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PROTECTION OF A RAT TRACHEAL EPITHELIAL CELL LINE FROM PARAQUAT TOXICITY BY INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Abstract—Transformed rat tracheal epithelial cells (U2) were found to be 3.6-fold more sensitive than lung fibroblasts (RLF) to paraquat. Although the toxic effects of paraquat are associated with the generation of very active superoxides, U2 cells contained higher levels of superoxide dismutase and catalase than RLF cells. On the other hand, the specific activities of both NADPH-cytochrome *c* reductase and glucose-6-phosphate dehydrogenase (G6DP) were 3- to 4-fold higher in U2 cells than in RLF cells. Treatment with dehydroepiandrosterone (DHEA) and epiandrosterone (EPI), G6PD inhibitors, significantly decreased the intracellular NADPH and protected U2 cells from paraquat toxicity. Since DHEA and EPI treatment did not affect the uptake of paraquat, our results suggest that paraquat sensitivity may depend on the redox cycling-associated activities of paraquat.

Paraquat (1,1′-dimethyl-4,4′-bipyridilium; CAS No. 1910-42-5; PQ†), a nonselective contact herbicide, is extremely toxic to mammals [1]. Since PQ is rapidly concentrated and retained by pulmonary tissue [2], the primary lesions in humans mainly occur in the lung. PQ intoxication usually leads to alveolar pulmonary edema and hemorrhage followed by pulmonary fibrosis and death [3]. Similar lung-directed toxicity has also been described in experimental animals [3–5]. The mechanism of PQ toxicity is generally attributed to the generation of very reactive oxygen species such as superoxides through a redox reaction between PQ radicals and molecular oxygen [5–8]. The deleterious oxygen species consequently attack protein and membranous organelles [9–11], inhibit macromolecular synthesis, and enhance lipid peroxidation [2, 12].

Although numerous radical scavengers have been considered as PQ antidotes, none of them were effective in animal trials [13]. Thus, it is still questionable whether the generation of harmful oxygen radicals is a crucial step for PQ to exert its toxicity. In our laboratory, we have noticed that a transformed rat tracheal epithelial cell line is more susceptible to PQ than are fibroblasts derived from rat lungs. To better understand the toxic mechanism of PQ, we examined the enzymatic activities of these cells which were suspected to participate in PQ metabolism. In this paper, we show that the regeneration of NADPH is a limiting factor for PQ radical formation and that inhibitors of glucose-6-phosphate dehydrogenase (G6PD), dehydroepiandrosterone (DHEA) and epiandrosterone (EPI), can effectively protect cultured cells from PQ toxicity.

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† Abbreviations: DHEA, dehydroepiandrosterone; EPI, epiandrosterone; FBS, fetal bovine serum; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; HBSS, Hank's balanced salt solution; PQ, paraquat; RLF, rat lung fibroblasts; SOD, superoxide dismutase; and U2, an epithelial cell line derived from rat tracheas.

MATERIALS AND METHODS

Chemicals. PQ, DHEA, and EPI were obtained from the Sigma Chemical Co (St Louis, MO). PQ was prepared freshly by dissolving it in distilled water. DHEA and EPI were dissolved in ethanol and then diluted with medium.

Cell culture. Media and chemicals used for cell culture were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was from HyClone Laboratories, Inc. (Logan, UT). Rat lung fibroblasts (RLF), provided by Dr. Tsing-Cheng Wang (Institute of Zoology, Academia Sinica, R.O.C.), were derived from the lungs of an 8-week-old male Sprague–Dawley rat. RLF cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS at 37° and 10% CO_2 in air. An ultraviolet light-transformed rat tracheal epithelial cell line (designated as U2) was established in our laboratory and incubated in Ham's F-12 medium with 10% heat-inactivated FBS, 5 μM hydrocortisone and 1 μg/mL insulin.

Cytotoxicity analysis. Due to the low colony-forming efficiency of U2 and RLF cells, the cytotoxic effects of PQ on these cells were measured by a proliferation assay. In brief, 2×10^5 cells were plated in 60-mm petri dishes in triplicate. After overnight incubation, the cultures were treated with PQ in serum-free medium for 4 hr and then washed twice with Hank's balanced salt solution (HBSS). The cells were further incubated for 3 days and the cell numbers were determined with an electronic cell counter after trypsinization.

Since treatment with G6PD inhibitors would retard the cell growth, their effects on PQ toxicity were analyzed by a colony-forming assay. To increase the plating efficiency, 500 U2 cells were plated onto

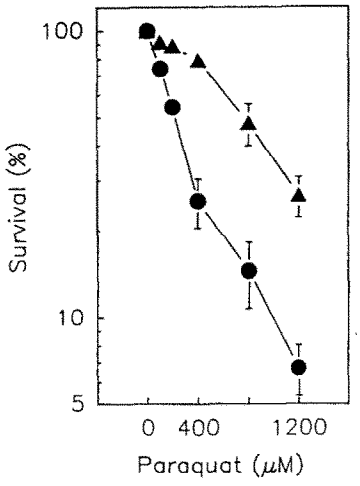

Fig. 1. Relative survival of U2 and RLF cells exposed to PQ. Cells were treated with various concentrations of paraquat for 4 hr. The cell numbers were counted after a 3-day incubation. Bars represent SD of three experiments. The averaged cell numbers of control cultures were 2.2×10^6 and 4.0×10^6 for U2 and RLF cells, respectively. Key: (●) U2 cells; and (▲) RLF cells.

Table 1. Comparison of antioxidant enzyme activities and glutathione level in U2 and RLF cells*

Parameters	Cell types RLF	U2
Catalase (mmol/min/mg protein)	10.4 ± 3.3	35.7 ± 3.2†
Superoxide dismutase (U/mg protein)	73.9 ± 6.0	93.3 ± 11.0
Glutathione peroxidase (nmol/min/mg protein)		
H_2O_2	15.2 ± 2.1	21.6 ± 4.2
Cumene peroxide	5.6 ± 0.2	14.4 ± 5.9‡
Glutathione *S*-transferase (nmol/min/mg protein)	52.1 ± 1.0	22.6 ± 5.2†
Glutathione (μg/mg protein)	3.0 ± 0.5	2.0 ± 0.2

* Results are means ± SD of 3 experiments.
†, ‡ Significantly different († $P < 0.01$ and ‡ $P < 0.05$, according to Student's *t*-test) from RLF values.

a 3T3 feeder layer which was prepared by plating 1.5×10^5 mitomycin C-treated NIH/3T3 cells in a 60-mm dish as described previously [14]. After overnight incubation, the cells were treated with drugs as indicated. At the end of treatment, the cells were washed twice with HBSS and allowed to grow for 7 days in complete medium. The colonies were then fixed with methanol, stained with a 10% Giemsa solution, and counted under a dissecting microscope.

Preparation of cell extracts. Nearly confluent cell cultures were washed twice with ice-cold phosphate-buffered saline, scraped off with a rubber policeman, and centrifuged at 200 *g* for 10 min at 4°. The cell pellets were resuspended in an appropriate volume (approx. 4×10^7 cells/mL) of 0.1 M potassium phosphate buffer, pH 6.8, and transferred into Eppendorf tubes. The cells were sonicated six times (10-sec burst with 1-min intervals) with a Heat System-Ultrasonics W-380 sonicator. Clear extracts were obtained by centrifugation at 12,000 *g* for 20 min at 4°, and kept on ice prior to enzymatic assays. The protein concentrations in cell extracts were determined by the method of Bradford [15] using bovine serum albumin as the standard.

Biochemical and enzymatic assays. Glutathione (GSH) levels were measured by a fluorometric method [16]. GSH transferase activities were measured by the methods of Habig *et al.* [17] and Habig and Jakoby [18], using reduced GSH and chlorodinitrobenzoate as substrates. GSH peroxidase activities, using hydrogen peroxide or cumene hydroperoxide as substrate, were analyzed in accordance with Lawrence and Burk [19]. Catalase activity was assayed by monitoring the decomposition of hydrogen peroxide spectrophotometrically at 240 nm [20]. Post-mitochondrial fractions, which were obtained by further spinning the cell extracts at 100,000 *g* for 30 min, were used for the assay of superoxide dismutase (SOD) [21]. NADPH-cytochrome *c* reductase activity was measured by reducing cytochrome *c*, an electron receptor, according to the methods of Prough *et al.* [22]. G6PD was assayed by using glucose-6-phosphate and NADP as substrates [23]. One unit of G6PD was defined as 1 μmol NADPH production/min/mg protein. Intracellular NADPH was measured by the HPLC method of Stocchi *et al.* [24] using a LiChrospher 100 RP-8 column (4 × 125 mm).

[^{14}C]*Paraquat uptake assay.* [*methyl*-^{14}C]Paraquat chloride (9.2 mCi/mmol) was obtained from NEN Research Products (Boston, MA). The [^{14}C]PQ uptake assay was performed according to a method described previously [25]. In brief, 4×10^5 U2 cells were plated in a 35-mm petri dish 2 days before experimental manipulation. The cultures were treated with 100 μM DHEA or EPI in serum-free medium for a time period as indicated. The control cultures were treated with serum-free medium only. Five dishes were used for each treatment. [^{14}C]PQ, 0.5 μCi/mL, was added to the medium during the last 30 min of incubation. Afterward, the cells were washed several times with HBSS and lyzed with 0.5 mL of 1 N NaOH at room temperature for 30 min. The lysate was neutralized by the addition of 0.5 mL of 1 N HCl, transferred to 15 mL Liquiscint (National Diagnostics, Manville, NJ), and counted in a liquid scintillation counter.

RESULTS

According to the results of the proliferation rate assay, U2 cells were 3.6-fold more sensitive than RLF cells to PQ (Fig. 1). The LC_{50} values of PQ for U2 cells were estimated to be 220 and 800 μM, respectively. Similar results were obtained by using the colony-forming assay (data not shown). Since the production of reactive oxygen radicals plays a

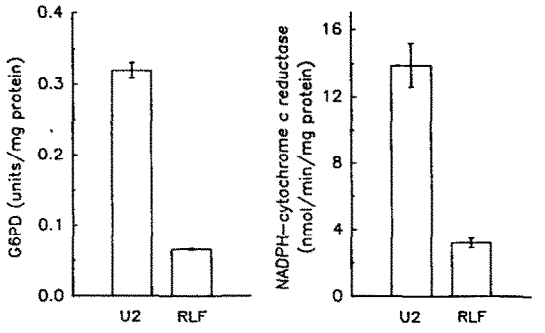

Fig. 2. Cellular levels of NADPH-cytochrome *c* reductase and G6PD in U2 and RLF cells. Crude extracts from these two cell types were assayed for enzymatic activity as described in Materials and Methods. Bars represent SD of three experiments.

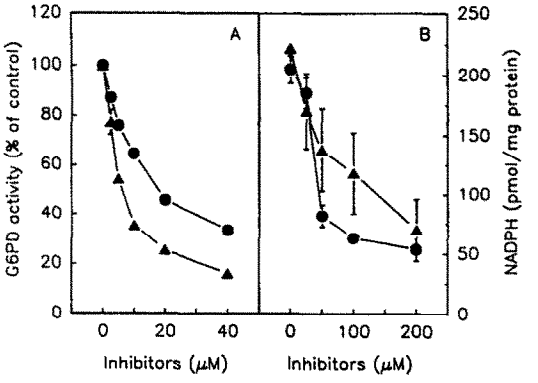

Fig. 3. (A) Inhibitory effects of DHEA and EPI on G6PD activity. Various concentrations of DHEA or EPI were added to the crude extracts of U2 cells 5 min prior to the G6PD activity assay. The control of G6PD activity was 0.32 ± 0.02 U/mg protein. (B) Depletion of intracellular NADPH by DHEA and EPI. U2 cells were treated with various concentrations of DHEA or EPI for 5 hr. The intracellular content of NADPH was determined by HPLC. Bars represent SD of three experiments. Key: (●) DHEA; and (▲) EPI.

crucial role in PQ toxicity, GSH levels and several enzymatic activities involved in free radical scavenging were compared between these two cell types. As summarized in Table 1, U2 cells contained higher levels of catalase and GSH peroxidase but less GST than RLF cells. SOD activity was slightly higher in U2 cells compared to RLF cells. GSH levels were not significantly different. GSH peroxidase, catalase and SOD are the major enzymes responsible for removing superoxide anions and hydrogen peroxide. Thus, the sensitivity of U2 cells to PQ toxicity was not due to less efficiency in removal of the oxygen radicals.

U2 cells were found to contain 3- to 4-fold higher levels of NADPH-cytochrome *c* reductase and G6PD activities than RLF cells (Fig. 2). These two enzymes are involved in PQ radical production and NADPH regeneration. Therefore, we suspected that the formation of PQ radicals was more efficient in U2 cells than in RLF cells. To confirm this hypothesis, we tested the PQ sensitivity of U2 cells with reduced intracellular NADPH content by inhibiting G6PD activity with DHEA and EPI.

As shown in Fig. 3A, DHEA and EPI clearly inhibited the G6PD activity in crude extracts from U2 cells. Treatment of U2 cells with 200 μM DHEA or EPI decreased intracellular NADPH levels by 65–75% (Fig. 3B). Addition of DHEA or EPI 1 hr prior to PQ treatment significantly protected U2 cells from the PQ toxicity (Fig. 4). The protective effects of DHEA and EPI were also observed when they were added 0.5 and 1 hr after PQ treatment (Fig. 5). As shown in Fig. 6, treatment with DHEA or EPI did not affect the uptake of ^{14}C-labeled PQ.

Due to the pleiotropic response of cells to DHEA and EPI, we also examined the intracellular activities of NADPH-dependent cytochrome *c* reductase and SOD after treatment with these two steroid hormones. DHEA and EPI treatment did not result in significant changes of these two enzyme activities. However, GST activity was increased (30–80%) by a 5-hr treatment with EPI or DHEA (Table 2). The increase of GST activity was more marked (77–168%) 24 hr after treatment with DHEA or EPI (Table 2).

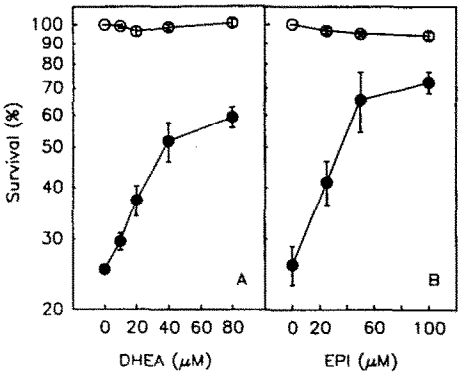

Fig. 4. Protection of U2 cells from PQ activity by pretreatment with DHEA and EPI. A colony-forming assay was used in this experiment. One hour prior to PQ treatment, various concentrations of DHEA or EPI were added to the culture medium. Without removing DHEA or EPI, the cells were further treated with 400 μM PQ for 4 hr. Afterward, the cultures were washed twice with HBSS and further incubated for 7 days. The colony numbers (> 50 cells) were determined under a microscope after staining. Bars represent SD of three experiments. Plating efficiency of control culture: 36.4 ± 5.8%. Key: (○) without PQ treatment; and (●) with PQ treatment.

DISCUSSION

Epithelial U2 cells, which contain higher levels of GSH peroxidase and catalase than RLF fibroblastic cells, are more susceptible to PQ. The PQ sensitivity

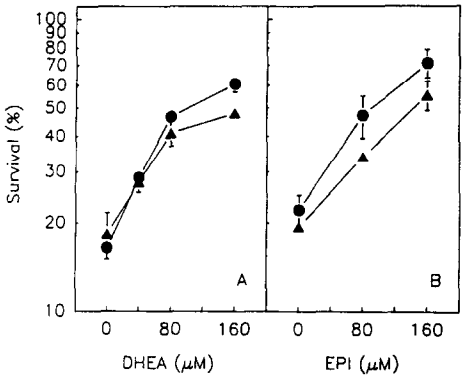

Fig. 5. Protection of U2 cells from PQ toxicity by posttreatment with DHEA and EPI. A colony-forming assay was used in this experiment. U2 cells were treated with 400 μM PQ for 4 hr. DHEA or EPI at various concentrations was added at 0.5 (●) or 1 (▲) hr after initiation of PQ treatment. The survival rate was determined as described in Fig. 4. Bars represent SD of three experiments. Plating efficiency of control culture: 48.8 ± 3.2%.

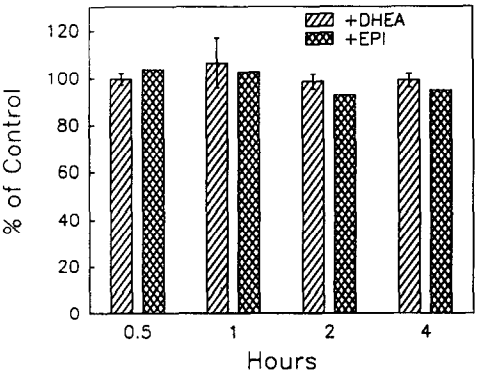

Fig. 6. Effects of DHEA and EPI on the uptake of ^{14}C-labeled PQ. U2 cells were treated with 100 μM DHEA (3 experiments) or EPI (2 experiments) in serum-free medium. The percentage of PQ uptake of drug-treated cultures was determined by comparing them to the control cultures at the time indicated. See Materials and Methods for details. The range of control values was 700 to 1500 cpm per dish. Bars represent SD of three experiments.

may be associated with some factors other than defensive activities against oxygen radicals. Our present study shows that U2 cells contained higher levels of NADPH-cytochrome *c* reductase and G6PD than the more PQ-resistant RLF cells. NADPH-cytochrome *c* reductase catalyzes the initial reduction of PQ generating deleterious free radicals [26–28], while G6PD is a key enzyme for NADPH generation. Since the PQ redox-cycle occurs continuously, the supply of NADPH could limit the rate of PQ reduction. As presented in this report, G6PD inhibitors (DHEA and EPI) significantly decreased the amount of intracellular NADPH and protected

Table 2. Enhancement of glutathione *S*-transferase activity by DHEA and EPI in U2 cells*

Hours after drug treatment	Glutathione *S*-transferase activity (nmol product/min/mg protein)		
	None	DHEA	EPI
0	16.9 ± 3.3	30.5 ± 6.7†	22.2 ± 1.5‡
24	16.2 ± 4.7	43.4 ± 2.6†	28.7 ± 1.1†

* U2 cells were treated with 50 μM DHEA or EPI for 5 hr. Cells were assayed for GST immediately or 24 hr after the drug treatment. Results are means ± SD of 3 experiments.

†, ‡ Significant difference († $P < 0.01$ and ‡ $P < 0.05$ according to Student's *t*-test, respectively) between the drug-treated and control cultures.

the cells from the killing effects of PQ. Since treatment with DHEA and EPI did not affect the uptake of [^{14}C]PQ, our results strongly suggest that the elimination of PQ toxicity could be achieved by limiting the regeneration of NADPH.

The inhibition of PQ reduction seems to be a more efficacious approach than destroying superoxide radicals after they have been produced. Numerous studies on the PQ-resistant cells lines have also revealed that the generation of deleterious PQ radicals through a redox cycle plays a crucial role in PQ activity. As reported by Kelner and Bagnell [29], a PQ-resistant cell line, derived from human HL-60 cells, was associated with the depletion of NADPH reductase. Our previous results also showed a significant decrease of G6PD activity in two PQ-resistant cell lines derived from Chinese hamster ovary cells [25]. Most PQ-resistant cells derived from rodent or human cell lines are not associated with increased antioxidant activities [25, 29, 30].

DHEA is one of the major secretory products of the human adrenal gland and has been demonstrated to possess several therapeutic effects, such as cancer prevention, and anti-autoimmune, anti-atherogenic, anti-obesity, and anti-diabetic activities [31]. Since DHEA is known to be a non-competitive inhibitor of mammalian G6PD [32, 33], the protective effects of DHEA have been considered to result from NADPH depletion [31]. Recently, DHEA has also been shown to induce the activities of GST [34], peroxisome-associated enzymes, and microsomal enzymes [35, 36]. Due to the pleiotropic effects of DHEA, the usage of DHEA and its analogs as antidotes to PQ poisoning will be an important question for further investigation. Besides DHEA and EPI, other G6PD inhibitors such as vanadium and aluminium chloride are also potential candidates for the inhibition of PQ toxicity in animal trials [37, 38].

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