Evaluation of Mitochondrial Function

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Summary: This protocol describes the procedures used by the AMDCC for evaluating mitochondrial function.

Protocol:

Protocols for Mitochondrial Analyses

Saponin-permeabilized fibers. Left ventricular cardiac fibers are prepared using previously described protocols (1,2). Briefly, small pieces (2-5 mg) of cardiac muscle are taken from the left ventricle and permeabilized with 50 μg/ml saponin at 4°C in buffer A containing (in mM) 7.23 K_2EGTA, 2.77 K_2CaEGTA, 6.56 $MgCl_2$, 20 imidazole, 0.5 dithiothreitol, 53.3 K-methanS, 20 taurine, 5.3 Na_2ATP, 15 PCr and 3 KH_2PO_4, pH 7.1 adjusted at 25°C. The fibers are then washed twice for 10 min in buffer B containing (in mM) 7.23 K_2EGTA, 2.77 K_2CaEGTA, 1.38 $MgCl_2$, 20 imidazole, 0.5 dithiothreitol, 100 K-methanS, 20 taurine, 3 KH_2PO_4 and 2 mg/ml BSA, pH 7.1 adjusted at 25°C.

Respiration measurements. The respiratory rates of saponin-permeabilized fibers are determined using an oxygen foxy probe in 2 ml solution B at 25°C with continuous stirring. Substrates used include 5 mM glutamate and 2 mM malate, which evaluates the integrity of the TCA cycle and electron transport chain, 10 mM pyruvate and 5 mM malate which evaluates flux through pyruvate dehydrogenase as well as the TCA cycle and electron transport chain and 20 μM palmitoyl-carnitine with 2 mM malate, which evaluates fatty acid beta oxidation. The solubility of oxygen in the solution B is 215 nmol O_2/ml. Oxygen consumption rates are expressed in nmoles O_2/min/mg dry weight. The following respiratory parameters are measured:

(1) State 2: Mitochondrial respirations in the presence of substrate but in the absence of any added ADP.
(2) State 3: Maximal stimulated mitochondrial respirations, which is initiated by adding 1mM ADP (in the presence of substrate).
(3) State 4: Mitochondrial respiration in the absence of ATP synthesis, which is achieved by adding the ATP synthase inhibitor oligomycin (1μg/ml). An increase in state 4 respiration would be suggestive of mitochondrial uncoupling.

(4) Respiratory control ratio (RC) is the ratio of state 3 to state 4 respirations. This ratio is used as a general index of the viability of the mitochondrial preparation. Low RC ratios raise the possibility of mitochondrial damage.

ATP measurements. ATP concentrations are determined by bioluminescence with the luciferin/luciferase reaction as described (3), using a commercially available ATP assay kit (Enliten ATP assay system, Promega, Madison WI).

Mitochondrial enzyme activity assays.

Mitochondrial Isolation: Hearts are minced and homogenized on ice in 10% (wt/v) isolation buffer containing (in mM) 20 HEPES (pH 7.4), 140 KCl, 10 EDTA, 5 $MgCl_2$ supplemented with 2-4 mg nagarse. The homogenate is then centrifuged at 500 g for 10 min. The debris is discarded and the supernatant centrifuged at 9,000 g for 35 min. The pellet is then washed with the isolation buffer without nagarse and suspended in 80 µl isolation buffer. Protein concentration is determined using the Micro BCA protein assay kit (Pierce Chemical Co., Rockford, Illinois, USA).

Carnitine palmitoyl transferase. Total CPT (CPT I and CPT II) activity is measured in isolated mitochondria (see above). Mitochondria (~200 µg) are assayed in 1 ml reaction buffer containing (in mM) 20 HEPES, 1 EGTA, 220 sucrose, 40 KCl, 0.1 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 1.3 mg/ml BSA, and 40 µM palmitoyl-CoA, pH 7.4 at 25°C. The reaction is started by adding 1 mM carnitine (omitted in baseline) and monitored at 412 nm for 4 min using an Ultraspec 3000 spectrophotometer. CPT II activity is measured using an identical reaction as total CPT but after adding 10 µM malonyl-CoA to inhibit CPT I activity. CPT I activity is calculated by subtracting the CPT II activity from the total CPT activity.

Citrate synthase. Small frozen pieces of hearts (~10 mg) are homogenized in 20% (wt/v) homogenization buffer containing (in mM) 20 HEPES, 10 EDTA, pH 7.4 on ice. The homogenates are then frozen for 1h to liberate citrate synthase from mitochondrial matrix and diluted 1:10. The reaction is then performed in 1 ml reaction buffer containing (in mM) 20 HEPES, 1 EGTA, 220 sucrose, 40 KCl, 0.1 DTNB, and 0.1 acetyl-CoA, pH 7.4 at 25°C. The reaction is started by adding 0.05 mM oxaloacetate (omitted at baseline) and monitored at 412 nm for 3 min using an Ultraspec 3000 spectrophotometer.

β-Hydroxyacyl-CoA dehydrogenase. Homogenates are prepared as used for the citrate synthase assay and diluted 1:4. The reaction is performed in 1 ml reaction buffer containing (in mM) 20 HEPES, 1 EGTA, 1 KCN, and 0.15 NADH, pH 7.4 at 25°C. The reaction is started by adding 0.1 mM acetoacetyl-CoA (omitted at baseline) and monitored at 340 nm for 4 min using an Ultraspec 3000 spectrophotometer.

References

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