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THE MEASUREMENT OF THE ROTENONE-SENSITIVE NADH CYTOCHROME *c* REDUCTASE ACTIVITY IN MITOCHONDRIA ISOLATED FROM MINUTE AMOUNT OF HUMAN SKELETAL MUSCLE

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Mitochondria isolated from minute amounts (100-500 mg) of human skeletal muscle displayed a very high rotenone-resistant NADH cytochrome *c* reductase activity. Moreover, compared to succinate cytochrome *c* reductase activity, a low rate of rotenone-sensitive NADH cytochrome *c* reductase activity was measured when using standard procedures to disrupt mitochondrial membranes. Only a drastic osmotic shock in distillated water as a mean to disrupt mitochondrial membrane was found to strongly increase the actual rate of the rotenone-sensitive activity. This was accompanied by a decrease in the rotenone-insensitive activity. Using such a simple procedure, the NADH cytochrome *c* reductase was found 70-80% inhibited by rotenone and roughly equivalent to 70-85% of the activity of the succinate cytochrome *c* reductase.

Reliable tests for the measurement of complex IV (COX) and complex II *plus* III (SCCR) activities are available and widely used to investigate respiratory chain disorders in human skeletal muscle (1,2). In contrast, major difficulties are encountered when trying to measure the NADH-dependent reductase activities, partially due to the high NADH cytochrome b_5 reductase activity mostly associated with endoplasmic reticulum (3). Indeed, the rotenone-sensitive NCCR activity (4) often represents a minor part of the total NCCR activity (5,6), and only about 30 to 40% of the SCCR activity (1,2). The measurement of NADH-ubiquinone reductase activity have been used as well (7). Unfortunately, it requires the commercially non-available short chain ubiquinones as acceptors. Moreover, a delicate balance between lipophilicity of quinone (conferring activity and specificity) and solubility in aqueous medium has to be handled. The

Abbreviations: BSA, bovine serum albumin; Cyt, cytochrome; COX, cytochrome *c* reductase; NCCR, NADH cytochrome *c* reductase; SCCR, succinate cytochrome *c* reductase.

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oxidation of Krebs cycle substrates generating NADH is also often taken as an index for the activity of complex I (8,9,10). However, this approach uses a relatively large amount of mitochondria and is dependent both on quality of the mitochondrial preparation and on activity of the NADH-generating dehydrogenases (11).

This was an incentive to work out experimental conditions for measuring the NCCR activity of mitochondrial preparation from minute amounts of skeletal muscles.

MATERIALS AND METHODS

Mitochondria were isolated by differential centrifugation from micro-biopsies (100 to 500 mg) under local anesthesia of the deltoid of children (less than 1 y of age). These biopsies were performed for histochemical investigations unrelated to respiratory chain disorders. Fresh tissue were washed in 5 ml of extraction medium (0.25 M sucrose, 40 mM KCl, 3 mM EDTA and 20 mM Tris-HCl, pH 7.4), quickly minced with scissors and disrupted in 30 ml of extraction medium in a Potter-Eljehvem (150 µM clearance) by 3 strokes of a motor-driven pestle (500 t/min). The homogenate was filtered through a 200 µM nylon net, centrifuged at 2,000 *g* for 10 min and the supernatant was kept on ice. The pellet was resuspended in 5 ml of extraction medium and homogenized in a Potter-Eljehvem (150 µM clearance, 3 strokes at 500 t/min). The second homogenate was centrifuged at 2,000 *g* for 10 min. The two supernatants were then pooled and the mitochondria spun down at 10,000 *g* for 10 min. The reddish pellet was layered on top of 1 ml of extraction medium containing 5% Percoll in 1.5-ml tube and centrifuged at 10.000 *g* for 10 min. Adding Percoll has been previously shown to increase the stability of the mitochondria (12). The mitochondria were resuspended in the smallest volume of extraction medium. From their impermeability to exogenous cyt *c*, more than 90% of the mitochondria appeared intact. In addition, no oxygen consumption triggered by the addition of exogenous NADH to mitochondria could be measured.

Enzyme activities were spectrophotometrically measured by following either the oxidation or the reduction of cyt *c* at 550 nm using standard procedures (4). COX activity (EC 1.9.3.1) was measured in 10 mM phosphate buffer (pH 6.5) containing 1 mg/ml BSA, using 10 µM reduced cyt *c*. SCCR was assayed in 10 mM phosphate buffer (pH 7.8) containing 1 mg/ml BSA, 0.2 mM KCN, 0.1 mM ATP, 3 µM rotenone and 40 µM oxidized cyt *c*. NCCR was assayed in 10 mM phosphate buffer (pH 8.0), containing 1 mg/ml BSA, 0.2 mM KCN, 40 µM oxidized cyt *c*. Malate dehydrogenase activity (EC 1.1.1.37) was measured as previously described (13).

Disruption of mitochondria by sonication (3x15s) was carried out in ice-cold tubes (1.5-ml) containing 10 mM phosphate buffer (500 µl) using an ultrasonic processor (VibraCell, Bioblock) equipped with a 2.5-mm microtip. For mitochondria disruption by the freeze-thaw method,the mitochondrial preparation was plunged into acetone mixed with solid carbon dioxide until completely frozen, and then allowed to stand at room temperature until completely thawed. The freeze-thaw cycle was repeated three times as fast as possible. Detergent treatments were carried out using anionic (cholic and taurocholate acid), zwitterionic (CHAPS) or non-ionic (Triton X-100, digitonin, Tween 80) detergents. For each detergent, the ratios of detergent to protein used were those causing the loss of the respiratory control of the mitochondria and those causing the complete release of the matrix malate dehydrogenase activity. Finally, disruption of mitochondrial membranes was obtained by incubating mitochondria (10 to 30 µg protein) in 1.6 ml of distillated water.

TABLE I. The effect of the disruption of mitochondrial membranes by the mean of different methods on the NADH cyt *c* reductase activity of mitochondria isolated from human skeletal muscle

Treatment	NADH cyt *c* reductase activity (nmol cyt *c* reduced/min/mg protein)		
	Total	Rotenone-sensitive	Rotenone-insensitive
Control			
a 10 mM Tris	345	115 (33%)[a]	230 (67%)
b 5 mM Tris	349	119 (34%)	230 (66%)
Sonication			
c 20 kHertz	361	98 (27%)	262 (73%)
d 30 kHertz	328	115 (35%)	213 (65%)
e 40 kHertz	246	115 (47%)	131 (53%)
f 50 kHertz	115	16 (14%)	98 (86%)
Freeze-thaw			
g 3 cycles	443	164 (37%)	279 (63%)
Detergent (Tween 80)			
h 0.1 mg/mg protein	234	34(15%)	200 (85%)
i 10 m/mg protein	263	33 (13%)	230 (87%)
Water treatment			
j 1 min	540	356 (66%)	184 (34%)
k 3 min	542	384 (71%)	158 (29%)
l 10 min	495	376 (76%)	119 (24%)
m 15 min	478	376 (79%)	102 (21%)

Experimental conditions are described under Materials and methods.
[a]percent of total NCCR activity.

Protein was determined by the method of Bradford (14) using BSA as a standard. All chemicals were of the highest purity available from Sigma Co., and Boehringer.

RESULTS

In the course of this study, the inhibition of the NCCR activity by rotenone (4) has been found to vary from a few percent to a maximum of 40% of the total NCCR activity according to the mitochondrial preparation. As a consequence, assays have to be made on the same mitochondrial preparation so as to allow comparison between the various permeabilizing

treatments. Table I presents a typical set of results obtained when measuring the activity of the NCCR in mitochondria supposedly made permeable to NADH by using various treatments. Mitochondria were first swollen in 10 mM phosphate buffer (line *a*). Both the SCCR (540 nmol/min/mg protein) and the COX (1560 nmol/min/mg protein) activities were maximal at such a low osmolarity, indicating the free access of cyt *c* to the inner mitochondrial membrane. Low osmolarity also made the inner membrane partially permeable to NADH, as shown by the partial sensitivity of the NCCR activity to rotenone (line *a*, 33% inhibition). Decreasing the osmolarity to a lower value (phosphate buffer 5 mM) did not significantly increase the rate of the rotenone-sensitive NCCR (line *b*). Mitochondria were therefore disrupted by sonication using increasing power output (lines *c* to *f*). A decrease in the total NCCR activity was measured when increasing the power output. This was accounted for by the loss of the rotenone-resistant activity. No significant change in the rotenone-sensitive activity was found, except at maximal power output, when a dramatic decrease occurred (line *f*). These data prompted us to look for other methods of membrane disruption (table I, line *g-m*). Subsequently, freeze-thaw cycles were used as an alternative method to disrupt mitochondrial membranes (line *g*).This enhanced both rotenone-sensitive and insensitive activities. The rotenone-sensitive NCCR activity still represented less than 40% of the total activity. The effect of detergents was next investigated. Results obtained using Tween 80, likely to have a low toxicity for mitochondria (15), are shown in Table I (line *h* and *i*). The level of the rotenone-sensitive activity after detergent treatment fell to only 10 to 15% of the total NCCR activity. Similarly, we tested various detergents (anionic, zwitterionic, non-ionic), but all gave very similar results, *ie.* a rapid loss of the rotenone-sensitive activity (not shown).

It thus appears that, in our hands, none of these classical methods of mitochondrial membrane disruption was successful in measuring a high rate of rotenone-sensitive NCCR when studying human skeletal muscle mitochondria. As the last resort, a drastic osmotic shock (*ie.* 2 µl of the mitochondrial suspension in 1.6 ml of distillated water) was used to disrupt mitochondrial membranes (Table I, line *k* to *m*). After 1 min of incubation at 37°C, both an increase in the rate of the rotenone-sensitive activity and a decrease in the rate of the rotenone-resistant NCCR activity were measured. As a result the rotenone-sensitive activity represented 70% of total. Increasing the duration of the incubation time neither enhanced nor decreased the rotenone sensitive activity, although still decreasing the rotenone insensitive one (line *k* to *m*). A similar increase in the rate of the rotenone-sensitive NCCR activity upon drastic

osmotic shock has been obtained when using rat muscle mitochondria (from 592 to 1361 nmol cyt *c* reduced/min/mg protein). However, in this latter case the percent of rotenone sensitivity was already high without osmotic shock in water (about 85%).

While only a drastic osmotic shock unveiled an active rotenone-sensitive NCCR, freeze-thaw as well as sonication brought about a nearly total release of matrix malate dehydrogenase (95 to 100% of the activity liberated by an osmotic shock in water). As a consequence, mitochondria should have been made permeable to NADH too. A potential loss of the rotenone-sensitive NCCR activity caused by these treatments was therefore next considered. The rotenone-sensitive NCCR activity was first released by a drastic osmotic shock. Then, the effect of either freeze-thaw or sonication on level of rotenone-sensitive NCCR activity was investigated (Table II). Both procedures resulted in a significant decrease of the rotenone-sensitive NCCR activity. This established the high unstability of this activity upon sonication or freeze-thaw.

Finally, the rotenone-sensitive NCCR activity of water-treated mitochondria was compared to the SCCR activity (Table III). As previously

Table II. Effect of sonication and freeze-thaw on the NADH cyt *c* reductase activities of human skeletal muscle

Treatment	NADH cyt *c* reductase activity (nmol cyt *c* reduced/min/mg protein)		
	Total	Rotenone-sensitive	Rotenone-insensitive
Control			
*a*Water treatment (3 min)	542	384(71%)[a]	158 (29%)
Freeze-thaw			
*A*Water treatment *plus* freeze-thaw (3 cycles)	427	279 (65%)	148 (35%)
Sonication			
*a*Water treatment *plus* sonication (40 kHertz)	263	181 (69%)	82 (31%)

Experimental conditions are described under Materials and methods.
[a]Percent of total NCCR activity.

Table III. Comparison between the activities of the rotenone-sensitive NADH cyt *c* reductase and the succinate cyt *c* reductase in mitochondria isolated from human skeletal muscle

Experiment	Succinate cyt *c* reductase	Rotenone-sensitive NADH cyt *c* reductase	Ratio NCCR/SCCR
	(nmol cyt *c* reduced/min/mg prot)		
n°1	188	156	0.83
n°2	199	166	0.83
n°3	542	384	0.70
n°4	152	130	0.85
n°5	393	278	0.71
	294 ± 155[a]	223 ± 109	0.78 ± 0.056

[a]average value and standard error.

reported when studying these enzyme activities in children muscle, relatively high standard errors were calculated (2,7). However, the ratio between the two activities appeared to vary to a lesser extent, as indicated by the much lower standard error associated with this parameter. According to this parameter, the activity of the rotenone-sensitive NCCR represented about 75% of the activity of the SCCR, a value rather similar to that found in mitochondria from other sources (3).

DISCUSSION

The measurement of the rotenone-sensitive NCCR activity of mitochondria requires a free access of NADH to the matrix side of the inner mitochondrial membrane (16). Low osmolarity, sonication and/or freeze-thaw have proved to be successful to measure this activity when using mitochondria from various sources (4,16). However, such hypotonic treatments appeared insufficient to allow a rapid diffusion of NADH in human skeletal muscle mitochondria. Attempts to disrupt the inner membrane by sonication, freeze-thaw, or detergent were also unsuccessful due to a rapid loss of activity of the rotenone-sensitive NCCR. Only a drastic osmotic shock in distillated water resulted in a net increase of the rate of the rotenone-sensitive NCCR activity. Under these conditions, this activity represented about 75% of the SCCR activity. Moreover, the rotenone-insensitive activity - NADH cyt b_5 reductase associated with endoplasmic reticulum and with the mitochondrial outer membrane (3) - was reduced to less than 40% of the total activity. Introducing a purification

step (centrifugation on saccharose or Percoll gradients, phase partition) could further reduce contaminating activities. Unfortunately, this would select a population of mitochondria (17), while a potential heterogeneity of the mitochondrial population associated with respiratory chain disorders has been repeatedly reported (18,19).

How the remarkable stabilization of inner mitochondrial membrane is achieved in human skeletal muscle remains to be elucidated. Interestingly enough, a very high resistance to hypotonic treatment of mitochondria from rabbit epididymal spermatozoa has also been reported (20). However in this latter instance, even the outer membrane of mitochondria treated with 10 mM phosphate was not made permeable to exogenous cyt *c*. Similarly, rat heart mitochondria have proved to be much less prone to swelling in hypotonic medium than rat liver mitochondria (21). In keeping with this, major difficulties to measure the rotenone-sensitive NCCR activity of human fibroblast mitochondria have been reported (22) a feature which could be related to the existence of a similar permeability barrier to exogenous NADH.

The determination of experimental conditions allowing the use of the sensitive and convenient rotenone-sensitive NCCR assay (complex I *plus* III) using unpurified muscle mitochondria should allow better detection of dysfunction of this segment of the respiratory chain in subcellular preparation of skeletal muscle tissues.

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