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SHORT COMMUNICATION

Paraquat Toxicity and Oxidative Damage

REDUCTION BY MELATONIN

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ABSTRACT. The ability of melatonin to protect against paraquat-induced oxidative damage in rat lung, liver, and serum was examined. Changes in the levels of malondialdehyde (MDA) plus 4-hydroxyalkenals (4-HDA) and reduced and oxidized glutathione concentrations were measured. Paraquat (50 mg/kg) was injected i.p. into either Sprague–Dawley or Wistar rats with or without the co-administration of 5 mg/kg melatonin. Paraquat alone increased MDA + 4-HDA levels in serum and lungs of both rat strains, with these increases being abolished by melatonin co-treatment. Paraquat also decreased reduced glutathione levels and increased oxidized glutathione concentrations in lung and liver; these changes were negated by melatonin. The effect of melatonin on paraquat-induced mortality was also studied. Paraquat at a dose of 79 mg/kg was lethal for 50% of animals within 24 hr; when administered together with melatonin, the LD_{50} for paraquat increased to 251 mg/kg. BIOCHEM PHARMACOL 51;8:1095–1099, 1996.

KEY WORDS. melatonin as an antioxidant; paraquat-induced lipid peroxidation; reduced and oxidized glutathione; paraquat LD_{50}; oxidative damage to lung and liver; free radical-induced tissue damage

Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) is a widely used herbicide known to cause fatal intoxication in both humans and animals. Although paraquat toxicity affects lungs, liver brain, kidneys, and other organs [1–4], experimental and clinical deaths are often caused by severe lung inflammation and interstitial fibrosis resulting in respiratory failure [1]. This is due, at least in part, to an energy-dependent accumulation of the herbicide in the lung [5, 6]. Paraquat undergoes a one-electron reduction by the flavoenzyme NADPH–cytochrome P450 reductase, which results in the generation of the paraquat radical [2]. In the presence of oxygen, the paraquat radical rapidly auto-oxidizes to produce a superoxide anion radical and regenerates the paraquat di-cation. Thus, in the presence of a sufficient supply of reducing equivalents, repeated cycles of herbicide reduction and re-oxidation can occur, producing large amounts of reactive oxygen species, oxidative stress, and lipid peroxidation [2, 7].

Recently, melatonin was proven to be a potent hydroxyl and peroxyl radical scavenger [8, 9]. The pineal hormone is highly lipophilic [10] and quite hydrophilic as well [11], and thus its pharmacological use may potentially protect against oxygen toxicity occurring anywhere in the organism [12, 13].

In the present study, we examined the ability of melatonin (5 mg/kg, i.p.) to protect against paraquat induced-oxidative damage in rat serum, lung, and liver. Both Wistar and Sprague–Dawley rats were used to determine whether there were strain differences in sensitivity to either paraquat or melatonin. Moreover, the effects of melatonin on paraquat toxicity were evaluated by injecting different doses of the herbicide (from 50 to 480 mg/kg) with or without melatonin co-treatment, to test whether melatonin prolongs survival following administration of this toxic agent.

MATERIALS AND METHODS

Chemicals

All reagents were of the highest quality available. Paraquat (purity = 98%) was purchased from Chem Service (West Chester, PA). Melatonin, saturated picric acid, NADPH tetrasodium salt, DTNB§, GSH, and glutathione reductase were obtained from the Sigma Chemical Co. (St. Louis, MO). 2-Vinyl-pyridine monomer was purchased from Fluka (Ronkonkoma, NY).

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§ *Abbreviations:* DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MDA, malondialdehyde; 4-HDA, 4-hydroxyalkenals; GSH, reduced glutathione; GSSG, oxidized glutathione; and c.l., confidence limits.

Animals and Methods

Sprague–Dawley and Wistar adult male rats (body weight 200–230 g) were obtained from Harlan (Houston, TX) and housed in Plexiglas cages with 3 animals per cage. The animals rooms were windowless with automatic temperature controls (22 ± 1°) and lighting (light on 7:00 a.m. and off 9:00 p.m.; 14 hr light/10 hr dark). The rats received standard laboratory chow and water *ad lib*.

Paraquat was dissolved in saline and administered i.p. Melatonin was dissolved in absolute ethanol (the alcohol concentration in the final solution was 4%) and also administered i.p.

Oxidative Stress Study

After 1 week of acclimation, 32 Sprague–Dawley and 32 Wistar rats were divided into 4 groups of 8 animals each: control group, paraquat-treated group, paraquat + melatonin group, and melatonin-treated group. Paraquat was administered i.p. at a single dose of 50 mg/kg at 10:00 a.m. The paraquat injections were standardized to 10.00 a.m. to take advantage of the low endogenous melatonin levels that exist at this time. Melatonin (1 mg/kg) was injected five times: 30 min before paraquat administration and four more times over an 8-h interval. The aim of this injection scheme was to keep constantly high melatonin levels during the interval immediately following paraquat administration. All animals were killed by decapitation 24 hr after paraquat administration.

Tissue Preparation and Assay

Upon decapitation, blood was collected, mixed with potassium EDTA (0.17 mM), and immediately centrifuged at 2500 *g* for 15 min at 4°. Plasma was frozen at −80° until the day of the assay. The lungs and liver were dissected from each rat and prepared as previously described [12]. The Bioxytech LPO-586 kit, purchased from Cayman Chemical (Ann Arbor, MI), was used for these measurements [12]. GSH and GSSG concentrations in lung and liver homogenates were determined as described by Griffith [14]. Proteins were measured using the procedure of Lowry *et al.* [15] with albumin as standard.

LD_{50} Study

To determine whether melatonin would protect against paraquat-induced mortality, 80 Wistar rats were used. After 1 week of acclimation, the animals were divide into 16 groups of 5 animals each. Paraquat was given as a single i.p. injection in the morning. Eight groups of rats received either 50, 70, 95, 135, 270, 340, 400, or 480 mg/kg paraquat; the other 8 groups of rats were treated with paraquat and also received a total of 50 mg/kg melatonin. Melatonin was injected five times (10 mg/kg melatonin at each injection): 30 min before paraquat and then 2, 6, 10, and 14 hr after the herbicide was administered. The number of surviving animals was counted 24 hr after paraquat administration.

Statistical Analysis

Lipid peroxidation and glutathione data were analyzed by ANOVA followed by the Student–Newman–Keuls test. The level of significance was accepted as $P < 0.05$. Data for the lethality study were analyzed according to Tallarida and Murray [16].

RESULTS

Oxidative Stress Study

In Sprague–Dawley and Wistar rats, paraquat (50 mg/kg), when followed by saline injections, increased the indices of lipid peroxidation in serum by 60 and 35%, respectively, above levels found in tissues of control animals, within 24 hr (Table 1). When rats were also treated with melatonin (1 mg/kg × 5), the increases induced by paraquat were prevented.

In both Sprague–Dawley and Wistar rats, paraquat itself increased lipid peroxidation in the lungs by roughly 30% above the levels found in control animals (Table 1). These increases were abolished by treatment with melatonin. Melatonin alone did not change the level of lipid peroxidation compared with control animals.

TABLE 1. Effect of melatonin (1 mg/kg × 5) on paraquat (50 mg/kg)-induced lipid peroxidation in serum, lung, and liver of Sprague-Dawley and Wistar rats

	MDA + 4-HDA concentrations (nmol/mg protein)					
	Serum		Lung		Liver	
	SD	WI	SD	WI	SD	WI
Control	10.0 ± 2.0	10.0 ± 1.0	2.3 ± 0.1	2.8 ± 0.8	0.9 ± 0.01	0.7 ± 0.03
Paraquat	16.0 ± 1.0*	13.5 ± 0.1†	3.0 ± 0.1†	3.7 ± 0.3‡	1.1 ± 0.02‡	0.9 ± 0.03‡
Paraquat + melatonin	11.0 ± 1	9.4 ± 0.3	2.1 ± 0.1	2.9 ± 0.1	0.8 ± 0.02	0.7 ± 0.20
Melatonin	10.2 ± 1.0	9.7 ± 1.0	2.3 ± 0.1	2.8 ± 0.2	0.9 ± 0.01	0.7 ± 0.10

Lipid peroxidation is represented by MDA + 4-HDA concentrations. Values are means ± SEM, N = 8. Abbreviations: SD, Sprague-Dawley; and WI, Wistar.
Significantly different from control: *$P < 0.005$, †$P < 0.001$, and ‡$P < 0.05$.

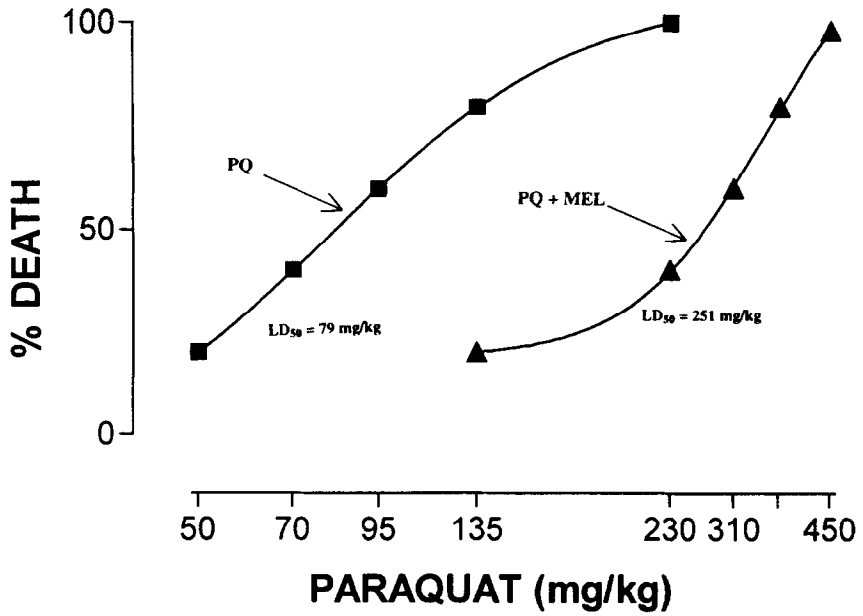

FIG. 1. Effect of melatonin (5 times 10 mg/kg body wt) on paraquat (50–450 mg/kg)-induced mortality. N = 5. Key: (■) paraquat; and (▲) paraquat + melatonin. Data were analyzed according to Tallarida and Murray [16]. Paraquat LD_{50}: 79 mg/kg body weight (c.l. 56–110); paraquat + melatonin LD_{50}: 251 mg/kg body weight (c.l. 230–270).

In the liver of Sprague–Dawley and Wistar rats, paraquat induced about 20 and 30% increases in lipid peroxidation, respectively, compared with that in control animals (Table 1). These effects were reversed by melatonin.

In the lungs and liver of both Sprague–Dawley and Wistar rats, paraquat elicited a decrease in GSH concentrations and a concomitant increase in GSSG levels (Table 2). Melatonin co-treatment abolished the alterations in the glutathione status induced by the herbicide.

LD_{50} *Study*

Mortality induced by paraquat and the protection conferred by melatonin were determined within 24 h following herbicide injection. The LD_{50} for paraquat alone was calculated as 79 mg/kg (c.l.56–110) (Fig. 1). The LD_{50} for paraquat plus melatonin was 251 mg/kg (c.l. 230–270) (Fig. 1).

DISCUSSION

Melatonin is known to be highly lipophilic and hydrophilic as well and to possess potent hydroxyl and peroxyl radical scavenging activity [8–10, 17]. *In vivo* and *in vitro* studies have shown that the pineal hormone counteracts the oxidative damage produced by different chemicals including bacterial lipopolysaccharide, kainate, and H_2O_2 [13, 18–20]. The protection conferred by melatonin against paraquat-induced toxicity as seen in the present studies is thus presumably dependent, at least in part, on its radical scavenging ability. This is supported by the findings described herein where lipid peroxidation products and the concentrations of GSH and GSSG were measured in lung, liver, and serum of both Sprague–Dawley and Wistar rats. In both rat strains, paraquat elicited a significant increase in lipid peroxidation as well as a decrease in GSH and a concomitant increase in GSSG concentration. These changes were

TABLE 2. Effect of melatonin (1 mg/kg × 5) on paraquat (50 mg/kg)-induced alterations in GSH and GSSG concentrations in the lungs and liver of Spraque-Dawley and Wistar rats

	GSH (μmol/g tissue)				GSSG (nmol/g tissue)			
	Lung		Liver		Lung		Liver	
	SD	WI	SD	WI	SD	WI	SD	WI
Control	1.2 ± 0.40	1.4 ± 0.11	4.7 ± 0.1	4.0 ± 0.2	45 ± 1	31 ± 1	125 ± 5	93 ± 2
Paraquat	0.6 ± 0.03*	1.0 ± 0.05†	3.5 ± 0.1†	3.0 ± 0.2†	51 ± 1*	45 ± 4†	170 ± 4*	119 ± 2‡
Paraquat + melatonin	1.0 ± 0.32	1.4 ± 0.01	4.6 ± 0.1	3.9 ± 0.1	39 ± 3	32 ± 1	128 ± 7	100 ± 5
Melatonin	1.0 ± 0.41	1.4 ± 0.05	4.6 ± 0.2	4.0 ± 0.2	42 ± 2	31 ± 2	117 ± 3	99 ± 2

Values are means ± SEM, N = 8. Abbreviations: SD, Sprague-Dawley; and WI, Wistar.
Significantly different from control: *$P < 0.001$, †$P < 0.05$, and ‡$P < 0.005$.

abolished *in toto* by melatonin co-treatment. The concentration of MDA + 4-HDA detectable in serum is accepted as an index of the general peroxidative damage to different tissues; thus, the high levels of these compounds detected in serum, in the present studies, is consistent with the findings of increased lipid peroxidation in both the lungs and liver. Also, the depletion of GSH with a concurrent rise in GSSG supports the involvement of reactive oxygen species in paraquat toxicity [21]. Depletion of GSH favors lipid peroxidation and consequently induces cell damage [22]. The results of the present studies correlate well with preliminary findings of increased lipid peroxidation in liver and lungs of rats treated with 70 mg/kg paraquat [12].

Results of the present studies also demonstrated that melatonin confers protection against paraquat-induced mortality. Of a total 40 rats (of both strains) that received paraquat of any dose, 30 of them died within 24 hr when not also given melatonin. Conversely, of 40 animals given both paraquat and melatonin, only 15 died within the 24-hr observation period. The 24-hr period was chosen because death occurring after this time period is primarily a result of organ dysfunction produced by the primary lesions and not directly correlated with the generation of free radicals. Most paraquat + melatonin-treated rats did eventually die within 64 hr following herbicide injection.

Studies of paraquat excretion, following its oral administration in rats, show that 64% of the herbicide is excreted in the feces and urine over 14 days. The remainder is retained in organs such as the lungs, liver, and kidney [23]. The half-life of melatonin is approximately 30 min. In our study, melatonin was administered to rats for only 14 hr after paraquat injection. Thus, it is possible that the protection of the pineal hormone against paraquat was eventually overcome by the herbicide which is normally present in the tissues for several days.

Prior to their death, paraquat-treated rats exhibited serious respiratory distress. Pulmonary toxicity which is a consequence of paraquat is well documented, but the exact biochemical mechanisms by which the herbicide causes cell damage in the lungs are not understood completely. The damage may be related, in part, to biological reduction-oxidation cycles of paraquat which involve molecular oxygen and reduced flavoprotein. As a result of the reduction-oxidation cycles of paraquat, oxygen radical generation, free radical-catalyzed lipid peroxidation, and membrane lysis presumably occur [7, 24–26]. *In vitro* experiments have demonstrated an enhancement of lipid peroxidation in lung, liver, and brain microsomes following paraquat administration [27–29]. *In vivo* studies have been less consistent, with both stimulation [30–34] and no effect on lipid peroxidation [35, 36] being reported.

Another hypothesis concerning paraquat toxicity assumes that the herbicide-induced tissue damage may be not necessarily or totally dependent on peroxidation of membrane phospholipids in cells. It has been suggested that NADPH is oxidized by oxygen free radicals generated in the cyclic reduction and re-oxidation of paraquat and that the resultant NADPH depletion may account for cell death by disturbing vital physiological and biochemical functions [37–39]. Because most GSSG is reduced rapidly by GSSG-reductase with NADPH as a reductant, GSSG increases when the NADPH supply becomes rate-limiting. In the case of a direct induction of lipid peroxidation or after an indirect impairment of cellular oxidative defenses by GSH depletion, an involvement of oxygen free radical reactions due to paraquat toxicity is very probable.

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