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Mitochondrial bioenergetics is affected by the herbicide paraquat

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Abstract

The potential toxicity of the herbicide paraquat (1,1-dimethyl-4,4′-bipyridylium dichloride) was tested in bioenergetic functions of isolated rat liver mitochondria. Paraquat increases the rate of State 4 respiration, doubling at 10 mM, indicating uncoupling effects. Additionally, State 3 respiration is depressed by about 15%, at 10 mM paraquat, whereas uncoupled respiration in the presence of CCCP is depressed by about 30%. Furthermore, paraquat partially inhibits the ATPase activity through a direct effect on this enzyme complex. However, at high concentrations (5–10 mM), the ATPase activity is stimulated, probably as consequence of the described uncoupling effect. Depression of respiratory activity is mediated through partial inhibitions of mitochondrial complexes III and IV. Paraquat depresses $\Delta\Psi$ as a function of herbicide concentration. In addition, the depolarization induced by ADP is decreased and repolarization is biphasic suggesting a double effect. Repolarization resumes at a level consistently higher than the initial level before ADP addition, for paraquat concentrations up to 10 mM. This particular effect is clear at 1 mM paraquat and tends to fade out with increasing concentrations of the herbicide.

Keywords: Paraquat; Mitochondrion; Herbicide toxicity; Superoxide

1. Introduction

Paraquat (1,1-dimethyl-4,4′-bipyridylium dichloride), the best known and most potent xenobiotic of the bipyridyl herbicides, is widely used in agriculture in more than 100 countries all over the world. The benefits of its use are, however, unfortunately accompanied by toxic effects in useful plants, animals and man [1,2].

The mechanism of action of paraquat has been suggested to be mediated by superoxide anions generated from paraquat radicals and by singlet oxygen derived from the anions [3–5]. Since the chemical and biological reactivity of the superoxide radical anion ($O_2^{\cdot-}$) in vitro and in vivo is relatively low, it is now widely accepted that superoxide formation may lead to the generation of the highly reactive hydroxyl radical through its reaction with various transition metal ions [6].

As judged from oral LD_{50} values, paraquat is considered of moderate toxicity (LD_{50} for rat 100 mg/kg) [1]. However, the frequent irreversibility of the injury produced by paraquat, the fact that humans may be more susceptible than the rat to this injury, and finally, the large number of human fatalities have directed much attention and interest of biochemical studies with this herbicide. Since the major oxidative metabolic processes of the cell take place in the mitochondrion and that this organelle is highly susceptible to the attack of active oxygen species, as those produced by paraquat metabolism, we tried to evaluate the effects of the herbicide paraquat on liver mitochondrial bioenergetics. Interactions on mitochondrial respiration, membrane potential and redox complexes activities are reported.

2. Methods

Chemicals. Paraquat (purity 98%) was a gift of ICI Agrochemicals, UK. All other chemicals were of the highest grade of purity commercially available.

Isolation of mitochondria. Mitochondria were isolated from liver of Male-Wistar rats (200–300 g) by conventional methods [7] with slight modifications. Homogenization medium contained 0.25 M sucrose, 5 mM Hepes (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA and 0.1% defatted bovine serum albumin. EGTA, EDTA and bovine serum

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albumin were omitted from the final washing medium, adjusted at pH 7.2. Protein was determined by the biuret method calibrated with bovine serum albumin [8].

Mitochondrial respiration. Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode [9] connected to a suitable recorder in a 1 ml thermostated water-jacketed closed chamber with magnetic stirring, at 25° C. The standard respiratory medium, adjusted to pH 7.2, consisted of 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM Hepes and 2 μM rotenone. Paraquat was added in aqueous solution to the reaction medium with mitochondria (1 mg protein) and allowed to incubate for 10 min before starting the reactions of energization. This time was chosen based on time-dependent herbicide effects, which were maximal at 10 min.

Membrane potential ($\Delta\Psi$) measurements. The mitochondrial transmembrane potential was estimated by calculating transmembrane distribution of TPP^+ (tetraphenylphosphonium) with a TPP^+-selective electrode prepared according to Kamo et al. [10] using a calomel electrode as the reference. TPP^+ uptake was measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Linear 1200 recorder. The voltage response of the TPP^+ electrode to log [TPP^+] was linear with a slope of 59 ± 1 mV, in close agreement with the Nernst equation. Calibration runs in the presence of paraquat excluded any direct interference of the herbicide on the electrode signal. Reactions were carried out in an open vessel, maintained at 25° C, with efficient magnetic stirring, in 1 ml of the standard respiratory medium supplemented with 4 μM TPP^+. This TPP^+ concentration was chosen in order to achieve high sensitivity in measurements and to avoid inhibitory effects on mitochondria reactivities and perturbations of $\Delta\Psi$ and ΔpH, since high concentrations would cause $\Delta\Psi$ to decline with a compensatory increase in ΔpH [11,12]. The $\Delta\Psi$ in mV was estimated by the following equation (at 25° C):

$$\Delta\Psi = 59\log(v/V) - 59\log(10^{\Delta E/59} - 1)$$

as indicated by Kamo et al. [10] and Muratsugu et al. [13]. v, V and ΔE stand for mitochondrial volume, volume of the incubation medium and deflection of the electrode potential from the baseline, respectively. This equation was derived assuming that TPP^+ distribution between mitochondria matrix and the medium follows the Nernst equation and that the law of mass conservation is applicable. A matrix volume of 1.1 μl per mg protein was assumed. No correction was made for the "passive" binding contribution of TPP^+ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in potentials rather than absolute values. As consequence, we can anticipate a slight overestimation for $\Delta\Psi$ values. However, the overestimation is only significant at $\Delta\Psi$ values below 90 mV, therefore, far from our measurements. Nevertheless, the non-specific binding of TPP^+ to the membrane was checked by using appropriate de-energizing inhibitors to demonstrate that herbicide does not affect this binding.

Enzymatic activities. Succinate dehydrogenase activity was measured polarographically [14] at 25° C in 1 ml of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 0.1 μg antimycin A, 1 mM KCN, 0.3 mg Triton X-100 and 0.5 mg mitochondria. The reaction was initiated by the addition of 1 mM phenazine methasulfate.

Cytochrome-*c* oxidase activity was measured polarographically [15] at 25° C in 1 ml of the standard reaction medium supplemented with 2 μM rotenone, 10 μM cytochrome *c*, 0.3 mg Triton X-100 and 0.5 mg mitochondria. The reaction was initiated by the addition of 5 mM ascorbate plus 0.25 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine).

Succinate-cytochrome-*c* reductase activity was measured spectrophotometrically [16] at 25° C by following the reduction of oxidized cytochrome *c* as an increase in absorbance at 550 nm. The reaction was initiated by the addition of 5 mM succinate to 2.0 ml of the standard reaction medium supplemented with 2 μM rotenone, 1 mM KCN, 54 μM cytochrome *c* and 0.8 mg of disrupted mitochondria (two cycles of freeze/thawing).

ATPase activity was determined by monitoring the pH change associated with ATP hydrolysis [17]. The reaction was carried out in 2 ml of the reaction medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM Hepes (at pH 7.2), supplemented with 2 μM rotenone and mitochondria (1 mg). The reaction was initiated by the addition of 3 mM Mg-ATP. The addition of oligomycin (2 μg) completely abolished H^+ production. H^+ production was calculated after an elapsed time of 3 min from the start of the reaction. Results obtained with solubilized mitochondria (addition of 0.6 mg of Triton X-100), provide higher levels of activities. However, the same relative effects were found for the effect of paraquat either in intact or solubilized mitochondria, as documented Table 2.

ATP-synthase activity was determined by monitoring the pH change associated with ATP synthesis [17], as described for ATPase. The reaction was carried out in 2 ml of the reaction medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$ and 2 mM KH_2PO_4 (at pH 7.2), supplemented with 5 mM succinate and 1 mg of protein. The reaction was initiated by the addition of 200–300 μM ADP. The addition of oligomycin (2 μg) completely abolished H^+ consumption. H^+ consumption was calculated after an elapsed time of 3 min from the start of the reaction.

Determination of thiobarbituric acid reactive substances. Lipid peroxidation was measured as thiobarbituric acid-reactive products, as described previously [18]. The

amount of reactive products formed was calculated by using an extinction coefficient of 156 mM^{-1} cm^{-1} at 530 nm [19].

Analysis of data and statistics. Results are presented as % of control ± S.E., from 4 to 6 independent experiments. Statistical evaluation was performed using the two-tailed Student *t*-test.

3. Results

3.1. Effects of paraquat on mitochondrial respiration

Toxic values are difficult to estimate, since the pharmacodynamics and kinetics of paraquat distribution are not fully understood. Fatal values as high of 300 mg/kg for humans have been reported [1], allowing an estimation of fluid concentration of about 340 mg/l. Considering that the compound is partially eliminated (20%) in feces [1] following subcutaneous administration, this give us a serum concentration of about 1.3 mM, a value within the range used in this study. Taking this information into account, we decided to start this study with concentrations in this range. However, considerably higher peak concentrations may be reached. Therefore, we extended our study to 15 mM.

Respiratory rates characteristics of State 4 (succinate alone), CCCP-stimulated respiration (uncoupled) and State 3 respiration (ADP-stimulated) are illustrated in Fig. 1. The inhibitory effect of paraquat on uncoupled respiration reflects its interaction with the mitochondrial redox chain. State 3 respiration is depressed by a little of 15%, at 10 mM paraquat, whereas uncoupled respiration in the presence of CCCP is depressed by about 30%. Addition of paraquat resulted in a concentration-dependent increase in the rate of State 4 respiration, indicating energy uncoupling. The respiratory rate triples at 10 mM paraquat. This uncoupling effect is presumably caused by the partition of paraquat causing the permeabilization of mitochondrial membrane to H^+. Lipid peroxidation induced by paraquat confirms the deleterious effect of the herbicide on membrane organization (Table 1). Even at 1 mM, paraquat increases by 10-fold the amount of peroxidation products reacting with thiobarbituric acid. Paraquat inhibits the ATPase activity for concentrations in the range of 1–2 mM (Table 2), suggesting the existence of a direct effect on ATPase complex. Although the ATPase rates are higher with solubilized mitochondria, as expected, similar relative effects of paraquat were observed for the ATPase of intact mitochondria (Table 2). For higher concentrations (5–10 mM), the ATPase activity is stimulated, probably as a consequence of the uncoupling effect of the herbicide that discharges the proton electroosmotic gradient build-up at the expense of ATP hydrolysis. Furthermore, ATP synthesis is strongly inhibited by the herbicide, as illustrated in Fig. 2. The inhibition increases sharply above 5 mM paraquat, i.e., for concentrations effective on ATPase stimulation as consequence of uncoupling.

Succinate cytochrome-*c* reductase and cytochrome-*c* oxidase (Fig. 3) are inhibited by 35 and 39% respectively, at 10 mM paraquat, whereas succinate dehydrogenase is

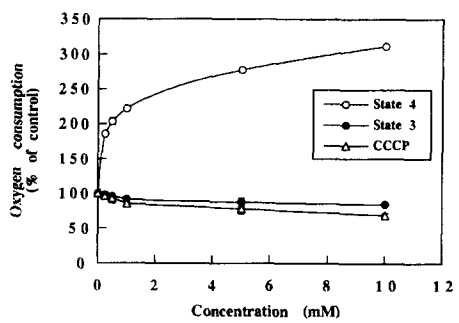

Fig. 1. Effect of paraquat on respiratory rates of mitochondria. Mitochondria (1 mg) were incubated in 1 ml of the respiratory standard medium. State 3 respiration energized by 5 mM succinate was initiated by the addition of 1.5 mM ADP. CCCP-stimulated respiration in the presence of 5 mM succinate was initiated by the addition of 2 μM CCCP. State 4 respiration was initiated by the addition of 5 mM succinate after 10 min of incubation with the herbicide. ADP or CCCP were added 2 min after the initiation of state 4 respiration. Values are the means ± S.E. of 4 to 6 independent experiments (when the error bars are absent, S.E. is encompassed by the size of symbols).

Table 1
Effect of paraquat on levels of TBA-reactive substances on rat liver mitochondria

Paraquat (mM)	TBARS (nmol/mg protein)
0	0.25 ± 0.002
1	2.50 ± 0.1
5	3.40 ± 0.15
10	7.10 ± 0.52

Mitochondria (1 mg) were incubated with different concentrations of paraquat for 10 min at 25° C. After this period, the level of TBARS is quantified as described in the Methods section.

Table 2
Effect of paraquat on ATPase activity of rat liver mitochondria

Paraquat (mM)	ATPase activity (nmol H^+ min^{-1} mg^{-1})
Normal	
0	30.8 ± 0.6
1	22.5 ± 0.8
2	24.1 ± 1.1
5	30.9 ± 0.7
10	39.7 ± 1.3
15	44.0 ± 0.8
Solubilized	
0	45.1 ± 0.4
1	37.6 ± 0.7
2	39.0 ± 1.0
5	45.6 ± 0.8
10	54.2 ± 0.6
15	60.1 ± 0.9

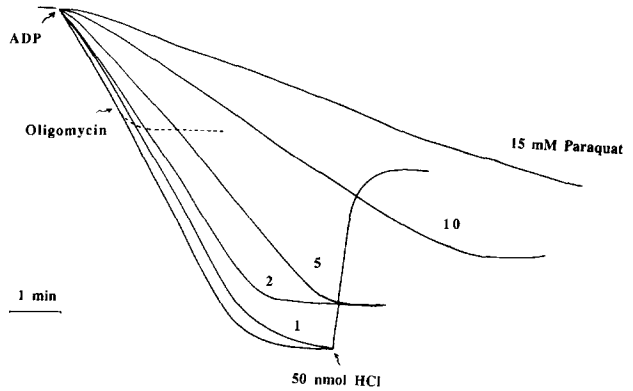

Fig. 2. Effect of paraquat on mitochondrial ATP-synthase. The reaction was carried out in 2 ml of reaction medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$ and 2 mM KH_2PO_4 (at pH 7.2), supplemented with 5 mM succinate and 1 mg of protein. The reaction was initiated by the addition of 200–300 μM ADP. Values are the means ± S.E. of 4 to 6 independent experiments (when the error bars are absent, S.E. is encompassed by the size of symbols).

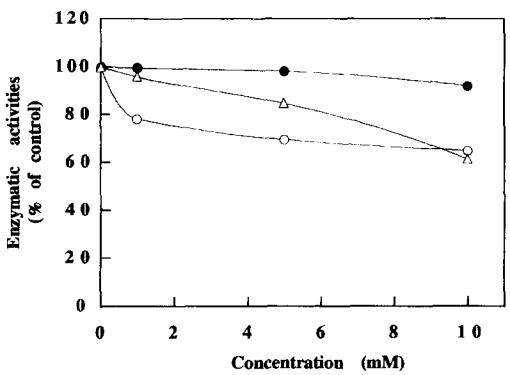

Fig. 3. Effect of paraquat on succinate dehydrogenase (●), succinate-cytochrome-*c* reductase (○) and cytochrome-*c* oxidase (△). Values are the means ± S.E. of 4 to 6 independent experiments (when the error bars are absent, S.E. is encompassed by the size of symbols).

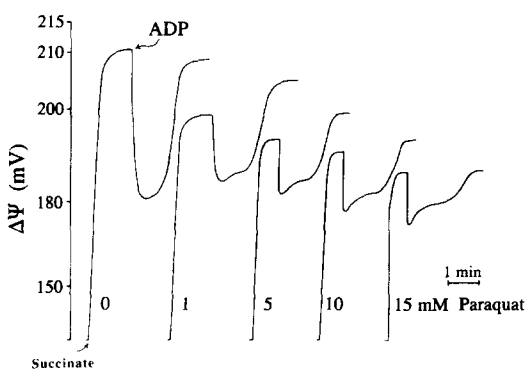

Fig. 4. Effect of paraquat on mitochondrial transmembrane potential ($\Delta\Psi$). Mitochondria (0.8 mg) in 1 ml of the standard respiratory medium supplemented with 4 μM TPP^+ were energized with 5 mM succinate. Addition of ADP (0.12 mM) induces State 3 conditions. The various concentrations of paraquat added are indicated on the traces. The traces represent typical direct recordings expressive of several independent experiments with different mitochondrial preparations.

Table 3
Effect of paraquat on the mitochondrial transmembrane potential

Paraquat (mM)	$\Delta\Psi$ (mV)		
	succinate energization	ADP depolarization	repolarization
0	207.4	17.9	206.1
1	199.0	14.1	205.0
5	194.7	12.4	199.3
10	191.5	11.8	194.7
15	187.4	11.4	187.8

not significantly affected. Therefore, paraquat partially inhibits the redox chain at mitochondrial complexes III and IV, resulting in depression of respiratory rates of State 3 and uncoupled respiration as previously described (Fig. 1).

3.2. Effects of paraquat on mitochondrial membrane potential

The effects of paraquat on the energization and phosphorylation capacities of mitochondria were investigated by following the transmembrane potential ($\Delta\Psi$) developed by mitochondria upon succinate oxidation (Fig. 4). After succinate addition, mitochondria develop a transmembrane potential of about −207 mV, i.e., a value in the range previously reported [37]. Upon ADP addition, the potential drops to −189 mV, owing to the use of $\Delta\Psi$ by ATP synthase to phosphorylate added ADP; after a short lag phase, at which ADP phosphorylation takes place, the transmembrane potential repolarizes close to its initial value (Table 3). In the presence of paraquat, $\Delta\Psi$ is depressed as a function of herbicide concentration. Additionally, the depolarization induced by ADP is decreased and the repolarization turns biphasic, suggesting a double effect of the herbicide. At the end of repolarization, the

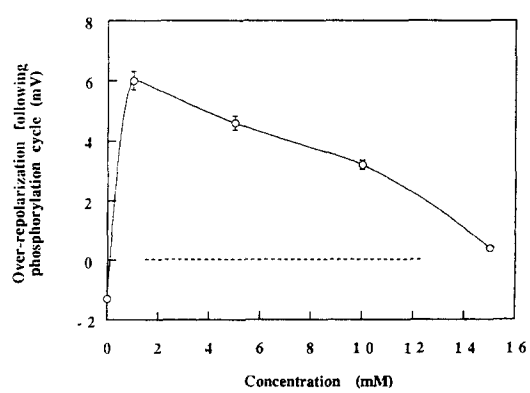

Fig. 5. Effects of paraquat on the level of repolarization following phosphorylation cycle. Experimental conditions were as described for Fig. 4. Over-repolarization values mean the differences in $\Delta\Psi$ levels after and before the phosphorylation of added ADP.

transmembrane potential exceeds by a small amount the value before ADP phosphorylation, an effect dependent on paraquat concentration up to 10 mM. The effect is quite clear at 1 mM herbicide but it is not discernible at 15 mM, as shown in Fig. 5 and described in Table 3.

4. Discussion

Mitochondria are provided with a variety of bioenergetic functions mandatory for the regulation of intracellular aerobic energy production and electrolyte homeostasis. Alteration of bioenergetic reactivities by the interaction of xenobiotics may have drastic consequences on cellular function through the perturbation of the bioenergetic charge and balance of the cell.

Paraquat is a non-selective contact herbicide used world-wide in agriculture with recognized benefits regarding food production [20]. However, it has been shown to be toxic not only to useful plants but also to animals, including humans [1–3,21], but the mechanism of toxicity is still controversial. The basis for the toxicity of paraquat appears to be related to reactive oxygen species that are produced when paraquat undergoes redox cycling. Paraquat can stimulate lipid peroxidation in intact microsomes [22,23], and this toxic action can be attenuated by the administration of superoxide dismutase, suggesting that the production of superoxide anion plays an important role in the toxicity mechanism of paraquat [24–26]. However, other mechanisms have been also proposed, namely the decrease in NADPH levels observed in the presence of the herbicide [27–29]. A decrease in NADPH levels will further impair the ability of antioxidant enzymes, e.g., glutathione peroxidase [30], to protect against the harmful effects of reactive oxygen species. Another mechanism has been proposed relating paraquat toxicity to a decrease in fatty acid synthesis [27,31].

Our data clearly demonstrate the ability of paraquat to impair mitochondrial bioenergetic reactions. Alteration of basic mitochondrial functions was monitored by the detection of changes induced in mitochondrial respiration and membrane energization ($\Delta\Psi$). Paraquat uncouples oxidative phosphorylation (stimulates State 4 respiration), and this effect is probably due to a slight permeabilization of mitochondrial membrane related with lipid peroxidation induced by paraquat (Table 1). The peroxidative process may permeabilize mitochondrial membrane to H^+, contributing to the uncoupling effect related with discharge of the transmembrane electroosmotic proton gradient. Other studies have also provided evidence that paraquat induces lipid peroxidation [21,25,31] that might uncouple mitochondrial electron transport [32]. In spite of this uncoupling action, an inhibitory effect of paraquat on mitochondrial ATPase for concentrations ranging from 1 to 2 mM was observed. For higher concentrations, the ATPase activity is stimulated probably as a consequence of the uncoupling action that discharges the proton electroosmotic gradient build up at expense of ATP hydrolysis. As consequence, the proton gradient that controls the ATP splitting activity of the enzyme is colapsed by paraquat, therefore releasing the enzyme system from the controlled activity imposed by the transmembrane electroosmotic energy. Therefore, the activity is expressed at an increased rate, since the energetic control is lost. Accordingly, ATP synthesis is strongly depressed when the enzyme system operates is in its right sense.

Paraquat depresses State 3 respiration and uncoupled respiration, indicating that the energetic inhibitory effect of the compound is not only related to the phosphorylation system, but also to the electron transport chain. In fact, succinate cytochrome-*c* reductase and cytochrome-*c* oxidase are partially inhibited, suggesting that paraquat affects the redox electron transfer chain at the level of complexes III and IV. Other studies also reported that the compound interacts with mitochondrial respiratory chain [22] and stimulates State 4 respiration [33]. Sata and co-workers, in a study using submitochondrial particles, have shown that paraquat stimulates lipid peroxidation, inhibits electron transport and affects oxidative phosphorylation [34]. Furthermore, paraquat induces mitochondrial structural breakage in rat lung and liver [35]. All these studies concur well with our data regarding mitochondrial mechanisms of paraquat toxicity.

Paraquat depresses the transmembrane potential ($\Delta\Psi$) during succinate oxidation and the repolarization is biphasic suggesting the existence of a double effect, that might be related with the uncoupling effect of paraquat accompanied by a direct effect on the ATPase. Finally, the repolarization resumes at a level higher than that before ADP addition, clearly shown for low concentrations ot paraquat (Fig. 4). This effect is tentatively explained as a coupling action of the herbicide exerted on the ATP synthase itself, decreasing the reflux of protons through the proton channel of the enzyme.

Alterations in mitochondrial bioenergetics have been previously described for other xenobiotic compounds, suggesting the interference with normal functions of mitochondria as a mechanistic event involved in the toxicity of several compounds, e.g., methyl isocyanate [36], DDT [37], Wy-14,643 [38] and nongenotoxic carcinogens [39]. The interest of these studies has developed owing to the use of mitochondria as a model for cell toxicity since most of the cell energy in eukaryotes is provided by mitochondria.

In conclusion, paraquat uncouples oxidative phosphorylation by inducing lipid peroxidation and has an inhibitory action on the redox chain and ATP synthase activity. Additionally, paraquat at low concentrations induces an over-repolarization following the phosphorylation cycle. This study calls attention to the possible role of mitochondria in the development of cellular toxicity by paraquat.

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