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Original Contribution

Temperature controls oxidative phosphorylation and reactive oxygen species production through uncoupling in rat skeletal muscle mitochondria

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ABSTRACT

Mitochondrial respiratory and phosphorylation activities, mitochondrial uncoupling, and hydrogen peroxide formation were studied in isolated rat skeletal muscle mitochondria during experimentally induced hypothermia (25 °C) and hyperthermia (42 °C) compared to the physiological temperature of resting muscle (35 °C). For nonphosphorylating mitochondria, increasing the temperature from 25 to 42 °C led to a decrease in membrane potential, hydrogen peroxide production, and quinone reduction levels. For phosphorylating mitochondria, no temperature-dependent changes in these mitochondrial functions were observed. However, the efficiency of oxidative phosphorylation decreased, whereas the oxidation and phosphorylation rates and oxidative capacities of the mitochondria increased, with increasing assay temperature. An increase in proton leak, including uncoupling protein-mediated proton leak, was observed with increasing assay temperature, which could explain the reduced oxidative phosphorylation efficiency and reactive oxygen species production.

Mitochondrial oxidative phosphorylation (OXPHOS) plays a key role in skeletal muscle energy homeostasis under various physiological conditions (see, e.g., [4,14,17,19,39]). However, both the rate of this metabolic process and its efficiency in skeletal muscle can be significantly affected by changes in muscle temperature [6,35], which at rest amounts to 35–36 °C in rats [6] and humans [3,29]. Various strains of rats can survive a decrease in their core temperature to approximately 15 °C and its increase to approximately 43 °C [12,25]. Interestingly, similar values of the highest and lowest survivable core temperatures have been reported for humans [7,33]. In rat skeletal muscle mitochondria, with increasing assay temperature (from 37 to 43–45 °C), respiratory acceleration and a pronounced decrease in ADP/O ratio have been observed [6,35]. With decreasing assay temperature (from 37 to 25 °C), a significant decrease in respiratory rate has been accompanied by no change in ADP/O ratio [6]. Surprisingly, the effects of the extremely high and very low temperatures on the function of skeletal muscle mitochondria, especially their phosphorylation activities, uncoupling, and reactive oxygen species (ROS) production, are still poorly understood.

It is well documented that mitochondria produce ROS, which are generated as a by-product of oxygen metabolism (for review see [11,20,24,26,28]. Enhanced ROS production seems to be involved in cell damage. On the other hand, there is a growing body of evidence that enhanced ROS production stimulates vital processes for maintaining muscle homeostasis and muscle adaptation to exercise, including mitochondria biogenesis [2,13,15,27,30]. Interestingly, in rat brain mitochondria, a decrease in temperature from 37 to 32 °C results in an increase in ROS production [1]. Surprisingly, to the best of our knowledge, no effect of hyperthermia or hypothermia on skeletal muscle mitochondrial ROS production has been studied so far. Similarly, the literature poorly documents on the role of mitochondrial membranes (via proton conductance) in the control of OXPHOS by varying the temperature.

Mitochondrial energy metabolism, especially the OXPHOS step, is strictly controlled by a proton electrochemical gradient resulting from oxidation of reducing fuels and from a proton leakage through the inner mitochondrial membrane. Similarly, mitochondrial ROS production is described to be a direct function of the proton electrochemical

Abbreviations: BSA, bovine serum albumin; CS, citrate synthase; COX, cytochrome *c* oxidase; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; ROS, reactive oxygen species; Q, quinone, coenzyme Q; UCP, uncoupling protein; ΔΨm, mitochondrial transmembrane electrical potential

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gradient and controlled by mild uncoupling via proton leakage [20]. These processes should be extremely important in skeletal muscle mitochondria that produce a significant amount of ATP in the mammalian body and that undergo physiological changes in surrounding tissue temperature. Therefore, in the present study, our aim was to establish the effect of experimentally induced hyperthermia (42 °C) and hypothermia (25 °C) on the function of isolated rat skeletal muscle mitochondria. Particularly, respiratory rate activities under phosphorylating and nonphosphorylating conditions, the yield of ATP synthesis, and, for the first time, mitochondrial membrane potential (ΔΨm), mitochondrial uncoupling (proton leakage), and ROS production were studied at temperatures of 25, 35, and 42 °C. Moreover, the oxidative capacity of rat skeletal muscle mitochondria was studied for the first time at the tested temperatures by measuring maximal activities of cytochrome *c* oxidase (COX) and citrate synthase (CS). The novelty of the present study lies in demonstrating that an increase in proton leak resulting from increasing assay temperature could account for reduced OXPHOS efficiency and ROS production.

Material and methods

Chemicals

All chemicals were the highest available grade and were purchased from Sigma–Aldrich unless otherwise mentioned.

Animals

The experiments were carried out on adult 4- to 6-month-old male Wistar rats weighing 475–530 g. The animals were bred in the animal house at the Poznan University of Medical Sciences, Poznan, Poland. They were given free access to water and pellet food and were housed under standard humidity and temperature conditions on a 12-h light/dark cycle. Experimental protocols involving animals, their surgery, and their care were approved by the local ethics committee on animal experimentation in Poznan, Poland, and were in compliance with the guidelines of the European Community Council Directive on the protection of animals used for scientific purposes. Animals were sacrificed, and all efforts were made to minimize suffering.

Tissue preparation and mitochondria isolation

The gastrocnemius, soleus, and quadriceps muscles were quickly excised and placed into an ice-cold isolation medium that contained 100 mM sucrose, 100 mM KCl, 50 mM Tris–HCl, 1 mM KH_2PO_4, 0.5 mM EDTA, 0.1 mM EGTA, and 0.2% bovine serum albumin (BSA), pH 7.2. All subsequent steps were performed at 4 °C. The excised muscles were trimmed to remove adipose and connective tissue, finely minced, and homogenized. First, muscle portions were homogenized using a Polytron homogenizer (T18 basic, IKA) (three times for 2 s, at 80% power) and then by four passes with a glass–Teflon homogenizer. The homogenate was filtered through a double layer of cheesecloth and centrifuged at 700*g* for 10 min. The resultant supernatant was filtered again and then centrifuged at 9000*g* for 10 min to pellet the mitochondria. The mitochondrial pellet was then resuspended in the isolation medium without BSA and centrifuged at 700*g* for 10 min. The supernatant was filtered and centrifuged at 9000*g* for 10 min. The final mitochondrial pellet was then suspended in a small volume (1–1.5 ml) of a suspension buffer containing 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, and 10 mM Tris–HCl, pH 7.2. Mitochondrial protein concentration was determined by the Bradford method with BSA as a standard [5].

Maximal CS and COX activities

The maximal CS and COX activities were assayed at various temperatures (25, 35, and 42 °C) as described previously [40]. CS activity was measured with a UV-1650 Shimadzu spectrophotometer at 412 nm with 100 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 40–60 μg of muscle mitochondrial protein. CS activity was expressed as nmol DTNB × min^{-1} × mg $protein^{-1}$. COX activity was measured polarographically using a Clark-type oxygen electrode (Hansatech) in 1 ml of a respiration medium containing 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 5 mM KH_2PO_4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% BSA, 10 mM Tris–HCl, pH 7.2, with 0.1–0.12 mg of muscle mitochondrial protein. COX activity was assessed in the presence of sequentially added antimycin A (10 μM), 8 mM ascorbate, 0.06% cytochrome *c*, and up to 1 mM *N*,*N*,*N*′*N*′-tetramethyl-*p*-phenylenediamine. COX activity was expressed in nmol O × min^{-1} × mg $protein^{-1}$.

Measurements of mitochondrial respiration and membrane potential

Measurements were performed at various temperatures (25, 35, and 42 °C). Oxygen uptake was determined polarographically using a Rank Bros. oxygen electrode or a Hansatech oxygen electrode in either 2.8 or 0.7 ml of the respiration medium (see above), with either 1 or 0.25 mg of mitochondrial protein (0.36 mg/ml). The temperature of the electrode chambers was tightly controlled with Lauda Ecoline 003 thermostats. Oxygen electrodes were calibrated after each change in assay temperature. The ΔΨm was measured simultaneous with oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode. The TPP^+-electrode was calibrated after each change in assay temperature by four sequential additions (0.4, 0.4, 0.8, and 1.6 μM) of TPP^+. After each run, 0.5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added to release TPP^+ for baseline correction. For the calculation of the ΔΨm value, the matrix volume of skeletal muscle mitochondria was assumed to be 2.0 μl × mg $protein^{-1}$. The values of ΔΨm were corrected for TPP^+ binding using the apparent external and internal partition coefficients of TPP^+ [36]. The correction shifted the calculated ΔΨm values to lower values (approx. 30 mV shift), but did not influence the changes in the resulting membrane potential (relative changes). The values of ΔΨm are given in millivolts.

Succinate (5 mM) with rotenone (2 μM) or malate (5 mM) plus pyruvate (5 mM) was used as respiratory substrate. OXPHOS studies were performed in the absence of Mg^{2+} to avoid the adenine nucleotide interconversion catalyzed by mitochondrial adenylate kinase. Phosphorylating respiration (State 3) was measured after an ADP prepulse (50 μM) using 150 μM ADP as a main pulse. The total amount of oxygen consumed during State 3 respiration was used for calculation of the ADP/O ratio. Measurements of ΔΨm allowed for fine control of the duration of State 3 respiration.

The proton-conductance response to a driving force can be expressed as the relationship between the oxygen consumption rate and the ΔΨm (flux/force relationship) when varying the potential by titrating with respiratory-chain inhibitors. Proton leak assessments during nonphosphorylating (resting, State 4) respiration were performed as previously described [31] with 5 mM succinate (plus 2 μM rotenone) as an oxidizable substrate in the absence of exogenous ADP and the presence of 1.8 μM carboxyatractyloside and 0.7 μg/ml (2 μg/mg of protein) oligomycin, which inhibited the activities of an ATP/ADP antiporter and ATP synthase, respectively. $MgCl_2$ (0.5 mM) was added to the respiration medium (see above). To induce uncoupling protein (UCP2/3) activity, linoleic acid (up to 16 μM) was used. To inhibit UCP2/3 activity, 2 mM GTP was applied. To decrease the rate of the quinone (Q)-reducing pathway, succinate dehydrogenase was titrated with malonate (up to 1.7 mM).