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Superoxide mediates the toxicity of paraquat for Chinese hamster ovary cells

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ABSTRACT **The roles of superoxide and H_2O_2 in the cytotoxicity of paraquat were assessed in Chinese hamster ovary cells. Neither catalase nor superoxide dismutase inhibited the loss of ability to form colonies when added to the medium. When introduced into the cells, superoxide dismutase but not catalase inhibited the toxicity of paraquat. That superoxide dismutase acted by its known catalytic action is shown by the loss of inhibition when the enzyme was inactivated by H_2O_2 before being introduced into the cells. The lack of inhibition by catalase, by dimethyl sulfoxide, and by desferoxamine suggests that the toxicity is not mediated by a reaction between H_2O_2 and superoxide to engender the hydroxyl radical. Exposure of Chinese hamster ovary cells to paraquat may be a suitable means to determine the effects of superoxide anion in cultured cells and the ways in which cells can resist this toxic action.**

The ability to introduce macromolecules into living cells has greatly enhanced the study of the mechanisms of intracellular events. The actions of drugs, for example, can now be studied in cells that have been enriched for enzymes, which, by the specificity of the reactions they catalyze, can reveal the necessary steps in the cytotoxic actions of the drug. One such chemical agent, the mechanism of whose action in mammalian cells is not yet clear, is paraquat, a compound of great interest because of its capacity to engender the superoxide anion (1–3).

The discovery that a cellular enzyme acted upon superoxide anion (4), catalytically scavenging this free radical derived from molecular oxygen, suggested that superoxide anion might exist in cells and that its effects might be deleterious. *In vitro*, an impressive array of potentially toxic effects has been documented (5). The study of the effects of superoxide anion *in vivo* has been hindered by the instability of superoxide anion in water, necessitating some mechanism for generating superoxide within cells. In *Escherichia coli* the toxicity of paraquat appears indeed to be mediated by superoxide anion. When grown in the presence of paraquat and molecular oxygen, *E. coli* are killed, but less rapidly if they are enriched in superoxide dismutase by prior induction of its synthesis (6) and not at all if oxygen, from which superoxide is derived, is excluded.

As a step toward determining the cellular effects of superoxide in mammalian cells we have studied the suitability of paraquat as an intracellular generator of superoxide. We have, therefore, studied the cytotoxic effects of paraquat in cultured cells in which the content of superoxide dismutase has been augmented by "scrape-loading" (7), a procedure for the introduction of macromolecules into cells from their medium. The cytotoxicity in these cells has been compared with the toxicity in cells subjected to the same procedure except that no enzyme, or catalytically inactive enzyme, was introduced. We find that paraquat is indeed cytotoxic for Chinese hamster ovary (CHO) cells and superoxide anion appears to mediate, at least partly, these cytotoxic effects.

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MATERIALS AND METHODS

Materials. Fluoresceinated dextran (M_r 70,000), diaminobenzidine, and horseradish peroxidase were obtained from Sigma. Paraquat was a gift from Imperial Chemical Industries (London, England). Bovine hepatic Cu/Zn-containing superoxide dismutase was supplied by Diagnostic Data (Mountain View, CA) and bovine hepatic catalase was obtained from Calbiochem–Behring. Enzymes were dialyzed against F-12 medium (Kansas City Biologicals, Lenexa, KS) without serum for 24 hr at 4°C before use for scrape-loading.

Inactivation of Superoxide Dismutase with H_2O_2. The Cu/Zn-containing superoxide dismutase was inactivated with H_2O_2 (8) by the addition of 1 μl of 30% H_2O_2 to 1 ml of a solution of superoxide dismutase (10 mg/ml) in 50 mM carbonate buffer (pH 10.2) at 37°C twice at hourly intervals. The native enzyme and the enzyme that had just been inactivated with H_2O_2 were both dialyzed against F-12 medium for 24 hr at 4°C following treatment with H_2O_2. The peroxide-inactivated enzyme and the native enzyme were then assayed by their abilities to inhibit the superoxide-dependent reduction of cytochrome *c* when superoxide was generated by the enzymic action of xanthine oxidase on xanthine (4).

Radioiodination of Superoxide Dismutase. Bovine Cu/Zn-containing superoxide dismutase from liver was radioiodinated with ^{125}I by the Iodo-Gen method (9). Unincorporated $^{125}I^-$ was separated from the radioiodinated enzyme by gel filtration on Sephadex G-10. No loss of activity due to iodination was detected. The iodinated enzyme was dialyzed against F-12 medium without serum for 24 hr at 4°C before scrape-loading into CHO cells. All of the radioactivity could be precipitated with 10% trichloroacetic acid. Autoradiography of cells after scrape-loading with ^{125}I-labeled superoxide dismutase was performed on glass coverslips (10) after allowing the cells to adhere overnight in 35-mm dishes.

Tissue Culture. CHO cells were obtained from the American Type Culture Collection (ATCC CCL 61) and were grown in Ham's F-12 medium with 5% donor/5% fetal calf serum (5% D/5% FCS). Scrape-loading was used to introduce macromolecules into CHO cells because of its simplicity and efficacy. In this method cells are removed from monolayers by mechanical forces with the macromolecule to be introduced present in the serum-free medium while the cells are scraped. Scrape-loading of enzymes into cells was performed as described by McNeil *et al.* (7), except that the cells were scraped at 0°C with a plastic device with a hinge between the handle and the scraping surface. The cells were resuspended and counted in the chamber of a hemocytometer after scraping and washing. The number of cells plated was the number of rounded, phase-bright cells; the cells that had

Abbreviations: CHO, Chinese hamster ovary; FCS, fetal calf serum; D, donor.

lost refractility and birefringence were ignored. The plating efficiency of cells counted in this manner gave values that were similar to those observed with the same cells subjected to trypsinization.

Assay of the Toxicity of Paraquat. CHO cells were trypsinized from monolayers, washed, resuspended, and seeded at 200 cells per 60-mm dish in quadruplicate to adhere overnight at 37°C. The next morning the medium was replaced with complete F-12 medium with 5% D/5% FCS containing the indicated concentrations of paraquat. Cells were rocked in the presence of paraquat for 24 hr at 37°C in 94% air/6% CO_2. The medium was then replaced with complete F-12 medium without paraquat, and colonies were allowed to form stationarily at 37°C. Colonies in plastic dishes were counted by rinsing the dish with phosphate-buffered saline (0.15 M NaCl/10 mM potassium phosphate, pH 7.8) and staining with 50% methanol/10% acetic acid/0.07% Coomassie blue G.

Gel electrophoresis in $NaDodSO_4$ was performed (11) in a 9–15% logarithmic gradient of acrylamide. The gels were fixed in 50% methanol/10% acetic acid overnight and stained in 0.07% Coomassie blue/25% isopropyl alcohol/10% acetic acid.

RESULTS

Toxicity of Paraquat for CHO Cells. To verify that paraquat was toxic for CHO cells, the ability to form colonies was assessed after exposure for 24 hr to several concentrations of paraquat. Indeed, striking loss of ability to form colonies was observed, which increased with increases in the concentration of paraquat (Fig. 1).

Effect of Superoxide Dismutase or Catalase in the Medium. To determine whether the cytotoxicity of paraquat was caused by superoxide or H_2O_2, CHO cells were exposed to 80 μM paraquat in the presence of either superoxide dismutase or catalase or both in the medium. The results (Table 1) indicate that neither enzyme diminished the toxicity of paraquat. Although the combination of superoxide dismutase and catalase in this experiment appeared to offer slight protection, in two repetitions, one at a concentration of enzymes of 20 μg/ml and the other at 57 μg/ml, neither enzyme nor the combination of the two protected the cells from paraquat at concentrations of 80 μM and 114 μM, respectively. This result suggested either that the cytotoxicity of paraquat was not caused by superoxide or H_2O_2 or that the enzymes in the medium could not act upon their substrates within the cells. To distinguish between these possibilities we compared the sensitivities to paraquat of cells in which the content of superoxide dismutase and/or catalase differed.

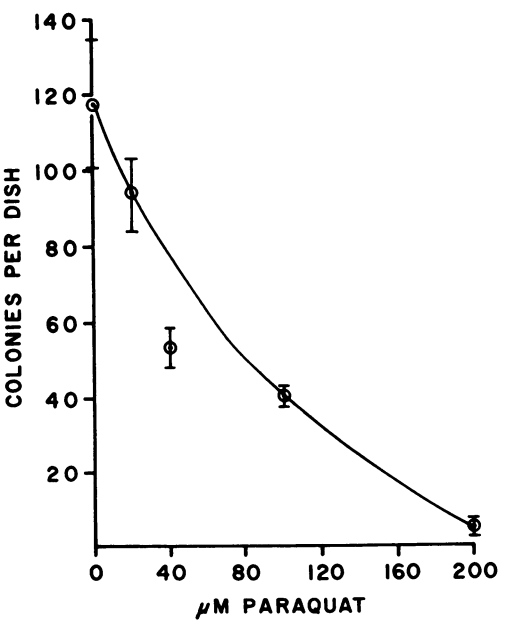

FIG. 1. Effect of paraquat on the survival of CHO K1 cells. CHO cells were exposed in 60-mm dishes at 37°C with gentle rocking of various concentrations of paraquat in F-12 medium with 5% D/5% FCS. After 24 hr of exposure the medium was replaced with fresh medium lacking paraquat and the cells were incubated stationarily for 8 days until colonies were visible.

Table 1. Effect of catalase, superoxide dismutase, or both in the medium on the cytotoxicity of paraquat for CHO cells

Group	SOD	CAT	PQ, μM	Colonies, no. per dish	% killed*	*P* vs. control
1	−	−	0	124.25		
2	−	−	80	60.25	52.5 ± 5.30	—
3	+	−	0	123.50		
4	+	−	80	63.00	49.0 ± 5.25	0.22
5	−	+	0	121.50		
6	−	+	80	62.00	49.8 ± 5.76	0.34
7	+	+	0	103.50		
8	+	+	80	66.50	35.8 ± 8.50	<0.02

CHO cells were grown in monolayers in F-12 medium with 5% D/5% FCS. They were trypsinized, collected in ice-cold F-12 medium with 5% D/5% FCS, sedimented by centrifugation, and washed in the same medium twice. They were inoculated in 60-mm dishes at 200 cells per dish and allowed to adhere overnight at 37°C in an incubator. The medium was removed and replaced with complete F-12 medium with 5% D/5% FCS with or without 80 μM paraquat (PQ). Superoxide dismutase (SOD) and catalase (CAT) were present, when indicated, at concentrations of 40 μg/ml. All dishes were incubated with rocking at 37°C in 7% CO_2 for 24 hr. The medium was then replaced in all dishes with complete F-12 medium with 5% D/5% FCS and the dishes were incubated stationarily at 37°C until stainable colonies were seen.
*Mean ± SD.

Loading of Enzymes into CHO Cells. To determine the suitability of scrape-loading (7) for introduction of macromolecules into CHO cells, we assessed the frequency of successful loading with fluoresceinated dextran. Subconfluent monolayers were subjected to scrape-loading in the presence of 20 mg of fluoresceinated dextran (M_r 70,000) per ml. The cells were plated in 35-mm plastic dishes containing glass coverslips and allowed to adhere overnight in F-12 medium with 5% D/5% FCS. The coverslips were removed and inverted in a drop of phosphate-buffered saline, and the fluorescent cells were counted with a fluorescent microscope. Eighty-one percent of the cells were brightly fluorescent. Fluorescence was diffuse, conforming to the outline of the entire cell. There was no outline of a nonfluorescent dark nucleus, suggesting that the nucleus, too, had received dextran.

This inference was confirmed by repeating the experiment with horseradish peroxidase in place of fluoresceinated dextran to detect the sites into which the macromolecules had been introduced. The CHO K1 cells were scraped in the presence or in the absence of horseradish peroxidase, allowed to adhere to glass coverslips overnight, fixed, and stained with diaminobenzidine (12) for the activity of horseradish peroxidase. Indeed, the brown reaction product was seen within the nuclei of 23% of the cells.

Since fluoresceinated dextran and horseradish peroxidase might not behave similarly to the Cu/Zn-containing superoxide dismutase, we assessed the amount of this enzyme introduced into CHO K1 cells by scrape-loading and the proportion of cells into which it was successfully introduced. Superoxide dismutase, radioiodinated by the Iodo-Gen method (9), was freed of unincorporated iodide first by gel

filtration over Sephadex G-10 and then by dialysis against F-12 medium without serum.

CHO cells were scrape-loaded with radioiodinated superoxide dismutase, washed, and seeded into dishes. One group of cells was allowed to adhere to coverslips in 35-mm plastic dishes overnight. The coverslips were rinsed eight times with phosphate-buffered saline, fixed with methanol/acetic acid (3:1), and processed for autoradiography. The proportion of cells into which superoxide dismutase had been successfully introduced was determined from the proportion of cells, stained with Giemsa, over which grains were observed microscopically. After 6 days of exposure, grains were found over 71% of the cells.

The other group of cells was seeded into 35-mm dishes without coverslips. After adhering overnight, the cells were rinsed eight times with phosphate-buffered saline, removed from the dish by trypsinization, sedimented by centrifugation at 250 × *g* for 10 min at 4°C, resuspended, and counted. Known numbers of cells were subjected to determination of radioactivity in a crystal scintillation counter. From the radioactivity in the iodinated superoxide dismutase per unit enzymic activity, the amount of enzyme associated with the cells could be determined from the cellular radioactivity. The increment in activity produced by scrape-loading was 30.2 units per 10 million cells, which is 4–9 times the total activity of superoxide dismutase in CHO cells. Taking into account the 71% of cells over which grains were found in the autoradiograms, the increment in activity produced by scrape-loading in those cells successfully loaded is 6–13 times the endogenous activity.

Effects of Superoxide Dismutase and/or Catalase Scrape-Loaded into CHO K1 Cells. To determine whether paraquat killed cells because of its ability to engender superoxide and/or H_2O_2, CHO cells were scrape-loaded with superoxide dismutase, with catalase, with both, or with neither ("dummy-loaded"). Both enzymes were present at a concentration of 10 mg/ml at the time of scraping. Other than the presence or absence of one of the two enzymes, the cells scraped in each way were treated identically. The results of such an experiment in which the cells were exposed in pentuplicate either to 0, 50, 60, or 80 μM paraquat (Table 2) indicate that the cells that had been scrape-loaded with superoxide dismutase were substantially protected from the cytotoxic action of paraquat. There was no protection, however, by catalase; indeed, catalase at 60 and 50 μM paraquat appeared to impair survival (groups C vs. groups A) and, at all concentrations, to antagonize slightly the protective effect of superoxide dismutase (groups D vs. groups B). This experiment was repeated at 100 μM paraquat with the same result; the survival of cells loaded with superoxide dismutase (35.5% ± 7.3%; mean ± SD) or with superoxide dismutase and catalase (32.0% ± 8.8%) was significantly greater ($P = 0.02$) than the survival of dummy-loaded cells (19.5% ± 7.3%) or of cells loaded with catalase alone (11.9% ± 2.0%).

Table 2. Effect of scrape-loading of catalase or superoxide dismutase on the cytotoxicity of paraquat for CHO cells

Group	Cells scraped: With SOD	Cells scraped: With CAT	PQ, μM	% survival*	*P* vs. control	% protection†
A2	−	−	50	79.4 ± 11.00		
B2	+	−	50	116.6 ± 22.22	<0.001	180.6
C2	−	+	50	63.4 ± 12.34	0.06	−77.8
D2	+	+	50	89.2 ± 8.44	0.13	47.6
A3	−	−	60	58.4 ± 8.92		
B3	+	−	60	94.7 ± 15.88	<0.01	87.3
C3	−	+	60	52.2 ± 4.74	0.15	−14.9
D3	+	+	60	79.2 ± 9.26	<0.01	50.0
A4	−	−	80	25.2 ± 6.13		
B4	+	−	80	55.8 ± 2.83	<0.00001	40.9
C4	−	+	80	29.0 ± 5.35	0.22	5.1
D4	+	+	80	46.6 ± 6.42	<0.001	28.6

SOD, superoxide dismutase; CAT, catalase; PQ, paraquat.
*Mean ± SD.
†% protection = 100(% survival in "x" − % survival in "A")/(100 − % survival in "A").

To verify that the effect of superoxide dismutase, scrape-loaded into CHO K1 cells, was due to its enzymic activity, we compared the effects of the native enzyme with that of the enzyme after inactivation with H_2O_2. Three types of scrape-loaded cells were prepared—one dummy-loaded, the second loaded with native Cu/Zn-containing superoxide dismutase, and the third loaded with the enzyme that had been inactivated with H_2O_2. The dummy-loaded cells were scraped in the presence of medium only, to which no enzymes had been added. The results (Table 3) show that, although native superoxide dismutase protected the cells from the cytotoxicity of paraquat, the peroxide-inactivated enzyme did not. At all three concentrations of paraquat, cells loaded with native superoxide dismutase were protected 60–80% from cytotoxicity. In contrast, the survival of cells loaded with the peroxide-treated enzyme was the same as cells that had been dummy-loaded. This experiment was repeated at 80 μM paraquat. Again, the survival of cells loaded with native superoxide dismutase (84.9% ± 5.5%) was significantly greater ($P < 0.001$) than that of either the dummy-loaded cells (63.8% ± 5.7%) or cells loaded with peroxide-inactivated superoxide dismutase (63.4% ± 3.2%).

To assess the roles of the hydroxyl radical in the toxicity of paraquat, we tested the ability of a scavenger of OH·, dimethyl sulfoxide (13), and of a chelator of ionic iron, desferoxamine (14, 15), to inhibit the loss of plating efficiency induced by paraquat. Neither 50 mM dimethyl sulfoxide nor 50 μM desferoxamine mesylate inhibited the toxicity of paraquat at concentrations of 100 μM, 150 μM, and 200 μM (Table 4).

DISCUSSION

The results of these experiments demonstrate that the cytocidal action of paraquat for CHO cells is caused, at least partly, by superoxide anion produced intracellularly. The lack of an effect of extracellular catalase or superoxide dismutase suggests that, if O_2^- or H_2O_2 escapes the cell, they do not injure cells from the medium. A different answer might be obtained if exposure to paraquat occurred at a greater density of cells at which a larger concentration of either O_2^- or H_2O_2 might accumulate in the medium. In these experiments <200 cells were present in a dish at the time the exposure to paraquat commenced, a number probably too small to have generated cytotoxic concentrations of either H_2O_2 or O_2^- in the medium.

The loss of inhibition by peroxide-treated superoxide dismutase suggests that the effect of the native enzyme was indeed exerted by its known catalytic action. The absence of an inhibitory effect of catalase does not necessarily mean, however, that H_2O_2 plays no role in the cytotoxicity of paraquat, particularly in other types of cells. CHO cells are relatively rich in catalase, possessing 5–10 times the activity found in HeLa cells (R.E.L., unpublished data). In other cells in which the content of catalase is less, cytotoxicity dependent on H_2O_2 might be seen. The lack of an effect of catalase might also result from inactivation of catalase by superoxide (16).

Lack of any inhibitory effect of catalase suggests that paraquat's toxicity for these cells is not mediated through a

Table 3. Comparison of the effects of scrape-loaded Cu/Zn-containing superoxide dismutase with those of the peroxide-inactivated Cu/Zn-containing superoxide dismutase on the cytotoxicity of paraquat

Group	Scraped	PQ, μM	% survival*	*P* vs. control	% protection†	Ratio to control (group A)
A1	—	0	(100) ± 9.98	—	—	—
B1	With SOD	0	(100) ± 12.30	—	—	—
C1	With perox-SOD	0	(100) ± 10.50	—	—	—
A2	—	60	77.9 ± 5.99	—	—	—
B2	With SOD	60	95.4 ± 6.24	<0.01	79.2	1.22
C2	With perox-SOD	60	78.7 ± 3.84	0.37	3.6	1.01
A3	—	80	57.5 ± 6.35	—	—	—
B3	With SOD	80	83.3 ± 13.05	<0.01	60.7	1.45
C3	With perox-SOD	80	55.3 ± 18.53	0.35	−5.2	0.96
A4	—	100	34.8 ± 2.05	—	—	—
B4	With SOD	100	78.7 ± 3.89	<0.000001	67.4	2.26
C4	With perox-SOD	100	33.7 ± 4.35	0.34	−1.7	0.97

Subconfluent monolayers of CHO cells were scrape-loaded with the Cu/Zn-containing superoxide dismutase (SOD), with the peroxide-inactivated superoxide dismutase (perox-SOD), or in the absence of any added enzymes (—). The concentration of superoxide dismutase was 9760 units/ml in the medium at the time of scrape-loading. Treatment with H_2O_2 reduced the activity to 11%. Two hundred phase-bright cells of each type were seeded in 60-mm dishes and allowed to adhere overnight in F-12 medium with 5% D/5% FCS. The medium was changed to F-12 medium with 5% D/5% FCS with the indicated concentrations of paraquat (PQ) and the cells were incubated at 37°C with rocking for 24 hr. The medium was again changed and the cells were incubated stationarily for 8 days. The colonies were fixed, stained, and counted.
*Mean ± SD.
†% protection = 100(% survival in "x" − % survival in "A")/(100 − % survival in "A").

reaction between H_2O_2 and superoxide anion catalyzed by ionic iron to form OH· (5, 17–19). The inability of dimethyl sulfoxide, which scavenges OH·, or of desferoxamine, which complexes ionic iron and scavenges OH·, to antagonize the toxicity of paraquat strengthens this contention.

The variation in the toxicity of paraquat between experiments deserves comment. The concentrations of paraquat used in these experiments are much greater than those that CHO cells can tolerate continually (about 15 μM), causing the viability to decline rapidly. Slight variations in the duration of exposure will, consequently, affect survival. In addition, the formation of superoxide from paraquat will depend on the concentration of oxygen in the medium, which itself may depend critically on the amplitude and frequency of rocking. Slight variations between experiments in the conditions of exposure may, therefore, produce variations in mortality. For these reasons we have restricted our conclusions to comparisons within a single experiment in which all of the cells were prepared at once, incubated at once in the same incubator on the same rocker, exposed to the same solutions of paraquat, loaded with the same solutions of enzymes, initiated together, and concluded simultaneously.

Because its cytotoxicity is exerted by virtue of its capacity to generate superoxide, paraquat may serve as a convenient vehicle to study the range of the effects of superoxide and the mechanisms by which they are produced. There may be disadvantages to the use of paraquat to increase the concentration of superoxide intracellularly. Superoxide may not be formed at the same intracellular site(s) where O_2^- is formed "normally," and the magnitude of the flux may exceed any that occurs in the absence of paraquat. However, until cells are available in which the activities of the superoxide dismutases can be selectively magnified and reduced, exposure to paraquat is a useful way to ascertain superoxide's actions on cells.

Table 4. Effect of desferoxamine and of dimethyl sulfoxide on the toxicity of paraquat for CHO K1 cells

Group	PQ, μM	Me_2SO, 50 mM	DFA, 50 μM	Colonies, no. per dish*	% survival
A	0	−	−	179.67	(100)
E	0	+	−	181.00	(100)
I	0	−	+	165.00	(100)
B	100	−	−	107.67	59.9
F	100	+	−	108.33	59.9
J	100	−	+	100.33	60.8
C	150	−	−	81.33	45.3
G	150	+	−	81.00	44.8
K	150	−	+	72.67	44.0
D	200	−	−	28.67	16.0
H	200	+	−	21.67	12.0
K	200	−	+	20.67	12.5

CHO cells were trypsinized from monolayers and distributed into 60-mm dishes at 200 cells per dish in triplicate. After overnight incubation to permit adherence, the medium was changed to F-12 medium with 5% D/5% FCS and the indicated concentrations of paraquat (PQ) and desferoxamine (DFA) or dimethyl sulfoxide (Me_2SO). The cells were exposed for 24 hr with rocking at 37°C. The medium was then changed to complete F-12 medium without the addition of chemicals and the cells were allowed to form colonies for 8 days stationarily at 37°C.
*Mean.

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