The effect of paraquat on the incorporation of ^{14}C-leucine and ^{14}C-palmitate into lung proteins and lung lipids of rats and rabbits

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Summary

The results of this comparative study show that paraquat added *in vitro* inhibited lipid and protein biosynthesis by lung slices from rats and rabbits during the first 2 hours of incubation. This inhibition was maintained in spite of an increased oxygen uptake and pentose pathway activity during this period.

Paraquat (1 mM) added *in vitro* caused significant inhibition of ^{14}C-leucine and ^{14}C-palmitate incorporation into the soluble proteins and total lipids respectively of rat and rabbit lung slices.

The effect of paraquat (27 mg/kg) administered intraperitoneally to rats and rabbits and assessed on the rate of ^{14}C-leucine incorporation *in vitro* 24 hours after injection, also showed a significant inhibition. However, after 48 hours, protein synthesis was stimulated in rat lungs, whereas paraquat had no effect on rabbit lungs.

A highly significant suppression of the rate of ^{14}C-palmitate incorporation 24 hours after intraperitoneal injection of paraquat was found with rat lung slices, whereas with rabbit lung slices the degree of inhibition was not significant. Forty-eight hours after injection a highly significant inhibition was maintained in rat lung slices while rabbit lung slices showed no inhibition. This finding points to a species difference in the effect of paraquat on metabolic processes and may explain the resistance of rabbit lung to damage by paraquat.

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There is growing concern about the continued use of paraquat, since ingestion of this herbicide is known to cause fatalities in both humans and animals. Paraquat (1,1-dimethyl-4-4-dipyridylium) is a very toxic herbicide and a specific lung toxicant. Regardless of the route of administration, the lung accumulates and retains paraquat at a higher concentration. In mammals, paraquat toxicity is characterized by death of type I and II alveolar cells, followed by oedema, acute degenerative pulmonary changes and subsequently intra-alveolar and interstitial fibrosis.[1] Previous workers[2] demonstrated an association between the amount of paraquat absorbed by the lungs and death. The selective uptake and retention of paraquat in the lung are mediated by an active energy-dependent transport process which might determine the relative toxicity of the herbicide.[3] Although an energy-dependent accumulation of paraquat has been demonstrated for rabbit lung, failure to induce lung lesions in rabbits after *in vivo* exposure suggest that this species is resistant to pulmonary damage by paraquat.[4,5]

Rats injected intravenously with a single dose of paraquat (20 mg/kg) developed alveolar damage, haemorrhage and oedema within 24 hours of administration. Large areas of consolidation were observed at 48 - 72 hours and some evidence of fibrosis at between 7 and 10 days. The intravenous administration of paraquat in doses up to 80 mg/kg in rabbits did not produce the characteristic lung lesions seen in rats and other species although the rabbit mortality rate was high during the first 24 hours.[6]

Lung damage in rats was accompanied by a decrease in the cytochrome P-450 concentration of the microsomes. In rabbits, however, paraquat had no effect on the P-450 system. The microsomal NADPH oxidation by rat lungs, 48 hours after administration, was completely blocked, whereas NADPH reductase was virtually unchanged.[6]

Although paraquat is preferentially localized in lung tissue, the rabbit is exceptional in that paraquat is only initially concentrated in the lung; later the rate of efflux of paraquat from the rabbit lung is more rapid than from the rat lung.[7]

Work in our laboratory showed that no species difference exists between rats and rabbits regarding the *in vitro* effects of paraquat on oxygen uptake, pentose pathway activity and microsomal reactions.[8,9] At a cellular level, the effect of paraquat toxicity on macrophages and fibroblasts differs markedly.[10]

The mechanism of paraquat toxicity in mammals is still unknown. It might involve the generation of superoxide and other highly reactive radicals with subsequent lipid peroxidation.[11] Positive evidence exists that paraquat deranges the surfactant system of the lung, possibly by interference with the synthesis of dipalmitoyl lecithin.[12] Paraquat intoxication also leads to a depletion of the NADPH pool[13] and of the NADPH/NADP ratio in tissues. This might contribute to some metabolic defects during biosynthetic processes.

Further research is necessary regarding: (*i*) the mechanism of paraquat toxicity; (*ii*) the species differences between rats and rabbits as regards paraquat *in vivo;* (*iii*) the induced increase in oxygen consumption and pentose pathway activity over the first 2 hours *in vitro*, and (*iv*) the significance of the reduction in the tissue pool of NADPH.

In the present study the *in vitro* and *in vivo* effects of paraquat on the lung tissue of rats and rabbits respectively were investigated in an effort to elucidate its possible role in metabolic processes.

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Materials and methods

New Zealand White rabbits (1,5 - 2,0 kg) and Wistar White rats (180 - 200 g) were used. The animals were anaesthetized with a sublethal dose of 2,5% thiopentone sodium (18 mg/kg body weight intravenously). The thorax was opened quickly and the lungs perfused *in situ* via the pulmonary artery with 15 ml (rat) and 50 ml (rabbit) isotonic saline at 4°C. Hereafter the lungs were removed, the large airways dissected and the lobes cut into 0,7 mm slices (McIlwain tissue slicer).

In vitro experiments

The *in vitro* effects of 1 mM paraquat (Sigma Chemical Co.) on the incorporation of ^{14}C-leucine into the soluble proteins of lung slices and of ^{14}C-palmitate incorporation into the total lipids of lung slices of rats and rabbits respectively were measured over an incubation period of 2 hours. A period of 2 hours was chosen to coincide with the period of accelerated oxygen uptake observed previously.[8,9] The experiments on rat and rabbit lungs were performed on separate days.

^{14}C-leucine incorporation

When rabbit lungs were used, six samples of slices of 100 mg each were weighed accurately and transferred to metabolic flasks, each containing 4 ml Krebs-Ringer bicarbonate (KRB) medium (pH 7,4) with glucose (10 mM), 50 μM each of 20 amino acids and radiolabelled ^{14}C-leucine (specific activity 54,4 mCi/mmol) (New England Nuclear). Total activity amounted to approximately 500 000 dpm/4 ml buffer. One hundred microlitres of saline was pipetted into three control flasks, and 100 μl paraquat in saline into each of the three experimental flasks to give a final concentration of 1 mM paraquat. The incubation flasks were transferred into a shaking waterbath (90 cycles/min) at 37°C for 2 hours. Thereafter the proteins extractable with low ionic strength buffers (referred to as soluble proteins) were extracted, precipitated and processed as described previously.[12]

When rats were used, the slices from three rat lungs were pooled. Six samples of 100 mg each were weighed accurately and processed as above. Three samples served as controls and to the other three paraquat was added to a concentration of 1 mM. All the flasks were incubated for 2 hours. After precipitation of the protein, it was dissolved in 1 ml 1M NaOH. An aliquot of 100 μl of the protein solution was mixed with 10 ml Instagel (Packard); 250 μl of 5% acetic acid was added and the samples were stored in the dark for 12 hours before counting in a Beckman Liquid Scintillation counter.

Total protein was determined on each sample by the Lowry method[14] using bovine serum albumin (BSA; Miles Laboratories) dissolved in 1M NaOH as standard reference. The amount of radioactive leucine incorporated into the soluble protein fractions was expressed in dpm/mg protein extracted.

^{14}C-palmitate incorporation

The experimental planning for the measurement of ^{14}C-palmitate incorporation into the total lipids of lung slices of rabbits and rats was similar to that for ^{14}C-leucine incorporation, except that 300 mg of lung slices was used for each flask. The incubation flasks contained 3,5 ml KRB (pH 7,4) to which 0,5 ml of an albumin-palmitate complex was added. The complex consisted of 36 mg cold palmitate and 6,0 μCi 1- ^{14}C-palmitate bound to 27,0 ml of 24% bovine serum albumin.[15] The flasks were incubated for 2 hours under similar conditions as for ^{14}C-leucine incorporation. Total lipids were thereafter extracted by the method of Folch *et al.*[16] as previously described.[12] Aliquots of the extract were transferred to counting vials and evaporated in a stream of nitrogen, whereafter 10,0 ml Instagel was added, shaken thoroughly and counted in a Beckman Liquid Scintillation counter. The amount of ^{14}C-palmitate incorporated into lung lipids was calculated and expressed as dpm per total lipids extracted from 300 mg lung tissues over 2 hours.

In vivo experiments

To investigate the *in vivo* effects of paraquat, rats and rabbits were injected intraperitoneally with paraquat in saline 27 mg/kg body weight. Control animals received saline only. After 24 and 48 hours' survival, the animals were sacrificed and their lungs processed as described for the *in vitro* experiments, except that no additional paraquat was added to the incubation flasks. The rates of incorporation of ^{14}C-leucine and of ^{14}C-palmitate were determined *in vitro* and the values compared with those of the control lungs injected with saline only.

Each experiment was repeated 4 times in triplicate and standard methods were used to compute the mean and standard error of the mean. Pair-wise comparisons were made, using a two-sided Student's *t* test.

Results

The results of the effect of paraquat on protein and lipid synthesis of lung slices of rats and rabbits are summarized in the following tables.

In Table I are recorded the effects of paraquat: (*a*) added *in vitro* (1 mM) and (*b*) injected intraperitoneally (27 mg/kg) 24 and 48 hours previously, on the *in vitro* incorporation of ^{14}C-leucine into the soluble proteins of 100 mg lung slices of rats and rabbits over a period of 2 hours. The results are expressed in dpm/mg protein extracted from 100 mg lung slices after 2 hours incubation *in vitro*.

The mean control values of the rates of ^{14}C-leucine incorporation into the soluble proteins of lung slices of both species are very similar. Paraquat *in vitro*, in a concentration of 1 mM, inhibited ^{14}C-leucine incorporation into the soluble lung proteins of both species significantly. However, the degree of inhibition in rat lung slices ($P<0,01$) was more marked than in rabbit lung slices ($P<0,05$). Paraquat injected intraperitoneally (27 mg/kg) 24 hours previously also caused a significant inhibition of the *in vitro* protein synthesis in both species. The values obtained 48 hours after paraquat injection showed that in rat lung tissue a significant stimulation was induced, whereas in the rabbit lung it had a non-significant effect.

In Table II the *in vitro* and *in vivo* effects of paraquat on the incorporation of ^{14}C-palmitate into the total lung lipids of rats and rabbits are summarized. The results are expressed in dpm/total lipids extracted from 300 mg lung slices after 2 hours' incubation.

Paraquat added *in vitro* in a concentration of 1 mM inhibited the ^{14}C-palmitate incorporation into the total lung lipids of both species significantly ($P<0,01$). However, the effects of paraquat administered intraperitoneally (27 mg/kg) and assessed *in vitro* 24 and 48 hours after injection were quite different in the two species. In the rat a highly significant inhibition of the ^{14}C-palmitate incorporation persisted up to 48 hours, whereas in the rabbit paraquat had a non-significant effect on this parameter at both time intervals.

Discussion

Previously reported work[8,9] from this laboratory showed that, over the first 2 hours, paraquat in a medium containing glucose caused an increase in the rate of oxygen uptake as well as an elevation of 1-^{14}C-glucose oxidation to $^{14}CO_2$ by lung slices, followed by a drastic decline of these parameters to below control

TABLE I. EFFECT OF PARAQUAT ON INCORPORATION OF ^{14}C-LEUCINE INTO SOLUBLE PROTEINS OF RAT AND RABBIT LUNG SLICES: (*a*) PARAQUAT ADDED *IN VITRO* (1 mM) AND (*b*) PARAQUAT INJECTED (27 mg/kg) AND ASSESSED *IN VITRO* AFTER 24 AND 48 HOURS (RESULTS ARE EXPRESSED IN dpm/mg PROTEIN EXTRACTED)

	In vitro		*In vivo*			
	Control	PQ	Control	PQ 24 h	Control	PQ 48 h
Rat	12 115	7 135	14 940	7 179	14 357	15 235
	13 910	8 032	14 157	7 361	13 539	16 378
	11 268	7 191	12 056	7 492	13 007	14 313
	14 329	7 706	13 780	7 350	13 734	15 006
Mean	12 906	7 516	13 733	7 346	13 659	15 233
SEM	± 1 454	± 429	± 1 218	± 128	± 558	± 858
		$P<0,01$		$P<0,01$		$P<0,05$
Rabbit	15 129	13 286	14 265	7 099	9 333	9 085
	13 861	9 553	11 077	7 873	9 698	10 773
	13 082	10 564	12 493	7 450	13 418	11 270
	12 822	6 485	11 196	8 171	13 102	11 567
Mean	13 724	9 972	12 258	7 648	11 388	10 674
SEM	± 1 036	± 2 809	± 1 484	± 470	± 2 171	± 1 108
		$P<0,05$		$P<0,01$		$P<0,05$

All estimations in triplicate.

TABLE II. EFFECT OF PARAQUAT ON INCORPORATION OF ^{14}C-PALMITATE INTO TOTAL LIPIDS OF RAT AND RABBIT LUNG SLICES: (*a*) PARAQUAT ADDED *IN VITRO* (1 mM) AND (*b*) PARAQUAT INJECTED (27 mg/kg) AND ASSESSED *IN VITRO* 24 AND 48 HOURS LATER (RESULTS ARE EXPRESSED IN dpm/TOTAL LIPIDS EXTRACTED FROM 300 mg LUNG SLICES OVER 2 HOURS)

	In vitro		*In vivo*			
	Control	PQ	Control	PQ 24 h	Control	PQ 48 h
Rat	66 654	43 698	55 911	31 399	69 559	39 968
	60 752	42 139	52 049	34 389	55 442	29 576
	65 596	51 686	65 261	33 373	45 831	24 522
	60 340	40 840	53 948	26 561	64 815	48 812
Mean	63 336	44 591	56 792	31 431	58 912	35 720
SEM	± 3 254	± 4 872	± 5 862	± 3 476	± 10 510	± 10 841
		$P<0,01$		$P<0,01$		$P<0,01$
Rabbit	52 479	43 592	49 032	36 240	64 355	66 784
	53 613	44 260	42 746	37 342	52 005	55 750
	52 372	46 654	47 466	48 326	67 715	47 927
	50 821	40 760	64 683	58 164	48 352	68 599
Mean	52 321	43 817	50 982	45 018	59 765	58 107
SEM	± 1 147	± 2 425	± 9 517	± 10 324	± 9 722	± 9 376
		$P<0,01$		$P<0,05$		$P<0,05$

All estimations in triplicate.

values. The magnitude of the increase and decrease appeared to be directly related to the paraquat concentration.

In the present study we investigated the effect of paraquat added *in vitro* on lipid and protein biosynthesis in normal lung slices from rats and rabbits over a period of 2 hours. Normally the incorporation of ^{14}C-leucine by control lung slices was linear over a period of 5 hours. However, in the presence of 1 mM paraquat in the incubation medium the *in vitro* incorporation of ^{14}C-leucine over a period of 2 hours into the soluble proteins of lung slices from rats and rabbits was significantly depressed, in spite of the increased oxygen uptake and pentose pathway activity reported previously. The degree of inhibition by 1 mM paraquat was slightly more marked in rats ($P<0,01$) than in rabbits ($P<0,05$).

These findings could possibly be explained by the observation of Rose *et al.*[2] who demonstrated a more active energy-dependent transport system for paraquat in rat lung than in rabbit lung. In our *in vitro* experiments we used 1 mM paraquat in the medium, a concentration that will promote diffusion of paraquat down a concentration gradient into the lung slices. If diffusion was the only determining factor of the amount of intracellular paraquat, one would expect the same concentration of paraquat in rat and rabbit lung slices. However, the kinetic constants for paraquat accumulation by lung slices from rats and rabbits differ, amounting to 300 and 200 nmol/g tissue/h, respectively.[2] The amount of paraquat that accumulates within 2 hours in rat lung slices when active transport is also involved will thus be higher than that in rabbit lung slices. The larger amount of paraquat will cause a more pronounced inhibition because of its dose-response relationship.

As far as the *in vivo* effects of paraquat on protein and lipid biosynthesis were concerned, marked differences were observed between the two species. After 24 hours' exposure to paraquat (27 mg/kg intraperitoneally), ^{14}C-leucine incorporation into the soluble proteins of lung slices of both species tested *in vitro* was significantly inhibited. After 48 hours' exposure to paraquat an insignificant difference was found in rabbit lung slices compared with the normal control values. On the other hand, in rat lung slices a significant stimulation was observed after this period.

Differences in the rate of protein biosynthesis between the two species after paraquat administration *in vivo* could be due, firstly, to the capacity of the lung cells to concentrate and to retain paraquat in the cells with time after injection, and, secondly, to the mobilization of mononuclear cells from the blood. These cells may be involved in protein biosynthesis together with the paraquat-affected lung cells and could explain the observed stimulation of protein biosynthesis in rat lungs 48 hours after paraquat administration *in vivo*. Intravenous administration of a LD_{50} dose of paraquat to rats induced lung lesions within 24 hours and also after intraperitoneal injection, although here a less consistent pattern of lesions was observed.[2] Evidence also exists that the efflux of paraquat from the lung cells of rabbits is faster than from rat lung cells,[7] an observation which is also in support of our present findings.

In rats, paraquat caused a highly significant inhibition of the incorporation of ^{14}C-palmitate into the total lipid of lung slices at both time intervals ($P<0,01$). Paraquat injected intraperitoneally into rabbits, however, had a non-significant effect after both 24 and 48 hours.

In view of the fact that type II alveolar cells are actively involved in surfactant biosynthesis, contributing a large percentage of the extractable lipids of the lung, these cells must particularly be affected by paraquat in rat lungs. From our observations it can be deduced that either the active uptake and retention of paraquat by type II alveolar cells are significantly different in the two species or that the efflux of paraquat from rabbit lung cells is much faster than from rat lung cells.

Montgomery[17] claimed that rat lung microsomes generated higher levels of superoxide radicals than rabbit lung microsomes when exposed to paraquat *in vitro*. If this is true, one would expect marked differences regarding the effect of paraquat on metabolic processes in the two species. However, protein biosynthesis is significantly inhibited by adding paraquat *in vitro* over 2 hours of incubation and also after 24 hours' *in vivo* exposure to paraquat in both species. This observation is also true for the *in vitro* effect of paraquat on the incorporation of ^{14}C-palmitate into the lung lipids of both species. Paraquat *in vivo*, however, appeared to be more harmful to lipid biosynthesis in rat lung than in rabbit lung. These cells, involved in lipid biosynthesis in rat and rabbit lung, therefore appear to have different capacities for concentrating or retaining intracellular paraquat.

Although the generation of superoxide and other highly reactive radicals with subsequent lipid peroxidation[11] is generally accepted as a means by which paraquat induces tissue damage, our results suggest an additional mechanism. In view of the fact that paraquat stimulates the cyanide-insensitive respiration of lung homogenates[18] and increases microsomal activity,[17] the rate of NADPH oxidation will be increased correspondingly, leading to a lowering of the NADPH/NADP ratio. The decrease in the NADPH/NADP ratio in turn stimulates the pentose pathway to generate more NADPH. The uncontrolled increase in the oxidation rate of NADPH by the activated microsomes might lead to a relative shortage of NADPH in the tissue pool, thereby retarding some reactions involved in lipid and protein biosynthesis. A decrease of the NADPH concentration in the tissues could also be induced by a transfer of electrons from NADPH by paraquat directly to molecular oxygen, which would also explain the increase in oxygen uptake[8] of tissues after paraquat exposure.

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