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Application of modular kinetic analysis to mitochondrial oxidative phosphorylation in skeletal muscle of birds exposed to acute heat stress

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ABSTRACT

We previously showed that heat stress stimulates reactive oxygen species (ROS) production in skeletal muscle mitochondria of birds, probably via an elevation in mitochondrial membrane potential ($\Delta\Psi$). To clarify the mechanism underlying the elevation of $\Delta\Psi$, modular kinetic analysis was applied to oxidative phosphorylation in skeletal muscle mitochondria of heat-stressed birds (34 °C for 12 h). In the birds exposed to heat stress, 'substrate oxidation' (a $\Delta\Psi$-producer) was increased compared to control (24 °C) birds, although there was little difference in 'proton leak' (a $\Delta\Psi$-consumer), suggesting that an elevation in the $\Delta\Psi$ at state 4 may be due to enhanced substrate oxidation. It thus appears that enhanced substrate oxidation plays a crucial role in the overproduction of ROS for heat-stressed birds, probably via elevated $\Delta\Psi$.

1. Introduction

The production of reactive oxygen species (ROS) occurs to a large extent in mitochondria [1–3]. The primary ROS generated by mitochondria is superoxide ($O_2^{\cdot-}$), as a result of the single-electron reduction of an oxygen molecule at the electron transport chain. ROS may have important roles in cellular signaling pathways [4], but excessive levels of ROS can cause irreversible molecular damage, leading to cellular dysfunction [5]. Therefore, a mechanistic understanding of the overproduction of mitochondrial ROS under physiological conditions is important.

The overproduction of superoxide may depend on mitochondrial membrane potential ($\Delta\Psi$). Skulachev [6] suggested that even a small decrease in mitochondrial proton motive force (Δp) could significantly lower superoxide production by mitochondria, a suggestion substantiated by others: Even a very low concentration of uncoupler strongly inhibits state 4-H_2O_2 production by mitochondria [7,8]. Therefore, factors resulting in a high $\Delta\Psi$ could enhance superoxide production by mitochondria.

Heat stress is an environmental factor responsible for stimulating superoxide production. We have already demonstrated that mitochondrial superoxide generation, detected by both electron spin resonance spectroscopy with 5,5-dimethyl-1-pyrroline *N*-oxide as a spin trap agent and lucigenin-derived chemiluminescence, was significantly enhanced in skeletal muscle of birds given acute heat stress [9], causing oxidative damage to mitochondrial proteins and lipids [10]. Enhanced $\Delta\Psi$ may be a mechanism underlying acute heat stress-induced overproduction of mitochondrial superoxide in chicken skeletal muscle. Indeed, the $\Delta\Psi$ of muscle mitochondria from heat-stressed bird is elevated, although the proton leak, one factor affecting $\Delta\Psi$, remained unchanged [11]. From these findings, we now postulate that another factor influencing $\Delta\Psi$ might be responsible for the elevated $\Delta\Psi$. Furthermore, heat-stressed birds have an elevated state 3-oxygen consumption rate reflecting increased substrate oxidation by the electron transport chain, a $\Delta\Psi$-producer [11]. Therefore, acute heat exposure may cause an increase in 'substrate oxidation', resulting in an elevated $\Delta\Psi$ in muscle mitochondria.

To test the hypothesis, we employed modular kinetic analysis to quantitatively evaluate under heat stress conditions mitochondrial oxidative phosphorylation divided into processes which produce

Abbreviations: ROS, reactive oxygen species; $\Delta\Psi$, membrane potential; UCP, uncoupling protein

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or consume $\Delta\Psi$: 'substrate oxidation' (a $\Delta\Psi$-producer), 'phosphorylation system' and 'proton leak' (two $\Delta\Psi$-consumers). We also characterized coupling efficiency of oxidative phosphorylation under heat stress conditions, as little information is available about the capacity of energy production systems in ROS-overproducing mitochondria.

2. Materials and methods

2.1. Animals and experimental design

Meat-type male chicks (1-day-old) were obtained from a commercial hatchery (Economic Federation of Agricultural Cooperatives, Iwate, Japan). They were housed in electrically heated batteries under continuous light, and provided with access ad libitum to water and commercial starter meat-type chick diet (crude protein, 23%; metabolizable energy content, 3050 kcal/kg). After 14 days, they were randomly divided into two groups (n = 12 birds per group). After a 7-day adaptation period, one of the two groups was exposed to 34 °C for 12 h, while the other group was maintained at 24 °C (humidity 55 ± 5%). Birds were provided with access ad libitum to water and diet during the treatments. After exposure to heat stress, eight birds in each group were selected and killed by decapitation. This method of killing was used in preference to overdose by general anesthetics, which are known to uncouple oxidative phosphorylation [12]. *Pectoralis superficialis* muscles were rapidly excised, and a sample of each muscle was placed in ice-cold isolation medium (100 mM KCl, 50 mM Tris/HCl, and 2 mM EGTA, pH 7.4) for mitochondrial isolation (see below). The Animal Care and Use Committee of the Graduate School of Agricultural Science, Tohoku University approved all procedures, and efforts were made to minimize pain or discomfort of the animals.

2.2. Isolation of skeletal muscle mitochondria

Muscle mitochondria were isolated by homogenization, protein digestion, and differential centrifugation at 4 °C, as previously described [11,13]. Muscle was trimmed of fat and connective tissue, blotted dry, weighed, and placed in isolation medium on ice. Tissue was shredded and minced with sharp scissors, rinsed with isolation medium three times, stirred for 5 min in protein digestion medium [100 mM KCl, 50 mM Tris/HCl, 2 mM EGTA, 1 mM ATP, 5 mM $MgCl_2$, 0.5% (wt/vol) bovine serum albumin (BSA), and 11.8 units of protease per gram of tissue (Sigma, substilisin type VIII), pH 7.4], and gently homogenized using a Polytron tissue homogenizer. The homogenate was stirred for 6 min before being mixed with the equivalent medium without protease to stop protease activity. The homogenate was rehomogenized in a Potter-Elvehjem homogenizer (six passages) and centrifuged at 500×g for 10 min. The supernatant was filtered through muslin and centrifuged at 10 400×g for 10 min. Mitochondrial pellets were resuspended in isolation medium and centrifuged at 10 400×g for 10 min, followed by 3800×g for 10 min, and were resuspended in isolation medium. Mitochondrial protein concentration was determined by the bicinchoninic acid (BCA) assay, with BSA as the standard [14]. One sample of mitochondria was isolated from two individual pooled muscles, and four samples of mitochondria were isolated in each group. All isolated mitochondria were used for the kinetic analysis on the day of the isolation.

2.3. Mitochondrial oxygen consumption and $\Delta\Psi$

Mitochondrial oxygen consumption and $\Delta\Psi$ were simultaneously measured using electrodes sensitive to oxygen and a potential-dependent probe, triphenylmethyl phosphonium cation ($TPMP^+$), respectively [13]. Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) maintained at 38 °C and calibrated with an air-saturated standard assay medium [115 mM KCl, 10 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 2 mM $MgCl_2$ and 0.3% (wt/vol) BSA, pH 7.2], assumed to contain 402 nmol of atomic oxygen per milliliter [15]. The $TPMP^+$ electrode was calibrated with sequential additions of 0.5 up to 2 μM $TPMP^+$. The electrode linearity was routinely checked by following the uncoupled respiration in the presence of 1 μM carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) from 100% to 0% air saturation. This uncoupling dissipated $\Delta\Psi$ and released TPMP back into the medium, allowing for correction of any small electrode drift. Mitochondria (0.35 mg protein/ml) were incubated in the standard assay medium with 0.1 μM nigericin (to collapse ΔpH) and 5 μM rotenone (to inhibit complex I), to which 4 mM succinate was used to initiate respiration. $\Delta\Psi$ values were calculated from the distribution of $TPMP^+$ across the mitochondrial inner membrane, using a binding correction factor of 0.45 per microliter milligram protein [16].

Respiratory control ratio (RCR) was calculated by dividing the oxygen consumption at maximal rate of ATP synthesis (state 3) by the oxygen consumption rate with no ATP synthesis (state 4).

2.4. Kinetics of proton leak, phosphorylation and substrate oxidation

To evaluate oxidative phosphorylation in skeletal muscle mitochondria of heat-stressed birds, we used modular kinetic analysis [17,18]. Mitochondrial oxidative phosphorylation can be divided conceptually into three modules connected by their common intermediate ($\Delta\Psi$): (i) the reactions that produce $\Delta\Psi$, which are called 'substrate oxidation'; (ii) the reactions that consume $\Delta\Psi$ and synthesize ATP, called 'phosphorylation system'; and (iii) the reactions that consume $\Delta\Psi$ without ATP synthesis, referred to as 'proton leak' [13]. To measure kinetic response of proton leak to $\Delta\Psi$, state 4 respiration (non-phosphorylation) of mitochondria was titrated with malonate (up to 3 mM) in the presence of 1.25 μM oligomycin (to prevent any residual ATP synthesis). In a similar way to the proton leak kinetics, the state 4 respiration was titrated with FCCP (up to 0.6 μM) for measurement of kinetic response of substrate oxidation to $\Delta\Psi$. State 3 (maximal rate of ATP synthesis) was obtained using an ADP-regenerating system (100 μM ATP, 20 mM glucose and 6 U/ml hexokinase). Titration of state 3 respiration with malonate (up to 1.5 mM) allowed measurement of kinetics of the $\Delta\Psi$-consumers (the sum of phosphorylation system and proton leak).

To quantitatively investigate changes in control of the oxidative phosphorylation system under heat stress conditions, flux control coefficients and concentration control coefficients of the oxidative phosphorylation modules were calculated over the subsystem fluxes and the $\Delta\Psi$, respectively [19]. Briefly, elasticity coefficients of the modules were calculated from the modular kinetic curves, and then the flux and concentration control coefficients were calculated from the elasticity coefficients and the respiration rates. Flux control coefficients describing control over a subsystem flux (substrate oxidation, proton leak and phosphorylation) add up to 1, with a value of one indicating complete control, a value of 0 indicating no control, and a value of 0.50 indicating 50% control. Concentration control coefficients quantify the control exerted by the individual components over the $\Delta\Psi$ and add up to 0. For concentration control coefficients, the components exerting the greatest control will have the largest absolute values.

2.5. Coupling efficiency of mitochondrial oxidative phosphorylation

Coupling efficiency of oxidative phosphorylation was calculated from the kinetic curves as the percentage of mitochondrial respiration rate at a given $\Delta\Psi$ used for ATP synthesis [18,20]. This is calcu-