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A comparative study of plant and animal mitochondria exposed to paraquat reveals that hydrogen peroxide is not related to the observed toxicity

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Abstract

Rat liver mitochondria are much more susceptible to protein oxidation induced by paraquat than plant mitochondria. The unsaturated index and the peroxidizability index are higher in rat than in potato tuber. The levels of superoxide dismutase and glutathione reductase are concurrent with the different sensitivities to paraquat, with higher activities in plant mitochondria. However, glutathione peroxidase and catalase activities are higher in rat mitochondria. Paraquat (10 mM) inhibited all the enzymatic activities; excluding catalase all the other activities were inhibited to a similar degree. The differential sensitivities of plant and animal mitochondria to paraquat correlate with fatty acid composition of mitochondrial lipids and a similar correlation was also established for some antioxidant enzymes. At the mitochondrial level, H_2O_2 is not a major factor of paraquat toxicity since rat liver mitochondria which exhibit higher activities of glutathione peroxidase and catalase are however more susceptible to paraquat.

Keywords: Paraquat; Mitochondria; Hydrogen peroxide; Antioxidant enzymes; Herbicide toxicity

1. Introduction

Paraquat, a quaternary nitrogen herbicide, is a strong toxicant for humans and animals and many cases of acute poisoning and death have been reported over the past decade (Bruyndonckx et al., 2002). In animals and plants, mitochondria are often taken as targets for paraquat toxicity (Lambert and Bondy, 1989). Paraquat treatment of pea seedlings induced lipid peroxidation, which resulted in the rapid loss of glycine-dependent respiration (Taylor et al., 2002). Mitochondrial bioenergetics of animal and plant show different sensitivities to paraquat toxicity. Differences in SOD activity and α-tocopherol content have been pointed out as the rationale for the different reactions to paraquat in plant and animal mitochondria (Vicente et al., 2001).

The mechanisms of paraquat toxicity (Suntres, 2002) are frequently related to the generation of the superoxide anion, which can lead to the formation of more toxic reactive oxygen species, e.g., hydrogen peroxide, often taken as the main toxicant (Farrington et al., 1973). As a consequence, either in plants (Bowler et al., 1991) or in mammals (Ogata and Manabe, 1990), protein cross-linking (Dean et al., 1991) of peptide radicals is also a putative mechanism of toxicity.

It is shown that the differential sensitivities to paraquat toxicity are dependent on antioxidant defences against damaging effects and also on the fatty acid membrane composition related with peroxidizability. Furthermore, generation of H_2O_2 is not a major factor of paraquat toxicity at mitochondrial level.

2. Materials and methods

2.1. Chemicals

Paraquat was supplied by Zeneca Agrochemicals UK. All the other chemicals were of analysis grade and of the highest available purity.

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2.2. Isolation of mitochondria

Animal mitochondria were isolated from the livers of male wister rats (200–300 g) by conventional methods (Gazzotti et al., 1997), with slight modifications. The homogenization medium contained 0.25 M sucrose, 5 mM hepes (pH 7.4), 0.2 mM EGTA and 0.1% defatted bovine serum albumin. EGTA, EDTA and bovine serum albumin were omitted from the final washing medium, adjusted at pH 7.2. Plant mitochondria were isolated from fresh potato tubers (*Solanum tuberosum* L.), obtained from local markets and stored at 4–6 °C. Isolation and purification involved a percoll gradient centrifugation as a terminal step (Neuburger et al., 1982). The mitochondrial fraction was washed twice by centrifugation at $30,000 \times g$ for 5 min in a medium containing 0.25 M sucrose and 10 mM hepes (pH 7.2). The pellet was gently resuspended in the same medium at a protein concentration of 20–30 mg/mL. Protein was determined by the Biuret method using BSA as standard (Gornall et al., 1949).

2.3. Determination of protein thiol contents

Mitochondria (1 mg protein) were incubated, with continuous stirring, in 1 mL medium containing 0.25 M sucrose, 30 mM KCl, 15 mM hepes (pH 7.2), 20 mM KCl, 5 mM K_2HPO_4, 2 mM $MgCl_2$, 0.2 mM EDTA and 0.05% BSA, using different paraquat concentrations, at 25 °C for 20 min. The mitochondrial suspension was then submitted to three subsequent freeze–thaw procedures to release matrix proteins and centrifuged for 2 min at $7200 \times g$. The pellet was treated twice with 500 μL 4% sulfosalicylic acid and centrifuged at $7200 \times g$ for 3 min to precipitate the protein. The pellet was then washed twice with 5 mL medium containing 0.5 mM EGTA and 0.5 M Tris (pH 8.3) and centrifuged at $7200 \times g$ for 5 min to precipitate the protein. The final pellet was resuspended in 1 mL medium containing 100 μM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 0.5 mM EGTA, and 0.5 mM Tris (pH 8.3). Absorption was measured at 412 nm, using cysteine for calibration.

2.4. Fatty acid analysis

Phospholipid extraction was performed according to Bligh and Dyer (1959) with some modifications. The chloroform phase was mixed in vortex with a double volume of KCl solution (0.1 M). After centrifuging for 5 min at 3000 rpm, the lower phase was collected and dried under a stream of N_2. The preparation of fatty acid methyl esters (FAME) was performed in the following system of Rosa and Catalá (1998) using 5% HCl in methanol as the derivatizing reagent, at 60 °C for 3 h. Fatty acid methyl esters were analyzed with a Perkin–Elmer gas chromatograph adapted for capillary columns with helium as the carrier gas. The linear velocity was 35 cm/s with a flow rate of 4.0 mL/min. We used an Alltech capillary column (AT-SILAR 30 m × 0.53 mm ID × 0.5 μ) and a temperature program as follows: 5 min at 140 °C; 4 °C/min to 210 °C; 10 min at the upper temperature. The results were calculated using an appropriate integration software (JCL 6000 chromatography data system).

The peroxidizability index (PI) was calculated according to the formula, PI = (% monoenoic acids × 0.025) + (% dienoic acids × 1) + (% trienoic acids × 2) + (% tetraenoic acids × 4) + (% pentaenoic acids × 6) + (% hexaenoic acid × 8) (Rosa and Catalá, 1998).

The unsaturation index (UI) was calculated according to the formula, UI = Σ% of unsaturated fatty acids × number of double bonds of each unsaturated fatty acid (Rosa and Catalá, 1998; Pamplona et al., 1999).

2.5. Determination of enzyme activities

Superoxide dismutase (MnSOD) activity was determined according to the method of Payá et al. (1992), using the xanthine–xanthine oxidase system. One unit of SOD activity was defined as the amount of SOD that inhibits the reduction rate of NBT by 50%. The effect of paraquat on the xanthine–xanthine oxidase system was evaluated by measuring spectrophotometrically the production of uric acid at 295 nm.

The mitochondrial glutathione reductase activity was performed according to Smith et al. (1988). The rate was calculated from the linear portion of the curve and expressed as nmol NADPH min^{-1} mg $protein^{-1}$. Glutathione peroxidase was assayed by the method of Flohé and Gunzler (1984). The activity of the enzyme was measured indirectly by determining the oxidation of NADPH, at 340 nm, induced by the action of glutathione reductase. The results were expressed as μmol GSH oxidized min^{-1} mg^{-1} protein. Catalase was assayed as described by Aebi (1983) and the activity was determined without disruption of mitochondria in the presence of 0.5% Triton X-100. Substrate dilutions were made in the same buffer from solutions freshly prepared before each assay.

2.6. Statistics

Data are expressed as mean ± s.d. of four–six independent experiments. Statistical significance was determined using the unpaired Student's *t*-test.

3. Results

Paraquat toxicity is often related with the generation of free radicals by a redox cycling mechanism (Hassan

and Fridovich, 1979). Therefore, different levels of protective agents in plant and animal mitochondria are expected to correlate with the relative paraquat toxicity (Vicente et al., 2001). Putative damages dependent on free radicals and intrinsic mitochondrial agents affording protection against oxidative damages were investigated, as described below. Paraquat 10 mM has been used, since at this concentration proved effective in rat liver and potato tuber mitochondria (Vicente et al., 2001).

3.1. Oxidation of protein thiol groups induced by paraquat

Paraquat, as prooxidant, may challenge cross-linking of several proteins, as consequence of oxidation of sulphydryl groups, with simultaneous decrease of membrane protein thiols, resulting in the formation of protein aggregates (Kuroda and Cuéllar, 1993; Castilho et al., 1996). Fig. 1 shows that proteins from rat liver mitochondria are much more susceptible to paraquat oxidation than potato mitochondria, in agreement with a higher level of protective agents afforded by plant mitochondria. These data further correlate with the different sensitivities of mitochondrial bioenergetics to paraquat, in which plant mitochondria is less susceptible to lipid peroxidation as compared with animal mitochondria (Vicente et al., 2001).

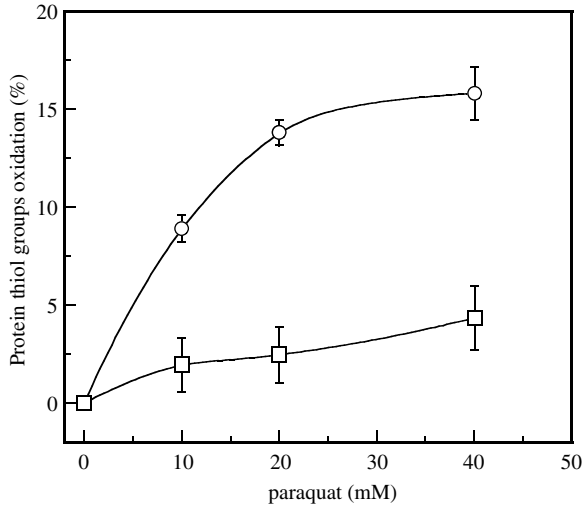

Fig. 1. Effect of paraquat on oxidation of mitochondrial protein thiol groups of potato (□) and rat fractions (○). Mitochondria (1 mg protein) were incubated for 30 min in a medium containing 0.25 M sucrose, 30 mM KCl, 15 mM hepes (pH 7.2), 20 mM KCl, 5 mM K_2HPO_4, 2 mM $MgCl_2$, 0.2 mM EDTA and 0.05% BSA. Control values for protein thiol groups are: 18.8 ± 2.51 for potato and 32.2 ± 1.53 for rat, expressed in nmol per mg of protein. All values are mean ± SEM of three independent experiments. Values statistically different from control: $* p < 0.05$.

3.2. Fatty acid composition

The fatty acid composition of mitochondrial membranes was determined (Fig. 2) since unsaturated fatty acids are prone to oxidation by prooxidants, like paraquat, and because of the higher reactivity of the double bonds to the abstraction of hydrogen atoms.

The major saturated fatty acids of rat liver mitochondrial membranes are palmitic (16:0) and stearic acids (18:0), and the unsaturated are linoleic (18:2) and arachidonic acids (20:4). In potato mitochondrial membranes, palmitic (16:0) is the major saturated fatty acid and linoleic (18:2) and linolenic acids (18:3) are the major unsaturated species (Fig. 2). The total number of double bonds (unsaturated index—UI) (Rosa and Catalá, 1998; Pamplona et al., 1999) is significantly higher in rat liver than in potato membranes. The calculated peroxidizability index (PI) (Rosa and Catalá, 1998) is also higher for rat liver (126.2 ± 11.5), than for potato tuber mitochondria (72.3 ± 2.4). Therefore, it is tempting to propose that animal membranes are more susceptible to peroxidation.

3.3. Oxidative stress protection by enzyme activities

Tuber mitochondria exhibit increased Mn–SOD activity when compared with liver mitochondria, as shown in Table 1. Therefore, the superoxide scavenging capacity of the potato fraction is increased by about 62% relative to rat mitochondria. Paraquat promotes a significant decrease of the Mn–SOD activity in both mitochondrial fractions. Glutathione reductase (GR) is

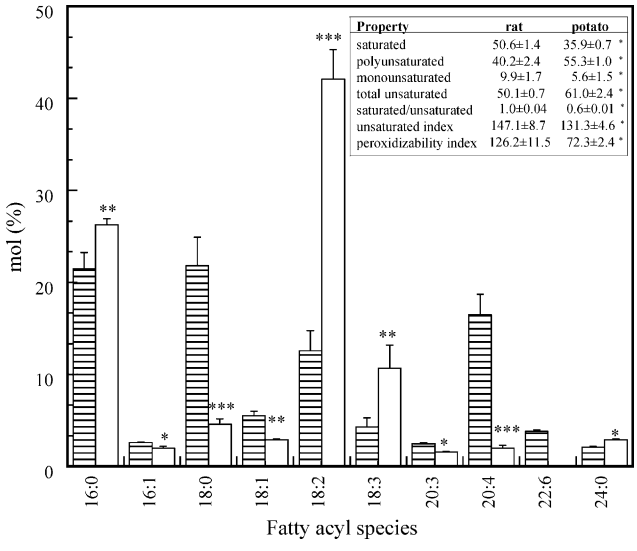

Fig. 2. Fatty acyl moieties of membrane lipids of rat (▤) and potato (□) mitochondria, determined by gas-liquid chromatography. Inset: the results are given in mol % of total contents. Data are mean ± SEM of three independent experiments. Values statistically different from control: $* p < 0.05$; $** p < 0.005$; $*** p < 0.0005$.

Table 1
Activities of superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase in potato tuber mitochondria and rat liver mitochondria

Enzymatic activity	Control	Paraquat (10 mM)
Superoxide dismutase		
Potato tuber	12.3 ± 0.2	9.1 ± 0.4*
Rat liver	7.6 ± 0.7	5.3 ± 0.7*
Glutathione reductase		
Potato tuber	64 ± 5	44 ± 3*
Rat liver	42 ± 3	30 ± 2*
Glutathione peroxidase		
Potato tuber	14 ± 7	10 ± 2*
Rat liver	288 ± 19	150 ± 6*
Catalase		
Potato tuber	0.42 ± 0.03	0.40 ± 0.02
Rat liver	1.3 ± 0.06	0.93 ± 0.03*

The activity of superoxide dismutase is expressed in units per min per mg of protein (1 unit of activity is defined as the amount of enzyme which inhibits the reduction rate of NBT by 50%). The activity of glutathione reductase is expressed in nmol NADPH oxidized per min per mg protein and the glutathione peroxidase activity was expressed in nmol GSH oxidized per min per mg of protein. Catalase activity is expressed in sec^{-1} per mg protein. Mitochondria were pre-incubated in presence of paraquat (10 mM) for 10 min. in the appropriated medium for the enzymatic assays, and the assays were performed as described in Section 2. The results are the mean ± SEM for five different preparations.
* $p < 0.05$ as compared with controls.

the enzyme responsible for the regeneration of GSH, i.e., the maintenance of the high level of GSH. Consequently, GR is required for the continuous operation of glutathione peroxidase (GPX). Table 1 shows that GR activity in the tuber fraction is about 35% higher than in the liver fraction. Paraquat (10 mM) promoted similar and significant decreases of about 30% for the GR activity in both mitochondrial fractions.

GPX and GR are related to the redox cycling of glutathione. GPX is involved in the removal of hydrogen peroxide and organic peroxides (Flohé and Gunzler, 1984) and GR is involved in the recycling of reduced glutathione, providing a constant intracellular level of GSH (Calbert and Mannervik, 1985), the main cell antioxidant (Meister, 1981; Alscher, 1989; Reed, 1990). Tuber mitochondria exhibit a mere 5% of the GPX activity found in the liver fraction (Table 1) but in both fractions paraquat incubation promoted a significant decrease in the GPX activity. Catalase activity either native or from putative contamination was measured in both mitochondrial fractions. Catalase (CAT) activity (Table 1) in the potato mitochondrial fraction was only 32% of the activity observed in the rat fraction. In the rat fraction paraquat induced a small but significant decrease in CAT activity; conversely, no statistically significant effect has been detected in potato fraction. The lowest sensitivity of catalase of plant mitochondria to paraquat may result from structural differences as previously demonstrated (Kirkman et al., 1987; Beaumont et al., 1990). The relative activities of glutathione peroxidase and catalase do not correlate with the different sensitivities for the effects of paraquat on plant and animal mitochondria (Vicente et al., 2001).

4. Discussion

Considerable controversy about the mechanism of paraquat toxicity in animals is documented in a large number of publications (Misra and Fridovich, 1972; Singer et al., 1973; Kornbrust and Mavis, 1980; Lambert and Bondy, 1989; Hoffer et al., 1997). The relevance of catalase on the resistance against paraquat is also controversial (Tsukamoto et al., 2000; Rohrdanz et al., 2001; Asma and Yesilada, 2002; Hauck et al., 2002; Mockett et al., 2003).

The effects observed in animals could, in principle, apply to plants. However, a comparative study of paraquat effects in animal and plant mitochondria reveals a stronger effect on the stimulation of oxygen consumption in state 4 and on dissipation of mitochondrial transmembrane potential of animal mitochondria, as compared to plant mitochondria (Vicente et al., 2001). These results are explained by differences in the extension of the oxidative processes which depend on the extent of antioxidant protective agents, namely α-tocopherol and SOD (Vicente et al., 2001).

The different levels of lipid peroxidation in animal and plant mitochondria (Vicente et al., 2001) are not exclusively related to the increased antioxidant protection in plants, but also with the differences in the fatty acid compositions of the mitochondrial membrane.

Lipid peroxidation end products from isolated mitochondria of pea leaves sprayed with paraquat (662.5 mg/L) reveal that the peroxidation induced by paraquat was only evident after 8 h and concomitant with the appearance of MDA. Following paraquat treatment, the alternative oxidase markedly increased (Taylor et al., 2002). Taylor et al. (2002) suggested that 4-hydroxy-2-nonenal (HNE), an end product of lipid peroxidation, is the main responsible for the inhibition of mitochondrial functions and, in particular, photorespiratory metabolism.

Concentrations up to 50 mM, HNE had no significant effect on pyruvate or 2-oxoglutarate-dependent respiration but inhibited glycine-dependent respiration by 75%. Furthermore, pyruvate and 2-oxoglutarate-dependent respiration in mitochondria isolated from leaves treated with paraquat for 12 h did not show any significant difference from the control. However, glycine-dependent respiration declined sharply to ca. 40% of the control (Taylor et al., 2002).

The alternative oxidase pathway induced in mitochondria of many plant tissues exposed to stress (Minagawa et al., 1992; Rhoads, 1993; Simons et al., 1999) offers an additional way of channelling electrons, bypassing the traditional electron pathway (Lambers, 1982). Taylor et al. (2002) showed that concomitant with the appearance of MDA following paraquat treatment, alternative oxidase expression was increased. However, potato mitochondria do not exhibit alternative oxidase activity in the actual experimental conditions.

The extension of lipid peroxidation (Vicente et al., 2001) and protein thiol oxidation (Fig. 1) promoted by paraquat are consistent with the observed effects on plant and animal mitochondrial bioenergetics (Vicente et al., 2001). As a consequence of the oxidation promoted by paraquat, proteins of submitochondrial particles (mol. wt. ca. 30 kDa) in rat liver were altered by paraquat radicals, aggregating in protein bands ca. 100 kDa, as observed by polyacrylamide electrophoresis, but a similar effect has not been observed in submitochondrial particles isolated from potato tuber. Therefore, lipid peroxidation and protein thiol oxidation may reflect paraquat toxicity.

Paraquat toxicity is associated to a redox-cycling of ROS generation, since no specific effect of paraquat has been observed in rat and potato tuber mitochondria; however a specific effect has been described for glycine-dependent respiration (Taylor et al., 2002), which is only observed in mitochondria isolated from photosynthetic tissues (Douce et al., 1977).

Owing that membranes are highly permeable to H_2O_2 (Chance et al., 1979), paraquat toxicity has been assigned to H_2O_2 production (Farrington et al., 1973; Hassan and Fridovich, 1979). Therefore, catalase should protect against paraquat toxicity and higher catalase and glutathione peroxidase activities are expected on potato fraction which is less sensitive to paraquat. Nevertheless, the stronger activity is found in the rat fraction. Our data are in agreement with data obtained for cells containing a reporter plasmid with the upstream region of the catalase gene, where the exposure to paraquat induced a decrease in the reporter gene activity (Rohrdanz et al., 2001).

Mitochondrial preparations purity was ascertained by electron microscopy confirming the absence of peroxisomes. Nevertheless, differences in the methodology of mitochondrial fractionation may affect the fraction purity. However, this does not invalidate the in vitro data obtained here, since contaminations of antioxidant protectors would account for the antioxidant capacities of the fractions.

Assuming that paraquat exerts its toxicity in an oxidant path, it is reasonable to suggest that peroxides are not the directly responsible agents for paraquat toxicity, since the activity of the glutathione peroxidase and the activity of catalase are much higher in the liver than in the potato tuber fraction. Moreover, our data point out to the importance of MnSOD and GR activities, which can be related to the protection against radical superoxide or other ROS. Vicente et al. (2001) have already reported a higher Mn–SOD activity in potato tuber mitochondria as compared with rat liver mitochondria, but they have reported a Mn–SOD stimulation by paraquat. Conversely, in our conditions, paraquat (10 mM) clearly inhibited the SOD activity. The higher scavenging of superoxide anions in the presence of paraquat reported by Vicente et al. (2001) is certainly the result of an inhibition on the xanthine–xanthine oxidase system promoted by paraquat (10 mM). The inhibition of the xanthine–xanthine oxidase system by paraquat 10 mM was evaluated spectrophotometrically at 295 nm, by the production of uric acid. To account for this inhibition, the SOD activity should be evaluated from experimental conditions in the absence and presence of paraquat (10 mM).

The fatty acid composition of plant and animal mitochondria favours a higher susceptibility of rat mitochondria to paraquat. A higher saturated/unsaturated fatty acids ratio for rat does not mean a low unsaturated level. Rather, the number of unsaturated bonds is determinant, since only the unsaturated bonds are susceptible to oxidation. Liver membranes have a higher content of unsaturated bonds and, as consequence, the peroxidizability index observed for rat is 1.8 times the index calculated for tuber membranes (Fig. 2—inset).

In conclusion, our data is consistent with the different sensitivities of the mitochondrial fractions to paraquat in terms of bioenergetic efficiency. The differences observed between potato tuber and rat liver mitochondria to paraquat toxicity are not exclusively assigned to the antioxidant capacity promoted by enzymatic antioxidant systems. The native aliphatic contents of the membrane phospholipids can also afford protection against paraquat toxicity, since bioenergetic efficiency is intimately dependent on the structural integrity of the membrane. Moreover, from the data obtained with catalase and glutathione peroxidase, it can also be concluded that peroxides are not major intermediates in paraquat toxicity at the mitochondrial level.

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