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Mechanism of paraquat-stimulated lipid peroxidation in mouse brain and pulmonary microsomes

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Abstract—Paraquat-stimulated NADPH-dependent lipid peroxidation in mouse brain and pulmonary microsomes was inhibited by superoxide dismutase and singlet oxygen quenchers, but not by catalase or hydroxyl radical scavengers. $MnCl_2$, which might form a salt with unsaturated lipid, inhibited the lipid peroxidation in brain microsomes, but not that in pulmonary microsomes. These findings suggest that activated oxygen species, especially superoxide and singlet oxygen, may play a major role in the stimulation of microsomal lipid peroxidation by paraquat in both brain and lung, and that the nature of the lipids exposed to peroxidative attack may be different in microsomes of the two organs.

Paraquat is a widely used, non-selective herbicide. Unfortunately, the herbicide is toxic and is often lethal to animals, including man (Smith & Heath 1976). The major lesion caused by paraquat is seen in the lung, and it has been proposed that this is due, at least in part, to an energy-dependent accumulation of the herbicide in this organ (Rose et al 1976). Lipid peroxidation following generation of superoxide anion derived from redox cycling of paraquat was suggested as a possible mechanism of the toxic effect of paraquat (Bus & Gibson 1984). However, in-vivo studies have failed to demonstrate a correlation between pulmonary injury and lipid peroxidation following paraquat treatment (Keeling & Smith 1982; Ogata & Manabe 1990), although high concentrations of oxygen enhance the paraquat toxicity (Selman et al 1985). In addition, there have been reports indicating both stimulation and inhibition of lipid peroxidation by paraquat in-vitro (Bus et al 1974; Misra & Gorsky 1981; Trush et al 1981; Cadenas et al 1983).

Recently, we (Hara et al 1991) have demonstrated that paraquat inhibited microsomal NADPH-dependent lipid peroxidation in mouse hepatic microsomes and stimulated it in brain microsomes, both in a dose-dependent manner. In mouse pulmonary microsomes, paraquat stimulated lipid peroxidation, though to a lesser extent than in brain microsomes, and only over a narrow concentration range. In addition, paraquat underwent an NADPH-dependent reaction in microsomes of mouse brain, but not in liver, lung or kidney (Hara et al 1989).

The present study was designed to examine the mechanism of stimulation of lipid peroxidation by paraquat in brain and pulmonary microsomes.

Materials and methods

Chemicals. Paraquat, superoxide dismutase (SOD) and catalase were purchased from Sigma Chemical Co., St. Louis, MO, USA. 2,5-Dimethylfuran was from Aldrich Chemical Co., Milwaukee, WI, USA. 2-Thiobarbituric acid (TBA) was from E. Merck, Darmstadt, Germany. NADPH was from Boehringer Mannheim GmbH, Mannheim, Germany. Other chemicals were from Wako Pure Chemical Industries, Tokyo, Japan.

Mannitol, benzoate and ethanol were used as hydroxyl radical scavengers (Kameda et al 1979; Misra & Gorsky 1981; Iba & Mannering 1987). 2,5-Dimethylfuran and histidine are singlet oxygen quenchers, although the latter quencher also has an ability to scavenge the hydroxyl radical (Misra & Gorsky 1981).

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Animals. Male ICR mice, 24–28 g, were purchased from Charles River Japan, Kanagawa, Japan, and were housed in an air-conditioned (22–24 C) room on a 12 h light-dark cycle, with free access to food and water.

Preparation of microsomes. Animals were killed by decapitation for preparation of brain microsomes or by exsanguination after they had been stunned by a blow on the neck for preparation of pulmonary microsomes. Blood was cleared from the lungs by infusing 1·15% KCl into the portal vein. Brains or lungs removed from about fifty mice were pooled and homogenized in 4 or 9 vols of ice-cold 1·15% KCl solution, respectively. The homogenate was centrifuged at 9000 *g* for 20 min. The supernatant was removed, and the pellet was again homogenized in an adequate volume of the solution and recentrifuged under the above conditions. The first and the second supernatants were combined and centrifuged at 105 000 *g* for 60 min. Microsomes were washed by suspension in 1·15% KCl and subsequent sedimention at 105 000 *g* for 30 min. The washed microsomes were suspended in the KCl solution and used for all experiments. These procedures were carried out at 0–4 C.

Protein concentration was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Determination of microsomal lipid peroxidation. Microsomal lipid peroxidation was estimated by measuring malondialdehyde (MDA) as TBA-reactive material. The incubation mixture consisted of microsomes (0·2 mg protein), 0·1 M phosphate buffer (pH 7·4), 30·8 mM KCl, 20 μM $FeSO_4$, paraquat and 0·5 mM NADPH in a total volume of 0·5 mL. Incubation was carried out at 37 C for 30 min. The concentrations of paraquat added were 1 and 0·1 mM for brain and pulmonary microsomes, respectively, since these concentrations induced maximum stimulation of lipid peroxidation in the respective microsomes (Hara et al 1991). The reaction was started by the addition of NADPH and terminated by the addition of 0·5 mL of ice-cold 10% trichloroacetic acid. After centrifugation, 0·25 mL of the protein-free supernatant was transferred to a tube containing 1·5 mL of 0·67% TBA solution. The mixture was boiled for 10 min and then quickly cooled to room temperature. MDA concentration was determined from the absorbance at 532 nm based on a molar extinction coefficient of $1{\cdot}56 \times 10^5$ (Wills 1969).

The data were expressed as means of two separate experiments. The difference between each datum and the corresponding mean did not exceed 10% of the mean.

Results

Paraquat stimulated NADPH-dependent lipid peroxidation in brain and pulmonary microsomes, resulting in 7·6- and 1·7-fold increases in MDA formation, respectively (Table 1).

Effects of scavengers and quenchers of active oxygen species on microsomal lipid peroxidation. SOD provided significant protection against lipid peroxidation in both brain and pulmonary

Table 1. Effects of superoxide dismutase and catalase on paraquat-stimulated lipid peroxidation in mouse brain and pulmonary microsomes.

Additions		nmol of malondialdehyde (mg protein)$^{-1}$/30 min Brain	Lung
None		2·31	5·00
Paraquat	(μg)	17·47 (100·0)	8·69 (100·0)
+superoxide dismutase	25	0·39 (2·2)	1·70 (19·6)
	50	0·55 (3·1)	1·92 (22·1)
+ catalase	50	25·66 (146·9)	11·51 (132·5)
	250	27·76 (158·9)	8·85 (101·8)

Numbers in parentheses represent per cent of the corresponding value in the presence of paraquat alone.

microsomes, while catalase enhanced lipid peroxidation (Table 1). Mannitol, benzoate and ethanol had little effect on the MDA formation in brain or pulmonary microsomes (Table 2); however, histidine and dimethylfuran, decreased MDA formation in both types of microsomes (Table 2).

Effects of EDTA and $MnCl_2$ on microsomal lipid peroxidation. Microsomal lipid peroxidation in the presence of paraquat was almost completely prevented by EDTA, in both brain and lung. $MnCl_2$ significantly decreased MDA formation in brain microsomes, whereas it slightly enhanced MDA formation in pulmonary microsomes (Table 3).

Table 2. Effects of hydroxyl radical and singlet oxygen scavenger/quenchers on paraquat-stimulated lipid peroxidation in mouse brain and pulmonary microsomes.

Additions		nmol of malondialdehyde (mg protein)$^{-1}$/30 min Brain	Lung
Paraquat	(mM)	17·86 (100·0)	8·91 (100·0)
+mannitol	3	19·75 (110·6)	9·52 (106·8)
	6	19·71 (110·4)	9·78 (109·8)
+benzoate	3	17·34 (97·1)	8·94 (100·3)
	6	17·66 (98·9)	8·40 (94·3)
+ethanol	6	18·49 (103·5)	8·91 (100·0)
	8	18·40 (103·0)	9·10 (102·1)
+histidine	6	11·57 (64·8)	7·05 (79·1)
	8	11·41 (63·9)	5·29 (59·4)
+dimethylfuran	10	14·55 (81·5)	5·61 (63·0)

Numbers in parentheses represent per cent of the corresponding value in the presence of paraquat alone.

Table 3. Effects of EDTA and $MnCl_2$ on paraquat-stimulated lipid peroxidation in mouse brain and pulmonary microsomes.

Additions		nmol of malondialdehyde (mg protein)$^{-1}$/30 min Brain	Lung
Paraquat	(mM)	17·86 (100·0)	8·91 (100·0)
+EDTA	0·1	0·23 (1·3)	0·55 (6·2)
+$MnCl_2$	1	2·28 (12·8)	9·94 (112·8)

Numbers in parentheses represent per cent of the corresponding value in the presence of paraquat alone.

Discussion

Bus et al (1974) reported stimulation of lipid peroxidation by paraquat in-vitro and suggested the involvement of superoxide anions and singlet oxygen. The present observations, that SOD and quenchers of singlet oxygen decrease the stimulation of lipid peroxidation by paraquat in both brain and pulmonary microsomes, support their suggestion. The significant suppression by a chelator, EDTA, of MDA formation in both brain and pulmonary microsomes may indicate an important role of ferric iron in the peroxidation. In view of the lack of protective effects of catalase and hydroxyl radical scavengers, however, it seems that hydrogen peroxide and the hydroxyl radical may have little or no role in the lipid peroxidation. Thus, the mechanism of stimulation of lipid peroxidation by paraquat in microsomes may not be similar to those associated with the iron-catalyzed Haber-Weiss and Fenton reactions generally accepted as the mechanisms of oxidative stress (Halliwell & Gutteridge 1986).

On the other hand, paraquat potentiated lipid peroxidation in brain microsomes more strongly than it did in pulmonary microsomes. Previous experimental evidence has shown that NADPH-cytochrome P450 reductase, which is responsible for reduction of paraquat, yielding the paraquat radical, is involved in the stimulation of lipid peroxidation (Bus et al 1974; Horton et al 1986). This also appears to be the case in both brain and pulmonary microsomes, as indicated by our recent studies (Hara et al 1991) in which paraquat-stimulated lipid peroxidation was suppressed by *N*-ethylmaleimide, a sulphydryl agent, in parallel with its inhibitory potency on NADPH-cytochrome P450 reductase activity in the two types of microsomes. However, the enzyme activity of pulmonary microsomes was about 7-fold higher than that of brain microsomes. Furthermore, paraquat radical formation determined under anaerobic conditions did not parallel the potency of paraquat to stimulate lipid peroxidation in both types of microsomes. Cadenas et al (1983) suggested that changes in electron flow through NADPH-cytochrome P450 reductase were caused by paraquat's effects on MDA formation and the appearance of chemiluminescence, associated with oxidative stress. Thus, the electron flow leading to lipid peroxidation might not be the same in brain and pulmonary microsomes in the presence of paraquat. In addition, Misra & Gorsky (1981) suggested that inhibition of lipid peroxidation by $MnCl_2$ might be due to the formation of a lipid-Mn complex, which would be more resistant to peroxidative attack. Therefore, the significant inhibition by $MnCl_2$ of lipid peroxidation in brain microsomes and the absence of such inhibition in pulmonary microsomes suggest that the nature of the lipids exposed to peroxidative attack may differ between the microsomes of the two organs.

We suggest that, under conditions where paraquat stimulates NADPH-dependent lipid peroxidation, generation of superoxide anion and singlet oxygen rather than hydrogen peroxide and the hydroxyl radical may play the major role in microsomal lipid peroxidation in both brain and lung. In addition, we hypothesize that the diferent degrees of vulnerability of brain and pulmonary microsomes to the paraquat-stimulated peroxidation might be at least in part due to differences in the nature of the lipids composing them. Thus, the effects of paraquat on various organs appear to be complex. Since it has been found that various organs, including brain and lung, are damaged during paraquat poisoning (Vale et al 1987), further studies are needed to verify whether paraquat toxicity is elicited by a uniform mechanism in all organs.

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The role of the gastric mucosal sulphydryls in the ulcer-protecting effects of sulphasalazine

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Abstract—The role of gastric mucosal sulphydryls in gastric ulceration, produced by restraint at 4°C (stress) for 2 h, and in the ulcer-protecting effects of sulphasalazine and its constituents (sulphapyridine and 5-aminosalicylic acid), have been studied in rats. Stress significantly raised the mucosal sulphydryl content, but sulphasalazine and sulphapyridine did not influence these changes; only 5-aminosalicylic acid decreased the mucosal sulphydryl concentration. These results indicate that depletion of mucosal sulphydryls does not occur in stress-induced ulceration, in contrast to what has been shown in other experimental ulcer models. The antiulcer effects of sulphasalazine or of any of its constituents may, therefore, not involve the sulphydryl mechanism.

The role of sulphydryls in gastric mucosal protection is unclear. Decreased stomach wall sulphydryls accompany ethanol-induced gastric mucosal damage (Szabo et al 1981); paradoxically, their depletion by diethylmaleate antagonizes ethanol-evoked lesions (Robert et al 1984). Thus, evidence for a direct relationship between gastric sulphydryls and mucosal susceptibility to ethanol-induced lesions is equivocal. We have studied the participation of mucosal sulphydryls in stomach ulcers produced by cold-restraint stress, and we have also looked at their relationship to the antiulcer action of sulphasalazine and its constituents (sulphapyridine and 5-aminosalicylic acid) (Garg et al 1990), which might be expected to be mediated through the sulphydryl mechanism because the drug possesses a sulphur atom in the side chain.

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Materials and methods

Female Sprague-Dawley rats, 150–190 g, were starved for 48 h before use but were allowed to drink a solution of sucrose 8% in NaCl 0·2% w/v which was removed 1 h before the experiment. Sulphasalazine (Sigma), sulphapyridine (Sigma) or 5-aminosalicylic acid (Sigma), freshly dissolved in 0·1 M NaOH, was injected s.c. 30 min before the animals were restrained in individual wire-mesh tubular cages at 4°C (stress) for 2 h; controls received a similar volume (5 mL kg^{-1}) of vehicle. Non-stressed rats were left in their cages (temperature 23 ± 1°C, humidity 65–70%). All animals were killed by a sharp blow on the head after 2 h. Their stomachs were removed, opened, rinsed in ice-cold sodium phosphate buffer (0·2 M, pH 8·0), and quickly placed on an ice-cold surface for scraping off the glandular mucosa. The scrapings were then suspended in 1 mL sodium phosphate buffer, homogenized, made up to 2 mL with buffer, and centrifuged at 5000 rev min^{-1} for 10 min at 4°C. The supernatant sulphydryl content was determined (Sedlak & Lindsay 1968); protein sulphydryl levels were obtained by subtracting non-protein sulphydryl values from that of the total sulphydryls. Light absorbance at 412 nm, against a reagent blank, was measured with a spectrophotometer (Varian, Cary 219). Sul-