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Impaired adaptability of in vivo mitochondrial energetics to acute oxidative insult in aged skeletal muscle

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ABSTRACT

Periods of elevated reactive oxygen species (ROS) production are a normal part of mitochondrial physiology. However, little is known about age-related changes in the mitochondrial response to elevated ROS in vivo. Significantly, ROS-induced uncoupling of oxidative phosphorylation has received attention as a negative feedback mechanism to reduce mitochondrial superoxide production. Here we use a novel in vivo spectroscopy system to test the hypothesis that ROS-induced uncoupling is diminished in aged mitochondria. This system simultaneously acquires ^{31}P magnetic resonance and near-infrared optical spectra to non-invasively measure phosphometabolite and O_2 concentrations in mouse skeletal muscle. Using low dose paraquat to elevate intracellular ROS we assess in vivo mitochondrial function in young, middle aged, and old mice. Oxidative phosphorylation was uncoupled to the same degree in response to ROS at each age, but this uncoupling was associated with loss of phosphorylation capacity and total ATP in old mice only. Using mice lacking UCP3 we demonstrate that this in vivo uncoupling is independent of this putative uncoupler of skeletal muscle mitochondria. These data indicate that ROS-induced uncoupling persists throughout life, but that oxidative stress leads to mitochondrial deficits and loss of ATP in aged organisms that may contribute to impaired function and degeneration.

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1. Introduction

Mitochondrial dysfunction is implicated in many age-related disorders, including loss of muscle mass and function (sarcopenia) (Marzetti and Leeuwenburgh, 2006), exercise intolerance (Conley et al., 2000), and neurodegeneration (Gibson et al., 2010). In humans, these effects are associated with diminished mobility, reduced quality of life, and stress on health care systems in countries with aging populations (Lang et al., 2010).

The accumulation of oxidative damage resulting from increased oxidative stress in aging tissues contributes to mitochondrial dysfunction in aging and disease (Harman, 1972; Harper et al., 2004; Jang et al., 2010). Mitochondria generate reactive oxygen species (ROS) in the form of superoxide anion at complexes I and III of the electron transport chain (ETC) as a normal byproduct of aerobic metabolism (St-Pierre et al., 2002; Jackson et al., 2007). As a result of their proximity to the ETC, mitochondrial proteins, lipids, and DNA incur significant oxidative damage. In skeletal muscle, evidence suggests that oxidative damage to mitochondria is linked to reduce oxidative phosphorylation capacity (Papa, 1996), degeneration of neuromuscular junctions (Jang et al., 2010), and apoptosis (Pollack et al., 2002).

Periods of elevated oxidative stress are a normal part of physiological processes. For example, mitochondrial ROS production is elevated following exercise (Anderson et al., 2007) and during high fat feeding (Anderson et al., 2009). Consequently, mitochondria have evolved intrinsic antioxidant

Abbreviations: ANT1, adenine nucleotide translocase 1; ATPase, resting rate of ATP hydrolysis; ATPmax, maximum rate of oxidative phosphorylation; Cr, total creatine; ETC, electron transport chain; FDI, first dorsal interosseous; Hb, hemoglobin; HNE, 4-hydroxy-2-nonenal; Mb, myoglobin; MIM, mitochondrial inner membrane; MR, magnetic resonance; NIR, near-infrared; PLS, partial least squares; PCr, phosphocreatine; P_i, inorganic phosphate; P/O, ATP produced per oxygen atom consumed; PQ, paraquat; ROS, reactive oxygen species; UCP3, uncoupling protein 3.

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defense mechanisms to maintain redox homeostasis. One such proposed mechanism is ROS-induced uncoupling of oxidative phosphorylation, whereby oxidative stress activates pathway(s) which dissipate the mitochondrial inner membrane (MIM) potential without generating ATP (Skulachev, 1996; Brand, 2000; Echtay et al., 2002), leading to a reduction in the ATP produced per oxygen atom consumed by the ETC (lower P/O). ROS production by the ETC is driven by a high MIM potential (Korshunov et al., 1997) due mainly to the slowed passage of electrons through complexes I and III leading to increased probability for the partial reduction of O_2 (Nicholls, 2004). Mild uncoupling increases electron flux through the ETC for a given rate of ATP production and causes a reduction in ROS production at the cost of reduced energetic efficiency. Evidence for this negative feedback hypothesis comes from recent work demonstrating that increased oxidative stress results in reduced in vivo P/O in skeletal muscle (Siegel et al., 2011), and studies suggesting that mitochondrial uncoupling is protective against age-related dysfunction (Amara et al., 2007) and associated with increased lifespan (Speakman et al., 2004; Caldeira da Silva et al., 2008; Andrews, 2010).

Despite the importance of ROS-induced uncoupling in maintaining redox homeostasis, little is known about the effect of aging on ROS-induced uncoupling. Age-related decreases in mitochondrial P/O and phosphorylation capacity (Conley et al., 2000; Marcinek et al., 2005; Amara et al., 2007) suggest that the flexibility of mitochondria to further uncouple in response to changing intracellular environments may be compromised. The diminishing utility of this defense mechanism with age would represent a significant loss of an antioxidant defense and may contribute to the age-related buildup of oxidative damage. Furthermore, the link between ROS-induced uncoupling and cellular energetics may present a unique challenge to old organisms that are already struggling to maintain energy balance (Wilson and Morley, 2003; Marcinek et al., 2005; Amara et al., 2007).

Here we test the hypothesis that age-related deficiencies in mitochondrial function disrupt the ROS-induced uncoupling mechanism, rendering old skeletal muscle less able to adapt to transient increases in oxidative stress. To address this hypothesis we introduce a novel multi-modal in vivo spectroscopy system capable of acquiring simultaneous magnetic resonance (MR) spectra and near infrared (NIR) optical spectra from skeletal muscle in the fully intact mouse hindlimb. We use a mild paraquat (PQ) treatment to induce an acute oxidative insult in mice at young, middle, and old ages to measure the effect of age on the ability of skeletal muscle mitochondria to adapt to this insult in vivo. PQ is a redox cycling agent leading to increased production of superoxide at both cytosolic and mitochondrial NAD(P)H oxidases (Cocheme and Murphy, 2008). We use UCP3 knockout mice to test whether the activation of UCP3 mediated proton leak is responsible for PQ-related changes in in vivo P/O observed in this study.

2. Methods

2.1. Animals

This study was approved by the Institutional Animal Care and Use Committee of the University of Washington. For aging studies, young (5–7 months), middle aged (20–21 months), and old (27–28 months) female C57BL/6 mice were purchased from the aged mouse colony maintained by the NIA at Charles River Laboratories (Wilmington, MA). Female UCP3$^{-/-}$ mice on a C57BL/6J background (Gong et al., 2000) were studied between 4 and 7 months of age. All mice were kept on a 12 h light/dark cycle at 22 °C and 20% humidity with free access to water and standard mouse chow until immediately prior to experimentation. Mice were warmed using forced air to maintain skeletal muscle temperature at 36 ± 1 °C throughout in vivo experiments.

2.2. Paraquat

Paraquat (Item 36541, Sigma, St. Louis, MO) was dissolved in 0.9% filtered saline at a concentration of 1 mg/mL and administered by intraperitoneal injection. Mice received either 20 mg PQ per kg of body weight or volume matched saline on the evening prior to in vivo experiments.

2.3. Multi-modal spectroscopy

Mice were anesthetized by intraperitoneal injection of 0.01 mL/g of 2.5% tribromoethanol (“Avertin”, Sigma) and the left distal hindlimb shaved using veterinary hair clippers. As illustrated in Fig. 1, mice were then suspended using Velcro straps in a custom built MR/optics probe. We developed custom probes for use with either 7T (Varian, Palo Alto, CA) or 14T (Bruker, Billerica, MA) vertical bore spectrometers. Both probes employed the same general design: the distal hindlimb is centered within a horizontal, solenoid MR coil tunable to both ^{1}H and ^{31}P with the foot fixed against a plastic post. Aligned fiber optic bundles are positioned on either side of the coil (perpendicular to the coil axis) to deliver light from a QTH source (Newport Corp, Irvine, CA) to the lateral surface of the distal hindlimb, collect light transmitted through the hindlimb, and deliver this transmitted light to a spectrograph coupled to a CCD camera (Princeton Instruments, Trenton, NJ). A custom-built, inflatable ischemia cuff was positioned around the hindlimb proximal to the MR/optics hardware so that blood flow to the distal hindlimb could be rapidly blocked and restored with an external sphygmomanometer during in vivo experiments. Complete short-term ischemia is used to perturb the energy homeostasis in the skeletal muscle for measurement of mitochondrial ATP and O_2 fluxes (Blei et al., 1993; Marcinek et al., 2004).

After positioning the mouse, MR signal was optimized by shimming the ^{1}H peak using tissue water and optical signal was optimized by adjusting acquisition time (typically 30–60 ms). A high signal to noise ^{31}P spectrum was acquired under fully relaxed conditions (32 transients, 4096 complex points, 10 kHz sweep width, 25 s interpulse delay at 7T; 80 transients, 8192 complex points, 20 kHz sweep width, 10 s interpulse delay at 14T). Dynamic optical (0.5 s delay) and MR spectra (45° flip angle, 4 transients, 4096 complex points, 10 kHz sweep width, 1.5 s interpulse delay, at 7T; 45° flip angle, 4 transients, 4096 complex points, 20 kHz sweep width, 0.6 s interpulse delay at 14T) were acquired continuously through periods of rest (2 min), ischemia (11 min), and recovery (7 min). After the first minute of rest mice breathed 100% O_2 for the remainder of each experiment. Experiment timing is summarized in Fig. 2. Spectra acquired using 7T and 14T systems were similar (Fig. S1) and flux rates and metabolite concentrations acquired on the two systems were not different.

2.4. Tissue preparation

Immediately following in vivo spectroscopy, mice were injected with a supplemental, non-lethal dose of Avertin and the skeletal muscles of the distal hindlimb were dissected and flash-frozen in liquid nitrogen. From the left leg, extensor digitorum longus, gastrocnemius, soleus, and tibialis anterior muscles were pooled and pulverized over liquid nitrogen for measurement of mixed muscle metabolite, hemoglobin (Hb), and myoglobin (Mb) concentrations. From the right leg, gastrocnemius was pulverized over liquid nitrogen and prepared for western blotting. All muscle samples were stored at −80° until the day of assay.

2.5. Metabolite concentrations

Tissue concentrations of ATP and total creatine (Cr) were determined in mixed muscle by HPLC (Waters, Milford, MA) using a protocol described in detail elsewhere (Wiseman et al., 1992).

2.6. Protein content

Absolute Hb and Mb concentrations were determined using Coomassie-staining with in-gel standards of known concentration. Aliquots of pulverized mixed muscle were combined 1:25 with Cellytic MT lysis buffer (Sigma #C3228) containing 0.1% protease inhibitor (Sigma #P8340), and homogenized at 4 °C using a Bullet Blender 24 (Next Advance, Averill Park, NY). The resulting lysate was combined 1:1 with tricine sample buffer (Bio-Rad #161-0739) containing 350 mM DTT and brought to 95 °C for 8 min. Proteins were separated on 10–20% gradient gels at 150 V for 2.5 h and then stained overnight in Coomassie Brilliant Blue stain (Bio-Rad). After destaining, gels were imaged using the Bio-Rad ChemiDoc imaging system and band intensities were analyzed using QuantityOne software (Bio-Rad).

All other protein analyses were accomplished using western blotting. Aliquots of pulverized gastrocnemius were combined 1:25 with lysis buffer containing 0.1% protease inhibitor (same as above) plus 1% phosphatase inhibitor (Thermo Scientific #78420), and homogenized at 4 °C using a Bullet Blender 24. The resulting lysate was combined 1:1 with Laemmli sample buffer (Bio-Rad #161-0737) containing 350 mM DTT and proteins were separated on 4–20% gradient gels at 200 V for 65–75 min. Proteins were transferred to nitrocellulose membranes, Ponceau stained to ensure uniform transfer and loading, and then immunoblotted as follows (block, primary, secondary): UCP3 (3%BSA 1 h at RT, 1:1K AbCam #3477 overnight at 4 °C, 1:10K Cell Signaling #7074 2 h at RT), ANT1 (5%BSA overnight at 4 °C, 1:1K Sigma #SAB2105530 2 h at RT, 1:5K Cell Signaling #7074 2 h at RT), ETC complexes I–V (3%BSA 1 h at RT, 1:1K MitoSciences #604 overnight at 4 °C, 1:10K Cell Signaling #7076), HNE (5%NFDM 1 h at RT, 1:1K Alpha Diagnostics #HNE12s, 1:2K Alpha

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