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EFFECT OF RUTHENIUM RED ON OXIDATIVE PHOSPHORYLATION AND THE CALCIUM AND MAGNESIUM CONTENT OF SKELETAL MUSCLE MITOCHONDRIA OF NORMAL AND BIO 14.6 DYSTROPHIC HAMSTERS

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SUMMARY

1. During the isolation of normal hamster skeletal muscle mitochondria large amounts of calcium were accumulated by the organelles if this ion was added at the concentration found in the homogenates of muscle from BIO 14.6 dystrophic hamsters.

2. Addition of ruthenium red (2.5 μM) during the isolation of the organelles inhibited the accumulation of Ca^{2+} by both normal and dystrophic muscle mitochondria. Respiration rates and oxidative phosphorylation were not affected in normal muscle mitochondria by the ruthenium red.

3. High Ca^{2+} levels were found in dystrophic muscle mitochondria, irrespective of whether they were isolated in the presence or absence of ruthenium red. These findings are consistent with the view that mitochondria *in situ*, in dystrophic muscle, have higher levels of Ca^{2+} than normal muscle organelles.

4. Normal Mg^{2+} levels were present in dystrophic muscle mitochondria. The mitochondrial content of this ion was not influenced by ruthenium red in preparations from both normal and dystrophic muscles.

INTRODUCTION

We have recently reported[1] that mitochondria isolated from necrosing muscle of BIO 14.6 dystrophic hamsters exhibit depressed respiration and uncoupling of oxidative phosphorylation. These defects, which were ameliorated by the addition of Mg^{2+}, were associated with an elevated mitochondrial Ca^{2+} content[2]. Similar Mg^{2+}-responsive abnormalities could be produced in normal muscle mitochondria by exposure to 200 μM Ca^{2+} during isolation. However, it was not clear whether the high Ca^{2+} levels in dystrophic organelles were present *in situ* or, as with heart mitochondria[3], Ca^{2+} was accumulated during the isolation of the organelles. The present work[4] was an attempt to isolate muscle mitochondria under conditions which limited both Ca^{2+} uptake and its release from the organelles during the isolation procedure. Thus, the aim of this study was to determine the Ca^{2+} levels in mitochondria of

normal and dystrophic muscle, avoiding as much as possible Ca^{2+} movements in and out of the mitochondria during the isolation procedure.

Ruthenium red inhibits *in vitro* Ca^{2+} accumulation by liver mitochondria without interfering with the energy conservation[5], which is necessary for retention of Ca^{2+} by liver mitochondria[6]. It is shown here that ruthenium red exhibits this effect also with mitochondria from skeletal muscle, and that it is enhanced by pyruvate–malate. The combination of ruthenium red and substrate also favoured the retention of Ca^{2+} by the mitochondria. Thus, the combined effect of the inhibition of Ca^{2+} accumulation by ruthenium red and of the retention of endogenous Ca^{2+} due to the available substrate, minimized changes in the calcium levels of the organelles during the isolation procedure. Application of these conditions to dystrophic muscle suggested that the endogenous mitochondrial Ca^{2+} levels in severely affected dystrophic muscle are 5–10 times higher than normal.

MATERIALS AND METHODS

Normal hamsters of the Lakeview-RB strain were obtained from Lakeview Hamster Colony, New Field, New Jersey. Dystrophic hamsters of the BIO 14.6 strain were obtained from the BIO-Research Institute, Cambridge, Mass. Additional animals were bred locally by random mating of dystrophic to dystrophic, and normal to normal animals.

Hamster skeletal muscle mitochondria were isolated at 0–4 °C in the presence of Nagarse by the method described previously[7]. Approximately 2.7–3 g of muscle were incubated for 5 min with frequent stirring in 15 vol. of a medium containing at pH 7.4: 0.21 M mannitol, 0.07 M sucrose and 0.1 mM EDTA (mannitol–sucrose–EDTA). To this medium was added 10 mM Tris–phosphate, 0.5 mg Nagarse per ml, *plus* the additions indicated in the tables. This was followed by homogenization for 30 s using a Teflon pestle (0.66 mm clearance). The homogenate was further incubated for 5 min and then diluted with an equal volume of mannitol–sucrose–EDTA, *plus* the additions indicated in the tables. The resulting suspension was homogenized for 45 s in a S37 Tri-R vessel, using a tight (0.15 mm clearance) glass-reinforced Teflon pestle. Cellular debris was removed by centrifuging this homogenate at $400 \times g$ for 5 min. Mitochondria were harvested by centrifugation of the supernatant from the previous step at $12000 \times g$ for 10 min. The mitochondrial pellet was rinsed with mannitol–sucrose–EDTA *plus* the additions shown in the tables to remove a fluffy layer. The pellet was suspended in 20 vol. of the same medium containing 10 mM Tris–chloride and centrifuged at $8000 \times g$ for 5 min. The pellet was finally suspended in mannitol–sucrose–EDTA medium containing 10 mM Tris–chloride.

Oxygen consumption by mitochondria was measured polarographically with a Clark oxygen electrode[7]. The respiratory control ratios and ADP/O ratios were calculated according to Chance and Williams[8]. The protein concentration of the mitochondrial suspension was determined by the method of Lowry *et al.*[9]. Ca^{2+} and Mg^{2+} were determined using a Perkin–Elmer atomic absorption spectrophotometer, Model 303.

All the chemical reagents were of analytical grade. Ruthenium red was purchased from K and K Laboratories Inc., Plainview, N.Y. Its concentration in the isolation media and in the isolated mitochondria was determined using the spectrophotometric method and the molecular extinction coefficient given by Luft[10].

RESULTS

Table I shows that the presence of 2.5 μM ruthenium red during the isolation procedures which resulted in about 1–2 nmoles of ruthenium red being bound per mg mitochondrial protein did not significantly affect the respiration rates, respiratory control ratios and ADP/O ratios.

TABLE I

EFFECT OF RUTHENIUM RED (RR) ADDITION DURING THE ISOLATION OF MUSCLE MITOCHONDRIA FROM NORMAL HAMSTER

Mitochondria were prepared in two identical batches from muscle mince of individual animals. O_2 rate and ATP formation in all mitochondrial preparations were measured at pH 7.2 in 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris–HCl, 0.02 mM EDTA and 5 mM potassium phosphate at 28 °C.

	Addition	
	None (control)	*RR 2.5 μM*
O_2 rates*	167 ±23 (7)	157 ±14 (7)
Respiratory control ratio	3.8 ± 0.4 (7)	4.0 ± 0.4 (7)
ADP/O	2.31 ± 0.05 (7)	2.40 ± 0.05 (7)

* State 3, expressed as μmoles oxygen consumed per min per g mitochondrial protein at 28 °C. The values presented are means ±S.E. (number of animals). RR has no effect ($P > 0.05$ in paired "t" tests) on the parameters of oxidative phosphorylation.

Table II shows the effect of this compound on the mitochondrial Ca^{2+} levels under various isolation conditions. These normal muscle mitochondria isolated in the presence of 0.2 mM $CaCl_2$ accumulated large amounts of Ca^{2+}. Such Ca^{2+}-loaded organelles exhibited slow respiration and uncoupling of oxidative phosphorylation at 28 °C. Addition of ruthenium red to the isolation media resulted in lower Ca^{2+} values, improved respiration and coupled phosphorylation. To ensure the availability of energy during the isolation of the organelles for the retention of endogenous Ca^{2+}, pyruvate–malate was added to the isolation media. The presence of substrate had no effect on the respiration and the parameters of oxidative phosphorylation of the organelles isolated with or without Ca^{2+} and/or ruthenium red present. However, the addition of substrate as well as ruthenium red in the presence of Ca^{2+} resulted in lower mitochondrial levels of Ca^{2+} than the addition of ruthenium red and Ca^{2+} alone. No clear explanation is apparent for this effect of substrate. However, it may be that the latter influences the effectiveness of ruthenium red in blocking Ca^{2+} uptake, possibly by affecting membrane conformation. The augmenting effect of pyruvate–malate on the action of ruthenium red permitted the isolation of mitochondria from a Ca^{2+}-rich medium without the trapping of large amounts of this ion by the organelles. On the other hand, when no exogenous Ca^{2+} was added, ruthenium red *plus* pyruvate–malate had little effect on the Ca^{2+} levels.

The specific activity of the Ca^{2+} of mitochondria isolated in the presence of $^{40}CaCl_2$ from animals injected with a tracer amount of $^{45}Ca^{2+}$ prior to sacrifice was

TABLE II

EFFECTS OF RUTHENIUM RED (RR) AND SUBSTRATE ADDITION DURING THE ISOLATION OF MUSCLE MITOCHONDRIA FROM NORMAL HAMSTERS IN THE PRESENCE OF Ca^{2+}

Mitochondria were isolated as described in Materials and Methods, and tested as in Table I. The hamsters were injected intraperitoneally 45 min before sacrifice, with approximately 0.14 mCi $^{45}Ca^{2+}$ (0.25 μmole) per 100 g body weight. Values shown are means ± S.E. (number of animals).

	Additions (mM)					
	None	*$CaCl_2$ 0.2*	*$CaCl_2$, pyruvate 5, L-malate 1*	*$CaCl_2$, RR 2.5·10⁻³*	*$CaCl_2$, RR, pyruvate, L-malate*	*RR, pyruvate, L-malate*
O_2 rates*	220 ±28 (3)	37 ± 5 (3)	65 ±13 (2)	193 ± 9 (2)	187 ±21 (6)	243 ±14 (3)
Respiratory control ratios	5.3± 0.9 (3)	1.0± 0.0 (2)	1.0± 0.0 (2)	4.6 (1)	3.8± 0.3 (6)	6.5± 1.2 (3)
ADP/O	2.4± 0.1 (3)	0 (2)	0 (2)	2.3 (1)	2.4± 0.1 (6)	2.5± 0.1 (3)
Mg**	34 ± 4 (2)	29 ± 2 (3)	34 ± 0.3 (3)	32 ± 1 (3)	31 ± 2 (3)	35 ± 9 (3)
Ca**	28 ± 7 (3)	358 ±45 (3)	355 ±45 (2)	94 ±17 (3)	39 ± 6 (8)	22 ± 6 (3)
Mitochondrial Ca^{2+} specific activity (cpm/nmole)	—	85 ±11 (3)	91 ± 7 (3)	155 ±17 (3)	311 ±60 (3)	—

* State 3, expressed as μmoles oxygen consumed per min per g mitochondrial protein.
** nmoles per mg mitochondrial protein.

highest in the organelles with the lowest Ca^{2+} content (Table II). This was consistent with the inhibition by ruthenium red of the accumulation of Ca^{2+} from the medium. The further addition of substrate doubled the specific activity and halved the Ca^{2+} levels seen in the presence of ruthenium red alone. It therefore appears that pyruvate–malate enhanced the inhibition by ruthenium red of $^{40}Ca^{2+}$ uptake by the mitochondria. The Mg^{2+} content of the organelles was not affected by the conditions in these experiments.

Table III illustrates the findings with muscle mitochondria from dystrophic BIO 14.6 hamsters at an age (65–85 days) when the necrosing lesions are most extensive and are visible as longitudinal white streaks in the skeletal muscle. The severity of the dystrophic process in such animals is quite variable[2] and the mitochondria may be essentially normal with respect to the parameters measured here (*e.g.* hamster No. 7), or may be severely affected (*e.g.* No. 10). As reported earlier[2] and as seen in Table III, the organelles from hamsters with extensive muscle streaking had depressed respiration and were uncoupled and had elevated Ca^{2+} levels, but a normal Mg^{2+} content. The presence of ruthenium red and pyruvate–malate during the isolation procedure, resulted in lower Ca^{2+} levels in the mitochondria and improved respiration and oxidative phosphorylation. In these experiments labelled Ca^{2+} was not injected into the animals prior to sacrifice, but tracer quantities of $^{45}Ca^{2+}$ were added to the homogenizing medium instead of 0.2 mM $CaCl_2$ (Table III). The specific

TABLE III

EFFECTS OF RUTHENIUM RED (RR) AND SUBSTRATE ADDITION DURING THE ISOLATION OF MUSCLE MITOCHONDRIA FROM DYSTROPHIC HAMSTERS

The hamsters were not injected with $^{45}Ca^{2+}$ as in Table II, but 375 μCi $^{45}Ca^{2+}$/l, ($5 \cdot 10^{-4}$ mM) was added to the homogenizing medium. The organelles were isolated as described in Materials and Methods, and tested as in Table I.

	Hamster No. 7		*Hamster No. 8*		*Hamster No. 9*		*Hamster No. 10*	
	*A**	*B***	*A*	*B*	*A*	*B*	*A*	*B*
O_2 rates***	209	176	149	244	32	196	64	184
Respiratory control ratios	4.5	5.3	2.3	3.7	1	3.8	1	3.3
ADP/O	2.47	2.56	1.95	2.38	0	2.16	0	2.24
†Mg Initial	45	41	42	39	25	34	42	40
†Mg Final (%)	70	71	46	70	54	67	13	62
†Ca Initial	43	25	111	65	262	240	335	166
†Ca Final (%)	100	36	63	24	—	16	70	17
Mitochondrial Ca^{2+} specific activity (cpm/nmole)	146	9	56	4	85	3	151	8

* *A*, $^{45}Ca^{2+}$ added to the homogenizing medium.

** *B*, additions: $^{45}Ca^{2+}$, RR ($2.5 \cdot 10^{-3}$ mM), pyruvate (5 mM) and L-malate (1 mM). 1–2 nmoles of RR were bound per mg mitochondrial protein in such experiments.

*** State 3, expressed as μmoles oxygen consumed per min per g mitochondrial protein.

† Ca and Mg "Initial" values are expressed as nmoles per mg mitochondrial protein. "Final" Mg and Ca values are expressed as % of "Initial" values and were estimated at the end of oxidative phosphorylation determinations.

activity values indicate that the lower Ca^{2+} levels resulted largely from an inhibition of Ca^{2+} accumulation by ruthenium red in the presence of substrate. However, it is evident that even under these conditions, mitochondria isolated from severely dystrophic animals had much higher Ca^{2+} levels than organelles from normal hamsters (Table III *vs* Table II).

It is noteworthy that dystrophic mitochondria isolated in the presence of ruthenium red and substrate were coupled at mitochondrial Ca^{2+} levels which often are associated with uncoupling (hamster No. 9 and No. 10, Table III; also Table II in ref. 2). This may have been due in part to the much greater loss of Ca^{2+} from the treated mitochondria during the course of the oxidative phosphorylation assays (see "Final" values, Table III). The polarographic tracings obtained during these assays showed a progressive improvement in the efficiency of coupling which was reflected in a small increase in the respiratory control ratios. In addition, the presence of ruthenium red and substrate during the isolation of the dystrophic organelles resulted in a better retention of Mg^{2+} during the oxidative phosphorylation determinations. It is uncertain whether the beneficial effect of the additives on phosphorylative coupling was due to Ca^{2+} loss, Mg^{2+} retention or some unidentified effect of ruthenium red.

DISCUSSION

Ruthenium red is a specific inhibitor of Ca^{2+} transport in mitochondria[5]. However, it was shown by Vasington *et al.*[11] that at high concentrations, (> 10 nmoles per mg mitochondrial protein), ruthenium red inhibited State 4 respiration markedly and decreased State 3 rates. The present findings indicate that under our experimental conditions ruthenium red did not interfere with State 3 or State 4 rates in normal skeletal muscle mitochondria (Table I). This may be due to the fact that during the isolation of the organelles only a small quantity of ruthenium red was bound to the mitochondria, but that this nevertheless was sufficient to inhibit the accumulation of Ca^{2+}. There was no difference in amount of ruthenium red bound to the organelles from normal or dystrophic skeletal muscle. These experimental conditions when applied to severely dystrophic muscle yielded organelles that had Ca^{2+} levels 5–10 times higher than normal. Even these values may be lower than *in vivo* levels, because the possibility of Ca^{2+} loss from the mitochondria during the isolation procedure has not been entirely excluded.

The improvement in oxidative phosphorylation coupling found in dystrophic mitochondria isolated in the presence of ruthenium red and substrate was associated with better retention of Mg^{2+} by the organelles and a greater loss of Ca^{2+} during the assay. It is possible that sufficient ruthenium red remained adsorbed to the organelles to impede re-entry of the Ca^{2+} lost to the medium during the determination of oxidative phosphorylation. As was shown earlier[2], oxidative phosphorylation can also be improved in dystrophic mitochondria by 3–5 mM Mg^{2+}. Interestingly, Mg^{2+} also enhances Ca^{2+} uptake in such preparations, rather than stimulating its release (Wrogemann, K., Jacobson, B. J. and Blanchaer, M. C., unpublished).

The finding described here occurs in hamsters as young as 30 days[2] and thus represents one of the earliest abnormalities reported in this type of muscular dystrophy. However, it cannot be decided at present whether this mitochondrial abnormality is the primary expression of this genetic disease or whether it is a secondary defect

resulting from a still unidentified primary lesion. Nevertheless the present study affirms that dystrophic muscle mitochondria do have elevated calcium levels prior to their isolation. Thus, it is possible that the mitochondria of such muscle have a decreased capacity for oxidative phosphorylation *in vivo*.

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