The Effect of Paraquat on the Respiration of Lung Cell Fractions

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SUMMARY

The in vitro effects of paraquat on the aerobic metabolism of lung and liver homogenates, as well as on the oxygen consumption of isolated rat and rabbit lung mitochondria, were investigated.

It was found that the endogenous oxygen uptake of a 40% lung homogenate was similar to that of a 10% liver homogenate, and that succinate (20 mM) was well oxidized by both homogenates. About 14% of the basal respiration rate was due to cyanide-insensitive oxidative systems in lung and liver homogenates. Paraquat (1 mM and higher concentrations) induced an acute and highly significant increase in the cyanide-insensitive oxygen utilization.

We were able to recover about 5% of the protein in the supernatant (1 000 g) of the original homogenate as mitochondrial protein. Only results obtained from mitochondrial preparations with a respiratory control ratio between 2.7 and 3.3 were used in the final analyses, and the normal state 3 respiratory values of rabbit and rat lung mitochondria were 45.2 ± 5.3 and 38.7 ± 2.9 nmol oxygen per mg protein per minute respectively. Paraquat in concentrations up to 0.5 mM had no significant effect, but higher concentrations up to 1 mM induced a highly significant inhibition of mitochondrial oxygen consumption. When slices were pre-incubated with 1 mM and with 0.01 mM paraquat in the incubation medium, the oxygen consumption of the mitochondria isolated after 2 hours and 4 hours respectively, was significantly reduced.


The pulmonary toxicity of paraquat is well documented, but the mechanism of its action has not yet been definitely established. Previous reports on the effects of paraquat on the in vitro oxygen consumption of lung slices indicated an initial sharp increase in the oxygen uptake which was followed by a gradual inhibition of the rate of oxygen utilization. The increase in aerobic metabolism seemed to be glucose-dependent, but the inhibitory action of paraquat appeared to be of the same magnitude, whether glucose was present or not. It was speculated that the most probable mechanism of inhibition lay at a mitochondrial level, because the contribution of the microsomal respiration to the total oxidative metabolism is so small that it could hardly explain the highly significant inhibition.

Despite many reports on the pathophysiological and histopathological changes in the lung after exposure to paraquat, very few studies have been published on the effects of paraquat on the functional state of lung mitochondria. After working with isolated rat liver mitochondria, Gage reported that the oxidation of 3-hydroxybutyrate was stimulated very poorly by paraquat, probably because paraquat did not penetrate the mitochondrial membranes. When mitochondrial fragments were partially inhibited by amytyl or antimycin A, paraquat stimulated oxygen uptake with reduced nicotinamide adenine dinucleotide (NADH) or 3-hydroxybutyrate as substrates, but not with succinate. Gage suggested that NADH dehydrogenase appeared to be involved in these stimulations.

Kopaczyk-Locke also found that paraquat stimulated the oxidation of some liver mitochondrial substrates, acted as an electron carrier and inhibited oxidative phosphorylation. Her results also showed that the in vitro effects of paraquat were essentially reproduced in vivo, when the reactions occurring in mitochondria isolated from the livers of rats injected with paraquat were investigated.

In view of the total lack of information on the effects of paraquat on the respiration of lung mitochondria, as well as the dual effect of paraquat on the oxygen uptake of lung slices, we decided to study its effect on the aerobic metabolism of lung homogenates and isolated lung mitochondria. We used both rat and rabbit lung cell fractions, in order to determine possible species differences.

MATERIALS AND METHODS

Male Sprague-Dawley rats (180 - 220 g) and male New Zealand White rabbits (1.2 - 2.0 kg) were used in the experiments. The rats were sacrificed by decapitation and exsanguination, and the rabbits were anaesthetized with a sublethal dose of 2.5% thiopental sodium through the marginal ear vein. The lungs were immediately perfused in situ with 15 ml (rat) and 50 ml (rabbit) ice-cold isotonic saline, quickly removed, dissected free of large airways and blood vessels and sliced (1 mm) with a McIlwain tissue chopper. Only lungs free of macroscopical signs of respiratory disease were processed further. For the preparation of rabbit liver homogenates, the livers were perfused with ice-cold isotonic saline until the red pigmentation had almost disappeared. The piece of liver which was used was removed from the same anatomical position throughout the investigation, gently blotted, and 1-mm slices were cut with a McIlwain tissue chopper.
Liver and lung homogenates were prepared in a medium containing 150 mM sucrose, 150 mM mannitol, 1 mM tris-HCl (pH 7.4) and 1 mM potassium EDTA (henceforth called homogenizing medium). The lung (40% w/v) and liver (10% w/v) were homogenized with a glass-Teflon homogenizer (0.15-mm clearance) in homogenizing medium using two strokes of the pestle. The homogenates were then filtered through a single layer of cheesecloth. All the preceding steps were carried out on ice. The filtered homogenate was then centrifuged at 1000 g for 10 minutes in a precooled bench centrifuge (IEC HN-S centrifuge) at 4°C. The supernatant was carefully drawn off with a Pasteur pipette and stored on ice.

To isolate mitochondria, the filtered lung homogenate was centrifuged at 1000 g for 10 minutes (J-21 B centrifuge; JA20 rotor; Beckman) to remove the nuclei and broken cell debris. This supernatant was then centrifuged at 9000 g for 10 minutes to sediment the mitochondrial fraction. The mitochondrial pellet thus obtained was washed once by resuspension in homogenizing medium and then centrifuged again at 5000 g for another 10 minutes. All the differential centrifugation steps were carried out at 4°C.

The oxygen consumption of both homogenates and mitochondria was measured polarographically at 30°C using an oxygraph (Gilson Medical Electronics, model K-IC) equipped with a Clark-type oxygen electrode. The reactions were carried out in a vessel (2.0-ml capacity) containing the basic incubation medium saturated with room air and maintained at 30°C. The incubation medium (pH 7.4) consisted of 150 mM sucrose, 150 mM mannitol, 1 mM tris-HCl, 10 mM KCl, 5 mM MgCl₂, 1 mM KH₂PO₄ and 1 mM potassium EDTA. Substrate (20 mM succinate), KCN (5 mM) and paraquat solutions (Aldrich Chemical Company, Milwaukee, Wis.) were added to the reaction mixture separately. Protein determinations (according to Lowrey et al.) were done on the same day on fresh preparations, with crystalline bovine serum albumin (Cohn fraction V; Koch-Light-Laboratories) and Versatol used as standards. The results were expressed in terms of nanomoles oxygen consumed per milligram homogenate or mitochondrial protein per minute. The state 3 and 4 respiration rates referred to in the text are those defined by Chance and Williams, except that state 3 rate (active respiration) was achieved from state 4 rate (resting respiration; incubation medium plus 20 mM succinate) by supplementing the medium with adenosine diphosphate (ADP 0.5 mM). Standard methods were used to compute the mean and standard error of mean, and pairwise comparisons (P values) were made using a two-sided Student’s t test.

To prepare the mitochondria for electron microscopy, 1-mm cubes of the pellet were fixed in 2% phosphate-buffered glutaraldehyde with 1% formaldehyde. Thereafter the pellet was rinsed in phosphate buffer and postfixed in 1% osmium tetroxide in veronal buffer (pH 7.4). After dehydration in graded alcohols and propylene oxide, the tissue was embedded in Spurr resin. Thin sections (LKB ultramicrotome) were stained with 3% uranyl acetate and Reynolds lead citrate, and studied with a Siemens Elmiskop I electron microscope.

**RESULTS**

Fig. 1A illustrates the endogenous oxygen uptake of a 10% liver and 40% lung homogenate, the effect of potassium cyanide (5 mM) on the endogenous oxygen consumption and the influence of a succinate substrate (20 mM) on the rate of oxygen utilization of these homogenates. The rate of oxygen uptake of lung homogenates is noticeably slower than that of liver homogenates. The addition of KCN
Reduced the rate of oxygen uptake of lung homogenates to about 12.6% (SEM ± 0.3) and of liver homogenates to 14.8% (SEM ± 2.8) of the original values. Succinate is well oxidized by both homogenates, and it increased the rate of oxygen consumption to about twice that of the control values.

The effect of paraquat on the cyanide-insensitive respiration of lung and liver homogenates is summarized in Fig. 1B. Paraquat, in concentrations of 0.1 mM and below, has no effect on the cyanide-insensitive oxygen consumption of either homogenate. Higher concentrations of paraquat (1 mM, 5 mM and 10 mM), however, induced an acute and significant increase in oxygen uptake.

Electron microscopical examination of the mitochondrial-rich fraction showed intact mitochondria, some identifiable microsomal vesicles and some densely stained lysosomal particles (Fig. 2).

We were able to recover about 5% of the protein in the 1000 g supernatant of the original homogenate. The effect of paraquat on the respiration of rabbit lung mitochondria. Only the results obtained from mitochondrial preparations with a respiratory control ratio between 2.7 and 3.3 were used in the final analyses. The mean normal control state 3 respiration values of rabbit and rat lung mitochondria were 45.2 (SEM ± 5.3) and 38.7 (SEM ± 2.9) nmol O2/mg mitochondrial protein/min respectively. The addition of 5 mM KCN totally inhibited the state 3 respiration. Table 1

**TABLE I. Effect of Paraquat on the In Vitro Respiration of Rat and Rabbit Lung Mitochondria (State 3 Respiration)**

<table>
<thead>
<tr>
<th>Paraoquat concentration</th>
<th>Number of experiments</th>
<th>Mean ± SEM</th>
<th>% stimulation</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>28</td>
<td>38.7 ± 2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 mM</td>
<td>9</td>
<td>36.9 ± 2.8</td>
<td>(-) 4</td>
<td>NS</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>7</td>
<td>35.9 ± 4.9</td>
<td>(-) 8</td>
<td>NS</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.75 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1.0 mM</td>
<td>7</td>
<td>4.7 ± 3.5</td>
<td>(-) 87</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

NS = not significant.

**TABLE II. Oxygen Consumption of Rat Lung Mitochondria Isolated from Lung Slices After In Vitro Incubation in Paraquat**

<table>
<thead>
<tr>
<th>Paraoquat concentration</th>
<th>Incubation (hrs)</th>
<th>Control Mean ± SEM</th>
<th>Number of experiments</th>
<th>Paraquat-treated Mean ± SEM</th>
<th>Number of experiments</th>
<th>% stimulation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 mM</td>
<td>1</td>
<td>39.8 ± 5.8</td>
<td>10</td>
<td>37.3 ± 4.1</td>
<td>10</td>
<td>(-) 6</td>
<td>NS</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>2</td>
<td>39.1 ± 4.1</td>
<td>10</td>
<td>26.1 ± 5.3</td>
<td>10</td>
<td>(-) 32</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>4</td>
<td>36.4 ± 3.9</td>
<td>8</td>
<td>28.6 ± 2.4</td>
<td>8</td>
<td>(-) 22</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

NS = not significant.
also summarizes the effects of various concentrations of paraquat on the oxygen uptake of rabbit and rat lung mitochondria.

Paraquat in concentrations up to 0.5 mM had no significant effect on the state 3 respiration of lung mitochondria, but higher concentrations up to 1.0 mM induced a highly significant inhibition of mitochondrial oxygen consumption. Very similar results were obtained in both species, and there seem to be no species differences in the effects of paraquat at a mitochondrial level.

Table II summarizes the oxygen consumption of isolated rat lung mitochondria after in vitro incubation of lung slices with 0.01 mM and 1.0 mM paraquat at 30°C for various time intervals. Paraquat (1.0 mM) had no significant effect on mitochondrial respiration after 1 hour of incubation, but caused a significant inhibition of mitochondrial oxygen utilization when lung slices were incubated for 2 hours. Even 0.01 mM paraquat, after 4 hours of pre-incubation with lung slices, gave a significant inhibition of the state 3 respiration of isolated lung mitochondria.

**DISCUSSION**

Homogenization of tissues destroys the normal compartmentation of cell contents, and causes the release of lysosomal and other enzymes. These enzymes may alter the metabolic pathways and disrupt the relationships that may control the oxygen utilization of homogenates. For example, lung homogenization results in the release of NADase which leads to a rapid decrease in the NAD content of the preparation. This, and other factors, may be responsible for the low oxygen consumption of lung homogenates compared with that of slices. This phenomenon discouraged most investigators from using lung homogenates for the study of lung oxidative metabolism. However, homogenates are used in our laboratory as a screening procedure to test the effects of toxic substances on oxidative metabolism in the absence of a cell membrane, and to determine the cyanide-insensitive respiration of tissues.

According to Mason, mitochondrial and non-mitochondrial respiration accounted for 86-94% and 6-14% of the total oxygen consumption of tissues respectively, and Estabrook et al. reported that 10-25% of the total cellular oxygen utilization may occur by means of the extramitochondrial oxidation pathways. We found that the cyanide-insensitive respiration amounted to about 14% of the total endogenous oxidative metabolism of both liver and lung homogenates.

The stimulation of the cyanide-insensitive respiration by high concentrations of paraquat (10 mM) is supported by results in the literature, showing an increase in liver microsomal NADPH oxidase activity. According to Gage, the stimulatory effect might involve a flavoprotein by a mechanism not requiring thiol groups, because it is not inhibited by carbon monoxide. However, it must be emphasized that the observed stimulation of oxygen consumption in our experiments (Fig. 1) was measured over the initial 2-4 minutes only, because the oxygen in the reaction flask was depleted within 3-5 minutes after the addition of paraquat. Re-oxygenation of the reaction mixture in an effort to follow the oxygen uptake over a longer period was abandoned because quantitation of the results became pointless.

In contrast to the stimulatory effect of paraquat on the substrate utilization of liver mitochondria, we found that paraquat gave a highly significant inhibition of lung mitochondrial respiration (Table I, Fig. 3).

This direct in vitro effect of paraquat was essentially reproduced when mitochondria isolated from tissue slices which were incubated in vitro with paraquat for various time intervals were investigated. These observations confirmed our speculations that the inhibition of oxygen consumption of lung slices was of such a magnitude that mitochondrial respiration might be involved in some way. It also indicated that no species differences existed as regards the effect of paraquat at a mitochondrial level.

Although Gage doubted whether the bipyridilium compounds did enter the mitochondria, the acute inhibition of mitochondrial respiration in our experiments suggested that paraquat, despite being a charged compound, has no difficulty in penetrating mitochondrial membranes. Judged by the similar inhibition of mitochondrial function after in vitro incubation of lung slices with paraquat, it seems reasonable to conclude that paraquat, in adequate concentrations, certainly affects intramitochondrial activities in time.

The generation of hydrogen peroxide has been shown in a series of vertebrate mitochondria to constitute a physiological event in the aerobic mitochondrial metabolism. Furthermore, various investigators have advanced hypotheses indicating that the cellular and subcellular toxicity of paraquat may be related to the increased formation of hydrogen peroxide. We also felt that the possibility of peroxidation of mitochondrial membrane lipids cannot be excluded, especially in view of the high degree of unsaturation of these lipids. On the other hand, the speed with which paraquat inhibited the oxygen consumption of mitochondria is such that some intramitochondrial enzyme system may be involved. Further investigations are currently in progress to try to localize the mitochondrial defect in more specific terms.

**REFERENCES**