the one-electron metabolic reduction of paraquat (PQ^{+2})§ to form the corresponding monocation radical, $PQ^{\cdot+}$ and the subsequent rapid reaction of $PQ^{\cdot+}$ with O_2 to yield $O_2^{\cdot-}$ and PQ^{+2} has been demonstrated in several *in vitro* test-systems [1–5], including *E. coli* cells [6–9]. Redox-cycling of paraquat was previously shown to enhance oxygen consumption and increase the generation of superoxide radicals and H_2O_2 which have been implicated in cellular damage [10]. The induction of superoxide dismutase (SOD) by paraquat and the protective effects afforded by SOD [5, 8] further corroborated the conclusion that $PQ^{\cdot+}$ produces $O_2^{\cdot-}$. Since neither SOD nor $O_2^{\cdot-}$ readily cross cell membrane [7], exogenously applied SOD would not be anticipated to protect against paraquat-mediated damage unless intracellularly produced $PQ^{\cdot+}$ radicals react outside the cell with oxygen forming extracellular $O_2^{\cdot-}$ [7, 11]. Because of the known limited chemical reactivity of $O_2^{\cdot-}$, paraquat-induced damage has been attributed to 'OH radicals [4] or 'crypto-OH' formed through direct reaction of $PQ^{\cdot+}$ with H_2O_2 [3]. It has also been proposed that site-specific superoxide-driven Fenton reaction, wherein the relatively unreactive $O_2^{\cdot-}$ is catalytically converted to highly reactive hydroxyl radical, is operative in paraquat-induced injury of bacteria and mammals [9–15]. On the other hand, in the absence of electron acceptors like oxygen or H_2O_2 transition metals can accept an electron from $PQ^{\cdot+}$ but cannot redox cycle.

In this study we examine several aspects of the metal-catalyzed mechanism of paraquat activity. To reconcile the protective effect of exogenous SOD with the reports that PQ^{+2} induces mainly production of intracellular $O_2^{\cdot-}$, the radicals distribution inside and outside the cell was examined. The focal points in the investigation are the role of copper, molecular oxygen, and the location of the produced radicals.

§ Abbreviations used: CrOx: potassium trioxalato-chromiate; DMPO: 5,5-dimethyl-pyrroline-1-oxide; DMPO-OOH: 5,5-dimethyl-2-hydroperoxy-1-pyrrolidinyloxyl; DMPO-OH: 5,5-dimethyl-2-hydroxy-1-pyrrolidinyloxyl; DTPA: diethylenetriamine-pentaacetic acid; ESR: electron spin resonance; PQ^{+2}: paraquat; $PQ^{\cdot+}$: paraquat monocation radical; SOD: superoxide dismutase.

MATERIALS AND METHODS

Chemicals and solutions. Methyl viologen (paraquat, PQ^{+2}), the spin trap DMPO, superoxide dismutase (SOD), catalase, and diethylenetriamine pentaacetate (DTPA) were obtained from Sigma Chemical Co. (St Louis, MO); Desferrioxamine (Desferal®) from Ciba-Geigy (Switzerland) and $CuSO_4 \cdot 5H_2O$ from J. T. Baker (NJ). H_2O_2, K_2HPO_4, and KH_2PO_4 were manufactured by E. Merck (Darmstadt, F.R.G.). Potassium trioxalato-chromiate (CrOx) was obtained from Pfaltz and Bauer, Inc. and ethanol and $(NH_4)_2SO_4$ from Frutarom (Israel). Glucose was obtained from BDH (Poole, U.K.), and $MgSO_4$ from Mallinckrodt (F.R.G.).

Triply-distilled water was used to prepare phosphate buffer, containing 0.5% (w/v) glucose and 0.1 mM $MgSO_4$. Experiments were carried out at room temperature. K-medium used for bacterial growth contained K_2HPO_4 (7 g/l), KH_2PO_4 (3 g/l), $(NH_4)_2SO_4$ (1 g/l), and casamino acids (10 g/l).

E. coli *preparation. E. coli* cells, type SR-9, were grown on K-medium, pH 7.4 to midlog phase, then harvested, washed three times, and suspended to a density of 10^9 cells/ml by dilution with phosphate buffer. When anoxic conditions were required, the stock suspension was flushed with high purity nitrogen and kept on ice until diluted.

Electron spin resonance (ESR). Measurements were made of cells placed in either quartz flat cuvette or gas-permeable Teflon capillary. In order to anaerobically introduce additives to preformed radicals, a Y-shaped extension, hooked to the aqueous flat cuvette was used. Cells were incubated under N_2 with paraquat in one arm of the Y-shaped cap while in the second arm other reagents were deoxygenated. Following radical formation, the solutions were mixed, placed within the cavity, and scanned for ESR signal. When an alternate exchange of gases was desired, the sample was drawn by a syringe into a gas-permeable Teflon capillary which was inserted into a 3 mm i.d. quartz tube, and placed in the ESR cavity. To maintain or change oxygen level during the experiment, gases of known composition were flown around the sample. ESR spectra were recorded on a Varian E4 X-band spectrometer, with field centered at 3357G, modulation amplitude of 0.5 G and nonsaturating power.

RESULTS

The $PQ^{\cdot+}$ radicals

Exposure of *E. coli* cells to 4 mM paraquat in phosphate buffer under anoxic conditions resulted in an ESR spectrum (Fig. 1) characteristic of $PQ^{\cdot+}$ [11, 16]. In the absence of oxygen, *ca.* 30 μM of the radical accumulated as determined by calibration vs 50 μM 3-carbamoyl-2,2,5,5-tetramethyl-3 pyrroline-1-yloxy stable spin-label. The $PQ^{\cdot+}$ radical persisted for several hours and was not affected by metal chelators such as DTPA or desferrioxamine. Similarly, no effect on the intensity of the ESR signal was observed when 5 mM H_2O_2 was anaerobically added to the cells either before or after the appearance of $PQ^{\cdot+}$. This result excludes significant direct reaction of H_2O_2 with $PQ^{\cdot+}$ which has been previously claimed [3, 4].

Copper effect on $PQ^{\cdot+}$ radical

In view of the marked increase in toxicity of paraquat in the presence of copper ions [12], the influence of Cu(II) on $PQ^{\cdot+}$ radical was examined. The addition of 1–10 μM Cu(II) to paraquat-treated cells did not affect the appearance or the decay of the $PQ^{\cdot+}$ ESR signal. However, when both copper and H_2O_2 were added to the reaction system, no $PQ^{\cdot+}$ signal was observed. The absence of $PQ^{\cdot+}$ signal in the presence of both copper and H_2O_2 (but not with either of them alone) could result from the combined effect on the formation and the decay of the $PQ^{\cdot+}$ radical. To discriminate between the two possibilities, the experiment was modified by adopting a two-stage mixing procedure. Following the appearance of the $PQ^{\cdot+}$ signal in the presence of either H_2O_2 or copper, the second additive (copper or H_2O_2, respectively) was anaerobically introduced as detailed in Methods and Materials. Consequently,

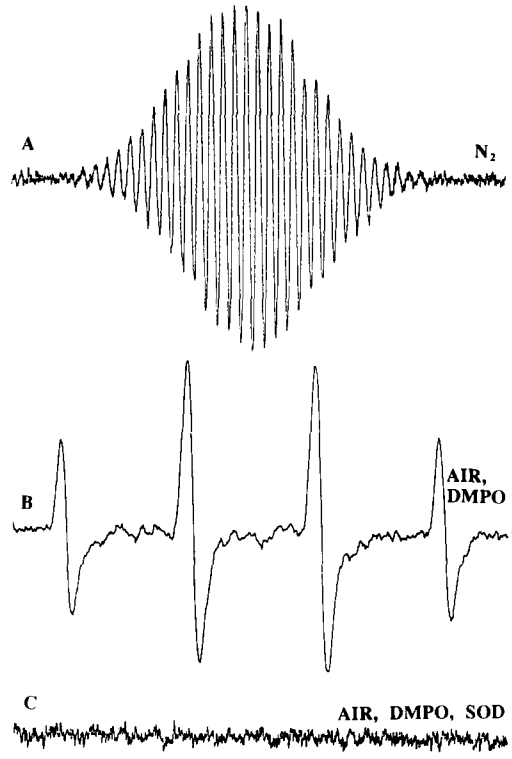

Fig. 1. Radicals generated by paraquat-treated cells. The ESR spectra observed upon incubation of *E. coli* (10^9 cells/ml) suspended in 15 mM phosphate buffer, pH 7.4, with 4 mM paraquat in the presence of 0.14 M DMPO: (A) paraquat monocation radical ($PQ^{\cdot+}$), under N_2; (B) DMPO-OH spin-adduct, under air; (C) as trace (B), with SOD (50 U/ml).

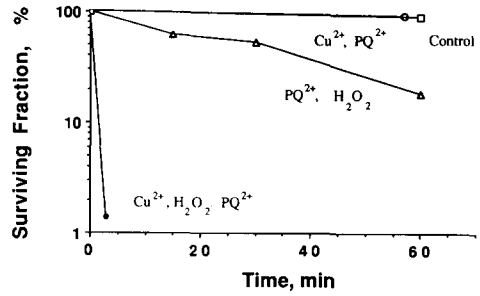

Fig. 2. Paraquat effect on cell survival. Survival curves of *E. coli* (10^9 cells/ml) following anoxic exposure to 4 mM paraquat, 10 mM H_2O_2, and 12.5 μM $CuSO_4$ in 15 mM phosphate buffer, pH 7.4, containing 0.1 mM $MgSO_4$, glucose (0.5% w/v), and 25 μM DTPA. (□) no additives; (○) 12.5 μM Cu(II); (△) 10 mM H_2O_2; (●) 10 mM H_2O_2 + 12.5 μM Cu(II).

the ESR signal of $PQ^{\cdot+}$ immediately disappeared, thereby showing that H_2O_2 in the presence of catalytic levels of copper facilitates $PQ^{\cdot+}$ decay.

To study the possible correlation between $PQ^{\cdot+}$ radical formation and the cytotoxic effect of paraquat, the cells were incubated with paraquat under anoxic conditions, then H_2O_2 and copper were sequentially introduced, either alone or together, and the appearance of the ESR signal of $PQ^{\cdot+}$ radical, along with cell killing were assayed. Under N_2, paraquat with and without copper, had no effect on cell survial, as shown in Fig. 2, whereas H_2O_2

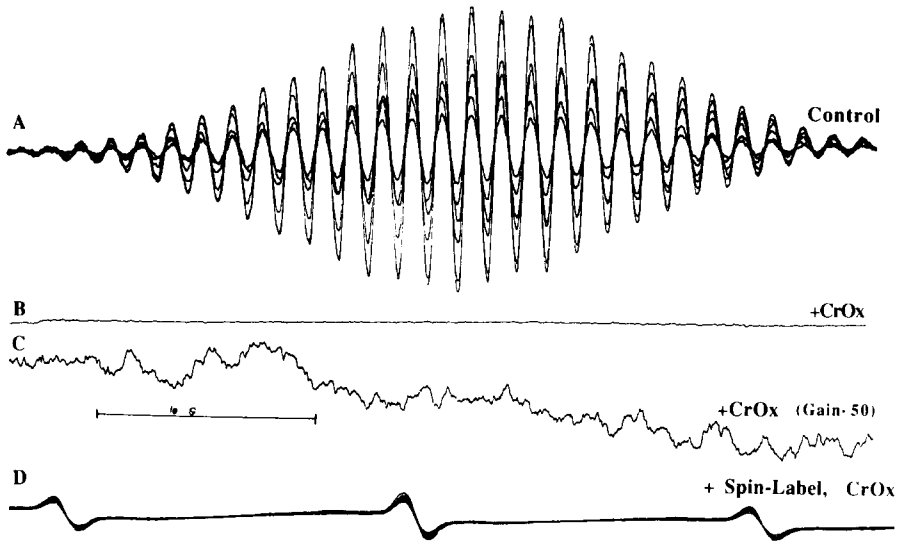

Fig. 3. Effect of CrOx on the ESR signal of $PQ^{\cdot+}$. The ESR signal of $PQ^{\cdot+}$ produced by paraquat-treated cells under anoxic conditions, with and without a subsequent addition of 75 mM CrOx (line broadening agent). Experimental conditions: 3×10^{10} *E. coli* cells/ml suspended in 15 mM phosphate buffer, pH 7.4, and exposed to 4 mM paraquat. The integrity of the cells was verified by the addition of 50 μM stable spin-label, 3-carbamoyl-2,2,5,5-tetramethyl-3 pyrroline-1-yloxy. (A) control; (B) with CrOx; (C) same as (B) but using 50-fold higher gain; (D) spin-label + CrOx.

(10 mM) caused marginal cell killing. However, the killing rate was greatly enhanced in the presence of both copper and H_2O_2, yet the $PQ^{\cdot+}$ ESR signal was not observable. It appears, therefore, as if the level of $PQ^{\cdot+}$ does not correlate with cell killing and that the radical itself is not toxic.

Distribution of paraquat monocation radical

The distribution of $PQ^{\cdot+}$ between the intracellular space and the extracellular bulk was evaluated by comparing its ESR signal intensity obtained in the absence and the presence of the line broadening agent, oxalato-chromiate (CrOx). Figure 3 shows the ESR spectrum of $PQ^{\cdot+}$ accumulated under anaerobiosis. The addition of CrOx, which is excluded from intracellular sites and makes the ESR signal of extracellular species invisible [17], caused the $PQ^{\cdot+}$ signal to disappear (Fig. 3B) and to remain undetectable even upon a 50-fold increase of the gain (Fig. 3C). This suggested that the $PQ^{\cdot+}$ radicals reside in the extracellular bulk. In order to ascertain that CrOx indeed does not cross the cell membrane and also masks $PQ^{\cdot+}$ inside the cell, we used the cell permeable spin label, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (Fig. 3D). When the spin-label was added to the *E. coli* suspension, its 3-line typical spectrum was observed. Subsequent addition of CrOx abolished the extracellular signal and eliminated ~98% of the ESR signal (Fig. 3D). In this experiment, with 3×10^{10} *E. coli* cells/ml and cell volume of 0.36 μ^3 [18], the calculated net intracellular volume is about 1% of the total suspension. The 2% residual signal indicates that: (a) the spin-label molecules indeed distribute between the intra- and extracellular compartments roughly according to their relative volumes, and (b) the cellular integrity is maintained in the presence of paraquat.

Spin-trapping of oxygen-derived radicals under aerobic conditions

When 10^9 cells/ml were incubated in gas-permeable capillary under N_2 with 2 mM paraquat and 0.1 M DMPO the $PQ^{\cdot+}$ signal accumulated. No difference was observed in the absence or in the presence of DMPO. Subsequent exchange of the N_2 with air resulted in the immediate disappearance of the $PQ^{\cdot+}$ signal and the appearance of the four-line signal characteristic of DMPO-OH shown in Fig. 1 [11]. The DMPO-OH signal formed during aerobic incubation was neither affected by catalase or H_2O_2 nor by the $^{\cdot}OH$-scavenger ethanol (0.2 M). By contrast, SOD fully prevented the appearance of the DMPO-OH signal (Fig. 1C). Thus, the possibility that authentic $^{\cdot}OH$ radicals are responsible for the signal is excluded. These results further substantiate the conclusion that only extracellular $PQ^{\cdot+}$ radicals are (a) observable and, (b) react with oxygen.

DISCUSSION

The present results, in accord with previous findings, show that paraquat is metabolically reduced by the cells, and that the resulting monocation radical readily reduces oxygen to $O_2^{\cdot-}$ [5]. Since the ESR signal of $PQ^{\cdot+}$ was not detectable in the presence of CrOx, even when a highly dense cell suspension was used, it appears that the $PQ^{\cdot+}$ signal derives exclusively from extracellular species. Our results thus indicate, contrary to previous assumptions [11], that the $PQ^{\cdot+}$ radicals reside primarily outside the cell. The present results do not contradict previous reports that $PQ^{\cdot+}$ radical readily crosses the cell membrane [18, 19], however, they do not imply whether paraquat is reduced intra- or extracellularly. Paraquat has been earlier reported to enter cells very

slowly and to be reduced in the periplasmic side of the cell membrane [19], yet, evidence was presented also for intracellular generation of $PQ^{\cdot+}$ [16].

It has been previously suggested that in the presence of paraquat, the extracellular $O_2^{\cdot-}$ radical is less than 5% of the corresponding intracellular generated $O_2^{\cdot-}$ [5, 6]. The present results, however, show that, both under oxic and anoxic conditions, only extracellular radicals accumulate. This well agrees with the identification of the cell-membrane as being the target organelle in paraquat-induced damage [20] and with the effect of exogenously added SOD.

Under aerobic conditions and in the presence of DMPO, only the DMPO-OH spin-adduct could be observed [11]. The failure of catalase and of OH-scavengers to affect the DMPO-OH signal substantiates the conclusion that DMPO-OH is not produced by trapping genuine $^{\cdot}OH$ radical, but rather it is formed through decomposition of the DMPO-OOH spin adduct [21]. The complete elimination of DMPO-OH signal by exogenous SOD further supports this conclusion.

Role of copper ions

Hydrogen peroxide did not eliminate the $PQ^{\cdot+}$ ESR spectrum, however, addition of copper resulted in the disappearance of the signal. This can be explained by assuming that Cu(II) serves as an electron acceptor which oxidizes $PQ^{\cdot+}$ to the parent PQ^{+2}. The newly formed Cu(I) ion is subsequently reoxidized in the Fenton reaction by H_2O_2.:

$$Cu(II) + PQ^{\cdot+} \rightarrow Cu(I) + PQ^{+2}. \quad (1)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\cdot}OH + OH^{-}. \quad (2)$$

Under a high and continuous flux of $PQ^{\cdot+}$, elimination of the $PQ^{\cdot+}$ signal by Cu(II) is not anticipated unless Cu(II) is *regenerated* thus playing a catalytic role. In the present study, the results indeed show that only in the presence of both copper *and* H_2O_2 was the $PQ^{\cdot+}$ signal eliminated. Therefore copper acts as a catalyst.

Accumulation of $PQ^{\cdot+}$ is relatively nontoxic to the cells (Fig. 2). On the other hand, H_2O_2 and trace amounts of copper are sufficient to produce $^{\cdot}OH$ at the expense of $PQ^{\cdot+}$ radicals. According to this mechanism, in the absence of oxygen the relatively innocuous $PQ^{\cdot+}$ species and H_2O_2 provide the respective reducing and the oxidizing moieties necessary for the metal-catalyzed cycling to induce site-specific damage. This explains the observed correlation of a higher killing rate of the cells concomitant with the disappearance of the $PQ^{\cdot+}$ species. However, contrary to previous reports [4], the present study excludes any significant direct effect of H_2O_2 on $PQ^{\cdot+}$.

The anoxic partitioning of $PQ^{\cdot+}$ between the intracellular volume and the extracellular bulk showed that despite the progressive cellular uptake of paraquat [16, 18, 19], $PQ^{\cdot+}$ radicals do not accumulate within the cells. Likewise, under aerobic conditions, $PQ^{\cdot+}$ reacts with molecular oxygen predominantly outside the cell, producing scavengable $O_2^{\cdot-}$. Under air, however, $PQ^{\cdot+}$ radicals are not expected to directly react with the transition metal ($k = 5.1 \times 10^6$/M/sec [22]) as they react faster with molecular oxygen ($k = 7.7 \times 10^8$/M/sec [23]). The conclusion, therefore, that DMPO-OH is derived from extracellular superoxide radicals is in accord with the total eradication of the DMPO-OH signal by SOD and with previous evidence indicating that intracellular DMPO-OH spin adducts are rapidly destroyed and do not survive detection [24, 25].

In conclusion, the present study proves the function of transition metals in paraquat toxicity and further shows that the paraquat monocation radical accumulates extracellularly, does not react directly with H_2O_2, and gives rise to extracellular oxygen-derived species through reaction with oxygen, H_2O_2, and metal ions, in accord with the identification of the membrane as a critical target.

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