Stimulation by paraquat of microsomal and cytochrome *P*-450-dependent oxidation of glycerol to formaldehyde

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Glycerol can be oxidized to formaldehyde by microsomes in a reaction that is dependent on cytochrome *P*-450. An oxidant derived from the interaction of H_2O_2 with iron was responsible for oxidizing the glycerol, with *P*-450 suggested to be necessary to produce H_2O_2 and reduce non-haem iron. The effect of paraquat on formaldehyde production from glycerol and whether paraquat could replace *P*-450 in supporting this reaction were studied. Paraquat increased NADPH-dependent microsomal oxidation of glycerol; the stimulation was inhibited by glutathione, catalase, EDTA and desferrioxamine, but not by superoxide dismutase or hydroxyl-radical scavengers. The paraquat stimulation was also inhibited by inhibitors, substrate and ligand for *P*-450E1 (pyrazole-induced *P*-450 isozyme), as well as by anti-(*P*-450E1) IgG. These results suggest that *P*-450 still played an important role in glycerol oxidation, even in the presence of

paraquat. Purified NADPH-cytochrome *P*-450 reductase did not oxidize glycerol to formaldehyde; some oxidation, however, did occur in the presence of paraquat. Reductase plus *P*-450E1 oxidized glycerol, and a large stimulation was observed in the presence of paraquat. Rates in the presence of *P*-450, reductase and paraquat were more than additive than the sums from the reductase plus *P*-450 and reductase plus paraquat rates, suggesting synergistic interactions between paraquat and *P*-450. These results indicate that paraquat increases oxidation of glycerol to formaldehyde by microsomes and reconstituted systems, that H_2O_2 and iron play a role in the overall reaction, and that paraquat can substitute, in part, for *P*-450 in supporting oxidation of glycerol. However, cytochrome *P*-450 is required for elevated rates of formaldehyde production even in the presence of paraquat.

INTRODUCTION

Glycerol was recently shown to be oxidized to formaldehyde by rat liver microsomes and by reconstituted systems containing NADPH-cytochrome *P*-450 reductase plus cytochrome *P*-450 in a reaction which requires the production of reactive oxygen intermediates [1–3]. The oxidation of glycerol to formaldehyde was inhibited by catalase, glutathione plus glutathione peroxidase and iron chelators such as EDTA and desferrioxamine, indicating that an oxidant produced from the interaction of H_2O_2 with iron was responsible for the reaction [2,3]. The lack of inhibition by superoxide dismutase, hydroxyl-radical ($\cdot\text{OH}$) scavengers and antioxidants such as trolox suggested that the direct oxidant was not superoxide radical, $\cdot\text{OH}$ or lipid radicals. Studies with inhibitors, ligands, antibodies and reconstituted systems containing reductase alone or reductase plus the pyrazole-induced cytochrome *P*-450 isoenzyme (*P*-450E1) showed the requirement for *P*-450 in the overall pathway of glycerol oxidation to formaldehyde [3]. Among the suggested roles for *P*-450 in the glycerol-oxidation pathway, production of the H_2O_2 and reduction of non-haem iron were considered to be very important [2,3]. Even though this reaction pathway is not likely to have any relevance for glycerol metabolism *in vivo*, our interest in this reaction system stems from the usual presence of glycerol in microsomal systems and reconstituted systems containing reductase and cytochrome *P*-450, and the possible use of this reaction as a simple sensitive assay for the detection of potent oxidants.

The goal of the current study was to evaluate the effect of paraquat on the oxidation of glycerol to formaldehyde by microsomes and reconstituted systems. Paraquat, in the presence of microsomes, NADPH and oxygen, undergoes redox cycling, with a subsequent increase in the production of reactive oxygen

intermediates, including $\text{O}_2^{\cdot-}$, H_2O_2 , $\cdot\text{OH}$ and oxidants that emit light [4–22]. Elevated production of H_2O_2 should increase the oxidation of glycerol to formaldehyde. On the other hand, by interacting with the reductase, paraquat uncouples the mixed-function oxidase system and decreases *P*-450-dependent oxidation of substrates. This could result in inhibition of glycerol oxidation to formaldehyde. Paraquat interacts with the purified reductase to produce reactive oxygen intermediates [23,24]. As reductase alone did not catalyse formaldehyde production from glycerol, it was considered that paraquat might be capable of substituting for *P*-450 in catalysing formaldehyde generation if a primary function for *P*-450 in the mechanism of glycerol oxidation was to generate H_2O_2 . Paraquat radical is also capable of reducing non-haem iron [25–28]; it is therefore possible that paraquat could replace *P*-450 as a reductant for iron and thereby catalyse oxidation of glycerol to formaldehyde in the presence of reductase.

MATERIALS AND METHODS

Sprague–Dawley rats were treated with 200 mg of pyrazole/kg body weight per day for 2 days, and liver microsomes were isolated by differential centrifugation, suspended in buffer (0.125 M KCl, 10 mM potassium phosphate, pH 7.4) and stored at -70°C at a protein concentration of about 15–20 mg/ml. *P*-450E1 was purified from liver microsomes isolated from rats treated with pyrazole as previously described [29]. The final *P*-450 preparation had a specific content of about 12 nmol of *P*-450/mg of protein, displayed dimethyl sulphoxide (DMSO)-binding spectrum, and one major band of apparent M_r 52000 was produced on SDS/PAGE. The *P*-450 was stored in 50 mM potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol at -70°C . NADPH-cytochrome *P*-450 reductase was

purified [30] to a final specific activity of 40 μmol of cytochrome *c* reduced/min per mg of protein, and stored at -70°C in 30 mM potassium phosphate, pH 7.7, containing 0.1 mM EDTA and 20% (v/v) glycerol.

The oxidation of glycerol by microsomes was determined at 37°C in a reaction system containing 0.1 M potassium phosphate, pH 7.4, 0.2 M glycerol, 0.001 M NaN_3 (to inhibit contaminating catalase) and microsomes equivalent to 1 nmol of total *P*-450/ml of reaction mixture. Reactions were initiated by the addition of an NADPH-generating system consisting of 0.3 mM NADP^+ , 10 mM glucose 6-phosphate, 10 mM MgCl_2 and 5 units of glucose-6-phosphate dehydrogenase and were terminated, after 20 min (except for time course experiments) by addition of trichloroacetic acid to a final concentration of 6% (w/v). Formaldehyde was determined on portions of the clear supernatant by the Nash reaction [31]. All values were corrected for zero-time controls in which the trichloroacetic acid was added before the NADPH. In some experiments, 4 mM dimethylnitrosamine (DMN) replaced glycerol as substrate.

The oxidation of glycerol to formaldehyde by reconstituted systems was determined [3] using a reaction system containing 100 mM potassium phosphate buffer, pH 7.4, 1 mM NADPH and the previously reconstituted system consisting of *P*-4502E1 (0.25 nmol), NADPH-cytochrome *P*-450 reductase (0.1 unit) and 30 μg of sonicated dilaurylphosphatidylcholine in 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 0.25 ml, or a system containing the above except that the *P*-4502E1 (reductase-dependent system) was omitted. The reconstituted system was incubated for 5 min at room temperature before being added to the reaction mixture. Glycerol was present in the reconstituted system at final concentrations of 80–150 mM, depending on the amount of reductase or *P*-450 added. However, 100 mM exogenous glycerol was added, making the final concentration of glycerol present in the reconstituted system equal to 180–250 mM. Reactions were carried out as described above for microsomes. The production of H_2O_2 by the reconstituted system was determined from the oxidation of 100 mM methanol to formaldehyde by the catalase- H_2O_2 complex.

Immunoinhibition studies were carried out with antibody raised in rabbits against the purified pyrazole-induced *P*-4502E1 as previously described [29,32]. The reconstituted system or microsomes were preincubated for 5 min at 37°C with the anti-(*P*-4502E1) IgG at concentrations of 5 or 7 mg of IgG/nmol of *P*-4502E1, followed by the addition of exogenous glycerol and NADPH.

The content of cytochrome *P*-450 was determined by the method of Omura and Sato [33]. All buffers and the water used to prepare solutions were passed through columns of Chelex-100 resin to remove metal contaminants. Glycerol (molecular biology grade) and paraquat were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The phosphate buffer was prepared from certified A.C.S.-grade reagents. Very low rates of glycerol oxidation were observed if lower than A.C.S.-grade phosphate was used, apparently because of the presence of significant amounts of heavy metals in lower-grade phosphate buffer, which compete with the iron necessary for the oxidation of glycerol to formaldehyde. Oxidation of DMN to formaldehyde was assayed by the Nash reaction. Results are from two experiments, carried out in duplicate; differences between the two experiments did not exceed 10%.

RESULTS

Effect of paraquat on microsomal oxidation of glycerol

In the presence of NADPH, rat liver microsomes catalysed the

oxidation of glycerol to formaldehyde in a time-dependent manner (Figure 1a). The addition of paraquat produced an approximately fourfold increase in the rate of formaldehyde generation (Figure 1a). Both the control rates and the paraquat-stimulated rates of glycerol oxidation to formaldehyde required the presence of glycerol, microsomes and NADPH. Control rates in the presence of NADH were about four-to-five-fold lower than with NADPH, and paraquat did not stimulate formaldehyde production with NADH as the microsomal reductant. In microsomes, paraquat has been shown to be relatively ineffective in stimulating production of reactive oxygen intermediates with NADH, as compared with NADPH [34]. A concentration curve for the stimulation by paraquat is shown in Figure 1(b); an approximately twofold increase was observed with 0.5 mM paraquat, and the stimulation continued as the concentration of paraquat was elevated over a 0.5–5.0 mM range. In contrast with the stimulation of formaldehyde production from glycerol, paraquat inhibited formaldehyde production from DMN (Figure 1b). DMN is a typical substrate for the mixed-function oxidase activity of cytochrome *P*-450, particularly cytochrome *P*-4502E1 [35,36], and paraquat is known to inhibit *P*-450-dependent metabolism probably by competition for reducing equivalents from NADPH-cytochrome *P*-450 reductase [37].

Effect of antioxidants, iron and *P*-450 ligands

Catalase nearly completely inhibited the control as well as the paraquat-stimulated rates of formaldehyde production (Table 1), indicating a requirement for H_2O_2 . There was little or no effect caused by superoxide dismutase or competitive $\text{OH}^\cdot$ scavengers such as ethanol or DMSO on formaldehyde production from glycerol in the absence as well as the presence of paraquat; however, GSH was inhibitory with both systems (Table 1). Inhibition by GSH probably reflects competition with glycerol for interaction with the oxidant generated from H_2O_2 and iron. Previous studies indicated a requirement for cytochrome *P*-450 in the overall pathway of glycerol oxidation to formaldehyde [3]. Miconazole, an inhibitor of *P*-450-dependent reactions [38], inhibited the control rate of formaldehyde production, and to a lesser extent, the paraquat-stimulated rate (Table 1). Substrates and ligands for *P*-4502E1, such as aniline and 4-methylpyrazole, were effective inhibitors of formaldehyde production in the absence as well as the presence of paraquat (Table 1). Tryptamine has recently been shown to inhibit the NADPH oxidase activity of *P*-4502E1 [39]. Tryptamine inhibited control and paraquat-stimulated rates of formaldehyde production (Table 1).

Desferrioxamine, a potent iron chelator, inhibits the control rate of glycerol oxidation to formaldehyde [2,3] (Table 2), suggesting a role for iron in the overall pathway of glycerol oxidation. Desferrioxamine also produced inhibition of the paraquat-stimulated rates of formaldehyde production, but much higher concentrations of desferrioxamine were required in the presence, than in the absence, of paraquat for effective inhibition (Table 2). The addition of a non-haem iron such as FeCl_3 did not stimulate rates of formaldehyde production in the absence or presence of paraquat, suggesting that sufficient iron is present in the reaction system to catalyse glycerol oxidation. Microsomes have been shown to contain pools of tightly bound non-haem iron, which can be made available during NADPH-dependent electron transfer to catalyse production of reactive oxygen radicals [40,41].

Production of formaldehyde from ethylene glycol

The simplest glycol, ethylene glycol, is also oxidized by micro-

Figure 1 Effect of paraquat on the oxidation of glycerol to formaldehyde by rat liver microsomes

(a) The oxidation of glycerol was determined in the absence (○) or presence (▼) of 1 mM paraquat as a function of time. (b) The effect of the indicated concentrations of paraquat on the oxidation of glycerol (■) or DMN (◆) by rat liver microsomes.

Table 1 Effect of various additives on the stimulation of glycerol oxidation by paraquat

The effect of the various additives on the oxidation of glycerol to formaldehyde by rat liver microsomes was determined in the absence and presence of 1 mM paraquat. Rates of formaldehyde production (nmol/min per nmol of *P*-450) were 4–7 for controls, and 13–17 with 1 mM paraquat.

Addition	Concentration	Effect on formaldehyde production (%)	
		Control	1 mM Paraquat
Catalase	100 units	–89	–95
Superoxide dismutase	20 units	–20	–20
GSH	10 mM	–69	–52
DMSO	25 mM	+4	–1
Ethanol	25 mM	+4	–11
Miconazole	0.25 mM	–99	–46
Tryptamine	2 mM	–22	–21
	5 mM	–43	–31
	10 mM	–61	–52
4-Methylpyrazole	5 mM	–45	–69
Aniline	5 mM	–48	–70

Table 2 Effect of iron and desferrioxamine on glycerol oxidation to formaldehyde

The oxidation of glycerol to formaldehyde by rat liver microsomes was determined in the absence and presence of paraquat and the indicated concentrations of desferrioxamine. Numbers in parentheses refer to the percentage inhibition caused by desferrioxamine.

Addition	Concentration (μM)	Rate of formaldehyde production (nmol/min per nmol of <i>P</i> -450)		
		Control	1 mM Paraquat	5 mM Paraquat
None	–	7.8	18.7	44.6
Desferrioxamine	6	0.5 (–94)	8.6 (–54)	44.2 (–1)
	9	0 (–100)	8.8 (–53)	41.9 (–6)
	25	0 (–100)	1.7 (–91)	10.7 (–76)

Table 3 Effect of paraquat on formaldehyde production from ethylene glycol

The effect of the various additives on the oxidation of 100 mM ethylene glycol to formaldehyde by rat liver microsomes was determined in the absence and presence of 2 mM paraquat. Numbers in parentheses refer to percentage inhibition caused by the additive.

Addition	Concentration	Rate of formaldehyde production (nmol/min per nmol of <i>P</i> -450)	
		Control	2 mM Paraquat
None	–	3.68	15.29
Superoxide dismutase	86 units	2.80 (–24)	11.71 (–23)
Catalase	86 units	0.42 (–89)	0.44 (–97)
GSH	10 mM	0.16 (–96)	1.89 (–88)
EDTA	0.05 mM	0.47 (–86)	3.73 (–75)
Desferrioxamine	0.01 mM	0.12 (–96)	11.72 (–21)
	0.02 mM	ND	9.22 (–38)
	0.04 mM	ND	3.72 (–75)
	0.08 mM	ND	1.64 (–89)

some to formaldehyde, and, analogous to results with glycerol, paraquat stimulated this reaction (Table 3). The sensitivity of the ethylene glycol system to various antioxidants in the absence or presence of paraquat was identical with the glycerol reaction system; catalase, GSH and EDTA were potent inhibitors, whereas superoxide dismutase had little effect. Desferrioxamine inhibited formaldehyde production from ethylene glycol, but, as with glycerol oxidation, higher concentrations of the iron chelator were required for effective inhibition in the presence than in the absence of paraquat (Table 3). In other experiments, paraquat also stimulated formaldehyde production from other vicinal glycols such as propane-1,2-diol, dihydroxyacetone and α -glycerophosphate (results not shown).

Effect of paraquat in reconstituted systems

Previous studies indicated that a reconstituted system containing phospholipid plus NADPH–cytochrome *P*-450 reductase was

Table 4 Effect of paraquat on glycerol oxidation to formaldehyde and H_2O_2 production by reconstituted systems with purified reductase and *P*-4502E1

The oxidation of glycerol to formaldehyde was determined in a reconstituted system containing phosphatidylcholine plus 0.1 unit of NADPH-cytochrome *P*-450 reductase (A), or 0.1 unit of reductase plus 0.25 nmol of cytochrome *P*-4502E1 (B) and the indicated additions of paraquat or $FeCl_3$ (0.025 mM final concentration when present). Results in (B) are means $\pm$ S.E.M. for three experiments. In (C), the production of H_2O_2 by the reconstituted systems was determined from the production of formaldehyde as a consequence of the oxidation of methanol by catalase- H_2O_2 .

Reaction system	Glycerol oxidation to formaldehyde (nmol/min)	Methanol oxidation to formaldehyde (nmol/min)
(A) Reductase	0.07	
Reductase + paraquat (1 mM)	1.08	
Reductase + $FeCl_3$	0.19	
Reductase + paraquat (1 mM) + $FeCl_3$	1.76	
Reductase + paraquat 5 mM)	1.62	
Reductase + paraquat (5 mM) + $FeCl_3$	4.0	
(B) Reductase	0.13 $\pm$ 0.04	
Reductase + paraquat (2 mM)	1.20 $\pm$ 0.22	
Reductase + <i>P</i> -4502E1	1.03 $\pm$ 0.32	
Reductase + <i>P</i> -4502E1 + paraquat (2 mM)	5.39 $\pm$ 1.27	
Reductase + <i>P</i> -4502E1 + $FeCl_3$	1.73 $\pm$ 0.14	
Reductase + <i>P</i> -4502E1 + $FeCl_3$ + paraquat (2 mM)	12.96 $\pm$ 3.04	
(C) Reductase		< 0.5
Reductase + paraquat (0.25 mM)		31
Reductase + <i>P</i> -4502E1		7.5
Reductase + <i>P</i> -4502E1 + paraquat (0.25 mM)		43.5
Reductase + paraquat (1 mM)		42
Reductase + <i>P</i> -4502E1		8
Reductase + <i>P</i> -4502E1 + paraquat (1 mM)		50.4

Table 5 Effect of anti-(*P*-4502E1) IgG on paraquat stimulation of the oxidation of glycerol to formaldehyde by microsomes and by reconstituted systems

The oxidation of glycerol to formaldehyde was determined in the absence or presence of 2 mM paraquat and either preimmune IgG or anti-(*P*-4502E1) IgG. (A) Reconstituted system; concentration of IgG was 5 mg/nmol of *P*-450. (B) Rat liver microsomes; concentration of IgG was 7 mg/nmol of *P*-450. At these concentrations of IgG, oxidation of DMN to formaldehyde was inhibited by about 85% with anti-(*P*-4502E1) IgG, and by less than 5% with preimmune IgG. Values in parentheses are percentage inhibition relative to preimmune IgG.

Addition	Rate of formaldehyde production (nmol/min per nmol of <i>P</i> -450)		Effect of addition (%)	
	Control	2 mM Paraquat	Control	2 mM Paraquat
(A) None	3.6	19.0	—	—
Preimmune IgG	2.5	9.5	—31	—50
Anti-(<i>P</i> -4502E1) IgG	0	1.3	—100	—93 (—86)
(B) None	5.5	19.4	—	—
Preimmune IgG	5.5	17.1	0	—72
Anti-(<i>P</i> -4502E1) IgG	1.4	4.5	—75	—77 (—74)

ineffective in catalysing oxidation of glycerol to formaldehyde, unless cytochrome *P*-450 was added [3]. As paraquat can interact with the reductase in the absence of *P*-450 to generate reactive oxygen intermediates [23,24], it was considered possible that the reductase could oxidize glycerol to formaldehyde in the presence

of paraquat, i.e. paraquat could substitute for *P*-450 in promoting formaldehyde production from glycerol. Results shown in Table 4 support this possibility. The reductase alone, with or without added iron, did not effectively produce formaldehyde. The addition of 1 mM or 5 mM paraquat resulted in elevated production of formaldehyde (Table 4, experiment A).

To evaluate whether *P*-450 plays a role in glycerol oxidation when paraquat is present, the above experiments were repeated in the presence of a reconstituted system containing reductase alone or reductase plus *P*-450. Under these experimental conditions, rates of formaldehyde production by the *P*-450 plus reductase system were similar to the rates for the reductase plus paraquat system (Table 4, experiment B). When *P*-450 was added to the reductase plus paraquat system, formaldehyde production was further increased, and rates were more than simply additive of the individual reductase plus paraquat and reductase plus *P*-450 rates.

In view of the critical role of H_2O_2 in the overall mechanism of glycerol oxidation to formaldehyde, rates of H_2O_2 production by the reconstituted system were determined. The reductase alone did not significantly produce H_2O_2 (Table 4C). The addition of *P*-4502E1 to the reductase or the addition of paraquat to the reductase resulted in H_2O_2 generation. In the presence of both *P*-450 plus paraquat, rates of H_2O_2 production appeared to be additive of the individual reductase plus *P*-450 and reductase plus paraquat rates (Table 4C).

Cytochrome b_5 can increase the metabolism of certain substrates by *P*-4502E1, e.g. DMN [42,43]. This may be due to provision of the second electron to the one-electron-reduced oxygenated *P*-450 complex. It was considered possible that cytochrome b_5 would inhibit the *P*-450-dependent oxidation of glycerol to formaldehyde, by promoting tighter coupling of the *P*-450–reductase system in contrast with the uncoupling caused by paraquat; this tighter coupling might prevent the stimulation by paraquat. At cytochrome b_5 /cytochrome *P*-450 molar ratios of 1:1 and 3:1, the oxidation of glycerol was decreased 20 and 30% respectively compared with *P*-450-dependent rates in the absence of cytochrome b_5 . This may reflect the ability of cytochrome b_5 to decrease the *P*-450-dependent production of H_2O_2 [44]. Paraquat produced the same increase in glycerol oxidation in the absence of cytochrome b_5 as it did in the presence of a 1:1 or 3:1 b_5 /*P*-450 molar ratio. It appears that cytochrome b_5 does not prevent the uncoupling produced by paraquat.

Effect of anti-(*P*-4502E1) IgG

To verify that the increase in formaldehyde production on the addition of *P*-4502E1 to the reductase plus paraquat system required functional catalytic activity of the added *P*-450, the effect of antibody raised against the purified pyrazole-inducible *P*-4502E1 was determined. At a concentration of 5 mg of globulin/nmol of *P*-450, this antibody produced about 80–90% inhibition of the oxidation of DMN and pyrazole by reconstituted systems containing *P*-4502E1 [29,45]. The antibody completely inhibited formaldehyde production by the reconstituted system in the absence of paraquat and strongly inhibited the stimulation by paraquat (Table 5, experiment A). Some inhibition was also noted with the preimmune control; the preimmune control had no effect on DMN oxidation by the reconstituted system [3], therefore the inhibition of glycerol oxidation is probably a consequence of non-specific free-radical scavenging by protein. Similar experiments were carried out with microsomes in order to determine the role of *P*-4502E1 in the stimulation of glycerol oxidation by paraquat. Anti-(*P*-4502E1) IgG strongly inhibited

Table 6 Reduction of FeCl_3 by reconstituted systems

The rate of reduction of 0.013 mM FeCl_3 by reductase alone or reductase plus *P*-4502E1 was determined in the presence of the indicated concentrations of paraquat. Ferrous production was assayed by following the increase in absorbance at 562 nm in the presence of 1 mM ferrozine.

Concentration of paraquat (mM)	Rate of ferric reduction (nmol of ferrous/min)	
	Reductase	Reductase + <i>P</i> -4502E1
0	0	15
0.1	18	37
0.5	44	54
1.0	46	60

formaldehyde production in the absence as well as the presence of paraquat (Table 5, experiment B). Most of the increase produced by paraquat was prevented in the presence of the antibody.

Reduction of FeCl_3 by purified reductase and *P*-4502E1

The inability of the reductase to catalyse glycerol oxidation is due to the lack of H_2O_2 production (Table 4C). It could also be due to the inability of the reductase to catalyse reduction of FeCl_3 . Reduction of 0.013 mM FeCl_3 by NADPH reductase was determined by monitoring the increase in absorbance at 562 nm in the presence of the ferrous chelator ferrozine [26,27]. The reductase itself could not reduce FeCl_3 (Table 6). FeCl_3 was reduced when *P*-4502E1 was added to the reductase (Table 6). It was also reduced when paraquat was added to the reductase (Table 6), probably reflecting reductase-dependent formation of paraquat radical, followed by reduction of the FeCl_3 by paraquat radical or paraquat-radical-derived superoxide radical. Paraquat stimulated reduction of FeCl_3 by reductase alone and by reductase plus *P*-4502E1 (Table 6). There was no synergism in reduction of FeCl_3 by paraquat plus *P*-4502E1 over rates by *P*-4502E1 alone or paraquat alone.

DISCUSSION

Paraquat can undergo one-electron reduction as catalysed by flavin reductases such as NADPH-cytochrome *P*-450 reductase to form the paraquat radical. In the presence of oxygen, the paraquat radical rapidly autoxidizes to produce superoxide radical, and subsequently H_2O_2 . In the presence of metal catalysts such as iron, paraquat radical can increase the production of $\cdot\text{OH}$ and other reactive oxygen species capable of initiating lipid peroxidation and chemiluminescence [11,13,18–22]. Paraquat radical has also been shown to catalyse the reduction of ferric iron [25–28]. Previous studies [2,3] showed that H_2O_2 and non-haem iron play critical roles in the glycerol-oxidation pathway and suggested that cytochrome *P*-450 may function to generate the H_2O_2 and reduce the non-haem iron, although additional roles for *P*-450 were also suggested. It was considered that paraquat could stimulate oxidation of glycerol to formaldehyde via either direct interaction of paraquat radical with the glycerol or paraquat-dependent increases in H_2O_2 production and ferric reduction. A more fundamental question was whether paraquat could effectively replace *P*-450 in the overall pathway of glycerol oxidation, thereby providing evidence that a major function of *P*-450 is to provide H_2O_2 and/or reduce the ferric catalyst.

Paraquat stimulated the oxidation of glycerol to formaldehyde; the stimulation was inhibited by catalase, EDTA and desferri-

oxamine, indicating that H_2O_2 and iron are required for glycerol oxidation in the presence (as well as the absence) of paraquat, thus ruling out a mechanism based on direct oxidation of glycerol to formaldehyde by paraquat radical. The lack of effect of superoxide dismutase indicates that elevated production of superoxide as a result of autooxidation of paraquat radical is not directly responsible for the increased formaldehyde production, although the elevated superoxide generation is likely to be the cause of increased H_2O_2 production. In the presence of iron, paraquat markedly increases $\cdot\text{OH}$ production by microsomes and NADPH-cytochrome *P*-450 reductase [23,24,34,37]. The lack of effect of $\cdot\text{OH}$ scavengers and the inhibition by EDTA indicates that elevated production of $\cdot\text{OH}$ is not responsible for the increase in formaldehyde generation. In the absence of glycerol, paraquat did not cause production of formaldehyde; paraquat is not significantly metabolized by mixed-function oxidation systems [46]. Paraquat does not bind bi- or tri-valent metal cations [8], and it stimulated glycerol oxidation even in the presence of large amounts of exogenously added iron. It would appear that elevated production of H_2O_2 and/or reduction of iron play important roles in the stimulation by paraquat of formaldehyde production from glycerol.

One major difference in the presence of paraquat was that the desferrioxamine inhibition curve was shifted so that much higher concentrations of desferrioxamine were required to inhibit formaldehyde production. Microsomes have been shown to contain pools of tightly bound iron [40,41], and it is possible that paraquat via its redox cycling activity may change the accessibility of the microsomal iron to desferrioxamine. Alternatively, desferrioxamine binds iron much more effectively in the ferric redox state (formation constant for ferric-desferrioxamine is $10^{30.7}$) than the ferrous redox state (formation constant for ferrous-desferrioxamine is 10^{11}) [47,48]. Reduction of iron by paraquat radical to the ferrous redox state may necessitate the addition of more desferrioxamine for inhibition of formaldehyde production in the presence than in the absence of paraquat. Moreover, it has been pointed out that desferrioxamine may be less effective in prevention of rapid Fenton-types of reaction between iron and paraquat radical and H_2O_2 because of the strong reducing potential of the paraquat radical [18,49]. Hence higher concentrations of the chelator may be required in the presence of paraquat for effective inhibition.

Can paraquat substitute for cytochrome *P*-450 in the glycerol-oxidation reaction system? Paraquat increases H_2O_2 production and reduces iron in the absence of *P*-450, and therefore should replace *P*-450 if the major functions of the haemoprotein are to generate H_2O_2 and reduce non-haem iron. Results indicate that paraquat can react with the reductase to catalyse the oxidation of glycerol to formaldehyde. The inability of the purified reductase to oxidize glycerol to formaldehyde relates to the lack of H_2O_2 production and reduction of FeCl_3 by this system, whereas production of H_2O_2 (Table 4) and the reduction of FeCl_3 (Table 6) occur when paraquat is added to the reductase. These results support the suggestion that cytochrome *P*-450 is necessary in the glycerol-oxidation pathway in order to produce H_2O_2 and to reduce FeCl_3 as addition of *P*-4502E1 to the reductase resulted in H_2O_2 generation (Table 4) and reduction of non-haem iron (Table 6). However, it appears that *P*-450 can further accelerate the oxidation of glycerol to formaldehyde by microsomes in the presence of paraquat. The paraquat stimulation was sensitive to miconazole, to substrates and ligands of *P*-4502E1 such as aniline and 4-methylpyrazole, to an inhibitor of the NADPH oxidase activity of *P*-4502E1 (tryptamine) and to antibody against *P*-4502E1. Although paraquat could indeed interact directly with the reductase and catalyse formaldehyde production, suggesting

that it at least to some extent can replace *P*-450, the addition of *P*-450 to the reductase plus paraquat system caused a further increase in formaldehyde production. The increase produced by *P*-450 was inhibited by anti-(*P*-4502E1) IgG, indicating a specific catalytic role for the *P*-450. The paraquat and *P*-450 seem to synergistically interact with each other to promote formaldehyde generation from glycerol, as rates in the presence of reductase plus *P*-450 plus paraquat (5.4 nmol/min; Table 4) are more than the sum of the rates for the reductase plus *P*-450 (1 nmol/min) and reductase plus paraquat (1.2 nmol/min), i.e. additive rates 2.2 nmol/min. These results suggest the possibility that the *P*-450, besides generating H_2O_2 and reducing non-haem iron, plays other roles in the mechanism of glycerol oxidation to formaldehyde, perhaps binding the substrate, the oxidant or paraquat or providing a favourable localized environment for interaction to occur. The more than additive effect on glycerol oxidation is not due to synergistic increases in H_2O_2 production or reduction of FeCl_3 . There are studies that suggest the interaction of paraquat or paraquat radicals with cytochrome *P*-450, as is evident from the use of paraquat to mediate the reduction of *P*-450 by dithionite [50]. In preliminary studies, we observed, in the absence of substrate, a spectral interaction on the addition of paraquat to *P*-4502E1, with a peak at about 435 nm and a trough at about 415 nm. Cytochrome *P*-450 has been shown to catalyse the one-electron reduction of quinones such as *N*-acetyl-*p*-benzoquinone imine or mitomycin [51,52]. In studies with quinones, reconstituted systems containing reductase plus *P*-450 were more effective in reducing the quinones and generating reactive oxygen radicals than systems containing reductase alone; moreover, with microsomes, quinone reduction and H_2O_2 generation were inhibited by ligands and inhibitors of *P*-450 [51,52]. These results are similar to those characterizing the ability of paraquat to increase formaldehyde production from glycerol, and suggest a role for *P*-450 in the overall reaction mechanism, even in the presence of paraquat.

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REFERENCES

- Winters, D. K. and Cederbaum, A. I. (1990) *Biochem. Pharmacol.* **39**, 697–705
- Clejan, L. A. and Cederbaum, A. I. (1991) *Arch. Biochem. Biophys.* **285**, 83–89
- Clejan, L. A. and Cederbaum, A. I. (1992) *FASEB J.* **6**, 765–770
- Bus, J. S., Aust, S. D. and Gibson, J. E. (1974) *Biochem. Biophys. Res. Commun.* **58**, 749–755
- Bus, J. S., Aust, S. D. and Gibson, J. E. (1976) *Environ. Hlth. Perspect.* **16**, 139–146
- Kappus, H. and Sies, H. (1981) *Experientia* **37**, 1233–1241
- Youngman, R. J. and Elstner, E. F. (1981) *FEBS Lett.* **129**, 265–268
- Trush, M. A., Mimnaugh, E. G., Ginsburg, E. and Gram, T. E. (1981) *Toxicol. Appl. Pharmacol.* **60**, 279–286
- Aldrich, T. K., Fisher, A. B., Cadenas, E. and Chance, B. (1983) *J. Lab. Clin. Med.* **101**, 66–73
- Trush, M. A., Mimnaugh, E. G., Ginsburg, E. and Gram, T. E. (1982) *Biochem. Pharmacol.* **31**, 805–814
- Cadenas, E., Brigelius, R. and Sies, H. (1983) *Biochem. Pharmacol.* **32**, 147–150
- Burk, R. F., Lawrence, R. A. and Lane, J. M. (1980) *J. Clin. Invest.* **65**, 1024–1031
- Steffen, C. and Netter, K. J. (1979) *Toxicol. Appl. Pharmacol.* **47**, 593–602
- Kornbrust, D. J. and Mavis, R. D. (1980) *Toxicol. Appl. Pharmacol.* **53**, 323–332
- Misra, H. P. and Gorsky, L. D. (1981) *J. Biol. Chem.* **256**, 9994–9998
- Steffen, C., Muliawan, H. and Kappus, H. (1980) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **310**, 241–243
- Sandy, M. S., Moldeus, P., Ross, D. and Smith, M. T. (1986) *Biochem. Pharmacol.* **35**, 3095–3101
- Borg, D. C. and Schaich, K. M. (1984) *Isr. J. Chem.* **24**, 38–53
- Kohen, R. and Chevion, M. (1985) *Biochem. Pharmacol.* **34**, 1841–1843
- Kohen, R. and Chevion, M. (1985) *Free Radic. Res. Commun.* **1**, 79–88
- Younes, M., Cornelius, S. and Siegers, C. P. (1986) *Res. Commun. Chem. Pathol. Pharmacol.* **51**, 89–99
- Puntarulo, S. and Cederbaum, A. I. (1989) *Biochem. Pharmacol.* **38**, 2911–2918
- Clejan, L. A. and Cederbaum, A. I. (1989) *Biochem. Pharmacol.* **38**, 1779–1786
- Yamazaki, I., Piette, L. H. and Grover, T. A. (1990) *J. Biol. Chem.* **265**, 652–659
- Sutton, H. C. and Winterbourn, C. C. (1984) *Arch. Biochem. Biophys.* **235**, 106–115
- Winterbourn, C. and Sutton, H. C. (1984) *Arch. Biochem. Biophys.* **235**, 116–126
- Vile, G. F., Winterbourn, C. C. and Sutton, H. C. (1987) *Arch. Biochem. Biophys.* **259**, 616–626
- Richmond, R. and Halliwell, B. (1982) *J. Inorg. Biochem.* **17**, 95–107
- Palakodety, R. B., Clejan, L. A., Krikun, G., Feierman, D. E. and Cederbaum, A. I. (1988) *J. Biol. Chem.* **263**, 878–884
- Guengerich, P. F. and Martin, M. V. (1980) *Arch. Biochem. Biophys.* **205**, 365–379
- Nash, T. (1953) *Biochem. J.* **55**, 416–421
- Clejan, L. A., Koop, D. R. and Cederbaum, A. I. (1989) *Drug Metab. Dispos.* **17**, 694–698
- Omura, T. and Sato, R. (1964) *J. Biol. Chem.* **237**, 2370–2378
- Dicker, E. and Cederbaum, A. I. (1991) *Biochem. Pharmacol.* **42**, 529–535
- Yoo, J. S. H., Cheung, R. J., Patten, C. J., Wade, D. and Yang, C. S. (1987) *Cancer Res.* **47**, 3378–3383
- Ko, I. Y., Park, S. S., Song, B. J., Patten, C., Tan, Y., Hah, Y. C., Yang, C. S. and Gelboin, H. V. (1987) *Cancer Res.* **47**, 3101–3109
- Belouqui, O. and Cederbaum, A. I. (1985) *Arch. Biochem. Biophys.* **242**, 187–196
- Yoshida, Y. and Aoyama, Y. (1987) *Biochem. Pharmacol.* **36**, 229–235
- Persson, Y., Terelius, Y. and Ingelman-Sundberg, M. (1990) *Xenobiotica* **20**, 887–900
- Vile, G. F. and Winterbourn, C. C. (1987) *FEBS Lett.* **215**, 151–154
- Minotti, G. (1989) *Arch. Biochem. Biophys.* **268**, 398–403
- Levin, W., Thomas, P. E., Oldfield, N. and Ryan, D. E. (1986) *Arch. Biochem. Biophys.* **248**, 158–165
- Patten, C. J., Ning, S. M., Lu, A. Y. H. and Yang, C. S. (1986) *Arch. Biochem. Biophys.* **251**, 629–638
- Ingelman-Sundberg, M. and Johansson, I. (1980) *Biochem. Biophys. Res. Commun.* **97**, 582–589
- Clejan, L. A. and Cederbaum, A. I. (1990) *Biochim. Biophys. Acta* **1034**, 233–237
- Daniel, J. W. and Gage, J. G. (1966) *Br. J. Ind. Med.* **23**, 133–136
- Aisen, P. (1977) Some physicochemical aspects of iron metabolism. *Ciba Found. Symp.* **51**, 1–19
- Rosen, H. and Klebanoff, S. J. (1981) *Arch. Biochem. Biophys.* **208**, 512–519
- Osheroff, M. R., Schaich, K. M., Drew, R. T. and Borg, D. C. (1985) *J. Free Radic. Biol. Med.* **1**, 71–82
- Petersen, J. A., White, R. E., Yasukochi, Y., Coomes, M. L., O'Keefe, D. H., Ebel, R. E., Masters, B. S. S., Ballou, D. P. and Coon, M. J. (1977) *J. Biol. Chem.* **252**, 4431–4434
- Van De Straat, R., De Vries, J. and Vermuelen, N. P. E. (1987) *Biochem. Pharmacol.* **36**, 613–619
- Vromans, R. M., Van De Straat, R., Groeneneld, M. and Vermeulen, N. P. E. (1990) *Xenobiotica* **20**, 967–978