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RESPIRATORY CONTROL IN KIDNEY AND LIVER MITOCHONDRIA ISOLATED FROM RATS TREATED WITH THE POTENT NEPHROTOGENIC AMINONUCLEOSIDE OF PUROMYCIN

CLARE C. JOHNSTON and PAUL BARTLETT*

Edsel B. Ford Institute for Medical Research,
Henry Ford Hospital, Detroit, Mich., U.S.A.

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Abstract—Rates of oxygen uptake of kidney mitochondria from normal and aminonucleoside-nephrotic rats have been determined by means of an oxygen electrode. The rates of oxygen uptake in state 3 (as defined by Chance and Williams) were lower for nephrotic rat kidney mitochondria than for normal kidney mitochondria. This change suggests that kidney mitochondrial enzymes from aminonucleoside-nephrotic rats may be decreased in amount, activity, or accessibility. It is also possible that mitochondria from aminonucleoside-nephrotic rats are less capable of maintaining structural integrity, and thus are damaged more during the isolation procedure. When livers of nephrotic rats were used as the source of mitochondria, impairment of respiratory control was not observed but, in fact, respiratory control ratios were higher than those of normal rat liver mitochondria. Respiratory rates were also obtained by preincubating normal kidney and liver mitochondria with aminonucleoside. No pronounced effect of aminonucleoside upon normal rat kidney mitochondria was observed *in vitro*.

REPEATED daily subcutaneous injections of the aminonucleoside of puromycin, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine, into rats produces a clinical picture and pathological lesions closely resembling those found in the nephrotic syndrome of children.[1] With the levels of aminonucleoside injected, proteinuria commences about the fifth day and progresses until, by the tenth day, large amounts of protein are being excreted. The structural similarity of the nephrotogenic aminonucleoside to naturally occurring adenosine, and the well-established involvement of the nucleotides of the latter in cellular bioenergetics, suggest that some of the metabolic effects of aminonucleoside may be due to induction of alterations in enzymatic processes by which adenosine triphosphate (ATP), required for the maintenance of normal cell function and structural integrity is formed; and/or to induction of alterations in reactions by which the energy of ATP is released. Indeed, previously reported observations of significant impairment in phosphorylation associated with the oxidation of several tricarboxylic acid cycle intermediates in kidney mitochondria of rats treated with aminonucleoside,[2] and of aminonucleoside inhibition and activation of adenosine triphosphatase activity of normal rat kidney mitochondria,[3] would appear to support this viewpoint. Study of respiratory control in kidney mitochondria from

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both normal and aminonucleoside- nephrotic rats furnishes an additional experimental approach that may be utilized in examining cellular bioenergetics at the subcellular level. The present report provides information on the respiration of kidney and liver mitochondria from both normal and aminonucleoside-nephrotic rats in metabolic states 3 and 4, as defined by Chance and Williams,[4] and on *in-vitro* effects of aminonucleoside on both kidney and liver mitochondria from normal rats in these metabolic states.

METHODS

Experiments in vivo

Sprague-Dawley virgin female rats were made nephrotic by 10 daily s.c. injections of 5·2 μmoles aminonucleoside/100 g body weight. Six experimentally nephrotic rats were paired with six 8-month old normal rats in experiment A, in order to compare the oxygen uptake rates in states 3 and 4 for normal and nephrotic rat kidney mitochondria. For confirmatory purposes, another experiment, B, was carried out with two pairs of 6-month old rats. In a similar study, experiment C, liver mitochondria from three pairs of 4-month old rats were used.

A normal and a nephrotic rat were fasted the terminal day. Urine samples were collected and analyzed for protein.[5] For purposes of comparison, mitochondria from a nephrotic rat and from a paired normal rat were prepared and incubated in parallel to minimize changes occurring with different preparations. When it was necessary to hold one partial preparation while an operation was being performed on the parallel sample, the sequence of operations was reversed in another experiment. Mitochondria were prepared essentially by the method of Weinbach,[6] washed twice in 2 ml of 0·25 M sucrose/g wet tissue and then resuspended in 1 ml of 0·25 M sucrose/g wet tissue.

In experiments B and C, the protein concentrations of the mitochondrial suspensions were determined on aliquot portions by a biuret procedure similar to that of Gornall *et al.*,[7] with a 15-min color development period at 38°. On the basis of this analysis, the mitochondrial suspensions were adjusted to essentially equal protein concentrations with 0·25 M sucrose. Equalization of protein concentrations was carried out in experiments B and C, because in experiment A the nitrogen content of the mitochondrial suspensions from the treated rats had averaged 67% that of the normal rat mitochondrial suspensions. This difference was caused, at least in part, by the greater wet weight of the nephrotic rat kidneys. (Changes in the observed respiratory control caused by varying the protein concentration of mitochondrial suspensions used in the incubation media have been reported in the case of α-glycerol phosphate as substrate with cockroach-muscle mitochondria.[8, 9]) During the half hour required to complete analyses on the aliquots and to adjust the concentrations of the mitochondrial suspensions to the same value, the suspensions were kept at 0°. Aliquots of the mitochondrial suspensions were later analyzed for nitrogen by a micro-Kjeldahl procedure.[10]

The oxidation of succinate by the mitochondrial suspension was followed in a GME Oxygraph cell at 30°, by means of a collodion-coated, vibrating platinum electrode. Oxygen uptake was measured alternately on mitochondria from normal and nephrotic rats, and the average of several values (usually triplicate) was obtained for each preparation of mitochondria from both normal and nephrotic rats.

With the exception of mitochondrial suspensions, all solutions used in the incubations were adjusted to pH 7·4 with either NaOH or HCl, except for the phosphate solution which was prepared with KH_2PO_4 and K_2HPO_4. The incubation medium contained per ml: 120 μmoles KCl, 20 μmoles glycylglycine buffer, and 8 μmoles $MgCl_2$. The following solutions were added in 0·1-ml volumes: (1) mitochondrial suspension (approx. 2 mg N/ml); (2) 5 μmoles phosphate; (3) 10 μmoles succinate (Calbiochem, disodium); (4) 0·5 μmoles adenosine diphosphate (ADP; Sigma, sodium salt). Oxygen uptake was recorded during these additions. After the succinate, but before ADP is added, the system is in state 4; after the addition of ADP but before it is completely phosphorylated, the system is in state 3.

Experiments in vitro

Half the mitochondria from a nonfasted normal rat was suspended in a solution of 0·30 M sucrose, 0·02 M glycylglycine, and 10^{-3} M Na_2 EDTA previously adjusted to pH 7·4 with NaOH. The other half of the same preparation of mitochondria was suspended in a solution of 0·25 M sucrose, 0·052 M aminionucleoside, 0·02 M glycylglycine, and 10^{-3} M Na_2 EDTA previously adjusted to pH 7·4 with HCl. The suspensions were held in ice for a half hour before determining oxygen uptake rates as described for the *in-vivo* experiments. The incubation medium contained 18 instead of 20 μmoles of glycylglycine buffer per ml.

RESULTS

The average oxygen uptake rates in states 3 and 4 for normal and nephrotic rat kidney mitochondrial preparations are shown in Table 1, experiments A and B. Results with liver mitochondria (experiment C) are also given in Table 1.

TABLE 1. OXYGEN UPTAKE RATES IN KIDNEY AND LIVER MITOCHONDRIA FROM NORMAL AND NEPHROTIC RATS

Experimental group	No. (*n*) of pairs of animals	Metabolic state	Oxygen uptake ± S.D.[a] (μmoles/mg N/min) (× 10)[b]		P < [e]
			Normal[c]	Nephrotic[d]	
Kidney					
A	6	4	3·3 ± 0·4	3·8 ± 0·5	0·02
A	6	3	11·9 ± 1·4	7·4 ± 1·2	0·01[f]
B	2	4	3·4 ± 0·3	4·0 ± 0·4	0·2
B	2	3	8·3 ± 0·6	5·9 ± 1·7	0·2[g]
Liver					
C	3	4	1·3 ± 0·1	1·3 ± 0·1	0·8
C	3	3	5·9 ± 0·3	7·4 ± 1·0	0·2[h]

[a] Average value using *n* rats and the standard deviation of the average value.

[b] To obtain actual values shift decimal points one place to the left.

[c] Average terminal daily urine protein excretion was 8 mg.

[d] Average terminal daily urine protein excretion was 390 mg.

[e] Significance level of difference between *n* concurrently-run paired variates (normal and nephrotic rat mitochondria prepared in parallel), obtained by the *t* test.[11, 12]

[f] P (unpaired, pooled variates)[11, 12] < 0·001.

[g] Although the small number of pairs of animals used in this experiment precludes a high test of significance by the *t* test, the results may be combined with those of experiment A in the "sign test"[12] (a less discriminating test but one that does not require the assumption of normality), in this case, P < 0·05.

[h] P (unpaired, pooled variates) < 0.1.

In state 4 (before ADP addition) the overall average values for oxygen uptake of kidney mitochondria for nephrotic rats were not very different from the values for normal rats; although, for five of the six pairs in experiment A and for the two pairs in experiment B, the rates of oxygen uptake by the mitochondrial preparations from nephrotic rats kidneys were slightly higher than rates obtained from the normal rat preparations. In liver mitochondrial preparations, there were no consistent differences in rates of oxygen uptake in state 4 between preparations from normal and from nephrotic rats.

In state 3 (after ADP addition) with kidney mitochondria, the average rate of oxygen uptake was appreciably lower for mitochondria from nephrotic rats than for mitochondria from normal rats. Since changes in state 4 were small, while changes in state 3 were appreciable, the respiratory control ratios (i.e. the ratios of the rates of oxygen uptake in state 3 to those in state 4) obtained with mitochondria from nephrotic rats were lower than those obtained with mitochondria from normal rats. With liver mitochondria on the other hand, the rates of oxygen uptake were slightly higher for nephrotic than for normal rats.

Experiments in vitro

Table 2 shows the rates of oxygen uptake obtained in three experiments (D) with normal rat kidney mitochondria preincubated at 0° in the presence and absence of aminonucleoside. The rates of oxygen uptake for similar *in-vitro* experiments with liver mitochondria (E) are also shown. No marked effects of aminonucleoside were seen but, in the case of liver mitochondria, there may have been a slight stimulation of oxygen uptake in state 4 in the presence of aminonucleoside.

TABLE 2. OXYGEN UPTAKE RATES IN NORMAL RAT KIDNEY AND LIVER MITOCHONDRIA PREINCUBATED WITHOUT AND WITH AMINONUCLEOSIDE

Experimental group	No. (*n*) of pairs of animals	Metabolic state	Oxygen uptake ± S.D.[a] (μmoles/ml/min) (×100)[b]		P <[c]
			Aminonucleoside		
			None	0·052 M	
Kidney					
D	3	4	4·4 ± 0·2	5·0 ± 0·8	0·3
D	3	3	13·7 ± 2·0	14·4 ± 1·6	0·6
Liver					
E	3	4	1·8 ± 0·3	2·3 ± 0·1	0·2[d]
E	3	3	9·4 ± 2·0	10·2 ± 1·2	0·4

[a] See footnote [a] of Table 1.
[b] To obtain actual values shift decimal points two places to the left.
[c] Significance level by the *t* test of difference between *n* concurrently-run paired variates (the same mitochondrial preparation preincubated without and with aminonucleoside).[11, 12]
[d] P (unpaired, pooled variates)[11] < 0·05.

DISCUSSION

In state 3, presumably the respiratory chain enzymes of the mitochondria are rate limiting; whereas in state 4, ADP is the rate-limiting component. Induction of aminonucleoside nephrosis results in a reduction of the rate of oxygen uptake in state 3 by kidney mitochondria when succinate is used as substrate. Therefore, nephrotic rat

kidney mitochondria may have a decreased respiratory chain activity compared to normal kidney mitochondria. This reduction could be caused by a decrease in amount, activity, or accessibility of enzymes in the respiratory chain. Also, in comparison with normal kidney mitochondria, kidney mitochondria from aminonucleoside-nephrotic rats may have reduced capabilities for maintaining structural integrity and during the isolation procedure suffer greater damage. In this case, the respiratory control of mitochondria of aminonucleoside-nephrotic rats may be as high *in situ* as that of normal rats. Further experiments are planned to test these possibilities.

Hess[13] has reported some swelling of mitochondria in cells of the middle and distal parts of the proximal convolution of the nephron on the fourth day of induction of aminonucleoside nephrosis, the extent of the mitochondrial swelling progressing daily to include the entire proximal convolution. Kidney tissue slices from aminonucleoside-nephrotic rats have a lower Q_{O_2} than those from normal rats.[14, 15] Furthermore, Fisher *et al.*[14] have histochemically demonstrated decreases in cytochrome oxidase and succinic dehydrogenase activities in kidney slices from aminonucleoside-nephrotic rats.

Liver was investigated to ascertain if induction of aminonucleoside disease also impaired the respiratory control of liver mitochondria. Mitochondria from livers of nephrotic rats, unlike those from kidneys, however, did not have lower respiratory control ratios than the liver mitochondria from normal rats but, indeed, had higher ones. Bartlett *et al.*[16] have reported that phosphorylation associated with the oxidation of succinate by liver mitochondria (as measured by the disappearance of inorganic phosphate) is elevated in aminonucleoside-nephrotic rats as early as the fourth day of induction of the disease.

The effects of aminonucleoside observed *in vitro* were not marked and can not be invoked to explain the *in-vivo* effects of aminonucleoside upon the kidney. There may be a slight elevation of oxygen uptake with liver mitochondria in state 4 in the presence of aminonucleoside. The observations of Bartlett *et al.* indicate that this may not be a simple uncoupling effect; incubation of normal rat liver mitochondria (but not kidney mitochondria) with aminonucleoside resulted in increased disappearance of orthophosphate associated with the oxidation of succinate.[16, 17]

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