MITOCHONDRIA IN SKELETAL MUSCLE HEALTH, AGING AND DISEASES

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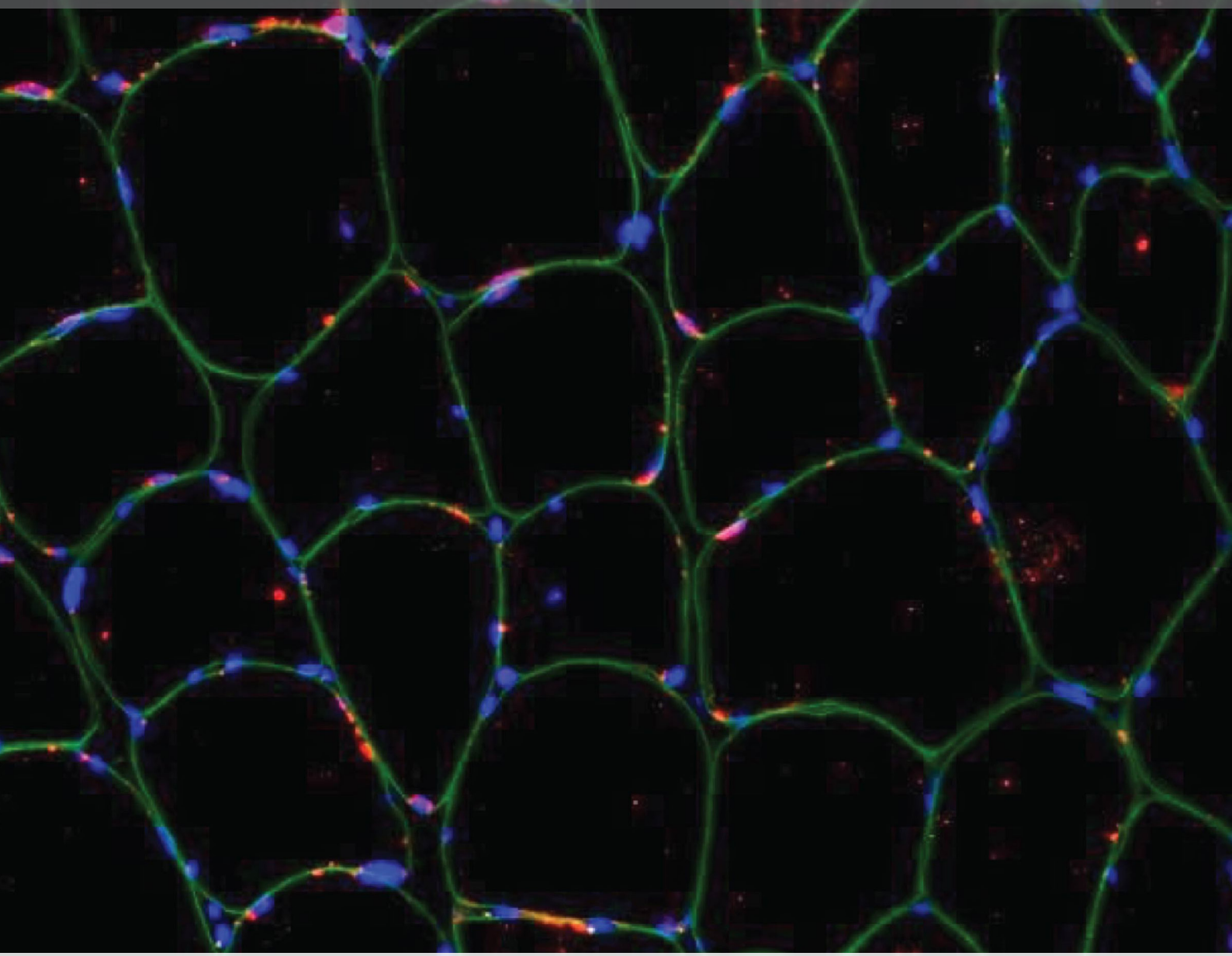

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MITOCHONDRIA IN SKELETAL MUSCLE HEALTH, AGING AND DISEASES

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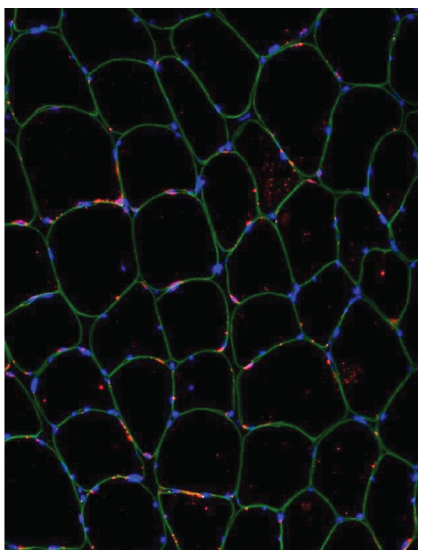

Immunolabeling for Endonuclease G (red; a mitochondrial pro-apoptotic protein translocating to nuclei when mitochondria trigger apoptosis), dystrophin (green), and nuclei (blue) obtained on a muscle cross section of an old men.

Image taken and adapted, with permission, from: Gouspillou G, Sgarioto N, Kapchinsky S, Purves-Smith F, Norris B, Pion CH, Barbat-Artigas S, Lemieux F, Taivassalo T, Morais JA, Aubertin-Leheudre M, Hepple RT. Increased sensitivity to mitochondrial permeability transition and myonuclear translocation of endonuclease G in atrophied muscle of physically active older humans. FASEB J. 2014 Apr;28(4):1621-33. doi: 10.1096/fj.13-242750. Epub 2013 Dec 26.

Skeletal muscle is the most abundant tissue of the human body, making up to 40 to 50% of the human body mass. While the importance of optimal muscle function is well recognized in the athletic field, its significance for general health is often underappreciated. In fact, the evidence that muscle mass, strength and metabolism are essential for our overall health is overwhelming. As the largest protein reservoir in the human body, muscles are essential in the acute response to critical illness such as sepsis, advanced cancer, and traumatic injury. Loss of skeletal muscle mass has also been associated with weakness, fatigue, insulin resistance, falls, fractures, frailty, disability, several chronic diseases and death. As a consequence, maintaining skeletal muscle mass, strength and metabolism throughout the lifespan is critical to the maintenance of whole body health.

Mitochondria are fascinating organelles regulating many critical cellular processes for skeletal muscle physiology, including for instance energy supply, reactive oxygen species production, calcium homeostasis and the regulation of apoptosis. It is therefore not surprising that mitochondrial dysfunction has been implicated in a large number of adverse events/conditions and pathologies affecting skeletal muscle health.

While the importance of normal mitochondrial function is well recognized for muscle physiology, there are important aspects of mitochondrial biology that are still poorly understood. These include mitochondrial dynamics (fusion and fission processes), morphology and processes involved in mitochondrial quality control (mitophagy). Defining the mechanisms regulating these different aspects of mitochondrial biology, their importance for muscle physiology, as well as the interrelations will be critical for expanding understanding of the role played by mitochondria in skeletal muscle physiology and health.

The present research topic provides readers with novel experimental approaches, knowledge, hypotheses and findings related to all aspects of mitochondrial biology in healthy and diseased muscle cells.

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EDITORIAL
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Editorial: Mitochondria in Skeletal Muscle Health, Aging and Diseases

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The Editorial on the Research Topic

Mitochondria in Skeletal Muscle Health, Aging and Diseases

Mitochondria are fascinating organelles regulating many critical cellular processes for skeletal muscle physiology. Indeed, they play central roles in muscle cell metabolism, energy supply, the regulation of energy-sensitive signaling pathways, reactive oxygen species (ROS) production/signaling, calcium homeostasis and the regulation of apoptosis (Brookes et al., 2004). Given these multifaceted roles of mitochondria in fundamental aspects of skeletal muscle cell physiology, it is not surprising that mitochondrial dysfunction has been implicated in a large number of adverse conditions affecting skeletal muscle health. This includes for instance the aging-related loss of muscle mass and function (Dirks and Leeuwenburgh, 2004; Short et al., 2005; Chabi et al., 2008; Gouspillou et al., 2010, 2014a,b; Picard et al., 2010; Hepple, 2014), disuse-induced muscle atrophy (Min et al., 2011), ventilator-induced diaphragmatic dysfunction (Picard et al., 2015), Duchenne and collagen muscular dystrophies (Godin et al., 2012; Bernardi and Bonaldo, 2013), long-term muscle dysfunction induced by chemotherapy treatment (Gouspillou et al., 2015; Power et al., 2016), and the development of insulin resistance (Goodpaster, 2013).

While the importance of normal mitochondrial function is well recognized for muscle physiology, there are important aspects of mitochondrial biology that are still poorly understood/investigated in the highly specialized muscle tissue. These include mitochondrial dynamics (fusion and fission processes), morphology and processes involved in mitochondrial quality control (mitophagy). Defining the mechanisms regulating these different aspects of mitochondrial biology, their importance for muscle physiology, as well as the interrelations existing between mitochondrial function, morphology, dynamics and mitophagy will be critical to further increase our understanding of the role played by mitochondria in skeletal muscle physiology and pathophysiology. The aim of the present research topic was therefore to bring together key experiments, advances, knowledge and new findings related to all aspects of mitochondrial biology in healthy and/or diseased muscle cells.

The accurate assessment of mitochondrial function is of crucial importance to dissect the role played by mitochondrial dysfunction in pathologies affecting skeletal muscles. It is also essential for the clear identification of mitochondrial adaptations to various interventions, such as nutritional or physical activity interventions. In the present research topic, Conley et al. provide a promising approach to assess mitochondrial oxidative phosphorylation *in vivo* by monitoring changes in mitochondrial NAD(P)H using ^{31}P NMR spectroscopy. In their study, Conley et al. show that a unique resonance (−11.05 ppm) in the *in vivo* ^{31}P spectrum provides a natural indicator of

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mitochondrial oxidation that is sensitive to the increase in mitochondrial phosphorylation rate induced by exercise training in elderly individuals.

One of the commonly used methods to assess mitochondrial function *in vitro* in skeletal muscle is the preparation of permeabilized myofibers (Kuznetsov et al., 2008). To be applied in humans, this technique requires biopsies to collect muscle samples. While the Bergstrom biopsy technique (Bergstrom, 1975) is the gold standard to collect skeletal muscle samples, a growing interest now surrounds microbiospsies due to their lower level of invasiveness (Hayot et al., 2005). However, no previous in-depth validation of this method for the assessment of mitochondrial function in permeabilized myofibers and for the assessment of muscle phenotype had been performed to date. Hughes et al. therefore compared mitochondrial bioenergetics in muscle sample obtained through Bergstrom biopsies vs. microbiopsies. They reveal that microbiopsies can provide a reliable assessment of mitochondrial bioenergetics only when assay conditions are supplemented with the myosin ATPase inhibitor Blebistatin. However, the authors highlight that caution should be taken when assessing muscle fiber type composition using the microbiopsy approach, since significant differences in fiber type proportion were observed between the two approaches (Hughes et al.).

Oxidative stress is thought to play an important role in skeletal muscle dysfunction and atrophy seen in aging, disuse, and many skeletal muscle pathologies (Powers et al., 2012; Johnson et al., 2013). Because they are considered as one of the main sources of ROS production, mitochondria are a key focus in the field of oxidative stress. While reactive oxygen and nitrogen species were initially only seen as detrimental for muscle cells, it is now recognized that these reactive species are essential for normal skeletal muscle physiology (Sohal and Orr, 2012), mainly through the reversible redox post-translational modifications they can induce. The ability to accurately quantify reversible redox post-translational modifications is therefore critical to investigate the mechanisms by which mitochondrial oxidative stress contributes to skeletal muscle dysfunction in diseases. In their article, Kramer et al. provide a detailed review of the available literature on reversible redox post-translational modifications and mitochondrial and skeletal muscle function. They then provide critical review on current approaches to assess reversible redox post-translational modifications (Kramer et al.).

Several studies have implicated altered kinetic properties of the adenine nucleotide translocator (ANT) in the aging-related impairment in mitochondrial energetics in skeletal muscle cells (Yan and Sohal, 1998; Gouspillou et al., 2014b). In the present research topic, Diolez et al. formulate the interesting hypothesis that these alterations in ANT could represent a protective mechanism to limit ROS production in aged muscle mitochondria while moderately disrupting mitochondrial energetics. Considering the importance of ROS as therapeutic targets, this hypothetical mechanism deserves further study.

The present research topic also provides readers with fundamental advancement in our understanding of the regulation of mitochondrial function in skeletal muscle cells. Indeed, in an elegant study, Lark et al. provide evidence that Protein Kinase A (PKA) can regulate mitochondrial energetics and H_2O_2 emission. Using PKA inhibitors and various mitochondrial substrates, they show that this regulation occurs at the level of Complex I. Finally, they provide new insights on how mitochondrial cyclic adenosine monophosphate (cAMP) production, cAMP being a positive regulator of PKA, is regulated (Lark et al.).

Understanding how nutrition modulates mitochondrial biology in muscle cells is of tremendous importance in the field of medicine. For instance, mitochondrial dysfunction has been suggested to be causally involved in obesity-induced insulin resistance and in the pathophysiology of type II diabetes (Goodpaster, 2013). Precisely defining how skeletal muscle mitochondria respond to obesogenic diet feeding is therefore of critical importance. In the present research topic, Putti et al. provide readers with a mini-review focused on the impacts of different dietary fat sources on mitochondrial bioenergetics, morphology and dynamics in skeletal muscle cells in the context of insulin-resistance. They also highlight the pressing need for mechanistic studies to confirm *in vivo* the relationship between mitochondrial morphology and dynamics and the development of insulin-resistance (Putti et al.).

Besides being of particular interest for the field of medicine, defining the impact of nutrition on mitochondrial biology is also an important research topic in the field of exercise physiology. The present research topic features two important review articles in this field. The first one, written by Craig et al. critically reviews the available literature on the utilization of small nutrients, such as caffeine, green tea extracts, polyphenols and amino-acids to enhance the impact of exercise training on mitochondrial biogenesis. They also provide recommendations and guidance for future studies that are required to explore the efficacy of these nutrients in humans, as well as the exercise setting in which they may prove beneficial (Craig et al.). The second review, written by Affourtit et al., first provides a state-of-the-art review of the available literature on the beneficial effects of dietary nitrate on human performance. Affourtit et al. then critically review the available experimental data in relation to the underlying mechanisms, with a particular emphasis on the impact of nitrate on mitochondrial bioenergetics.

It is now well established that low birth weight is associated with an increase in the risk of developing disease in later life such as coronary heart disease, diabetes, hypertension and stroke (see de Boo and Harding, 2006; Gluckman et al., 2008 for detailed reviews). Maternal undernutrition ranks as one of the most important causes that can lead to low birth weight (de Boo and Harding, 2006). In the present research topic, Beauchamp and Harper review the available evidence indicating that *in utero* undernutrition results in a metabolic reprogramming in both cardiac and skeletal muscles, characterized for instance by a reduction in mitochondrial content and respiration in the offspring. They also provide insights into the underlying mechanisms and provide a rationale linking the metabolic alterations resulting from *in utero* undernutrition to the increased risk of developing metabolic diseases (Beauchamp and Harper).

As mentioned earlier, mitochondrial dysfunction has been implicated in many conditions associated with muscle atrophy, including aging, cancer cachexia, and disuse-induced muscle atrophy (Dirks and Leeuwenburgh, 2004; Short et al., 2005; Chabi et al., 2008; Gouspillou et al., 2010, 2014a,b; Picard et al., 2010, 2015; Min et al., 2011; Hepple, 2014; Argilés et al., 2015). While the field to date has mainly focused on mitochondrial function (i.e., energetics, ROS production and mitochondrial mediated apoptosis), a growing interest now surrounds mechanisms involved in mitochondrial quality control (i.e., mechanisms responsible for the degradation of damaged / dysfunctional mitochondria). In the present research topic, Romanello and Sandri provide readers with a thorough and critical review on the current knowledge linking mitochondrial function, dynamics and quality control in the regulation of muscle mass. They also highlight several research avenues and challenges that will undoubtedly stimulate the field (Romanello and Sandri).

In the present research topic, Ryan et al. contributed a very interesting review paper focused on skeletal muscle and endothelial cell mitochondria in the setting of Critical Limb Ischemia. The latter is the most severe clinical presentation of peripheral arterial disease and manifests as chronic ischaemic "rest pain" and/or ischaemic skin lesions (Minar, 2009). Unfortunately, there is no effective way to treat the muscle myopathies caused by this disease. In their review, Ryan et al. first highlight the importance of skeletal muscle in the manifestation of Critical Limb Ischemia, and then provide a strong and exciting rationale for a role for endothelial cell and skeletal muscle mitochondria in the patient outcomes. As such, they propose that limb muscle and endothelial cell mitochondria should be considered as targets for novel therapeutic intervention (Ryan et al.).

Skeletal muscles have an impressive capacity to adapt to mechanical and physiological challenges by changing their phenotype in terms of size, fiber type, capillarization levels and aerobic capacity. Although skeletal muscle plasticity has been the focus of intense research effort, the molecular mechanisms underlying muscle plasticity are still incompletely understood. Since the seminal paper published by Spiegleman's group in 2002 (Lin et al., 2002), in which PGC-1α over-expression was shown to result in an increase in the proportion of slow-oxidative fibers, PGC-1α has attracted sustained attention. In the present research topic, Kupr and Handschin discuss recent advances in our understanding of how PGC-1α regulates skeletal muscle cell plasticity in health and disease. They also highlight further avenues of research to fully decrypt how PGC-1α influences muscle plasticity in health and disease (Kupr and Handschin).

Although often overlooked, our endocrine system exerts an important control on the regulation of mitochondrial mass and function in skeletal muscle cells, especially through thyroid hormones (Salvatore et al., 2014). Amongst thyroid hormones exerting control on mitochondrial biology in skeletal muscle cells, 3,5,3′-Triiodo-L-thyronine (T3) has been extensively studied (Salvatore et al., 2014). In the present research topic, Lumbardi et al. review the experimental evidence indicating that 3,5-diiodo-L-thyronine (T2), and emerging iodothyronines also impact mitochondrial metabolism in skeletal muscle cells (Lombardi et al.). These novel aspects of thyroid physiology reviewed in Lombardi et al. open new perspectives for understanding the involvement of skeletal muscle mitochondria in systemic consequences of hypo- and hyper-thyroidism.

Because of the diversity and the quality of the articles compiled herein, we feel the present research topic was a success. We hope that it will not only provide readers with new insights and viewpoints on the role played by mitochondria in skeletal muscle health, aging and diseases, but will also serve as a platform to stimulate new ideas, experiments and research projects for further advances in the field.

AUTHOR CONTRIBUTIONS

GG and RH co-wrote and approved this Editorial.

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ORIGINAL RESEARCH
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Mitochondrial NAD(P)H *In vivo*: Identifying Natural Indicators of Oxidative Phosphorylation in the ^{31}P Magnetic Resonance Spectrum

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Natural indicators provide intrinsic probes of metabolism, biogenesis and oxidative protection. Nicotinamide adenine dinucleotide metabolites (NAD(P)) are one class of indicators that have roles as co-factors in oxidative phosphorylation, glycolysis, and anti-oxidant protection, as well as signaling in the mitochondrial biogenesis pathway. These many roles are made possible by the distinct redox states (NAD(P)$^+$ and NAD(P)H), which are compartmentalized between cytosol and mitochondria. Here we provide evidence for detection of NAD(P)$^+$ and NAD(P)H in separate mitochondrial and cytosol pools *in vivo* in human tissue by phosphorus magnetic resonance spectroscopy (^{31}P MRS). These NAD(P) pools are identified by chemical standards (NAD$^+$, NADP$^+$, and NADH) and by physiological tests. A unique resonance reflecting mitochondrial NAD(P)H is revealed by the changes elicited by elevation of mitochondrial oxidation. The decline of NAD(P)H with oxidation is matched by a stoichiometric rise in the NAD(P)$^+$ peak. This unique resonance also provides a measure of the improvement in mitochondrial oxidation that parallels the greater phosphorylation found after exercise training in these elderly subjects. The implication is that the dynamics of the mitochondrial NAD(P)H peak provides an intrinsic probe of the reversal of mitochondrial dysfunction in elderly muscle. Thus, non-invasive detection of NAD(P)$^+$ and NAD(P)H in cytosol vs. mitochondria yields natural indicators of redox compartmentalization and sensitive intrinsic probes of the improvement of mitochondrial function with an intervention in human tissues *in vivo*. These natural indicators hold the promise of providing mechanistic insight into metabolism and mitochondrial function *in vivo* in a range of tissues in health, disease and with treatment.

Keywords: magnetic resonance spectroscopy, ^{31}P MRS, nicotinamide adenine dinucleotide, NAD$^+$, NADP$^+$, muscle, exercise

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INTRODUCTION

Nicotinamide adenine dinucleotide is a co-enzyme that is integral to cell and mitochondrial metabolism (Williamson et al., 1967; Stubbs et al., 1972; Nicholls and Ferguson, 2002; Ying, 2008; White and Schenk, 2012) and has a signaling role in mitochondrial biogenesis (Imai, 2011; Canto and Auwerx, 2012). NADH is a key player in oxidative phosphorylation in mitochondria

(Nicholls and Ferguson, 2002) and in glycolysis (Williamson et al., 1967; Stubbs et al., 1972), while the related metabolite, NADP, is important in anti-oxidant protection (Ying, 2008). Optical methods detect the sum of these compounds (NAD^+ and $NADP^+ = NAD(P)^+$) and can resolve their cellular location to provide natural indicators of mitochondrial function and cytosolic redox state ($NAD(P)H/NAD(P)^+$) (Jobsis and Duffield, 1967; Barlow and Chance, 1976; Wendt and Chapman, 1976; Scholz et al., 1995; Mayevsky and Rogatsky, 2007; Gandra et al., 2012; Claflin et al., 2015). These measures have lead to insight into the role of NAD(P) redox in oxidative stress (Ying, 2008; Murphy, 2009; Aon et al., 2010; Massudi et al., 2012a), as an index of oxygenation states (Barlow and Chance, 1976; Mayevsky and Rogatsky, 2007), and as a probe of mitochondrial (dys)function in age and disease (Heikal, 2010; Massudi et al., 2012b; Claflin et al., 2015). However, optical measures are typically limited to isolated tissues and are limited in depth penetration *in vivo*. A non-invasive measure of NAD(P) *in vivo* would open a window on cell and mitochondrial metabolism in age and disease as well as the impact of treatments to reverse metabolic dysfunction.

Non-invasive magnetic resonance methods now make detecting $NAD(P)^+$ and NAD(P)H *in vivo* possible both in animals and humans (de Graaf and Behar, 2014; Zhu et al., 2015). A distinct spectral position (chemical shift, CS) in the phosphorus spectrum (^{31}P MRS) is found for each metabolite representing the $NAD(P)^+$ and NAD(P)H redox states (Lu et al., 2014). It may also be possible to distinguish the cellular location of these NAD(P) redox states since several metabolites are reported to show distinct CS in the ^{31}P MR spectrum between these two compartments (Garlick et al., 1983; Hutson et al., 1989, 1992). For example, both ATP and $NADP^+$ show striking differences in CS with Mg^{2+} binding at the low metal concentration in the cytosol vs. the 10-fold higher level found in mitochondria (Mas and Colman, 1984; Gout et al., 2014). Inorganic phosphate also shows a significant chemical shift *in vivo* as a result of the pH difference in cytosol vs. mitochondria (Garlick et al., 1983; Hutson et al., 1992; Kan et al., 2010). There are also promising studies suggesting that NAD(P) redox states can be distinguished between the mitochondria vs. the cytosol. These studies found that phosphoryl compounds related to NAD(P) showed CS differences between erythrocytes vs. the bathing solution that are large enough to be detected by ^{31}P MRS (Kirk and Kuchel, 1988a,c). Thus, it may be possible to detect a NAD(P)H CS difference inside mitochondria relative to the cytosol in the ^{31}P MR spectrum thereby providing a natural indicator of key player in oxidative phosphorylation.

Here we describe a unique resonance in the ^{31}P MRS spectrum that conforms with the properties of NAD(P)H in mitochondria in human muscle *in vivo*. The aim of this study was to test the nature of this new resonance and determine if it provides a natural indicator of mitochondrial oxidative function. The first test compared this unique resonance to chemical standards of metabolites known to be present in the same region of the ^{31}P MR spectrum, including NAD^+, $NADP^+$, NADH, and NADPH (Kushmerick et al., 1986). No overlap in the spectral position (chemical shift) of the unique resonance vs. that of known metabolites was found. Two additional tests were performed to determine whether dynamics elicited in this peak by exercise conformed with changes expected for mitochondrial NAD(P)H. This functional test was designed to elevate oxidative phosphorylation and involved 40 elderly subjects before and after exercise training. The pre-training test revealed a reciprocal decline in the unique resonance vs. elevation in $NAD(P)^+$, which are changes consistent with oxidation of mitochondrial NAD(P)H to $NAD(P)^+$. The post-training test found a greater change in the unique resonance after exercise training, which is consistent with elevated oxidation and the greater phosphorylation rate reported in these subjects. Taken together, these results provide evidence that this MRS detectable resonance conforms to the properties of mitochondrial NAD(P)H. Thus, this unique resonance holds promise as the first natural indicator of the inner workings of oxidative phosphorylation in the ^{31}P MRS spectrum. It also holds promise as a sensitive indicator of the mitochondrial response to treatments designed to improve mitochondrial function.

METHODS

Subjects

An elderly group consisted of 40 subjects (18 male, 22 female) ranging in age from 65 to 80 years (68.8 ± 5.9 years, means ± S.E.M.). Subjects were not involved in formal exercise training, were in good health and had no significant cardiac, neurological, or musculoskeletal disease, as we have described (Conley et al., 2000; Jubrias et al., 2001). All subjects voluntarily gave informed, written consent as approved by the University of Washington Human Subjects Review Committee and in accordance with the Declaration of Helsinki.

MR Methods

A General Electric 1.5 T Signa imager/spectrometer was used for the *in vivo* spectra as described (Conley et al., 2000). A 9 cm diameter surface coil tuned to the phosphorus frequency (25·9 MHz) was placed over the vastus lateralis muscle of the thigh. The B_1 field homogeneity was optimized by off resonance proton shimming on the muscle water peak. The unfiltered PCr linewidth (full width at half-maximal height) was typically 4–8. Each subject had a high resolution ^{31}P MR spectrum of the resting vastus lateralis muscle taken under conditions of fully relaxed nuclear spins (16 free-induction decays (FID) with a 16 s interpulse delay) using a spectral width of ±1250 Hz and 2048 data points. Since volume selective methods were not used in collection, the spectra represent signal from a hemispherical volume defined by the coil radius. No volume selective methods were used to collect the spectra. Dynamic changes during stimulation and recovery were made using a standard 1-pulse experiment with partially saturated nuclear spins (1.5 s interpulse delay). Artifacts due to movement were reduced by stabilizing the limb during the muscle contractions. All fully relaxed spectra were zero filled from 2048 to 4096 points, Fourier transformed with 15 Hz apodization, baseline corrected, and manually phased using the Mnova software package (Mestrelab Research, Santiago de Compostela (Spain)). No other spectral treatment was employed.

Chemical Phantoms

Metabolites that resonate in the NAD(P) region of the ^{31}P MR spectrum were identified from muscle extracts (Kushmerick et al., 1986). Solutions containing these chemicals were prepared using binding constants taken from the USA National Institute of Standards and Technology (NIST) Critically Selected Stability Constants of Metal Complexes Database (see Kushmerick, 1997). All solutions contained (in mmol/L): EGTA 15, MOPS 80, free Mg^{2+} 1, Na^+ 83, and K^+ 52. The following were varied in individual solutions: ATP, ADP, P_i, Creatine Phosphate (PCr), ADP, NAD^+, NADH, $NADP^+$, or UDP-Glucose. The ionic strength was maintained at 0.175 M, pH = 7.0 (36°C) in all solutions. High-resolution MR spectra of individual solutions in an NMR tube were taken at 4.7T (sweep width = 10,000 Hz, 16 K complex points, 128 FIDs, 5 s delay between pulses).

Peak Simulations

Resonances were simulated at 1.5T using the spin parameters and chemical shifts for the simulations determined from NAD^+, $NADP^+$, and NADH in physiological solution at 4.7T. The Spin Simulation feature of the Mnova software (Mestrelab Research, Santiago de Compostela (Spain)) permitted determining the spectral properties of these resonances. The line-width of the simulated resonances were fixed to that of α-ATP due to the similar chemical environment of the two phosphate groups in NAD(P). The details of this process have been described (Lu et al., 2014).

NAD(P) Region *In vivo*

The −10.4 to −11.3 region containing NAD^+ and $NADP^+$ was extracted from the fully relaxed spectra by fitting a Lorenzian line shape to the α–ATP peak (**Figure 1**). The spectral intensity of this region was integrated as well as three sub-regions: −10.5 to −10.68 ppm (NADH), −10.69 to −10.92 ($NAD(P)^+$) and −10.93 to −11.30 (hereafter, −11.05 ppm). The dynamic changes in these regions were determined by subtraction spectra normalized to the α–ATP level between stimulated vs. resting muscle and during recovery vs. resting muscle.

Stimulation and Recovery Protocol

The quadriceps muscles were activated by transcutaneous electrical stimulation of the femoral nerve, as previously described (Blei et al., 1993; Conley et al., 2000). Spectra were collected during rest, stimulation and recovery to measure the PCr, P_i, ATP, and the NAD(P) region peaks. Spectra were averaged during each of the three periods to analyze the NAD(P) region peaks:

Control period (60 s, 10 spectra): Baseline data were obtained during resting muscle conditions to establish initial metabolite peak levels.

Stimulation period (120 s, 20 spectra): A 3 Hz electrical stimulation period was used to decrease [PCr] and activate NAD(P)H synthesis.

Aerobic recovery (300 s, 50 spectra): upon cessation of stimulation, PCr recovery was followed until restoration of baseline levels during which NAD(P)H oxidation was determined.

Statistics

Pre- vs. post-training differences relative to zero were tested with a 2-tailed Student's *t*-test. No adjustment for multiple comparisons was made as per reference (Perneger, 1998). Statistical differences are reported at $P < 0.05$. Means are reported ±SEM.

RESULTS

Here we show evidence for a unique resonance in the ^{31}P spectrum corresponding to NAD(P)H in mitochondria. To identify this resonance, we first compare the unique resonance against known metabolites in muscle. Second, we test the link between the unique resonance vs. the change in mitochondrial content that came with exercise training in these subjects. Third, we use an acute exercise bout to activate NAD(P)H oxidation to test for changes in the resonance and determine whether these changes (oxidation) parallel the improvement in phosphorylation found after exercise training in these subjects.

Unique Resonance in ^{31}P Spectrum *In vivo*

The presence of an unidentified peak in the ^{31}P MR spectrum of human muscle is illustrated in **Figure 1**. An expansion of the spectrum in the α–ATP region shows the NAD(P) peak region from −10.4 to −11.3 ppm. This spectrum was taken from an elderly vastus lateralis muscle under MR conditions in which peak areas reflect their chemical content (i.e., fully relaxed conditions). The resonances for chemical standards of NADH (−10.6 ppm), NAD^+ (−10.75 ppm), and $NADP^+$ (−10.84 ppm) are superimposed on the NAD region from the *in vivo* spectrum in Panel B [UDP-glucose is also present (Kushmerick et al., 1986) and resonates in this region at −10.84 in *in vitro* solutions that emulate *in vivo* conditions]. This comparison highlights the presence of an additional resonance centered at −11.05 ppm in the NAD(P) region. Thus, the unique resonance at −11.05 ppm does not correspond with resonances for metabolites in this spectral region that have been identified in tissue extracts (Kushmerick et al., 1986).

Unique Resonance is 40% of Total NAD(P) Integral

The NAD(P) integral is 11% of the area of α–ATP (11.5 ± 1.0%), which agrees with the total NAD per ATP content (7–12%; Sahlin, 1983; Henriksson et al., 1986) reported from biochemical analyses in human vastus lateralis muscle (Sahlin, 1983; Henriksson et al., 1986; Ren et al., 1988). Based on the ATP level in these muscles (5.8 mM; Conley et al., 2000), the full integral (0.7 mM) contains 0.4 mM NAD(P) (60% of the integral) vs. 0.5 mM measured for total NAD in young muscle (Henriksson et al., 1986). The unique resonance at −11.05 ppm is the second largest peak in the NAD(P) integral (**Figure 1C**), which translates to 0.25 mM (38% of the total). This is close to the value (0.26 mM) estimated from the NADH content of muscle mitochondria (heart, 10 nmole/mg mitochondrial protein, Alano et al., 2007) and the mitochondrial volume density of the elderly muscle in this study [3% (Conley et al., 2000), i.e., 10 nmole/mg × mg

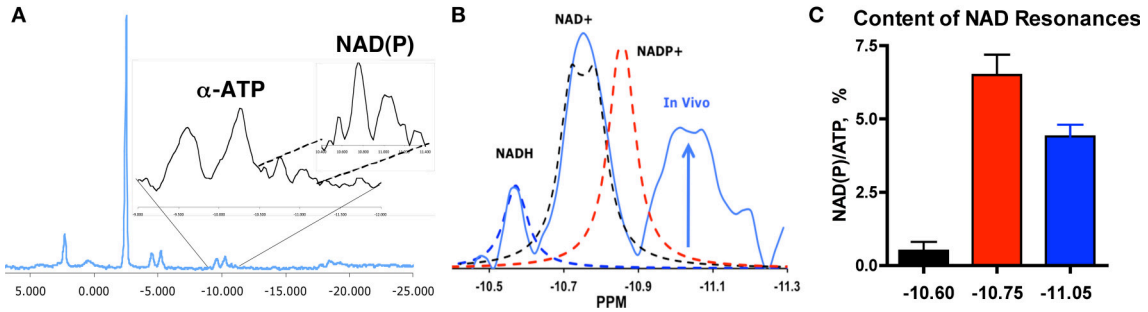

FIGURE 1 | NAD(P) resonances *in vivo* in human muscle. (A) ^{31}P magnetic resonance spectrum from human vastus lateralis at 1.5T with expansion of α–ATP and NAD(P). **(B)** Resonances for NADH, NAD^+, and $NADP^+$ (dashed lines) vs. the *in vivo* spectrum (blue) reveal an unidentified peak (arrow, −11.05 ppm). **(C)** Content of the three resonances *in vivo* [NAD(P)] per ATP content, mean % ± SEM, $n = 39$ (Conley et al., 2000).

protein/μl (Vinnakota and Bassingthwaighte, 2004) × 0.03 μl mito/μl muscle = 0.26 nmole/μl or 0.26 mM]. Thus, two key segments of the NAD(P) region *in vivo* are consistent in size with the contents of total NAD^+ assayed in muscle and NADH estimated from mitochondrial volume density of human muscle.

In contrast, the small resonance at −10.6 ppm associated with NADH in solution was 5% of the total integral in the NAD(P) region or ∼0.01 mM. A low NADH content in the cell is expected from the oxidized NAD redox state maintained by enzyme equilibria linked to the phosphorylation potential (Williamson et al., 1967; Stubbs et al., 1972). This low value agrees with the NADH levels in older bioluminescent assays on human vastus lateralis tissue (0.02 mM at rest, Henriksson et al., 1986). These findings suggest that the chemical standard for NADH at −10.6 ppm identifies the resonance for the small cellular NADH pool, while the larger −11.05 ppm resonance is more consistent with the mitochondrial NAD(P)H pool. Thus, the metabolite content represented by the −10.4 ppm to −11.3 ppm integral, which includes the unique resonance at −11.05 ppm, is in reasonable agreement with the total NAD(P) content reported from biochemical analysis of vastus lateralis muscle tissue.

Increase in −11.05 ppm Peak and Mitochondrial Volume with Exercise Training

Our second test of the nature of the unique resonance took advantage of the increased mitochondrial content that accompanied a 6-mo exercise training program in these subjects (Jubrias et al., 2001). We tested whether the −11.05 ppm resonance increased in proportion to the elevation in mitochondrial volume density found with resistance training (RT) in these subjects (Δ31%). **Figure 2** shows the significant changes in the −11.05 ppm resonance with RT in elderly subjects (Δ39%), while no change was found on average in either property in control (CN) or endurance trained (ET) groups. Also, no change was found in the NADH peak area at −10.60 ppm in the RT group (0.1 ± 0.5% ATP). Thus, the adaptation in the −11.05 ppm peak was exclusively associated with that of mitochondrial volume density with exercise training.

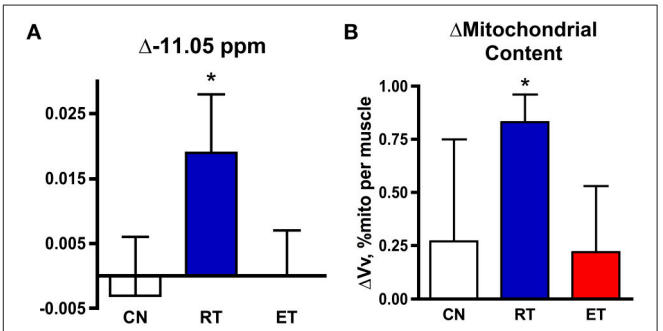

FIGURE 2 | Changes in NAD(P) resonances and mitochondrial volume in control (CN), resistance trained (RT, blue), and endurance trained (ET, red) groups. Change with exercise training in **(A)** the −11.05 ppm peak area, and **(B)** mitochondrial volume density. * – $P < 0.05$ post- vs. pre-training.

Reciprocal Change in −11.05 ppm Peak vs. $NAD(P)^+$ with Elevated Oxidation

A third test involved an exercise and recovery bout designed to alter the NAD(P) resonances by elevating oxidative phosphorylation, as shown in **Figure 3**.

Figure 3A shows the changes in creatine phosphate (PCr), a natural indicator of cell ATP use and re-synthesis, during an exercise and recovery bout. Exercise consisted of a 2 min muscle stimulation period that increased ATP use resulting in depletion of PCr level from the resting state (red line). The curvilinear shape of the PCr breakdown curve indicates that activation of ATP synthesis by mitochondria reduces the net breakdown toward the end of the stimulation period. Once stimulation ends and contractile ATP demand ceases, oxidative phosphorylation predominates (blue line) and PCr is resynthesized to quickly return to resting levels.

Figure 3B shows the ^{31}P spectra of the NAD(P) region taken from 10 subjects during these two periods. The spectrum collected from resting muscle (black line) represents the low oxidative phosphorylation condition. The spectrum collected from muscle during stimulation is shown in by red lines (left hand panels) and that collected during the recovery period of elevated oxidative phosphorylation is shown by the blue lines

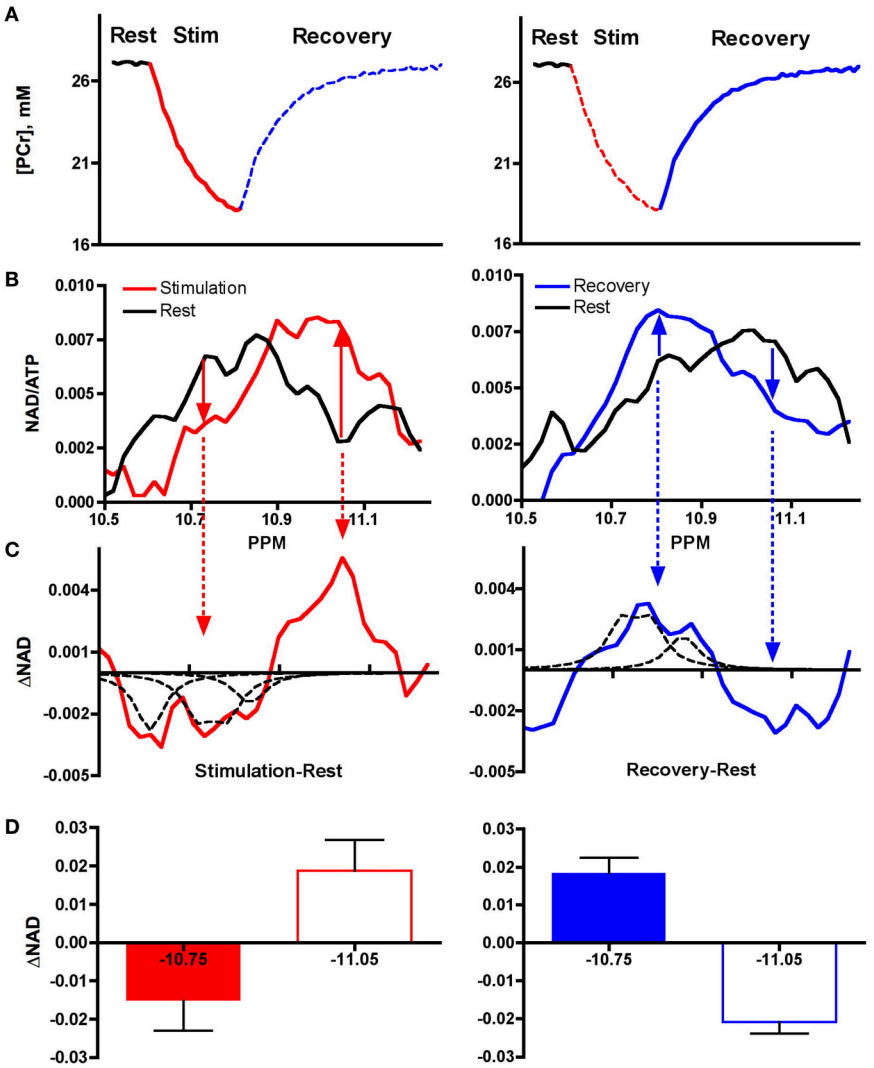

FIGURE 3 | Spectral changes during stimulation and recovery from exercise. (A) Exercise elicits elevated ATP use (PCr decline, red line) and oxidative phosphorylation (PCr re-synthesis, blue line). **(B)** Altered ^{31}P Spectra in stimulation (red) and recovery from exercise (blue) vs. resting muscle (black). **(C)** Subtraction of spectra region in resting (black) vs. stimulated muscle (red) and vs. recovering (blue) muscle reveals reciprocal changes in −10.75 ppm and in −11.05 ppm regions. The dashed peaks represent the resonances for the chemical metabolites in solution: NADH (−10.6 ppm), NAD^+ (−10.75 ppm), and $NADP^+$ (10.83 ppm). **(D)** Reciprocal changes in integrated areas of −10.75 ppm and −11.05 ppm regions.

(right hand panels). The solid arrows show the reciprocal changes in the −10.75 ppm and in the −11.05 ppm region.

Figure 3C shows the net differences in these regions with subtraction of these spectra. The change in the −10.75 ppm region is outlined by several dashed peaks. These peaks represent the resonances of the following chemical metabolites in solution: NADH (−10.6 ppm), NAD^+ (−10.75 ppm), and $NADP^+$ (10.83 ppm). Note that the decline in the −10.75 ppm region with stimulation (red arrows) is matched by an equal rise in the −11.05 ppm region. The opposite changes are seen during recovery from stimulation (blue line): the −11.75 ppm region rises and the −11.05 ppm region declines.

Figure 3D shows the integration of the peak areas in the difference spectra. Equal and opposite change in the −10.75 vs. −11.05 ppm region is confirmed by no significant difference in a paired *t*-test of the absolute integrated areas of the two regions in stimulation or recovery ($P > 0.15$ each). Such reciprocal changes are expected for a trade-off of oxidized and reduced forms of $NAD(P)^+$. The correspondence of the dashed peaks representing the resonances for NAD^+ and $NADP^+$ and the change in the −10.75 ppm region is strong support that this region represents net changes in $NAD(P)^+$. Similarly, the reciprocal change in the −11.05 ppm region suggests that change in NAD(P)H occurs in the region of the unique resonance. These changes are consistent with the dynamics expected for reduction and oxidation of mitochondrial NAD(P)H. Taken together, these results point to mitochondrial NAD(P)H dynamics occurring at the downfield spectral position (−11.05 ppm) from the resonance defined by the chemical NADH (−10.6 ppm) that likely defines the cell NADH.

Exercise Training Accelerates NAD(P) Dynamics and Phosphorylation

Our final test was whether the −11.06 ppm peak changes with an independent measure of oxidative phosphorylation. These subjects showed increased phosphorylation capacity after endurance (ET) and resistance (RT) training (Jubrias et al., 2001). This capacity was measured during the stimulation-recovery experiment shown in **Figure 3**, which was undertaken in each subject and repeated after exercise training. **Table 1** shows the changes in the −11.05 ppm peak in the three groups in this training experiment and reveals that both ET and RT groups show a net decline in the −11.05 ppm peak after training. No change is apparent in the control group. A corresponding net rise that balances the decline in the −11.05 ppm peak area is apparent in the −10.6 ppm peak (ET) or the −11.75 ppm peak (RT) with training. This rise in the integrated peaks in the −10.5 to −10.9 ppm region vs. the −11.05 ppm region demonstrates a net stoichiometry in the changes of the NAD(P) peaks with training. This net decline in the −11.05 ppm region during elevated oxidation following training further supports this spectral region as representing the mitochondrial metabolites, NAD(P)H.

Figure 4 shows that the significant decline in the −11.05 ppm region (−10.9 to −11.3 ppm) mirrored the net increase in phosphorylation with training in these subjects. These reciprocal changes suggest that the Δ-11.05 ppm region reflects the greater oxidation of NAD(P)H that is expected with the elevated phosphorylation (ΔPCr) found after training. Together, these results support that mitochondrial NADH oxidation *in vivo* is represented by the decline in the unique resonance at −11.05 ppm in the NADP region. Further, these results indicate that the −11.05 ppm peak is a sensitive indicator of the impact of an intervention on improving mitochondrial oxidation in elderly muscle.

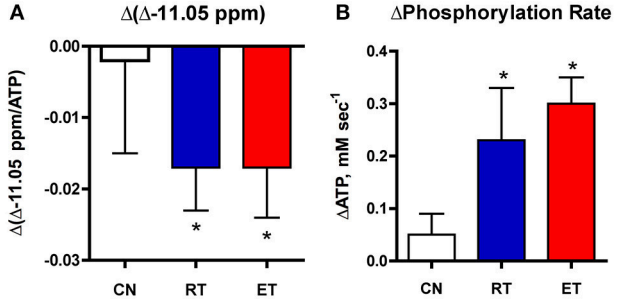

FIGURE 4 | Changes in the −11.05 ppm peak (A) and phosphorylation (B) with exercise training. Net decrease in the −11.05 peak and net elevation in oxidative phosphorylation during recovery is shown in muscle after resistance (RT) and endurance (ET) training vs. controls (CN). *$P < 0.05$.

TABLE 1 | Change in peak areas during the exercise recovery period in post- vs. pre-trained muscle.

Group	Δ−10.60 ppm	Δ−10.75 ppm	Δ−11.05 ppm
CN	2.3 ± 6.3	−1.0 ± 0.5	−0.2 ± 1.3
RT	0.3 ± 0.7	**1.5 ± 0.4***	**−1.7 ± 0.7***
ET	**1.5 ± 0.6***	0.1 ± 1.4	**−1.7 ± 0.6***

Units are % NAD per α–ATP peak for three resonances at distinct chemical shifts (ppm) in the NADP region. Values are means ± SE. Δ defines post- vs. pre-training changes in the integral of area of each resonance per integral of the α–ATP resonance.
The groups are: CN, control; RT, resistance training; ET, endurance training groups.
Δ−10.6, Δ−10.75, and Δ−11.05 are the chemical shifts (ppm) for the resonances defined by external standards for the reduced (NADH) and oxidized (NAD(P)$^+$) forms and a third resonance not identified by external standards, respectively.
**Bold font, $P < 0.05$ post- vs. pre-training.*

DISCUSSION

The metabolic role and redox state of the NAD(P) metabolites depend on their cellular location as illustrated in **Figure 5**. NADH is a critical substrate for oxidative phosphorylation and predominates over NAD^+ in mitochondria. The opposite is true for the oxidized cytosol in which enzyme equilibria linked to the phosphorylation potential holds NAD(P)H content low and $NAD(P)^+$ high (Williamson et al., 1967; Stubbs et al., 1972). Here we describe a unique resonance at −11.05 ppm in the *in vivo* ^{31}P MR spectrum that conforms with the properties of mitochondrial NAD(P)H. This unique resonance has the high chemical content and the dynamic changes with oxidative phosphorylation that are expected for the NAD(P) redox state in mitochondria. In contrast, a second peak identified by an NADH chemical standard (at −10.6 ppm) is consistent with the low metabolite content expected for cytosolic NADH. Three results summarize the evidence that the −11.05 ppm peak is a natural indicator of the content and dynamics of mitochondrial NAD(P)H and thereby provides a intrinsic probe of oxidative phosphorylation *in vivo*:

i) Quantitative agreement: A correspondence is found between the chemical content represented by the −11.05 ppm peak and the NAD(P)H level estimated from the mitochondrial content of these muscles and their changes with exercise training.
ii) Reciprocal dynamics with $NAD(P)^+$: The equal and opposite change in the −11.05 ppm peak vs. NAD^+ with exercise and recovery reflects the expected trade-off between reduced (NAD(P)H) and oxidized ($NAD(P)^+$) redox states in mitochondria.
iii) Dynamics parallel phosphorylation: A net decline in the −11.05 ppm peak mirrors the greater phosphorylation after exercise training, which supports this unique resonance as a sensitive natural indicator of changes in mitochondrial NAD(P)H oxidation with an intervention.

NAD(P) *In vivo* vs. *In vitro*

Several lines of evidence indicate good agreement of the NAD(P) metabolite levels determined in the ^{31}P MR spectrum *in vivo* and those reported in isolated human muscle by *in vitro* methods. The metabolite content represented by the peaks in the NAD(P) region (−10.5 to −11.3 ppm; 11% NAD/ATP) is in agreement with biochemical assays of NAD in human vastus lateralis (Sahlin, 1983; Henriksson et al., 1986). Chemical identification of the peaks in this region reveals that $NAD(P)^+$ accounts for 60% of the total peak area while only 5% of the total is accounted

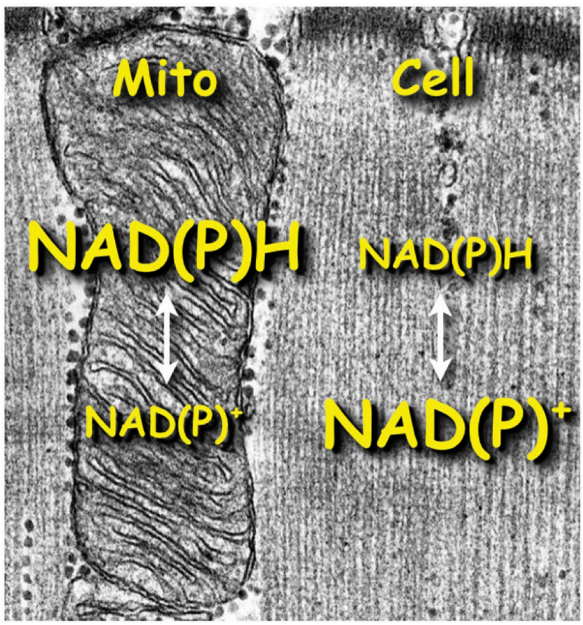

FIGURE 5 | Scheme illustrating the disparity of NAD(P) redox states expected in the cytosolic vs. mitochondrial compartments.

for by the chemically defined NADH (−10.6 ppm). The chemical content of the $NAD(P)^+$ region (0.4 mM) is consistent with estimates for NAD^+ derived from biochemical assays in young muscle (0.5 mM). The remaining 38% of the peak area of the NAD(P) region is represented by a peak centered at −11.05 ppm. Both the MR determination and that derived from independent measures estimate a NAD(P)H content of ∼0.26 mM.

Two Resonances Reflect NAD(P)H *In vivo*

A large difference in peak areas is expected from *in vitro* results if cytosolic vs. mitochondrial NAD(P)H can be distinguished in the ^{31}P MR spectrum (Williamson et al., 1967; Stubbs et al., 1972). The chemical content represented by the −10.60 ppm peak was low (5% of NAD(P) integral) relative to the unique resonance at −11.05 ppm (38%) as expected for cytosolic NADH, which is maintained at a low level by enzyme equilibria linked to the phosphorylation potential (Williamson et al., 1967; Stubbs et al., 1972). The larger chemical content of the −11.05 ppm corresponds with the predominance of mitochondrial NAD(P)H pool. This agreement of the size of two ^{31}P MR resonances with the expected NAD(P)H contents provides evidence that the cytosolic vs. mitochondrial NADH pools can be distinguished by MRS *in vivo* (Krebs and Veech, 1969; Stubbs et al., 1972).

Compartmentalization of NAD(P)H

The basis of the distinct spectral positions of NAD(P) is likely due to the changes in physical properties when the metabolite is located inside a compartment, such as in mitochondria. For example, enhanced fluorescence efficiency occurs with NAD(P)H inside mitochondria due to binding to complex I of the electron transport chain (Blinova et al., 2008), which permits highlighting mitochondrial NAD(P)H by optical spectroscopy and imaging.

A second example is the different chemical shift in the ^{31}P MR spectrum seen with phosphoryl compounds located inside erythrocytes (Kirk and Kuchel, 1988a,c; Larkin et al., 2007). Compounds related to NAD(P) show a Δ−0.5 ppm CS in the red blood cell, which is similar to the shift of the −11.05 ppm vs. −10.6 ppm NAD(P)H peak *in vivo* in muscle (Kirk and Kuchel, 1988a,c; Larkin et al., 2007). The mechanism for this downfield CS in red cells is reported to be elevated hydrogen bonding resulting from the high protein concentration of the erythrocyte (predominantly hemoglobin content) (Kirk and Kuchel, 1988b; Larkin et al., 2007). A high protein content is also characteristic of mitochondria (Vinnakota and Bassingthwaighte, 2004). Thus, the segregation of phosphoryl compounds inside a high protein environment provides a physical mechanism for the downfield CS of the −11.05 ppm peak (reflecting NAD(P)H in mitochondria) relative to NAD(P)H in the cell (−10.6 ppm) *in vivo*.

Increase in −11.05 ppm Peak and Mitochondrial Content with Exercise Training

A test of the identity of the −11.05 ppm peak is provided by the changes in the peaks of the NAD(P) region and in mitochondrial properties with exercise training (**Figure 2**). A shift in NAD(P) metabolite levels in correspondence with changes in mitochondria have been reported in several tissues with calorie restriction in mice in *in vitro* assays (Chen et al., 2008). One mechanism for an NAD^+ increase is a rise in the enzyme responsible for NAD^+ biosynthesis—NAMPT—that accompanies calorie restriction and exercise training (Costford et al., 2009). These adaptations in NADH and mitochondria were not uniform across tissues in calorie restricted mice (Chen et al., 2008). They also differed between training modes in the human muscle (**Figure 2**). A higher −11.05 ppm peak (Δ39%) was found in proportion to the greater mitochondrial content with RT (Δ31%), but neither property changed significantly with ET (**Figure 2**). In contrast to the −11.05 ppm peak, the chemically defined NADH peak at −10.6 ppm did not change despite the increased mitochondrial content in RT. This stability of the peak at −10.6 ppm in the face of a large increase in mitochondrial content is consistent with this resonance representing NAD(P)H in the cytosol. In contrast, a mitochondrial origin for the −11.05 ppm peak is supported by the unique rise of this resonance with mitochondrial volume density after exercise training.

Activating NAD(P)H Generation and Oxidation

A functional test of the identity of the −11.05 ppm peak is provided by the dynamics of the peaks in the NADP region with exercise and recovery. These dynamics are elicited by a protocol that activates increased energy use (as shown by PCr depletion from the resting state) followed by a period of elevated oxidative phosphorylation after exercise (as shown by restoration of PCr, **Figure 3A**). The peaks in the NAD region change in concert with these PCr dynamics during exercise and recovery (**Figure 3B**) to reveal the trade-offs in the reduced vs. oxidized redox states

of NAD(P) (**Figure 3C**) (Jobsis and Duffield, 1967; Wendt and Chapman, 1976). Such trade-offs are evident from the changes in the −11.05 ppm peak, which are matched by equal and opposite changes in oxidized NAD(P) (**Figure 3D**). Similar changes are apparent in optical fluorescence studies that indicate reduced mitochondrial NAD(P)H with muscle stimulation and oxidized NAD(P)H during elevated oxidative phosphorylation in recovery (Jobsis and Duffield, 1967; Wendt and Chapman, 1976; Gandra et al., 2012; Claflin et al., 2015). A rise in NAD(P)H generation under intense exercise has also been found in glycolytic type II fibers of human muscle in biochemical studies (Ren et al., 1988), while reduced NADH is found during sustained exercise in the oxidative, type I fibers of humans (Ren et al., 1988). Thus, the functional dynamics of the −11.05 ppm peak parallel the changes in mitochondrial NAD(P)H during exercise cycles reported in independent studies. These reciprocal changes in spectral regions are consistent with the −11.05 ppm peak representing mitochondrial NAD(P)H.

Sensitive Index of Mitochondrial Oxidative Improvements

An *in vivo* measure of mitochondrial NAD(P)H holds the promise of providing a natural indicator of oxidation adaptations with an intervention in human muscle. A test of the −11.05 ppm peak as such an indicator is provided by the results of exercise training that raised oxidative phosphorylation in these subjects (Jubrias et al., 2001). **Figure 4** shows a net drop in the −11.05 ppm resonance in an exercise bout after exercise training. This net decline parallels the net increase in phosphorylation rate and suggests that the −11.05 ppm peak reflects increased oxidation of mitochondrial NADH in the trained subjects. Independent findings of faster oxygen uptake after exercise training support these results. Human muscle O_2 uptake during recovery from exercise is increased and the kinetics of whole-body oxygen uptake is more rapid at the onset of exercise after training in adult and elderly subjects (Zoladz et al., 2006; Murias et al., 2010). The alternative is a higher ATP flux without increased O_2 uptake as a result of improvement in the coupling of generating ATP per O_2, which also occurs with exercise training (Conley et al., 2013). A recent analysis concluded that both mechanisms are activated: greater electron transport chain flux and more efficient generation of ATP appeared to contribute nearly equally to the phosphorylation improvements in these subjects (Conley, 2016). The results in **Figure 4** support this independent analysis to demonstrate that elevated oxidation is a part of the improvement in mitochondrial capacity to generate ATP after training. These results indicate that the dynamics of the −11.05 ppm peak captures the mitochondrial oxidation improvements that underlie greater phosphorylation. Importantly, both the oxidation and phosphorylation measures come from the same ^{31}P MR spectra collected in a single exercise test in human muscle *in vivo*.

Limitations and Future Directions

The low chemical content of NAD(P) related metabolites (<1 mM) *in vivo* is a limitation to detecting this natural indicator by ^{31}P MRS. Measurements are possible at the low field strength of 1.5T because of the trade-off with high B_1 field homogeneity at 1.5T, which provides good resolution of the individual NAD(P) peaks despite the low signal-to-noise. The large diameter coil (9 cm) used in this study and large muscle mass of the quadriceps group (∼1 kg) also helped to improve the signal for detecting these low concentration metabolites. Nonetheless, the high standard errors on some of the determinations may be due to this combination of low chemical content and low MRI field strength. However, two advances allow improved signal-to-noise over that found in the study. The first advance is the higher field strength (3T) MRIs that are now available in most medical centers that focus on human metabolic research. A second advance is the ability to decouple protons from phosphorus to increase the signal-to-noise of the NAD(P) peaks. Decoupling is a method derived from high-resolution MR spectroscopy that is now a standard feature of 3T MRIs with multi-nuclear detection (e.g., ^{31}P MRS) capability. Thus, the availability of the higher 3T field strength for human studies and the ability to further enhance signal-to-noise with proton decoupling will make detecting of the NAD(P) peaks and their dynamic changes with an intervention possible for clinical research studies.

SUMMARY

Here we show that a unique resonance in the *in vivo* ^{31}P spectrum provides a natural indicator of mitochondrial oxidation and its improvement with treatment. This assignment comes from the known compartmentalization of NADH between the highly oxidized cell (−10.6 ppm) vs. more reduced mitochondria (−11.05 ppm). A functional test that followed NAD(P) dynamics during exercise and recovery cycles confirmed that the −11.05 ppm peak reflected oxidation and reduction of mitochondrial NAD(P)H. Finally, the greater −11.05 ppm peak changes in the exercise test paralleled the faster phosphorylation rate in these subjects after exercise training. This correspondence provides further support that this unique resonance is a natural indicator of mitochondrial oxidation. Thus, intrinsic probes are present in the ^{31}P MR spectrum that reveal the NAD(P) redox disparity between mitochondria and cell and provide a natural indicator of oxidative phosphorylation that is sensitive to improvements in mitochondrial function with treatment.

AUTHOR CONTRIBUTIONS

KC, SJ, and ES conceived and designed the study; all authors contributed to the collection, analysis, and interpretation of data; KC and ES were involved in drafting or revising the paper.

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Mitochondrial Bioenergetics and Fiber Type Assessments in Microbiopsy vs. Bergstrom Percutaneous Sampling of Human Skeletal Muscle

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Microbiopsies of human skeletal muscle are increasingly adopted by physiologists for a variety of experimental assays given the reduced invasiveness of this procedure compared to the classic Bergstrom percutaneous biopsy technique. However, a recent report demonstrated lower mitochondrial respiration in saponin-permeabilized muscle fiber bundles (PmFB) prepared from microbiopsies vs. Bergstrom biopsies. We hypothesized that ADP-induced contraction (rigor) of smaller length microbiopsy PmFB causes a greater reduction in maximal respiration vs. Bergstrom, such that respiration could be increased by a myosin II ATPase-inhibitor (Blebbistatin; BLEB). Eleven males and females each received a 2 mm diameter percutaneous microbiopsy and a 5 mm diameter Bergstrom percutaneous biopsy in opposite legs. Glutamate/malate (5/0.5 mM)—supported respiration in microbiopsy PmFB was lower than Bergstrom at submaximal concentrations of ADP. 5 μM BLEB reduced this impairment such that there were no differences relative to Bergstrom ± BLEB. Surprisingly, pyruvate (5 mM)-supported respiration was not different between either biopsy technique ±BLEB, whereas BLEB increased succinate-supported respiration in Bergstrom only. H_2O_2 emission was lower in microbiopsy PmFB compared to Bergstrom PmFB in the presence of BLEB. Microbiopsies contained fewer type I fibers (37 vs. 47%) and more type IIX fibers (20 vs. 8%) compared to Bergstrom possibly due to sampling site depth and/or longitudinal location. These findings suggest that smaller diameter percutaneous biopsies yield lower glutamate-supported mitochondrial respiratory kinetics which is increased by preventing ADP-induced rigor with myosin inhibition. Microbiopsies of human skeletal muscle can be utilized for assessing mitochondrial respiratory kinetics in PmFB when assay conditions are supplemented with BLEB, but fiber type differences with this method should be considered.

Keywords: mitochondria, respiration, permeabilized fiber, muscle biopsy, blebbistatin

INTRODUCTION

The Bergstrom muscle biopsy technique has been an essential tool for the direct assessment of human skeletal muscle responses to a variety of physiological perturbations (Bergstrom, 1975). More recently, percutaneous microbiopsy needles have been adopted as an alternative approach to sample skeletal muscle in humans. Anecdotally, microbiopsies are often perceived as less invasive than Bergstrom given they obtain smaller samples and do not require an incision on the skin and fascia for the common procedure of sampling human vastus lateralis skeletal muscle. While these arguments have not been fully validated, interest in microbiopsies has increased nonetheless, particularly for the assessment of skeletal muscle metabolism (Hayot et al., 2005; Jorge et al., 2011; Krause et al., 2012; Votion et al., 2012). As such, validation of this methodology for metabolic assays in skeletal muscle is required given the potential influence of the sampling method on experimental results.

Permeabilized muscle fiber bundles (PmFB) are commonly used to assess mitochondrial bioenergetics in skeletal muscle. This technique requires careful separation of muscle fibers with fine forceps followed by chemical permeabilization with cholesterol-binding detergents (Kuznetsov et al., 2008; Pesta and Gnaiger, 2012; Perry et al., 2013). Surprisingly, it was recently reported that microbiopsies of pig skeletal muscle yielded lower mitochondrial respiration in PmFB which appeared to be positively correlated to needle diameter (Isner-Horobeti et al., 2014). While the exact cause of this lower respiration was not identified, it is possible that the smaller fiber length of PmFB from microbiopsies pose a challenge to preparing intact PmFB that can withstand the magnetic stirring during respirometric assessments. Loss of PmFB integrity could impair respiratory assessments given it has previously been established that preservation of mitochondrial structure and morphology are critical for optimizing respiratory assessments in PmFB (Veksler et al., 1987; Kuznetsov et al., 2008; Picard et al., 2011).

A key factor influencing PmFB intactness is a phenomenon of ADP-induced rigor, such that the very nature of assessing ADP-stimulated respiratory kinetics also induces contraction and disintegration *in vitro* (Ventura-Clapier and Vassort, 1985; Perry et al., 2011, 2013). Such PmFB contraction influences respiratory kinetics but can be prevented by the addition of Blebbistatin (BLEB), a myosin II ATPase inhibitor, to the assay media (Perry et al., 2011, 2012, 2013). Blebbistatin binds to the active site of subfragment 1 ATPase when ADP and phosphate are bound which stabilizes the intermediate state (Kovacs et al., 2004) lowers force production (Fedorov et al., 2007; Farman et al., 2008; Minozzo et al., 2012) and prevents shortening of muscle cell length (Fedorov et al., 2007; Farman et al., 2008; Ebrahim et al., 2013) during contraction. In fact, unpublished observations within our laboratory revealed drastic differences in mitochondrial respiration rates of PmFB in the presence (+BLEB) and absence (−BLEB) of BLEB from samples obtained using the microbiopsy technique. Moreover, microbiopsy PmFB appeared to be more prone to fiber disintegration and impaired respiratory kinetics following ADP-induced contraction than Bergstrom PmFB. If true, these observations would not only further highlight the importance of PmFB conformation when measuring mitochondrial function, but also suggest BLEB may be an effective tool for rescuing impaired respiration in microbiopsies.

The purpose of this investigation was to compare mitochondrial bioenergetics in samples obtained with the Bergstrom and microbiopsy techniques using BLEB as a tool to control the contractile state of PmFB *in vitro,* and furthermore, to characterize the fiber type composition of the samples obtained using each biopsy technique. We hypothesized that BLEB would increase respiration in microbiopsy PmFB *in vitro* by normalizing respiratory kinetics to Bergstrom PmFB. Likewise, we hypothesized that BLEB would result in similar mitochondrial H_2O_2 emission rates between microbiopsy and Bergstrom PmFBs. Finally, we expected fiber type composition analyses would be similar despite differences in biopsy size between both techniques.

MATERIALS AND METHODS

Human Participants and Muscle Biopsies

Eleven healthy, recreationally active males ($n = 5$) and females ($n = 6$) were recruited to participate in this investigation. Their mean ± standard error of the mean (SEM) age, height, weight and BMI were 25.3 ± 0.6 years, 171.7 ± 2.4 cm, 70.6 ± 4.8 kg, and 23.8 ± 1.3 kg·m^{-2}, respectively. All participants were non-smokers, free of disease and not taking prescription medications or supplements. Participants were given both oral and written information about experimental procedures before giving informed consent. All experimental procedures with human participants were approved by the Research Ethics Board at York University and conformed to the Declaration of Helsinki.

With the participant lying supine on a bed, a skeletal muscle sample was obtained from the lateral aspect of the right vastus lateralis by percutaneous needle biopsy technique using a spring-loaded 14 gauge (∼1.5 mm) Medax Biofeather microbiopsy disposable needle (San Possidonio, MO, Italy) under local subcutaneous anesthesia (∼2 ml of 2% xylocaine without norepinephrine). A 12 gauge (∼2 mm) cannula was used to puncture the skin at ∼30° from the surface to a depth of 2 cm and guide the needle to an additional depth of 2 cm longitudinally along the vastus lateralis. Four to five cuts (10–20 mg each) were sampled with the needle rotating ∼30–40° between cuts over a period of ∼1 min. Each piece was removed from the needle with sterile forceps or surgical blades before the subsequent cut was made. The first three samples were used for preparation of fiber bundles, and the remaining were used for fiber type analysis (described below). After applying a bandage to the ∼2 mm diameter biopsy site, a second skeletal muscle sample was obtained from the left vastus lateralis using the Bergstrom needle biopsy technique (Bergstrom, 1975) with manual suction (Shanely et al., 2014). Three to four cuts (100–150 mg total) were sampled over a period of ∼10 s. Approximately 40 mg of sample was used for preparation of PmFB, ∼20 mg was used for fiber type analysis and the remaining sample was frozen for future

work. The sequence of microbiopsy and Bergstrom sampling was randomized across participants.

Preparation of Permeabilized Muscle Fibers (PmFB)

This technique is partially adapted from previous methods (Kuznetsov et al., 1996; Tonkonogi et al., 1998) and has previously been described (Anderson et al., 2007; Perry et al., 2011, 2012). Briefly, small portions (∼25 mg) of muscle were dissected from each biopsy and placed in ice-cold BIOPS, containing (in mM): 50 MES, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 imidazole, 0.5 dithiothreitol (DTT), 20 taurine, 5.77 ATP, 15 PCr, and 6.56 $MgCl_2 \cdot 6\ H_2O$ (pH 7.1). The muscle was trimmed of connective tissue and fat and divided into several small muscle bundles (∼2–7 mm, 2–5 mg wet weight). Each bundle was gently separated along the longitudinal axis with a pair of anti-magnetic needle-tipped forceps under magnification (Zeiss 2000, Germany). Bundles were then treated with 30 μg/ml saponin in BIOPS and incubated on a rotor for 30 min at 4°C. Saponin at 30 μg/ml has previously been shown to optimize respiration in human skeletal muscle (Kane et al., 2011). Saponin is a mild, cholesterol-specific detergent that selectively permeabilizes the sarcolemmal membranes while keeping mitochondrial membranes, which contain little cholesterol, intact (Veksler et al., 1987; Kuznetsov et al., 2008). PmFB designated for pyruvate supported H_2O_2 emission (described below) were also treated with 1 μM CDNB during the permeabilization process to remove endogenous glutathione and better isolate emission kinetics, as previously described (Treberg et al., 2010; Fisher-Wellman et al., 2013). Following permeabilization, the PmFB were washed in either MiR05 containing (in mM): 0.5 EGTA, 10 KH_2PO_4, 3 $MgCl_2 \cdot 6\ H_2O$, 60 K-lactobionate, 20 Hepes, 20 Taurine, 110 sucrose and 1 mg/ml fatty acid free BSA (pH 7.1) for respiration experiments, or Buffer Z containing (in mM): 105 K-MES, 30 KCl, 10 KH_2PO_4, 5 $MgCl_2 \cdot 6\ H_2O$, 1 mM EGTA, 5 mg/ml BSA, (pH 7.1) for H_2O_2 emission experiments at 4°C until measurements were initiated (<30 min).

Mitochondrial Respiration in Permeabilized Muscle Fiber Bundles

High-resolution O_2 consumption measurements were conducted in 2 ml of respiration medium (MiR05) using the Oroboros Oxygraph-2k (Oroboros Instruments, Corp., Innsbruck, Austria) with stirring at 750 rpm. Respiration medium contained 20 mM Cr to saturate mitochondrial creatine kinase (Saks et al., 1991, 1994, 1995; Walsh et al., 2001; Anmann et al., 2006). For ADP-stimulated respiratory kinetics, either 5 mM glutamate or 5 mM pyruvate, accompanied by 0.5 mM malate, were added as complex I substrates (via generation of NADH to saturate electron entry into complex I) followed by a titration of submaximal ADP (225 and 750 μM) and maximal ADP (5 mM). Succinate (20 mM) was then added under state three conditions to saturate electron entry into complex II. Cytochrome *c* was added to test for mitochondrial membrane integrity, with all experiments demonstrating <10% increase in respiration. All experiments were conducted in either the presence (+) or absence (−) of 5 μM BLEB in the assay media which allowed for the comparison of respiratory kinetics in contracted or relaxed states for each biopsy technique. Each protocol was completed before the oxygraph chamber [O_2] reached 150 μM. Polarographic oxygen measurements were acquired in 2 s intervals, with the rate of respiration derived from 40 data points, and expressed as pmol/s/mg wet weight. PmFB were weighed in ∼1.5 ml of tared BIOPS (ATP-containing relaxing media) to prevent rigor that occurs when weighing PmFB in open air (unpublished observations).

Mitochondrial H_2O_2 Emission in Permeabilized Muscle Fiber Bundles

Mitochondrial hydrogen peroxide (H_2O_2) emission was determined fluorometrically (QuantaMaster 40, HORIBA Scientific, Edison, New Jersey) in a quartz cuvette with continuous stirring at 37°C, in 1 mL of Buffer Z supplemented with 10 μM Amplex Ultra Red, 0.5 U/ml horseradish peroxidase, 1 mM EGTA, 20 mM creatine, and 40 U/ml Cu/Zn-SOD1. Either 10 mM succinate or 10 mM pyruvate were added to stimulate H_2O_2 emission followed by a titration of ADP in step wise increments to progressively attenuate emission. All measurements were made in the presence of 5 μM BLEB to allow for the comparison of H_2O_2 emission in the relaxed state between biopsy techniques. Due to tissue limitations with the microbiopsy, no H_2O_2 emission measurements were made in -BLEB. The rate of H_2O_2 emission was calculated from the slope (F/min), after subtracting the background, from a standard curve established with the same reaction conditions and normalized to fiber bundle wet weight as described above.

Microscopic Imaging of PmFB Conformation

Images of Bergstrom and microbiopsy PmFB conformation were captured prior to, and immediately following respiration experiments. Photographs were captured with a Samsung Galaxy S5 camera (South Korea) placed face-down on a transparent acrylic surface at 2.4-4X zoom focused on PmFB placed in a culture dish containing MiRO. Video recordings with the same device were made to document the change in conformation of both Bergstrom PmFB and microbiopsy PmFB +BLEB and -BLEB following the addition of 2 mM ADP (with 5 mM pyruvate and 2 mM malate) in MiR05 buffer. The media temperature was maintained at 33–37°C by a pre-heated metal block.

Immunofluorescence Analysis of Fiber Type

Immediately after removal from the needles, muscles were embedded in O.C.T. compound (Tissue Tek), frozen in liquid nitrogen-cooled isopentane and stored at −80°C, until analysis. 10 μm thick cryosections were cut with a cryostat (Thermo Electronic), maintained at −20°C and transferred onto static-free microscope slides. Immunofluorescent detection of myosin heavy chain isoforms was performed as previously

described (Bloemberg and Quadrilatero, 2012). In addition, fiber membranes were visualized by staining for dystrophin (DSHB, University of Iowa, Iowa City, USA). Fiber type composition analysis was performed on image composites by counting all fibers across the entire cross section. Slides were visualized with an Axio Observer Z1 fluorescent microscope equipped with an AxioCam HRm camera and associated AxioVision software (Carl Zeiss, Germany).

Statistics

Results are expressed as mean ± SEM. The level of significance was established at $P < 0.05$ for all statistics. The D'Agostino—Pearson omnibus normality test was first performed to determine whether data resembled a Gaussian distribution. Due to failed normality, Friedman's non-parametric One-way ANOVA test was performed for all respiration data. When a significant F-ratio was obtained, Dunn's multiple comparisons *post-hoc* analysis was performed. Paired *t*-tests were performed to assess differences amongst biopsy techniques with respect to H_2O_2 emission rates in presence of BLEB as well as fiber type proportions.

RESULTS

Comparison of ADP-Induced Contraction in Skeletal Muscle PmFB

We first compared qualitative appearances of PmFB conformation from Bergstrom vs. microbiopsy sample in the relaxed state prior to ADP-stimulated respiration (**Figure 1A**). Bergstrom PmFB appeared to have longer and more connected individual fibers forming a more cohesive bundle while microbiopsy PmFB had much shorter individual fibers. Following titrations of ADP over a period of 45–60 min at 37°C in +BLEB or −BLEB, PmFB were retrieved from the respirometer chambers and imaged. The Bergstrom PmFB in −BLEB appeared less separated and more compact than those from +BLEB, suggesting greater ADP-induced rigor in the absence of BLEB (**Figure 1B**). However, 41% of microbiopsy PmFB from −BLEB disintegrated into multiple pieces, many of which were so small that they could not be removed from the oxygraph chamber for imaging (**Figure 1B**) whereas nearly all PmFB in +BLEB for either biopsy could be removed after the experiments.

Using video capture in separate PmFB, we observed a progressive contraction in response to ADP in −BLEB from Bergstrom (Supplementary Video Figure 1) as previously reported (Perry et al., 2011, 2012) that was also apparent in PmFB from microbiopsy (Supplementary Video Figure 3). ADP-stimulated contraction was not observed +BLEB from either biopsy type (Supplementary Video Figures 2, 4). All videos were 4 min in duration and may therefore underestimate the effect of ADP on contraction that occurs during typical respiratory protocols lasting >30 min.

While force production was not measured, these results suggest the change in PmFB conformation in response to ADP was contraction, given the response was prevented by BLEB.

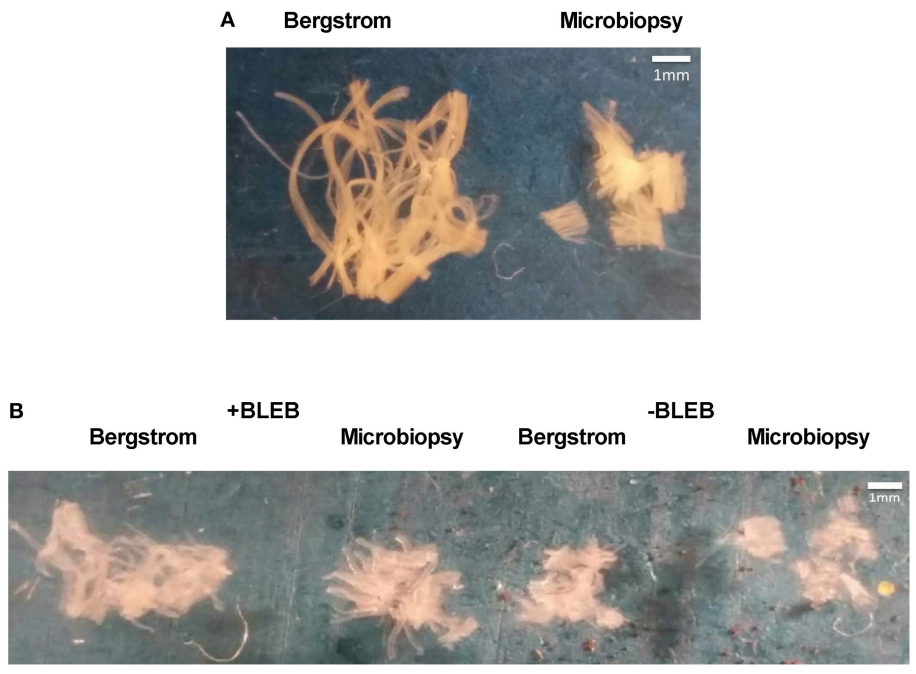

FIGURE 1 | Effect of apparent ADP-stimulated respiration on human PmFB conformation. PmFB conformation was imaged **(A)** prior to exposure to ADP-stimulated respiration in Bergstrom and microbiopsy PmFB (4x zoom) and **(B)** following 45–60 min of ADP-stimulated respiration in the presence (+) and absence (–) of BLEB (2.4x zoom).

BLEB Increases ADP-Stimulated Respiration in a Substrate Specific Manner

In the absence of BLEB, microbiopsy respiration was 51% lower than Bergstrom when supported by glutamate and malate at 225 μM ADP ($p = 0.06$) and 51% lower at 750 μM ADP ($p < 0.05$) with no differences at 5 mM ADP (**Figure 2A**). When 5 μM BLEB was added to the assay media, the impaired glutamate/malate-supported respiration in microbiopsy PmFB was increased at all ADP concentrations compared to microbiopsy −BLEB (**Figure 2A**). Furthermore, there were no significant differences in respiration between microbiopsy +BLEB, Bergstrom +BLEB or −BLEB (**Figure 2A**) indicating BLEB normalized microbiopsy respiration to both Bergstrom conditions. Contrary to the findings with glutamate/malate-supported respiration, there were, surprisingly, no differences in any condition during pyruvate-supported respiration between microbiopsy and Bergstrom (**Figure 2B**). BLEB increased succinate-supported respiration in Bergstrom by 84–166% vs. both microbiopsy −BLEB and Bergstrom −BLEB, but did not significantly affect microbiopsy respiration (**Figure 2C**).

Lower Mitochondrial H_2O_2 Emission Rates in Microbiopsy PmFB

Due to tissue limitations, mitochondrial H_2O_2 emission was measured in both biopsies only in the presence of BLEB. Succinate-induced H_2O_2 emission was 37% greater in Bergstrom +BLEB vs. microbiopsy +BLEB at 500 μM ADP ($p < 0.05$) and trended greater at 0 μM ADP ($p = 0.08$), with no difference at 50 μM ADP (**Figure 3A**). Similarly, pyruvate induced H_2O_2 emission rates were 51–66% greater in Bergstrom vs. microbiopsy at all ADP concentrations ($p < 0.05$, **Figure 3B**).

Fiber Type Analysis Reveals Differences in Myosin Expression Between Microbiopsy and Bergstrom Biopsies

Immunoflourescent analyses of fiber type composition revealed microbiopsy samples have a significantly lower proportion of type I fibers vs. Bergstrom (37.3 vs. 46.7%, $p < 0.05$, **Figure 4**) and a significantly higher proportion of type IIX fibers (20.1 vs. 7.6%, $p < 0.05$, **Figure 4**). However, no differences in relative proportion of type IIA fibers were observed (42.7 vs. 45.7%, **Figure 4**).

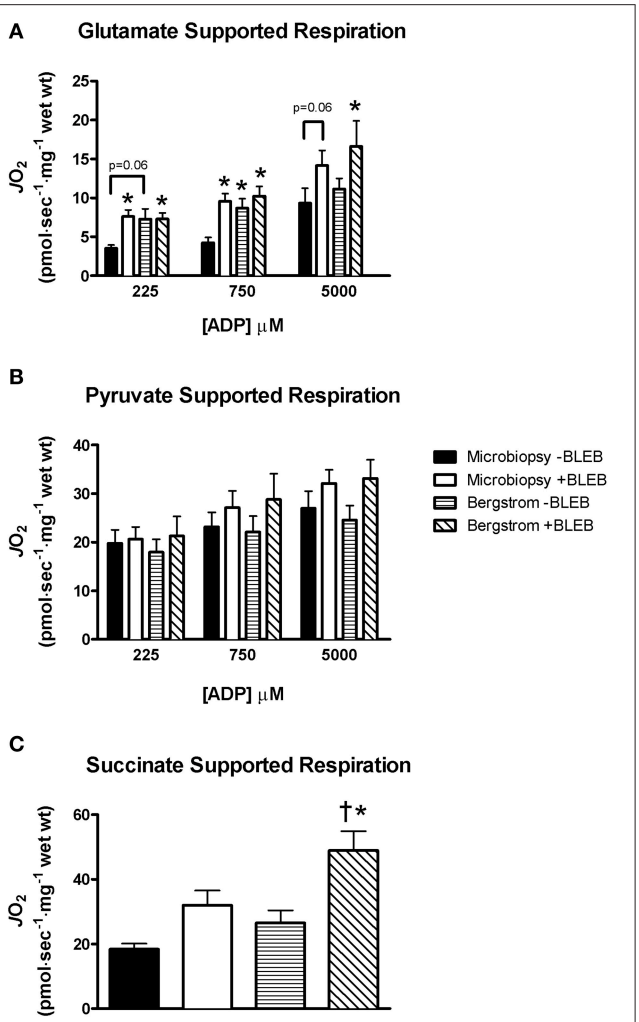

FIGURE 2 | Effect of apparent ADP-induced contraction on mitochondrial respiration in PmFB. ADP-stimulated respiration rates were supported by **(A)** glutamate (5 mM), **(B)** pyruvate (5 mM), and **(C)** succinate (20 mM) in microbiopsy and Bergstrom PmFB. Results represent means ± S.E.M.; $n = 8$–11; $^*P < 0.05$ compared to microbiopsy−BLEB within the same [ADP] condition, $^\dagger P < 0.05$ compared to Bergstrom−BLEB.

DISCUSSION

This investigation demonstrates that myosin inhibition in PmFB is a novel solution to resolving the reduction in respiratory kinetics observed in microbiopsy samples of human skeletal muscle. Specifically, in −BLEB, PmFB from Bergstrom biopsies appeared more compacted and less separated following ADP-stimulated respiration whereas PmFB from microbiopsies had a greater occurrence of disintegration into fragments. This *in vitro* change in morphology lowered glutamate-supported respiratory kinetics in PmFB from microbiopsies moreso than in Bergstrom. As hypothesized, the lower respiration in microbiopsy PmFB were increased with the myosin II ATPase inhibitor Blebbistatin, suggesting ADP stimulated contraction of PmFB in -BLEB was responsible for the change in morphology in response to ADP and reduction in respiration vs. Bergstrom. Microbiopsies also contained fewer type I and greater type IIX fibers. However, this does not explain the lower glutamate-supported respiration given kinetics were similar with other substrates in all conditions. Mitochondrial H_2O_2 emission rates were detectable in samples obtained using the microbiopsy technique but absolute values were lower relative to Bergstrom, even in the presence of BLEB. Collectively, these results highlight the importance of considering needle sampling approaches when assessing mitochondrial bioenergetics in human skeletal muscle and clearly demonstrate that microbiopsies can be used to assess mitochondrial respiration in PmFB when apparent ADP-induced contraction is prevented by BLEB.

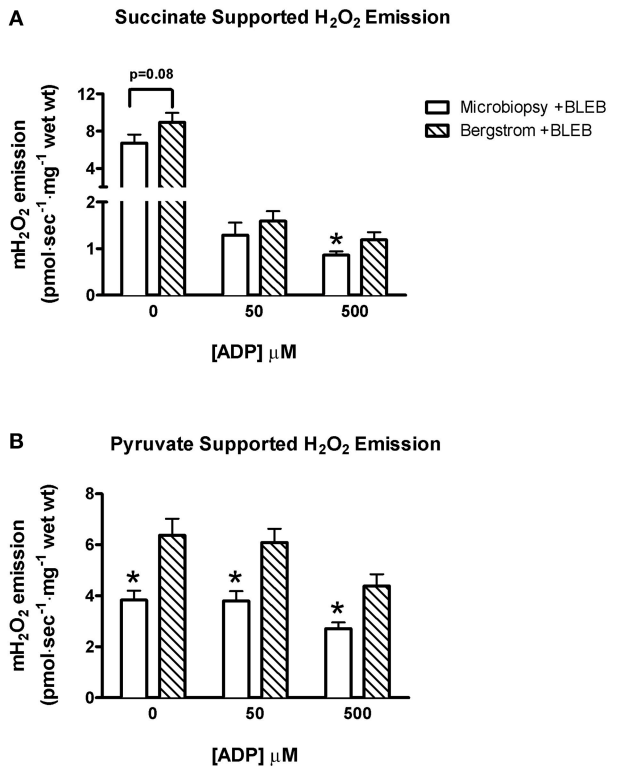

FIGURE 3 | H_2O_2 emission rates of PmFB sampled from microbiopsy and Bergstrom biopsy techniques in the presence of 5 μM BLEB. Mitochondrial H_2O_2 emission rates at varying concentrations of ADP titrated after stimulation by 10 mM **(A)** succinate and **(B)** pyruvate in PmFB from microbiopsy and Bergstrom samples. Results represent means ± S.E.M.; $n = 11$; $^*P < 0.05$ compared to Bergstrom of same [ADP].

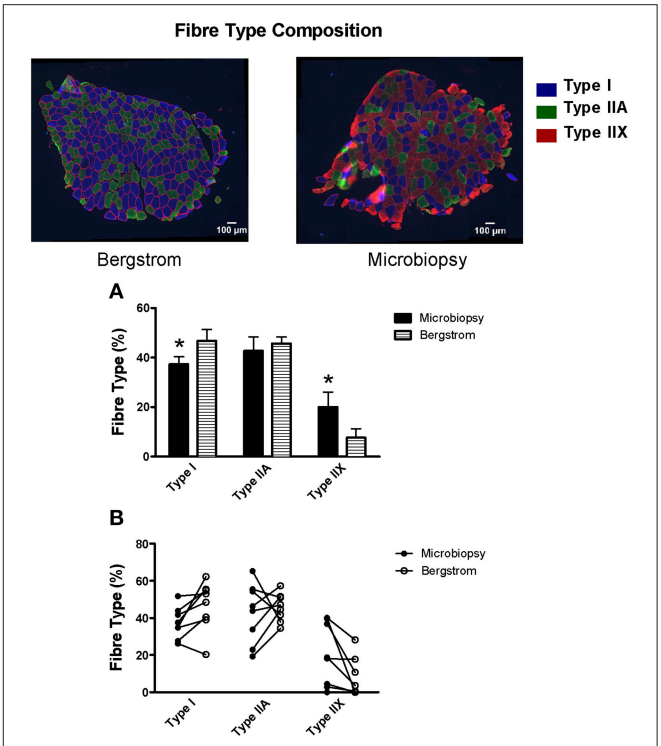

FIGURE 4 | Fiber type composition of microbiopsy and Bergstrom muscle samples. Relative percentage of fiber type from samples obtained using the microbiopsy and Bergstrom biopsy technique are represented with blue indicating type I, green indicating type IIA and red indicating type IIX. Results represent **(A)** means ± S.E.M and **(B)** individual data; $n = 8$–9; $^*P < 0.05$ compared to Bergstrom of same fiber type.

Preventing Microbiopsy ADP-Induced Contraction *in vitro* Improves Glutamate-Supported Respiration

Microbiopsy sampling is seen as an attractive alternative to Bergstrom biopsies in part, because of the smaller diameter of the needle. However, concerns about the length of fibers obtained from a smaller diameter needle have been raised (Isner-Horobeti et al., 2014). Previous work demonstrating a positive correlation between needle diameter and maximal rates of respiration in PmFB suggested that this impairment in respiration is related to fiber length (Isner-Horobeti et al., 2014). This notion is supported by differences in bundle morphology observed in the present study (**Figure 1A**), which demonstrates shorter myofiber length and a disorganized conformation in microbiopsy relative to Bergstrom. Previous work with Bergstrom PmFB revealed that spontaneous ADP-induced contraction results in drastic changes in conformation including rigor and fragmentation (Perry et al., 2011, 2012). This followed previous work showing ADP causes rigor in PmFB from rodent skinned cardiac fibers (Ventura-Clapier and Vassort, 1985). Given the small fibers in microbiopsies pose a challenge to preparing intact PmFB, it is not surprising that this same apparent contraction results in more consistent and complete disintegration of PmFB (**Figure 1B**). However, similar to previous findings in Bergstrom PmFB (Perry et al., 2011, 2012), BLEB prevents this apparent ADP-induced contraction and partially retains microbiopsy PmFB conformation, myofiber length and structural integrity (**Figure 1B**). This result demonstrates that BLEB corrects the observed reductions in glutamate-supported respiration reported previously (Isner-Horobeti et al., 2014) and therefore permits the use of microbiopsies for respiratory assessments in PmFB.

Surprisingly, these disintegration-related impairments in respiration were substrate-specific. While we generally observed similar degrees of contraction regardless of the substrate utilized (unpublished findings), this disintegration did not appear to have an impact on pyruvate-supported respiration rates given respiration was similar between both biopsy techniques with or without BLEB present (**Figure 2B**). However, succinate-supported respiration was increased by BLEB in Bergstrom PmFB which was greater than the rate obtained in microbiopsies PmFB. Speculating on the substrate-specific differences in respiration between biopsies is difficult but might suggest contraction exerts a regulatory influence on specific metabolic pathways through mechanisms that might be retained in PmFBs but are yet to be realized. While the reason for these substrate-specific differences is unclear, this finding highlights the necessity

of considering all experimental parameters when choosing a biopsy technique and the subsequent protocols that will be used. Given BLEB improved glutamate-supported respiration and did not impair kinetics with pyruvate, we suggest that BLEB should be supplemented in assay media during respiratory assessments supported by any of these substrates. In our hands, this approach allays concerns regarding the use of microbiopsies of PmFB as reported previously (Isner-Horobeti et al., 2014).

It should be noted that previous work demonstrating impaired respiration in microbiopsy PmFB was performed at 22°C (Isner-Horobeti et al., 2014) whereas the present study was conducted at 37°C. While we cannot conclude whether BLEB would normalize microbiopsy respiration to Bergstrom at 22°C, we have previously shown BLEB is more effective at influencing respiratory kinetics in human PmFBs at 37°C vs. lower temperatures (Perry et al., 2011). Nevertheless, it may be preferable to conduct PmFB respiration experiments at or near body temperature given that temperature is a critical regulator of metabolic control *in vitro* and *in vivo*.

Mitochondrial H_2O_2 Emission is Lower in Microbiopsy vs. Bergstrom PmFB

It is tempting to speculate that the lower H_2O_2 emission in microbiopsies were a result of fewer mitochondria related to the greater proportion of type IIX fibers and reduced type I fibers. However, succinate- and pyruvate/malate-supported H_2O_2 emission has been shown to be higher in rat red gastrocnemius muscle [predominantly type IIA and IIX fibers (Bloemberg and Quadrilatero, 2012)] than soleus [predominantly type I fibers (Bloemberg and Quadrilatero, 2012)] when normalized to muscle mass (Anderson and Neufer, 2006). Surprisingly, this apparent inverse relationship between H_2O_2 emission and assumed mitochondrial content is opposite to our observations of lower H_2O_2 emission in microbiopsies with greater type IIX content. Thus, based on previous findings (Anderson and Neufer, 2006), it would appear that factors independent of mitochondrial content and fiber type might explain the lower H_2O_2 emission in microbiopsies.

Anecdotally, it is known that PmFB must be separated as much as possible without losing bundle integrity in order to optimize the detection of H_2O_2 emission. Specifically, we found Bergstrom fibers can be separated to a greater degree than microbiopsy PmFB without losing integrity (**Figure 1A**). This potentially allows for greater diffusion of substrates and ADP within the fiber, and may indicate that H_2O_2 emission measurements are more sensitive to the degree of separation than perhaps previously recognized. Hence, it is possible that the relatively lower degree of separation required to maintain integrity in microbiopsy PmFB may result in under-estimations of H_2O_2 emission.

These measurements were made in the presence of BLEB and suggest that preventing PmFB contraction does not normalize microbiopsy PmFB H_2O_2 emission to Bergstrom. Whether BLEB still improved this measurement in comparison to untreated PmFB is uncertain as we were not able to compare ±BLEB due to tissue limitations in microbiopsies. Ultimately, H_2O_2 emission is detected quite clearly in microbiopsy +BLEB which does not rule out the use of microbiopsies for this measurement. Nevertheless, potential underestimations relative to Bergstrom should be considered.

Fiber Type Differences in Microbiopsies vs. Bergstrom: Does This Influence Respiratory Assessments?

An intriguing difference in fiber type composition existed between samples obtained from the two needles. Specifically, 7/8 subjects demonstrated an increase in percentage of type I fibers and a decrease in percentage of type IIX fibers in the Bergstrom sample relative to microbiopsy. It is well-established that the human vastus lateralis has a heterogeneous composition (Johnson et al., 1973; Komi and Karlsson, 1978; Lexell et al., 1984; Taylor and Bachman, 1999; Staron et al., 2000). In fact, the distribution varies mainly as a function of depth in that the superficial vastus lateralis contains more type II fibers whereas a greater proportion of type I fibers are found in deeper portions of the muscle (Lexell et al., 1983; Lexell and Taylor, 1989). Given the microbiopsy technique was performed at a shallower depth in the present study (∼30° insertion angle relative to the skin), these differences in fiber type composition might be attributed to the difference in needle depth. However, we cannot rule out potential contributions of the longitudinal location between the two needles given the shallow insertion angle of the microbiopsy generally sampled at a location more proximal to the knee.

It is tempting to speculate that the lower glutamate-supported respiration with microbiopsies was due to the lower type I and higher type IIX content, as discussed above for H_2O_2 emission. However, there were no differences in pyruvate or succinate-supported respiration between either needle in ±BLEB. This suggests the fiber type differences in human muscle were too small to influence the kinetics even though large differences in fiber type in rodents have been shown to influence respiration (Kuznetsov et al., 1996). Nonetheless, it would appear that the small differences in fiber type do not explain the lower glutamate-supported respiratory kinetics or H_2O_2 emission in microbiopsy PmFB -BLEB. We cannot rule out that such composition differences between needles could influence other metabolic assessments.

Ultimately, the differences in fiber type between sampling methods does not necessarily pose a problem for skeletal muscle metabolic assessments *per se*. Rather, the interpretation of results should be made in the context of sample fiber type composition inherent in each sampling method. Of course, fiber type composition could differ within a sampling approach due simply to variations in sampling depth or longitudinal consistency within trials, highlighting the need for consistency in sampling procedure regardless of needle type.

Selecting a Muscle Biopsy Approach: Important Considerations

Categorizing one procedure as superior to the other must be considered in the context of specific parameters. The obvious advantage of the Bergstrom method is a larger sample yield

with typical ranges of ∼50–250+ mg depending, in part, on the diameter of the needle, the use of suction and the number of cuts. However, we obtained a typical yield of ∼70–90 mg (and up to 120 mg) with microbiopsy by rotating the needle ∼30 degrees to uncut areas of muscle between five separate cuts while applying light pressure on the skin prior to each cut. A fast procedure is required to minimize blood contamination of muscle (<60 s including removal of muscle after each cut). Additionally, participant comfort level must also be considered. While microbiopsies are smaller in diameter, these five separate cuts also require separate insertions into the same cannula guide and therefore take longer to obtain. Conversely, the Bergstrom procedure permits multiple cuts over several seconds with a single insertion. However, 8 out of 11 participants reported preferring the microbiopsy technique over the Bergstrom when questioned following both procedures. Furthermore, reduced risks of infection are sometimes marketed by commercial suppliers of microbiopsy needles due specifically to the fact that they are disposable, thereby eliminating the need for subsequent sterilization. However, proper sterilization of Bergstrom needles obviously ameliorates this concern. These claims must also be balanced with the fact that the single-stage cut with microbiopsies requires removal of skeletal muscle from the needle with sterile forceps prior to re-insertion of the needle into the muscle for additional cuts. This requires considerable care in preventing contamination of the needle in between samples from the same entry site.

It should also be noted that our conclusions are specific to a 14 gauge needle from a specific supplier. Other models of disposable microbiopsy needles could influence the results based on needle sharpness or length of time required to obtain a sample given some needles do not have a cannula "guide" that accelerates the procedure as in the present study. Likewise, we have not compared the influence of multiple gauges, although previous work suggests a positive correlation exists between PmFB respiration and needle diameter (Isner-Horobeti et al., 2014). Finally, experimental procedures requiring intact muscle fibers should carefully consider the requirement for specific fiber lengths independent of sample size. Overall, the advantages and disadvantages to both procedures must be considered within the context of specific study designs rather than focusing on needle diameter and sample yield as the sole criteria for selecting a needle.

CONCLUSIONS

In conclusion, this investigation reveals that PmFB prepared from 14 gauge microbiopsies provide similar glutamate-supported respiratory kinetics to Bergstrom samples when assay media are supplemented with 5 μM BLEB to prevent apparent ADP-induced contraction and maintain PmFB integrity. The similar pyruvate-supported respiration between biopsy approaches further suggests that microbiopsies can be used to measure respiratory kinetics in PmFB, although Bergstrom +BLEB provided the greatest rate of succinate-supported respiration. However, observed reductions in H_2O_2 emission and altered type I:type IIX fiber types should be considered when adopting microbiopsies. These findings highlight critical experimental parameters to consider when selecting biopsy needles and underscores the importance of preventing ADP-induced PmFB contraction by myosin inhibition with Blebbistatin *in vitro*.

AUTHOR CONTRIBUTIONS

MH, BG, JQ, DK, and CP. contributed to study design. MH, SR, PT, AN, HT, RL, and CP conducted clinical trials and/or experiments while MH, BB, JQ, DK, and CP analyzed and interpreted the data. MH and CP were the primary writers of the manuscript while all authors contributed to critical interpretation and manuscript preparation. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00360

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REVIEW
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The Measurement of Reversible Redox Dependent Post-translational Modifications and Their Regulation of Mitochondrial and Skeletal Muscle Function

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Mitochondrial oxidative stress is a common feature of skeletal myopathies across multiple conditions; however, the mechanism by which it contributes to skeletal muscle dysfunction remains controversial. Oxidative damage to proteins, lipids, and DNA has received the most attention, yet an important role for reversible redox post-translational modifications (PTMs) in pathophysiology is emerging. The possibility that these PTMs can exert dynamic control of muscle function implicates them as a mechanism contributing to skeletal muscle dysfunction in chronic disease. Herein, we discuss the significance of thiol-based redox dependent modifications to mitochondrial, myofibrillar, and excitation-contraction (EC) coupling proteins with an emphasis on how these changes could alter skeletal muscle performance under chronically stressed conditions. A major barrier to a better mechanistic understanding of the role of reversible redox PTMs in muscle function is the technical challenges associated with accurately measuring the changes of site-specific redox PTMs. Here we will critically review current approaches with an emphasis on sample preparation artifacts, quantitation, and specificity. Despite these challenges, the ability to accurately quantify reversible redox PTMs is critical to understanding the mechanisms by which mitochondrial oxidative stress contributes to skeletal muscle dysfunction in chronic diseases.

Keywords: redox signaling, post-translational modification, skeletal muscle, mitochondria, myofibrils, glutathionylation

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INTRODUCTION

Oxidative stress plays an important role in skeletal muscle health and disease. In the past, most attention has been focused on skeletal muscle dysfunction caused by oxidative damage to proteins, lipids, and DNA. This damage alters the structure of macromolecules in an irreversible manner and requires turnover and production of new macromolecules to reverse the effects. However, there is growing appreciation of the role of reactive oxygen and nitrogen species (ROS/RNS) in reversible redox post-translational modifications (PTMs) in both muscle adaptation and the effects of chronic disease and aging on skeletal muscle (Sohal and Orr, 2012). Normal skeletal muscle physiology is characterized by transient periods of elevated ROS production due to contractile activity

(Powers et al., 2010). In response to repeated bouts of contraction these transient periods of elevated ROS production play an important role in mediating the adaptive benefits of exercise through activation of redox sensitive transcription factors (Powers and Jackson, 2008). On the opposite extreme, chronic elevation of oxidative stress underlies some of the pathological changes associated with disuse atrophy (Min et al., 2011; Powers et al., 2011).

In addition to these longer term adaptive changes in muscle, it is now clear that the intracellular redox environment of skeletal muscle also exerts dynamic control over skeletal muscle physiology on the time scale of seconds to hours. Mild elevation of oxidants increases skeletal muscle force production, while further increases in oxidative stress reduce force and play a role in skeletal muscle fatigue (Reid et al., 1993; Andrade et al., 1998; Matuszczak et al., 2005). Manipulation of muscle oxidative stress also induces rapid effects on mitochondrial energetics. Within 24 h of treatment to induce a mild oxidative stress in mice, paraquat impairs *in vivo* mitochondrial energetics and induces an energy stress similar to that observed in aged skeletal muscle (Siegel et al., 2012). Reducing mitochondrial oxidative stress by treating with the mitochondrial targeted peptide SS-31 has the opposite effect. Within 1 h of treatment with SS-31 improved mitochondrial energetics and fatigue resistance were associated with a more reduced GSH/GSSG redox state in aged mouse skeletal muscle (Siegel et al., 2013). The rapid and reversible nature of the control of this activity indicates that these effects are not dependent on turnover of oxidatively damaged molecules or protein translation, but rather are the result of a more dynamic interaction between the redox environment and skeletal muscle physiology (**Figure 1**), most likely through reversible redox-dependent PTMs. In this review we will highlight key mitochondrial, myofibrillar, and excitation-contraction (EC) coupling proteins whose activities are modified in a redox dependent manner and discuss the challenges and recent developments in strategies to quantify redox PTMs.

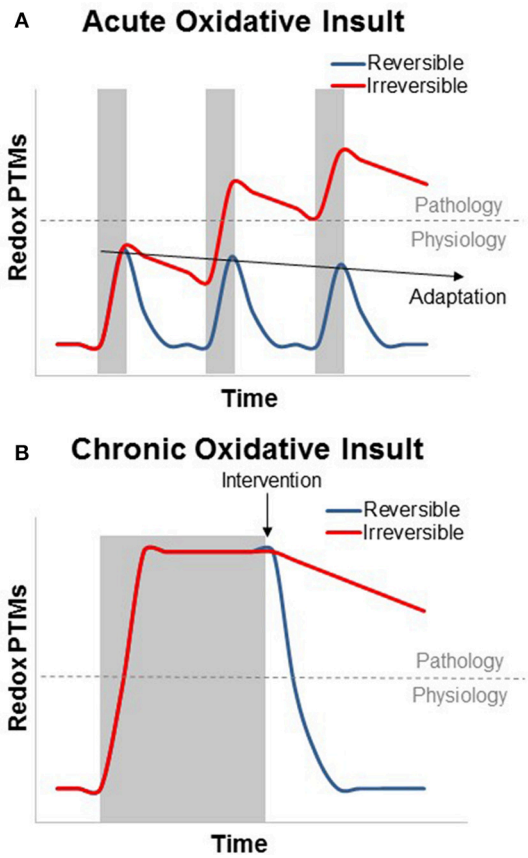

FIGURE 1 | Theoretical graph of the transient and additive nature of reversible and irreversible redox PTMs in the context of skeletal muscle physiology and disease. (A) Acute oxidative insults (shaded area) can cause a swift and transient increase in reversible oxidative PTMs. Irreversible PTMs rely on protein degradation and turnover, while most reversible modifications can be cleared by the GSH/GSSG and Trx(red)/Trx(ox) redox couples. Subsequent acute oxidative insults can be deleterious if the modifications become irreversible, but this is not the case for reversible modifications, which, can cause transcriptional activation of the antioxidant response (adaptation, preconditioning, or hormesis) to suppress PTM formation. **(B)** Chronic oxidative insults can result in an oxidized redox status which persists throughout the duration of the stress. As with aging, many metabolic and functional skeletal muscle defects that result from this oxidized redox status can be rapidly reversed by restoring a more reduced redox environment. In contrast, irreversible oxidative modifications, however, can persist after the end of the oxidative insult.

SOURCES OF ROS IN SKELETAL MUSCLE

Skeletal muscle produces ROS from both mitochondrial and non-mitochondrial sources. During muscle contractile activity there is an increase in muscle oxidant production. Mitochondria are frequently cited as the primary source for oxidant production in skeletal muscle. However, growing evidence supports a primary role for non-mitochondrial oxidant by membrane bound NADPH oxidases and, to a lesser extent, extracellular xanthine oxidase production during muscle contraction (Gomez-Cabrera et al., 2005; Frasier et al., 2013; Wadley et al., 2013; Alleman et al., 2014; Sakellariou et al., 2014). In fact, recent work demonstrates that under conditions of increased mitochondrial respiration, as in working muscle, mitochondrial superoxide generation is decreased compared to resting conditions (Anderson and Neufer, 2006; Goncalves et al., 2015). NAD(P)H oxidase enzyme complexes are found in the sarcoplasmic reticulum (SR), the transverse tubules, and plasma membrane. Superoxide produced by SR NAD(P)H oxidases has been implicated in regulation of SR Ca^{2+} dynamics, while plasma membrane NAD(P)H oxidases, along with xanthine oxidase are the main contributors of contraction induced extracellular superoxide production as reviewed by Powers and Jackson (2008). This transient and cyclical contraction induced ROS plays an important role in regulating muscle contraction and inducing adaptive responses to exercise in skeletal (McArdle et al., 2004; Ristow et al., 2009; Powers et al., 2010) and cardiac muscle (Frasier et al., 2013).

In contrast, elevation of superoxide production associated with chronic degenerative disease appears to be primarily mitochondrial in origin. Mitochondrial superoxide or hydrogen peroxide (H_2O_2) production is elevated in aging skeletal muscle (Siegel et al., 2013), neurodegenerative disease (Manczak et al.,

2011), cardiac pathology (Adlam et al., 2005; Dai et al., 2011a), and following high fat diet. Reducing mitochondrial H_2O_2 production with either genetic or pharmacological interventions targeted to the mitochondria reverses or protects against mitochondrial deficits in these conditions (Dai et al., 2011b; Siegel et al., 2013). The mitochondria contain several sites of superoxide production associated with the electron transport system (ETS) and tricarboxylic acid cycle. Complex I of the ETS appears to be the dominant source of mitochondrial superoxide under most conditions with contributions from complex II and III increasing under conditions simulating more active muscle (Goncalves et al., 2015). Complex I and II produce superoxide in the matrix of the mitochondria, while complex III generates superoxide on both sides of the inner mitochondrial membrane (Goncalves et al., 2015). Multiple dehydrogenase enzymes, including pyruvate dehydrogenase and oxoglutarate dehydrogenase, also contribute to superoxide production in the matrix (Mailloux et al., 2014a). Superoxide produced by the mitochondria is rapidly converted to H_2O_2 by either MnSOD (matrix) or CuZnSOD (inner membrane space). H_2O_2 then interacts with the GSH-thioredoxin redox buffering system or is rapidly decomposed to water and oxygen by the enzyme catalase in the cytosol.

REDOX BUFFERING SYSTEM

Cellular redox status, as discussed in this review, is defined as the relationship of ROS producers and scavengers. A common method of determining the redox status of a cell is the reduced to oxidized glutathione ratio (GSH:GSSG). Increasing evidence suggests that redox regulation of cellular processes is the result of signaling rather than non-specific oxidation (Matés et al., 2008). H_2O_2, for example, interacts with the redox buffering system through the GSH/GSSG and thioredoxin (Trx_{red}/Trx_{ox}) redox couples that are present in both the cytosolic and mitochondrial matrix compartments. This redox system, for which NADPH/$NADP^+$ provides reducing power, transduces information on the redox status to the cell by interacting with the proteome through the thiol group on cysteine (Cys) residues. Of the greater than 200,000 Cys residues present in the proteome over 20,000 are thought to be redox sensitive, meaning that they can undergo reversible redox modification as described above (Jones and Go, 2011). The susceptibility of the thiol groups on Cys residues, referred to as redox switches, to oxidative modification varies according to their local biochemical environment. The deprotonated thiol, the thiolate anion, is much more susceptible to oxidation than the thiol group. The pKa of thiols is high (>8); however, its proximity to positively charged amino acids in proteins, arginine, and lysine, can function to decrease their pKa values and make them more reactive at physiological pH (Winterbourn and Hampton, 2008). Redox PTMs of thiols are classified as reversible or irreversible, largely depending on the degree of oxidation. The oxidation states of a low pKa thiol (−2), for example, can be readily oxidized to a reversible sulfenic acid (0) by a 2 electron oxidation with H_2O_2, and subsequently to sulfinic (+2) and sulfonic acid (+4) which are considered irreversible (**Figure 2**). These redox PTMs can modify protein function by altering protein structure or modifying interactions with other proteins, ligands, or DNA (Forman et al., 2014).

THIOL-BASED REDOX PTMS

In addition to S-sulfenylation, S-nitrosylation (SNO) and S-glutathionylation (SSG) are two of the most studied reversible redox PTMs (**Figure 2**). These modifications have been well-recognized as a fundamental mechanism of redox signaling by modulating enzyme activities and protein functions in a variety of cellular activities, such as signaling, metabolism, gene expression, and apoptosis (Janssen-Heininger et al., 2008; Winterbourn and Hampton, 2008; Brandes et al., 2009; Antelmann and Helmann, 2011). The types or levels of PTMs depend on the presence of ROS and RNS, and the redox buffering system, and the enzymatic reduction systems. Collectively, these modifications represent a continuum that relates the form of modification to the extent of ROS/RNS damage and functional consequence. However, our

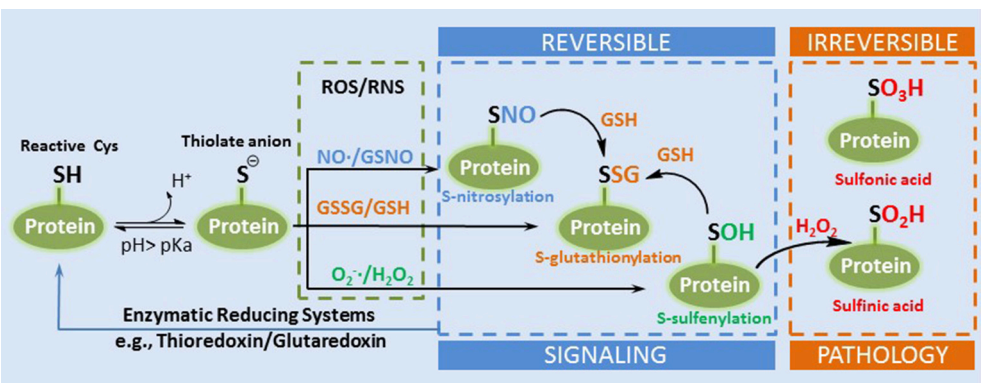

FIGURE 2 | A simplified diagram of the formation of thiol redox PTMs. A reactive Cys can exist as thiolate anion under physiological pH. The thioate anion is reactive with different ROS or RNS to form several types of reversible redox PTMs such as SNO, SSG, and SOH. SOH can be further oxidized into irreversible sulfinic and sulfonic acid (Adapted from Filomeni et al. Cell Death and Differentiation 2005, Filomeni et al., 2005).

current knowledge of redox PTMs and their significance is still relatively limited due to the technical challenges associated with the measurement of these labile modifications.

APPROACHES FOR MEASURING REVERSIBLE REDOX PTMS

To measure endogenous redox PTMs, one of the major challenges is how to preserve the modifications during sample storage and prevent artificial oxidation during sample preparation. There are two general processes of introducing artifacts: (1) thiol oxidation by air through sample processing; (2) reduction of PTMs by antioxidant enzymes during sample storage or processing (Held and Gibson, 2012). Therefore, special care and sample processing protocols are required in order to accurately measure *in vivo* redox PTMs. To minimize oxidation artifacts during cell or tissue processing, two techniques are commonly applied. These are trichloroacetic acid (TCA) quenching and cell lysis directly with a relative high concentration of alkylation reagents, such as N-ethylmaleimide (NEM) and iodoacetamide (IAA), to block free thiols (Leichert and Jakob, 2006). TCA quenching is a fast process, which can cause precipitation and denaturation of proteins, thus suppressing reactivity of thiolate anions by lowering the pH below their pKa. In contrast, alkylation with NEM and IAM is applied to covalently block free thiols quickly, thus preserve the redox PTMs for downstream processing and measurements. NEM reacts with thiols via a faster and more specific Michael addition reaction than the nucleophilic substitution reaction with IAM. NEM is also less pH dependent compared to IAM, which makes NEM often the preferred blocking reagents for measuring redox PTMs. For measuring redox PTMs in tissues such as muscle, a detailed optimization of tissue handling and processing is particularly important to make sure that the endogenous PTMs are well-preserved during this process.

Approaches for measuring redox PTMs include both indirect and direct detection methods. Given the lack of direct detection approaches of redox PTMs, the indirect approaches are still a popular choice. **Figure 3** illustrates the general chemistry principles of most indirect and direct measurement approaches. Basically, the free thiols are initially blocked by alkylation with NEM in a denaturing buffer during tissue homogenization or cell lysis. For indirect methods (**Figure 3A**), the different types of redox PTMs are selectively reduced to free thiols by using individual sets of reagents where ascorbate is commonly used to reduce SNO (Derakhshan et al., 2007; Su et al., 2013); a glutaredoxin (Grx) reduction cocktail for SSG (Shelton et al., 2005; Zhang et al., 2011), and DTT for total reversible oxidation (Leichert et al., 2008; Paulech et al., 2013). Then, the pre-processed protein samples containing free thiols can be subjected to different enrichment strategies and to either gel-based or mass spectrometry (MS)-based approaches for profiling redox PTM. It should be noted that the selective reduction strategies used in most indirect detection methods are not perfect in terms of specificity. For example, besides reducing SNO, ascorbate is also reported to reduce disulfides in a certain degree (Kuncewicz et al., 2003; Dahm et al., 2006). Similarly, even with the use of mutated form of Grx to reduce its chance for disulfide reduction, non-specific reduction of the Grx enzyme cocktail was still observed (Su et al., 2014). In this regard, the inclusion of proper negative or positive controls when applying these indirect methods is important for more confident identifications of endogenous redox modifications (Guo et al., 2014a).

The concept of indirect detection of redox PTMs was first reported as the biotin-switch technique (BST) for S-nitrosylated proteins (Jaffrey and Snyder, 2001; Jaffrey et al., 2001). This approach was also modified to detect other redox PTMs including SSG (Lind et al., 2002; Reynaert et al., 2006) and total reversible thiol oxidation (Leichert and Jakob, 2004). In this approach, the reduced thiols were labeled with reagents containing a biotin tag and labeled proteins were enriched by avidin affinity purification for biochemical (e.g., Western blotting) analyses. For example, redox differential in gel electrophoresis (redox DIGE) allows the comparison of the levels of oxidation between two samples on the same gel by using iodoacetamido or maleimide-based cyanine fluorophores (Leichert and Jakob, 2004; Hurd et al., 2007).

With the advances of MS-based proteomics a number of liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics approaches have been developed for identifying and quantifying redox PTMs at the proteome level. For example, in BST the biotinylated peptides (formerly SNO-modified) were enriched by avidin chromatography and the biotin tags were removed to allow the detection of SNO modified Cys sites (Hao et al., 2006). Similarly, the OxICAT approach was developed to measure the levels of total reversible thiol oxidation by labeling the reduced and oxidized thiols with two versions of alkylating ICAT (isotope-coded affinity tags) reagents (Leichert et al., 2008). The differentially labeled thiols with ICAT are purified by biotin-avidin purification and followed by LC-MS/MS to quantify the levels of thiol oxidation on Cys residues based on the light and heavy-labeled peptide intensity ratios. Although the BST or biotin-based approaches are still popular, these approaches generally suffer from the issue of non-specific binding during enrichment. Alternatively, our laboratory developed a resin-assisted capture (RAC) approach (originally called quantitative cysteinyl-peptide enrichment technology, Liu et al., 2004, 2005) for high-efficiency enrichment of cysteine-containing peptides using Thiopropyl Sepharose 6B thiol-affinity resin. More recently, we and others have demonstrated the effectiveness of enriching several types of redox PTMs including SNO, SSG, and total oxidation using this approach (Forrester et al., 2009, 2011; Liu et al., 2010; Paulech et al., 2013; Su et al., 2013, 2014; Guo et al., 2014a,b). Compared to the biotin-based approaches, the resin-assisted procedure provides a simpler workflow, a very high enrichment specificity (>95% of peptides as Cys-peptides), better sensitivity than biotin avidin-based enrichment as demonstrated by a side-by-side comparison (Forrester et al., 2009). The RAC also provides the flexibility for enabling multiplex quantification by allowing on-resin digestion and isobaric labeling. For example, 4-plex (iTRAQ, isobaric tags for relative and absolute quantification), 6-plex and 10-plex TMT

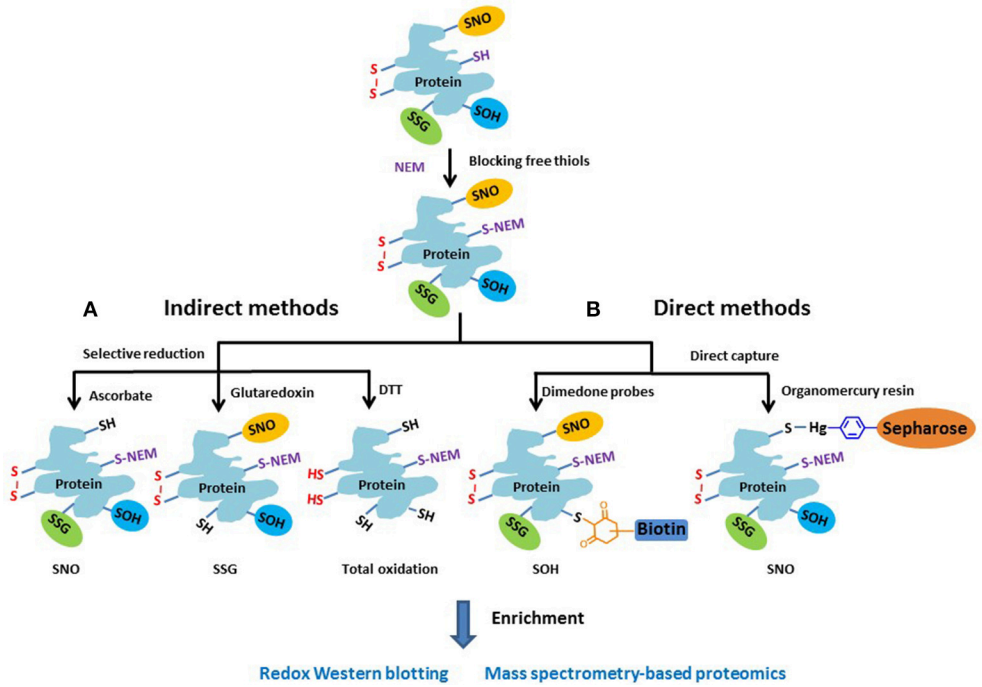

FIGURE 3 | Schematic of the general approaches for measuring different reversible thiol-based redox PTMs. The free thiols are typically blocked by alkylation with NEM as the initial step. **(A)** In the indirect methods, the different types of modified cysteines are selectively reduced to free thiols by using individual sets of reagents. **(B)** In the direct methods, dimendone probe is often used to react with SOH, and organomercury resin for SNO. The reduced or labeled protein samples are then subjected to enrichment and measurement by either gel-based or MS-based proteomic approaches.

(tandem mass tags) can be applied to enable MS-based site-specific identification and quantification of redox PTMs across 4–10 biological conditions (Forrester et al., 2009; Su et al., 2013, 2014; Guo et al., 2014a,b). More recently, a new isobaric reagent iodoTMT that contains an iodoacetyl reactive group was developed to directly label free thiols to enable multiplex quantification of multiple redox PTMs (Pan et al., 2014; Qu et al., 2014). In this approach, the labeled peptides are enriched by an anti-TMT resin. Both the resin-assisted and iodoTMT approach offer the flexibility of multiplexed quantification; however, the resin-assisted approach should provide higher enrichment specificity due to the covalent capture process compared to the non-covalent immunoaffinity enrichment.

Besides the popular indirect methods, several direct measurement strategies (**Figure 3B**) have been developed for redox PTMs. For example, dimedone-based probes have been shown to specifically label SOH (Charles et al., 2007; Leonard et al., 2009). By incorporating a clickable dimedone probe with a UV-cleavable biotin, MS-based global profiling of SOH modified Cys sites was recently demonstrated (Yang et al., 2014, 2015). For SNO, organomercury resin has recently been applied for direct and site-specific identification of SNO-containing peptides by first blocking the free thiols with MMTS and capturing SNO directly by organomercury resin (Doulias et al., 2010, 2013; Raju et al., 2015). Compared to indirect methods, the direct measurement strategies are less prone to potential artifacts for redox PTMs; however, to date the indirect approaches such as RAC or iodoTMT still provide the most flexibility in multiplexed quantification over many biological samples.

Besides these modifications shown on **Figure 3**, protein S-sulfhydration (SSH) has recently been reported as a novel thiol-based PTM similarly to SNO (Paul and Snyder, 2012). A modified version of the BST method was applied to detect proteins modified by S-sulfhydration (Mustafa et al., 2009); however, the blocking reagent MMTS (methyl methanethiosulfonate) was shown to react with both free thiols and persulfides (Pan and Carroll, 2013). More recently, a tag-switch assay was reported for more specific SSH detection (Zhang et al., 2014). Further, studies are necessary to validate the specificity and effectiveness of these approaches for SSH modification.

In the study of redox PTMs, it is also important to be able to be quantify the stoichiometry of redox PTMs (i.e., ratios oxidized vs. reduced thiols) in order to better identify functionally important Cys sites. To date, such measurements were only demonstrated by a few approaches such as OxICAT (Leichert et al., 2008; Knoefler et al., 2012) and the RAC approach coupled with TMT (Guo et al., 2014b) in different biological systems. This would be an important area of further development. Furthermore, targeted quantification of redox PTMs by MS is also an interesting development in its ability to perform precise multiplexed quantification of many site-specific PTMs. The proof-of-concept was demonstrated by coupling differential alkylation of free/oxidized thiols using NEM and d5-NEM and multiple reaction monitoring (MRM) to quantify site-specific

Cys oxidation status of an endogenous protein p53 (Held et al., 2010). The targeted quantification strategy should have a good potential to complement Western blotting to verify redox PTMs in specific proteins.

CONTROL OF PROTEIN STRUCTURE AND FUNCTION BY REDOX PTMS

Changes in protein structure due to an oxidative environment, can affect the localization, proteasome clearance, and the function or activity of a protein depending on the site, type, and extent of the oxidative modification. The formation of a disulfide bond, for example, can affect the proteins tertiary structure, making it a target for proteases. Many proteases have evolved to target oxidatively modified proteins, thus turning over many of the dysfunctional or structurally abnormal proteins (Jung et al., 2013). Lon protease, for example, has been shown to preferentially target oxidized mitochondrial aconitase in skeletal muscle (Bota et al., 2002). However, heavily oxidized proteins, like aconitase, can form aggregates and become inaccessible to the proteasome (Bota and Davies, 2002). In muscle, creatine kinase has been shown to be oxidative modified in aged mice, resulting in protein aggregates and the loss of enzyme activity (Nuss et al., 2009). Oxidative and other PTMs can have a direct effect on protein function independent of structural changes. Actin, for example, has many reported PTMs that affect its polymerization, organization, and stability (Chung et al., 2013; Terman and Kashina, 2013). These PTMs can form in an enzyme active site with similar functional consequences as will be discussed in this review.

A less recognized role of oxidative PTMs is their ability to regulate the formation of other PTMs associated with normal physiological signaling. This occurs primarily by oxidative modification to a kinase or phosphatase, thus regulating its ability to change the phosphorylation status of a protein. Increasing evidence points to the significance of cross-talk between redox and phosphorylation based signaling systems (Duhé et al., 1998; Filomeni et al., 2005; Chiarugi and Buricchi, 2007; Kemble and Sun, 2009). Indeed, oxidative modifications to the cysteine of protein tyrosine phosphatases has been shown to suppress their activity and lead to the increase in total phosphorylation (Rhee et al., 2000). Intracellular redox circuitry is regarded as a master regulator of phosphorylation and de-phosphorylation because nearly all classes of protein phosphatases and many kinases contain redox sensitive Cys residues for regulating their activities (Chiarugi, 2005; Fisher-Wellman and Neufer, 2012). In general, a shift in cellular redox state such as in aging (Sohal and Orr, 2012) would impact the activities of many phosphatases and stress-sensitive Ser/Thr kinases (e.g., JNK/ASK1, IKKβ, NF-κB), many of which are implicated in insulin resistance (Boura-Halfon and Zick, 2009). Indeed, redox modulation of phosphatase activity and phosphorylation in intact skeletal muscle has been reported (Wright et al., 2009). Moreover, the redox regulation of JNK activation and inactivation of MAP kinase phosphatase (MKP-1) has been reported in cellular aging (Dasgupta et al., 2010).

The changes in protein function and their ability to interact with other proteins, macromolecules, and DNA, due to oxidative PTMs is broadly termed redox signaling. However, this is distinguishable from oxidative stress by the degree of oxidative insult, the irreversibility (damage) associated with the PTM, and the specificity of target (Schieber and Chandel, 2014). Aging is an example of a progressive increase in oxidative stress in which physiological redox signaling is disrupted. A redox proteomic analysis has recently demonstrated that the cysteine thiol proteome in aged mouse skeletal muscle is significantly more oxidized than in young adult mice (McDonagh et al., 2014). An important insight from this study was that the number of cysteines that were either reversibly oxidized or available for redox signaling, defined as being partially oxidized, decreased in the aged muscles suggesting reduced flexibility of the redox signaling system in aged muscle. Many mitochondrial and myofibrillar proteins were among the proteins with redox sensitive thiols detected in this study (McDonagh et al., 2014). Below we discuss evidence for the modulation of activity for many of these mitochondrial and myofibrillar proteins by redox PTMs with an emphasis on SSG modification (**Figure 4**).

REDOX PTMS AND MITOCHONDRIAL FUNCTION

Mitochondria are attractive targets for redox control. As discussed above, the mitochondria are an important source of superoxide and H_2O_2 production in the cell. Therefore, modulation of cell energetics and function by direct control over mitochondrial function by redox PTMs would co-localize the source and targets of oxidant production and allow rapid cellular response to changes in redox status. Mitochondria also contain high concentrations of glutathione and an active thioredoxin (Trx)/GSH redox buffering system that would facilitate translation of oxidative stress to redox PTMs. Finally, many mitochondrial proteins, such as 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase, Complex I, and glutaredoxin isoforms 1 and 2, have conserved cysteine residues associated with dehydrogenase or redox transfer reactions (Mailloux et al., 2014a). Dynamic interaction between mitochondrial respiration and redox biology has been demonstrated in isolated mitochondria, muscle cells, and *in vivo*. Electron flux through the ETS plays an important role in regulating mitochondrial redox status due to the important role of electron flux in controlling NADH/NAD and NADPH/NADP and thus reducing power. An oxidized GSH/GSSG ratio was observed in isolated brain mitochondria under low mitochondrial respiration (rotenone, antimycin A, or no ADP) compared to state 3 respiration (ADP and substrates), however, the GSH/GSSG ratio of freshly isolated mitochondria in the absence of respiratory substrates was more oxidized than either condition (Garcia et al., 2010). The more oxidized GSH/GSSG state was associated with increased protein SSG of several mitochondrial proteins. In a separate study, increases in SSG of mitochondrial proteins reduced basal mitochondrial ATP-linked oxygen consumption as well as mitochondrial

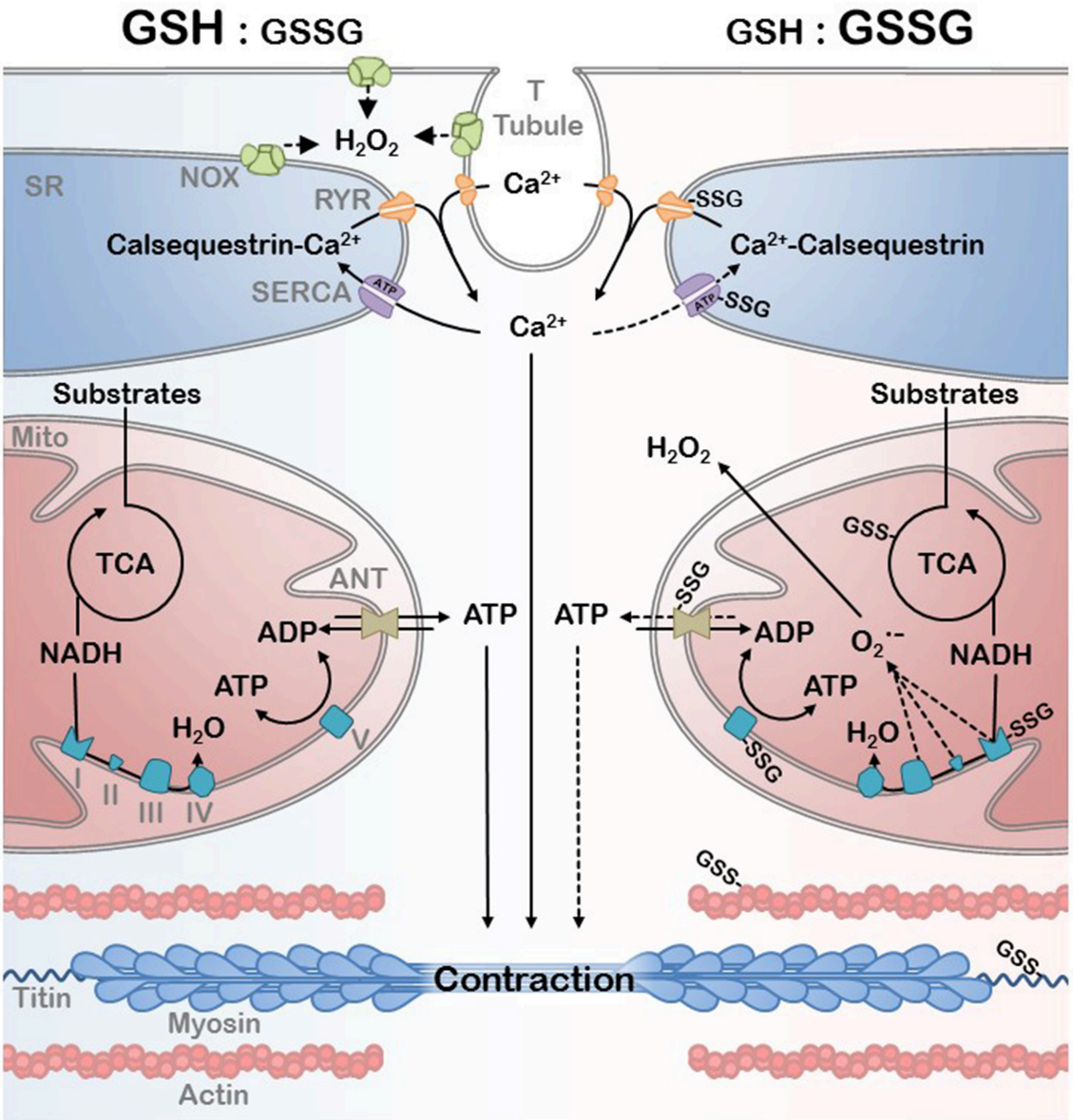

FIGURE 4 | EC coupling and bioenergetics network in a reduced (high GSH:GSSG ratio) and oxidative (low GSH:GSSG ratio) redox environment. Ca^{2+} handling by the SR and the ATP generated by the mitochondria are the primary substrates of myofibril contraction. Oxidative PTM can affect the mitochondrial efficiency as well as EC coupling during redox signaling and the oxidative stress associated with disease. Protein S-Glutathioylation of critical mitochondrial, SR, and myofibrillar proteins are depicted as well as the primary sources of H_2O_2.

reserve capacity in a dose-dependent and reversible manner in smooth muscle cells (Hill et al., 2010). This dynamic interaction between redox status and mitochondrial function also translates to *in vivo* energetics in skeletal muscle. Treatment with paraquat inhibited *in vivo* resting mitochondrial ATPase rate and maximal ATP production within 24 h in aged mice in the absence of changes in mitochondrial protein expression or accumulation of 4-Hydroxynonenal as a marker of oxidative damage (Siegel et al., 2012). Support for an important role of redox regulation of energetics in skeletal muscle pathology also comes from the reversal of mitochondrial deficits in aging muscle following treatment with the mitochondrial targeted peptide SS-31 to reduce mitochondrial oxidant production. One hour after treatment SS-31 increased mitochondrial coupling ratio (P/O) and maximal ATP production (Siegel et al., 2013). This was associated with a more reduced GSH/GSSG ratio in the treated aged muscles. The absence of an observed effect of this treatment in the young muscles or in permeabilized muscle fibers from the treated aged mice suggests that the inhibition of function that was reversed by SS-31 was dependent the interaction between

the more oxidized cell environment in the aged muscle and the mitochondria.

Protein SSG of an oxidized cysteine (sulfenic acid), which can be reversed by glutaredoxin, prevents the irreversible oxidation to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid. In the case of complex I, irreversible oxidation results in loss of enzyme activity (Hurd et al., 2008). Thus, reversible PTMs can serve as a protective modification during transient oxidative stress. The prevention of further oxidation by SSG was suggested as a mechanism to explain the protective effect of elevated Grx2 expression on doxorubicin-induced cardiotoxicity (Diotte et al., 2009). This study was unique in demonstrating increased SSG of mitochondrial proteins with overexpression of Grx2 under both normal and doxorubicin exposed conditions (Diotte et al., 2009). In contrast, other studies have demonstrated that reduced expression of Grx2 resulted in increased SSG of complex I in the human eye (Liu et al., 2015) and mouse heart (Mailloux et al., 2014b).

In addition to protecting against further oxidation, SSG can also modify protein function. Complex I is one of the most well-studied mitochondrial proteins in terms of redox regulation of activity. The attention given to complex I is likely due in part to its position as the entry point for electrons into the ETS from NADH and its role as a major source of ETS derived superoxide. Therefore, it plays a key role in regulating mitochondrial function and is the complex most commonly implicated in mitochondrial diseases associated with genetic, idiopathic, and lifestyle. Of the 130 cysteine residues contained in the 45 subunits of complex I, 30 were sensitive to reversible oxidation following diamide treatment in mitochondria isolated from brain (Danielson et al., 2011). Six of these residues in brain mitochondria were sensitive to reversible oxidation *in vivo* following depletion of GSH with buthionine sulphoximine (BSO). Two of these residues, cys-531 and 704 on the Ndufs1 subunit were also found to be oxidized in oxidatively stressed bovine hearts (Hurd et al., 2008).

Increased SSG of complex I in heart mitochondria by diamide treatment (Hurd et al., 2008) or by knocking out Grx2 (Mailloux et al., 2014b) leads to reversible inhibition of complex I activity. However, other groups report that the inhibition of complex I activity by SSG was only partially reversible when oxidized under severe oxidizing conditions, suggesting that the SSG may have initiated structural modifications or protein interactions of complex I that were less reversible than the PTM itself (Taylor et al., 2003). Despite the inhibition of complex I NADH reducing activity, SSG of complex I results in elevated superoxide production which is converted to H_2O_2 in the intact mitochondria (Taylor et al., 2003). The elevation of superoxide production occurs much more rapidly than inhibition of NADH reducing activity and was acutely reversible with exposure to a reducing agent suggesting that these two effects of SSG on complex I function have different biochemical mechanisms. These functional effects suggest a model whereby an initial disturbance in redox balance could initiate SSG of complex I leading to further oxidation of the mitochondrial environment and inhibition of mitochondrial ATP production, as well as other targets discussed below.

In addition to complex I, the activities of several other proteins involved in oxidative phosphorylation are modified by reversible redox PTMs. F1FO ATPase is another target of redox PTM in the mitochondria. In heart mitochondria disulfide formation and SSG are elevated at Cys 294 on the alpha subunit of the F1 complex, while S-nitrosylation is reduced, in dyssynchronous heart failure (Wang et al., 2011). Cardiac resynchronization therapy that improves cardiac function also reverses the redox PTM at this site. Working with brain and liver mitochondria, Garcia et al demonstrated F1FO ATPase was S-glutathionylated in response to changing mitochondrial GSH/GSSG ratios induced by altering the respiratory state of the mitochondria (Garcia et al., 2010). The increased SSG associated with low respiratory flux reversibly inhibited ATPase activity (Garcia et al., 2010). The supply of reducing equivalents to the respiratory chain can also be modified by redox PTMs. Succinyl CoA transferase (Garcia et al., 2010) and alpha-ketoglutarate dehydrogenase (Applegate et al., 2008) are also reversibly inhibited by SSG in brain and heart mitochondria, respectively. Alpha ketoglutarate dehydrogenase is an NADH generating step in the Kreb's cycle thus inhibition of this enzyme and complex I would reduce mitochondrial capacity by both directly inhibiting the supply of NADH and the entry of NADH into the ETS.

SSG may also exert control over bioenergetics by controlling the coupling of electron transport to ATP generation through the membrane potential. A high potential across the inner mitochondrial membrane under low flux or reductive stress leads to elevated mitochondrial superoxide production (St-Pierre et al., 2002; Nicholls, 2004). Uncoupling proteins 2 and 3 (UCP2 and UCP3) serve as part of the antioxidant defense system by facilitating proton leak across the inner mitochondrial membrane and dissipating the membrane potential to reduce mitochondrial superoxide production (Echtay et al., 2002; Echtay and Brand, 2007). It has been known for several years that oxidative stress induces increased proton leak through UCP3 (Echtay et al., 2002; Echtay and Brand, 2007). Recently Harper and colleagues demonstrated that this proton leak is under the control of the SSG state of Cys 25 and Cys 259 (Mailloux et al., 2011). In contrast to other examples of redox PTM of mitochondrial proteins, where increased SSG is associated with an oxidized GSH/GSSG status, UCP3 appears to be deglutathionylated directly by GRx1 under high oxidative stress conditions (Mailloux et al., 2011). Deglutathionylation of UCP3 led to an increase in state 4 (non-phosphorylating respiration) in mitochondria isolated from skeletal muscle, while treatment with diamide to increase SSG had the opposite effect. Glutathionylation state had a similar effect on the control of UCP2-mediated proton leak in mitochondria from mouse kidney and thymocytes (Mailloux et al., 2011).

Other proton leak pathways may also be regulated by reversible PTMs. The adenine nucleotide transporter (ANT) also plays an important role in regulating mitochondrial ROS production through control of the membrane potential (Brand et al., 2005). There is some evidence that the ANT activity is also affected by SSG (Queiroga et al., 2010), although the evidence for this is not as strong as for other mitochondrial proteins.

Modulation of ANT function by redox PTM would provide another route by which the redox status could feedback and control both mitochondrial redox state and energy metabolism of the mitochondria. Thus, SSG of proteins involved in oxidative phosphorylation may represent a dynamic reversible response of the mitochondria to minimize the production of superoxide by the mitochondria or irreversible damage under periods of elevated oxidative stress, such as conditions of low respiratory flux, high reductive stress (high fat feeding), or intermittent exercise (Anderson et al., 2007, 2009). As noted above, SSG can provide a defense against excess mitochondrial superoxide production by modifying the supply of reducing equivalents to the ETS, electron flux through the ETS, and the membrane potential of the mitochondria. However, under conditions of sustained oxidative stress such as chronic disease or aging, these same responses may contribute to mitochondrial energetic deficits and contribute to tissue dysfunction. This suggests that directly altering the redox environment of the mitochondria may be an effective strategy to rapidly improve muscle mitochondrial energetics in the context of chronic disease (Marcinek and Siegel, 2013; Siegel et al., 2013).

It has also been suggested that mitochondrial dynamics, at the level of mitochondrial ultrastructure, are controlled by redox signaling within a cell, which has a direct impact on mitochondrial function (Willems et al., 2015). In C2C12 myocytes, the change in mitochondrial morphology associated with H_2O_2 and possibly exercise, is thought to be due to mitochondrial depolarization (Fan et al., 2010), however, increasing evidence of oxidative modifications of mitochondrial fission and fusion proteins (Drp1, Mfn1, Mfn2, and OPA1) suggests redox PTMs may be responsible for regulating mitochondrial dynamics in oxidative environments (Willems et al., 2015). In elderly subjects with a markedly low muscle function, OPA1 protein, mitochondrial respiration and cytochrome c oxidase activity, as well as PGC-1α and Sirt3 protein content were decreased compared to young subjects, suggesting that muscle dysfunction with aging is significantly correlated with changes in mitochondrial function, dynamics, and biogenesis (Joseph et al., 2012).

REDOX PTMS AND MUSCLE FORCE GENERATION

The important interaction between ROS, redox status, and muscle force production and fatigue is well-established. Depletion of basal levels of oxidants in resting muscle reduces maximal force, while small elevation of ROS can enhance muscle force production (Reid et al., 1993; Andrade et al., 1998). Several reports have also shown that ROS contribute to muscle fatigue, especially during repetitive submaximal contractions (Reid et al., 1994; Matuszczak et al., 2005). Experimental evidence suggests that ROS may exert their greatest effect on muscle contraction through altering E-C coupling or Ca^{2+} sensitivity of the myofibril (Moopanar and Allen, 2005, 2006), although recent evidence suggests a role for redox PTMs in muscle stiffness (Alegre-Cebollada et al., 2014). Despite the well-established role for redox modulation of muscle contraction, the specific molecular targets for redox modification are less well-defined. Below we discuss recent evidence for effect of redox PTMs on activity of specific E-C coupling and myofibrillar proteins.

Redox modifications of some components of the myofibril appear to be dependent on the contractile state of the protein. This has been hypothesized to be due to structural changes in the proteins that either bury or expose cysteine residues to the cytosolic environment (Gross and Lehman, 2013; Alegre-Cebollada et al., 2014). When cardiac myofibrils were exposed to cysteine oxidizing reagents under rigor, basal myofibrillar ATPase rates were significantly higher than controls, but there was no effect on maximal ATPase activity (Gross and Lehman, 2013). In contrast, myofibrils exposed under relaxing conditions have a significantly lower maximal ATPase activity with no effect on the basal rate. The reduced ATPase rate in the resting condition was associated with increased cysteine oxidation of the myosin heavy chain compared to control and rigor. These effects were similar for different oxidative modifications induced by NEM, SNAP, and DTDP suggesting that the type of modification to the MHC thiols may not be critical for the effect on ATPase activity. Redox modifications of titin also appear to be determined by changes in protein structure (Alegre-Cebollada et al., 2014). Titin is an approximately 3000 kD protein that stretches between the M-line and Z-line and anchors the thick filament of the myofibril to the z-line. It has a highly coiled structure at rest that is stretched during lengthening of striated muscle sarcomeres (Tan et al., 1993). This stretching provides stability and elasticity. Titin has multiple cysteine residues that are buried on the interior of the protein under relaxed conditions, but are exposed during stretching. Alegre-Cebollada et al found that stretching the protein made these cysteines susceptible to SSG which prevented its refolding and modulated the elasticity of cardiac sarcomeres (Alegre-Cebollada et al., 2014). Similarly, increased mechanical stability of titin caused by disulfide bond formation within the cardiac-specific N2-Bus region of the protein is believed to result in the passive stiffness in human heart myofibrils under oxidizing conditions (Grützner et al., 2009). This reduced elasticity of titin by reversible PTM may contribute to impaired relaxation and diastolic dysfunction under conditions of chronic oxidative stress such as aging.

Redox PTMs may affect muscle force production by modulating both Ca^{2+} dynamics and Ca^{2+} sensitivity of the myofiber. Redox proteomic analysis indicates that both alpha and beta isoforms of tropomyosin can undergo reversible redox modification as well as Troponin I, Myosin light chain 1/3, Myosin regulatory light chain 2, and Myosin-4(McDonagh et al., 2014). Following myocardial infarction or ischemia reperfusion in pig, mice, and rats, Cys190, the only cardiac tropomyosin cysteine, showed significant oxidation and was at least partly implicated in the resulting contractile dysfunction by a H_2O_2 dependent mechanism (Canton et al., 2004, 2006; Avner et al., 2012).

The effects of redox modulation on Ca^{2+} release from the SR by RyR and reuptake by SERCA may be the most well studied effects of redox PTMs on muscle function. Ca^{2+} reuptake into the SR by the SERCA proteins determines the rate of relaxation

of muscle and the ability to sustain repeated muscle contractions. In the mouse heart, SERCA Cys 674 glutathionylation resulted in increased activity; however, irreversible sulfonation resulted in decreased Ca^{2+} reuptake and relaxation and was prevented by catalase overexpression (Tong et al., 2010; Qin et al., 2013). Similarly, treatment of rat ventricular myocytes with H_2O_2 resulted in SR Ca^{2+} depletion due to SERCA oxidative inhibition and the oxidative activation of Na+/Ca^{2+}-Exchanger (Kuster et al., 2010).

Ca^{2+} release from the SR by RyR is also affected by the oxidation state of its cysteine residues. Stamler et al demonstrated that RyR has multiple cysteine residues that have different levels of susceptibility to oxidation (Sun et al., 2013). Under high pO2 conditions to induce an oxidative stress, cysteine residues in regions of the protein that interact with the L-Type Ca^{2+} channel form disulfide bonds, which may disrupt the interaction of the RyR with this protein. Interaction with the L-type Ca^{2+} channel is responsible for Ca^{2+} induced Ca^{2+} release and disruption of this interaction by thiol oxidation may inhibit full Ca^{2+} release from the SR. Marks and colleagues have also found disruption of the calstabin 1- RyR interaction by both reversible and irreversible PTMs in aged skeletal muscles (Umanskaya et al., 2014) and heart failure (Rullman et al., 2013). This oxidation was prevented by reducing mitochondrial-derived oxidative stress with mitochondrial-targeted catalase expression. Destabilization of the calstabin-1-RyR interaction led to increased open probability of the RyR and greater Ca^{2+} leak from the SR. This chronic leak reduced the Ca^{2+} released from the SR and therefore muscle force production upon muscle stimulation in aging muscle. The chronic Ca^{2+} leak induced by RyR oxidation also induces a feedforward cycle by increasing mitochondrial oxidative stress and muscle atrophy due to increased mitochondrial Ca^{2+} uptake. Both of these effects in aging muscle were reduced in aged mice overexpressing mitochondrial targeted catalase to reduce oxidative stress (Umanskaya et al., 2014).

CONCLUSIONS

Skeletal muscle physiology is determined by EC coupling, myofibrillar force production, and bioenergetics. In this review we have highlighted how each of these processes can be acutely controlled by changes in redox homeostasis through reversible redox sensitive PTMs. These redox PTMs provide a mechanism for cells to rapidly respond to redox changes to prevent irreversible oxidative damage, modulate protein activity or signal adaptive responses to stress. The GSH/GSSG and thioredoxin systems provide the biochemical link between the cellular redox environment and protein thiol groups. Thus, reversible thiol modifications provide a mechanism for cells to rapidly respond to redox changes, which during transient stress can serve important signaling or cell-protective roles by altering protein function. However, under conditions of chronic redox stress that create conditions, where these normally transient modifications persist, these same signaling or protective responses can inhibit cell function and lead to pathology. We have attempted to highlight some potential targets in the skeletal muscle mitochondria, E-C coupling and myofibrillar systems where persistent redox sensitive PTMs may contribute to muscle pathology in chronic diseases and aging. In the mitochondria it is becoming increasingly clear that redox PTMs exert control over both entries of electrons into the ETS as well as flux through the system, thereby dynamically influencing both mitochondrial energetics and oxidative stress. These changes to the mitochondria can then feedforward to influence redox PTMs on E-C coupling and myofibrillar proteins to influence Ca^{2+} sensitivity, relaxation, and force production (**Figure 4**). Thus, reversible redox PTMs provide a mechanism by which the mitochondria can influence muscle force production that is parallel to, and in some ways, independent of changes in ATP flux. Another important point is that because these changes rapidly respond to changes in the redox environment of the cell they may provide a mechanism to rapidly reverse functional deficits in muscle performance associated chronic disease. We are relatively early in our mechanistic understanding of the contribution of redox PTMs to skeletal muscle dysfunction in chronic disease. As interest continues to grow and technical challenges to the quantitative measurements of these changes *in vivo* are overcome we expect that this area will lead to new insights into the mechanisms of skeletal muscle dysfunction and identify exciting new directions for development of interventions to improve quality of life in this growing patient population.

AUTHOR CONTRIBUTIONS

All authors contributed to the design, writing, and editing of this review, with PK and DM writing the sections on redox PTMs and skeletal muscle physiology and disease, and WQ and JD writing the sections on methods and technical challenges. PK created **Figures 1**, **4**, and JD and WQ created/modified **Figures 2**, **3**.

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Hypothesis on Skeletal Muscle Aging: Mitochondrial Adenine Nucleotide Translocator Decreases Reactive Oxygen Species Production While Preserving Coupling Efficiency

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Mitochondrial membrane potential is the major regulator of mitochondrial functions, including coupling efficiency and production of reactive oxygen species (ROS). Both functions are crucial for cell bioenergetics. We previously presented evidences for a specific modulation of adenine nucleotide translocase (ANT) appearing during aging that results in a decrease in membrane potential - and therefore ROS production—but surprisingly increases coupling efficiency under conditions of low ATP turnover. Careful study of the bioenergetic parameters (oxidation and phosphorylation rates, membrane potential) of isolated mitochondria from skeletal muscles (gastrocnemius) of aged and young rats revealed a remodeling at the level of the phosphorylation system, in the absence of alteration of the inner mitochondrial membrane (uncoupling) or respiratory chain complexes regulation. We further observed a decrease in mitochondrial affinity for ADP in aged isolated mitochondria, and higher sensitivity of ANT to its specific inhibitor atractyloside. This age-induced modification of ANT results in an increase in the ADP concentration required to sustain the same ATP turnover as compared to young muscle, and therefore in a lower membrane potential under phosphorylating—*in vivo*—conditions. Thus, for equivalent ATP turnover (cellular ATP demand), coupling efficiency is even higher in aged muscle mitochondria, due to the down-regulation of inner membrane proton leak caused by the decrease in membrane potential. In the framework of the radical theory of aging, these modifications in ANT function may be the result of oxidative damage caused by intra mitochondrial ROS and may appear like a virtuous circle where ROS induce a mechanism that reduces their production, without causing uncoupling, and even leading in improved efficiency. Because of the importance of ROS as therapeutic targets, this new mechanism deserves further studies.

Keywords: adenosine nucleotide translocator, mitochondrial membrane potential, muscle energetics, oxygen free radicals, mitochondria, skeletal muscle aging

INTRODUCTION

Mitochondria, because of their central role in ATP supply through oxidative phosphorylation, but also in oxygen radical production and ion homeostasis, play a crucial role in skeletal muscle function. During last decades, a growing interest has been focused on the mechanisms linking dysfunctions of the mitochondrial oxidative phosphorylation and the aging process. One of the current leading hypothesis to explain the decrease in muscle energetics with aging is the mitochondrial theory of aging. The now accepted view hypothesizes that the reactive oxygen species (ROS) production linked to the activity of respiratory chain is responsible for the damages accumulated with aging to several components of the oxidative phosphorylation machinery and mitochondrial DNA (Dirks et al., 2006; Gonzalez-Freire et al., 2015) and triggers atrophy of the muscles (Dirks et al., 2006). These damages are mainly thought to result in mitochondrial dysfunction which disables the capacity of mitochondria to fulfill cellular ATP demand during contraction (Dirks et al., 2006; Bratic and Trifunovic, 2010). Therefore ROS production and related damages are considered responsible for loss of muscle mass and function during aging.

Indeed, assessed by *in vivo* $^{13}C/^{31}P$ NMR spectroscopy at rest, a decrease of mitochondrial oxidative phosphorylation activity was found in skeletal muscle of elderly (Conley et al., 2000; Petersen et al., 2003), explained by a decrease of mitochondrial mass and activity (Conley et al., 2000). However, other authors studying the oxidative capacity in tibialis anterior did not find any modification during aging (Kent-Braun and Ng, 2000). *In vivo* in human skeletal muscle during contraction of high intensity, no dysfunction of oxidative phosphorylation was evidenced, but only a reduced participation of glycolysis (Lanza et al., 2005). Conflicting results have also been obtained on isolated mitochondria from aged muscles. The decrease in the activity of different respiratory chain complexes has been measured in skeletal muscle of aged mice (Kwong and Sohal, 2000). Maximal oxidation rate was also found to decrease during aging in skeletal muscle of mice (Mansouri et al., 2006), rats (Kumaran et al., 2005), and from biopsies of human skeletal muscle (Tonkonogi et al., 2003), as well as ATP synthesis in skeletal muscle of rats (Drew et al., 2003) and humans (Short et al., 2005). *A contrario*, other authors demonstrated the absence of alteration of oxidative phosphorylation in mitochondria isolated from human muscles biopsies (Rasmussen et al., 2003), and several other studies did not evidence any alteration in the maximal oxygen consumption rate in rats with aging (Kerner et al., 2001; Chabi et al., 2008). The absence of consensus emerging from these results obtained at different levels of integration essentially demonstrate the complexity of the mechanisms involved in aging muscle, and of their consequences on mitochondrial functions and cell energetics (Lopez-Otin et al., 2013).

These considerations emphasize again the importance of integrative approaches for the understanding of pathologies in organ functions, a step forward from isolated enzymatic activities toward regulation of biological functions. We developed for several years the top-down elasticity analysis (Brown et al., 1990; Hafner et al., 1990) of muscle and heart energetics (Diolez et al., 2007, 2015; Arsac et al., 2008), after having applied this approach on isolated mitochondria, to allow better understanding of the very mechanisms at the origin of dysfunction occurring during hypothermic to normothermic reperfusion in rat liver (Leducq et al., 1998) and the description of the effect of temperature on oxidative phosphorylation (Dufour et al., 1996).

We previously investigated mitochondrial bioenergetics *in vivo* in the gastrocnemius muscle of aged (21 months) and young adult (6 months) rats with modular control analysis and ^{31}P magnetic resonance spectroscopy of energetic metabolites (Arsac et al., 2008). Because it gives real access to the integrated organ function, this approach brings out a new type of information—the "elasticities," referring to internal responses to metabolic changes—that may be a key to the understanding of the processes involved in pathologies. We revealed by this integrative approach that the *in vivo* "elasticity" (responsiveness) of oxidative phosphorylation to the increase in ATP demand induced by contraction is significantly reduced in skeletal muscle from aged rats, a dysfunction especially marked for low contractile activity (Gouspillou et al., 2014).

We further applied the Top-Down control analysis to isolated mitochondria (Brown et al., 1990; Hafner et al., 1990) to canvass the alterations in the regulation of oxidative phosphorylation that may occur during aging. Top-down control analysis is perfectly designed to evidence modification in regulation that have effective consequences on integrated organ function, since it is insensitive to any changes that have no significant functional outcome (Brand, 1998). We use this property to determine which module(s) may be responsible for the mitochondrial dysfunctions occurring during physiological (Dufour et al., 1996) or pathophysiological (Leducq et al., 1998) events. Therefore, this approach is useful to obtain a precise description of which of the changes occurring during aging have functional consequences for the regulation of oxidative phosphorylation.

In the present paper, we show how this integrative approach evidenced an alteration in the regulation of the mitochondrial adenine nucleotide translocator (ANT) with aging. The analysis of the flow-force relationships under variable phosphorylation rates (mimicking variable cellular ATP demand) revealed that for equivalent ATP demand the energetic intermediate of oxidative phosphorylation (mitochondrial membrane potential difference, $\Delta\psi$) is lower in aged mitochondria as compared to young ones. Decrease in mitochondrial membrane potential is known to reduce proton leak (Brand et al., 1994) and also ROS production (Echtay et al., 2003; Echtay and Brand, 2007; Toime and Brand, 2010). However, our results evidence that the decrease in $\Delta\psi$ is not due to uncoupling but interestingly to a decrease in sensitivity of ANT to ADP and appears therefore as a new mechanism for ROS production decrease induced by aging.

MATERIALS AND METHODS

Animals

Experiments were conducted on male Wistar rats aged 6 and 21 months. Rats were housed in an environmentally controlled

Abbreviations: GAS, gastrocnemius muscle; ANT, Adenine nucleotide translocator.

facility (12-h/12-h light/dark cycle, 22°C) and received water and food *ad libitum* until *in vitro* experiments were performed. All experiments were conducted in agreement with the National and European Research Council Guide for the care and use of laboratory animals. P. Diolez has a permanent license to conduct experiments on animals by the "Service Vétérinaire de la Santé et de la Protection Animale du Ministère de l'agriculture et de la Forêt" (03/17/1999, license number 3308010).

Isolation of Skeletal Muscle Mitochondria

Male Wistar rats were anesthetized by isoflurane inhalation and killed by intraperitoneal injection of pentobarbital (60 mg kg^{-1}). The GAS muscle of the right leg was then dissected and washed in the isolation medium containing 100 mM sucrose, 180 mM KCl, 50 mM Tris, 5 mM MgCl2, 10 mM EDTA, and 0.1% (w/v) BSA (pH 7.2). Before homogeinization, muscles were minced and exposed for 5 min to protease (2 mg mL^{-1} of isolation medium, Sigma P8038; Sigma-Aldrich, Saint- Quentin Fallavier, France). Mitochondria were extracted as previously described (Gouspillou et al., 2010). Mitochondrial protein concentration was determined by the Bradford method using BSA as standard (Bradford, 1976).

Experimental Determination of Mitochondrial Affinity for ADP

Oxidation rates were determined polarographically with a Clark electrode (Rank Brothers, Cambridge, UK). The use of a combined enzymatic system composed of glucose (5 mM), hexokinase (2.5 U mL^{-1} Sigma H4502), glucose-6-phosphatase dehydrogenase (2.5 U mL^{-1}, Sigma G6378), $NADP^+$ (1.6 mM) allowed the determination of phosphorylation rate since the NADPH production by this system is stoichiometrically linked to mitochondrial ATP synthesis rate. Changes in the rate of NADPH production were monitored at 340 nm by placing an optic fiber connected to a spectrophotometer in the oxygraphic vessel (Cary 50; Varian, Grenoble, France). Conversion of NADPH absorbance into NADPH concentration was performed using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. Bioluminescent assays were used to measure both ADP and ATP concentration during each experiment (Gouspillou et al., 2011). This method allowed the determination of the oxidation (KmVox) and phosphorylation (KmVp) rates affinities for ADP, as well as the determination of mitochondrial coupling efficiency as the ratio of phosphorylation over oxidation rates.

Simultaneous Monitoring of Oxidation Rate, Membrane Potential, and Phosphorylation Rate

Oxygen consumption, membrane potential, and ATP synthesis were monitored simultaneously (cf. **Figure 2**) in a glass vessel (final volume 3 ml) in a medium containing 140 mM sucrose, 100 mM KCl, 1 mM EGTA, 20 mM MgCl2, 10 mM KH2PO4, and 0.1% (wt./vol.) BSA (pH 7.2). Mitochondrial protein concentration used in the measurement vessel was approximately 0.3 mg ml^{-1} unless for the proton leak flux determination where the protein concentration used was approximately 0.6 mg ml^{-1}. Succinate (5 mM+rotenone 2 μg ml^{-1}) was used as a substrate for the monitoring of oxidative phosphorylation parameters (i.e., oxidation rate, membrane potential, and phosphorylation rate) and top-down control analysis experiments. To inhibit residual adenylate kinase activity, excess of P1,P5-Di(adenosine-5) pentaphosphate (AP5A, 20 μM) was added to the measurement medium in all experiments. State III oxidation rates were obtained by adding ADP (250 μM) and state IV oxidation rate was measured after complete ADP phosphorylation. Oxidation rates were determined polarographically with a Clark electrode (Rank Brothers) at 25°C. Concentration in air-equilibrated medium was taken as 240 μM (Dufour et al., 1996). Membrane potential was monitored using a homemade tetra-phenylphosphonium (TPP^+) electrode coupled to an Ag/AgCl- saturated reference electrode (Tacussel Mi402; Goubern et al., 1990; Dufour et al., 1996; Gouspillou et al., 2010). The phosphorylation rate was experimentally determined by continuously monitoring the pH variations of the measurement medium (Valerio et al., 1993).

Top-down Elasticity Analysis

We previously applied top-down elasticity control analysis (Hafner et al., 1990) to study alterations in mitochondrial oxidative phosphorylation in mitochondria (Dufour et al., 1996; Leducq et al., 1998). Oxidative phosphorylation of mitochondria from skeletal muscle was described as three large modules linked by a common thermodynamic intermediate (**Figure 1**). In this system, the substrate oxidation module (substrate translocases, dehydrogenases, and respiratory chain complexes) generates the proton-motive force (Δp), the common thermodynamic intermediate, which is consumed by the phosphorylation module (Pi translocator, ANT, and ATP synthase) to produce ATP and by the proton leak module (passive permeability of the mitochondrial inner membrane to protons and any cation cycling reactions; see Gouspillou et al., 2010 for details). The top-down control analysis lies on the experimental determination of the "elasticity" coefficients over Δp (approximated by the measurement of the membrane potential Δψ)—the response to changes in Δψ—of each module. As a consequence, the complete and accurate elasticity analysis of the regulation of skeletal muscle mitochondrial oxidative phosphorylation requires the experimental determination of the flux through each of the three modules and the measurement of the intermediate concentration (Δψ) under defined conditions. **Figure 2** presents a typical experiment of simultaneous determination of oxidation and phosphorylation rates with Δψ in isolated GAS muscle of an aged rat.

Elasticity coefficients of each module are calculated using modular kinetic analysis (Amo and Brand, 2007). This analysis consists in modifying the value of the intermediate (Δψ) by an adequate titration of a module that differs from the module under consideration. To cover the whole range of phosphorylation rates (from state IV to state III), these titrations were performed using succinate as a substrate at different concentrations of atractyloside in presence of an excess of

ADP. The elasticity of a module toward the intermediate $\Delta\psi$ at a given phosphorylation rate is the relative slope of the relationship between the activity of the module and $\Delta\psi$ obtained with the adequate titration. The elasticity depends on oxidative phosphoryation activity and for each module we obtain an elasticity curve when expressed as a function of the intermediate (see **Figure 4**). Any change in the regulation of the module under consideration will be evidenced by a change in the elasticity curve of this very module. The complete description of the experimental protocol used to determine the kinetic response of each module to membrane potential and the calculation of elasticity coefficients for each module has been described elsewhere (Dufour et al., 1996; Leducq et al., 1998; Gouspillou et al., 2010).

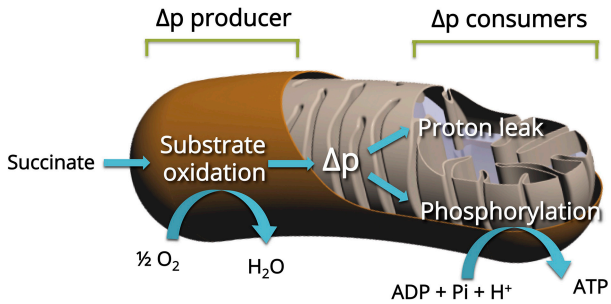

FIGURE 1 | Principle of the top-down elasticity analysis of the regulation of mitochondrial oxidative phosphorylation. The analysis considers the electrochemical H^+ gradient (Δp, estimated by his major component $\Delta\psi$) as the intermediate between respiratory chain (Producer) and two Consumers: Proton leak and ATP phosphorylation (see Material and Methods for details).

Statistics

Experimental between adult and aged rats were made using unpaired bilateral Student's *t*-tests. P values = 0.05 and 0.01 were considered significant.

RESULTS

The experiments performed on mitochondria isolated from aged and young rats did not evidence any difference in maximal membrane potential values (Gouspillou et al., 2010). Membrane potential values were not affected with aging, either under state III (maximal phosphorylation) or under state IV (no net phosphorylation) conditions. However, while the oxidation rate under non-phosphorylating conditions was identical between aged and young rats, the values of phosphorylating (State II) oxidation rate as well as the respiratory control ratio were significantly decreased in aged rats. Maximal phosphorylation rate was also decreased under state III conditions in aged rats (Gouspillou et al., 2010). Here, we only present the kinetic responses of the phosphorylation module to changes in membrane potential determined on mitochondria isolated from young and aged rat muscles (**Figure 3**). The main observation to be made here is represented by the dashed lines, which illustrate that for a given phosphorylation rate (equivalent to cellular ATP demand) the membrane potential value is always

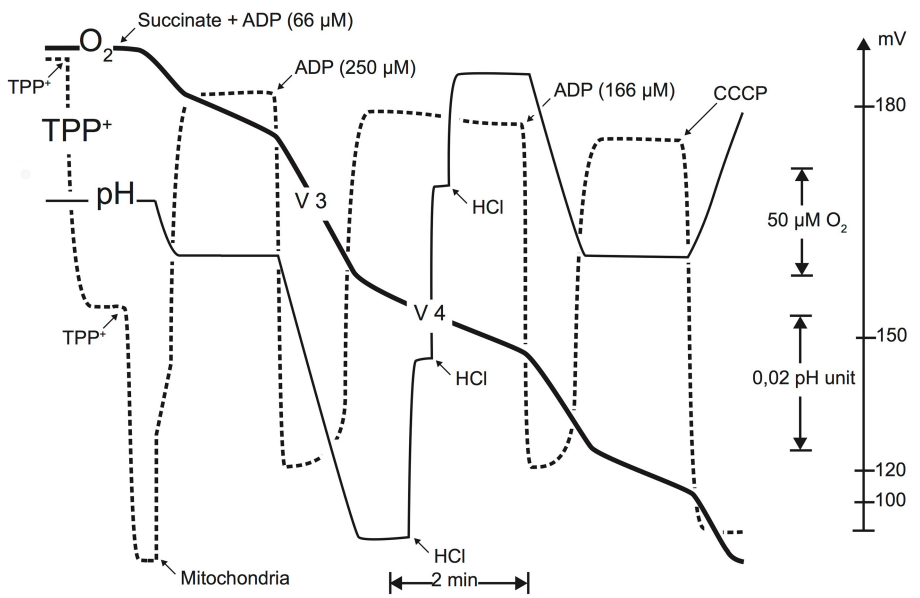

FIGURE 2 | Typical experimental simultaneous determination of oxidation rate, membrane potential (TTP^+-specific electrode), and phosphorylation rate (pH measurement). These recordings were performed using succinate as a substrate (5 mM). Membrane potential was monitored using a TPP+-sensitive electrode, allowing to follow the TPP+ concentration in the medium. This electrode was calibrated by two successive additions of 1.67 µM TPP+. At the end of each experiment, an uncoupler (1 µM CCCP) was added to obtain the membrane potential baseline. Phosphorylation rate was monitored using a pH electrode, calibrated using successive additions of a titrated HCl solution (0.1 mM) (cf. Materials and Methods Section for details; figure from Gouspillou et al., 2010).

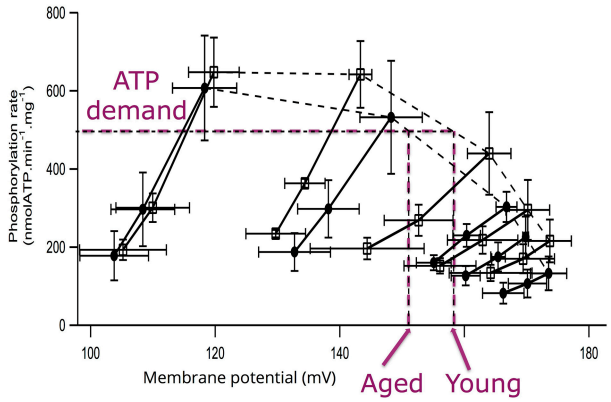

FIGURE 3 | Flow-force relationships obtained during modular kinetic analysis of mitochondrial oxidative phosphorylation. For each measurement, saturating ADP (1.7 mM) was present in excess and succinate was used as a substrate. The different phosphorylation rates were obtained by inhibition of the phosphorylation activity obtained by adding increasing atractyloside concentration for young (squares) and aged (circles) rats. Under each condition, respiratory chain activity was further progressively inhibited by increasing malonate concentrations. Purple dashed lines exemplify the differences in membrane potential values for young and aged rats corresponding to a given phosphorylation activity (ATP demand; Data and figure from Gouspillou et al., 2010).

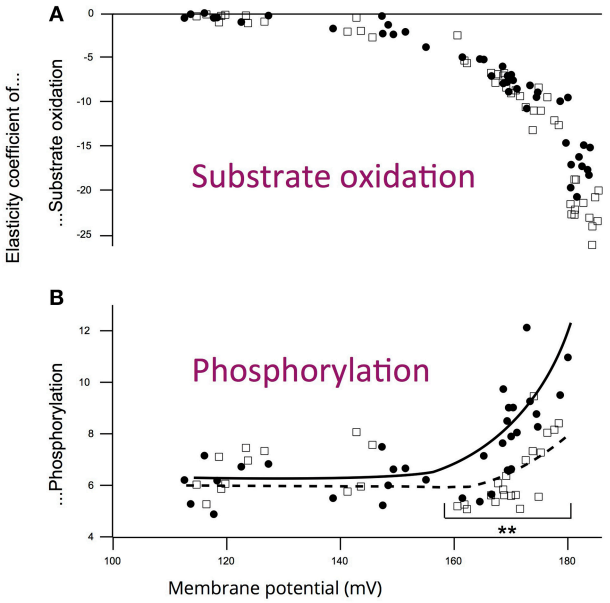

FIGURE 4 | Influence of aging on the elasticity curves of (A) substrate oxidation and (B) phosphorylation modules toward membrane potential for young (squares) and aged (circles) rats. Data from experiments similar to those presented in **Figure 3**. $^{**}p < 0.01$ between young ($n = 7$) and aged groups ($n = 7$). Art drawing has been added to emphasize the difference between young and aged rats (Data and figure from Gouspillou et al., 2010).

lower in mitochondria from aged rats. These data allows the determination of the elasticity coefficients of phosphorylation (**Figure 4**). The complete set of experiment for the determination of the elasticity of substrate oxidation has been detailed elsewhere (Gouspillou et al., 2010). We could observe that the elasticity of both modules changed with membrane potential value, but while the curves are superimposed for substrate oxidation, they appeared significatively different for phosphorylation, especially for high values of $\Delta\psi$, ranging from 160 to 180 mV, corresponding to low phosphorylation activity and higher proton leak, likely to be the most common situation *in vivo*.

Since a lower $\Delta\psi$ value is usually interpreted as uncoupling, i.e., increase in proton leak through inner mitochondrial membrane, we investigated phosphorylation coupling efficiency in mitochondria isolated from aged skeletal muscle (Gouspillou et al., 2014). This was possible thanks to our specific *in vitro* experimental setup, since we obtain simultaneous recording of oxidation and phosphorylation rates and therefore direct access to mitochondrial coupling efficiency (ATP to O ratio). ATP to O ratio was determined under conditions of constant ADP concentration across the whole range of mitochondrial oxidative phosphorylation activity (Gouspillou et al., 2014). Results are shown in **Figure 5** for young and aged rat muscle mitochondria. The results demonstrate that maximal ATP to O ratio was not diminished in aged rat mitochondria. Same result was obtained concerning the dependence of coupling efficiency on the ADP concentration. Most interestingly, when the ADP to O ratio was plotted as a function of the phosphorylation rate, we found that for any given phosphorylation rate aged mitochondria displayed a (non-significative) trend for higher coupling efficiency (results not shown, Gouspillou et al., 2014).

DISCUSSION

The purpose of the present paper is to describe a new mechanism of protection against ROS production appearing in skeletal muscle mitochondria during aging. This mechanism involves an alteration of oxidative phosphorylation due to mitochondrial ANT remodeling and results in a decreased ROS production in aged mitochondria for a given ATP synthesis rate. The role of mitochondrial ROS production in ANT remodeling remains to be elucidated.

Being partly composed of glycolytic fibers, well known to be affected during aging (Lexell, 1995) the gastrocnemius muscle is highly sensitive to sarcopenia (Martin et al., 2007), and therefore appeared physiologically relevant for this study of mitochondrial alteration induced by aging. The current theory to explain skeletal muscle aging relies on the accumulation of oxidative damages to mitochondrial DNA and proteins, due to mitochondrial ROS production (Dirks et al., 2006; Lesnefsky and Hoppel, 2006). Since phospholipids are also important targets of ROS, it has been proposed that an increased conductance to proton may occur during aging, and therefore a decrease in $\Delta\psi$. However, such decrease in $\Delta\psi$ would reduce mitochondrial ROS production (Brand, 2000). The increased proton leak found in aged mitochondria in some studies performed on rat (Kumaran et al., 2005) and human muscles (Tonkonogi et al., 2003) has been interpreted as a compensatory mechanism to decrease ROS production. A role for the uncoupling proteins has also been suggested (Brand and Esteves, 2005).

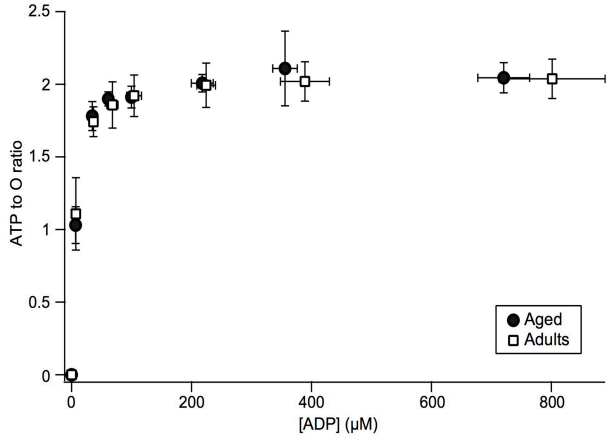

FIGURE 5 | Changes in the ATP to O ratio as a function of ADP concentration and phosphorylation rate in adult and aged mitochondria. ATP to O ratio was determined by calculating the ratio of phosphorylation to oxidation rates and plotted as a function of ADP concentration. Data for adult ($n = 7$) and aged ($n = 5$) rats are presented as mean ± SD (Figure from Gouspillou et al., 2014).

Thanks to integrative approaches applied at different integration levels—i.e., muscle *in vivo* and isolated muscle mitochondria—we could demonstrate that (i) the responsiveness (elasticity) of energy production is effectively reduced in the GAS of aged rats (Gouspillou et al., 2014) and (ii) that this alteration of muscle energetics is correlated with a modified regulation of phosphorylation module in mitochondria (**Figure 4** and Gouspillou et al., 2014). In other words, in the aging muscle, a greater rise in ADP, concomitant with a greater drop in PCr level is required to induce equivalent activation of ATP/PCr synthesis in aged compared to young muscle. Since the elasticity coefficients are intrinsically linked to the kinetics of the modules (Brand and Curtis, 2002) and because we investigated only *in vivo* low contractile activities of the muscles—unlikely to require the maximal oxidative phosphorylation capacity, we could previously demonstrate that a decrease in maximal oxidative phosphorylation capacity (or in mitochondrial content) is unlikely to explain the decrease in the elasticity of the energy-supply module found in aged rats (Gouspillou et al., 2014). Therefore, the altered regulation of muscle energetics strongly suggest the existence of an alteration in mitochondrial function related to muscle aging and sarcopenia. This *in vivo* impairment of mitochondrial bioenergetics in aged muscle was further completed by the *in vitro* integrative study of mitochondrial bioenergetics for better understanding of the cellular basis. Indeed, while maximum oxidation and phosphorylation rates were both decreased in aged mitochondria, only phosphorylation did show modified elasticities (**Figure 4**).

We recently reported a significant decrease in mitochondrial affinity for ADP in mitochondria isolated from aged GAS muscle as compared to young ones (Gouspillou et al., 2014), which is perfectly in line with the age-related reduction in the *in vivo* activation of mitochondrial oxidative phosphorylation in response to an increase in ATP demand (decrease in elasticity of energy supply). In fact, a decreased affinity for ADP indicates that for a given rate of mitochondrial oxidative phosphorylation to match ATP demand, a higher increase in the ADP concentration is necessary. This lower ADP sensitivity of mitochondria in aged rats fits well the regulation of phosphorylation (elasticity) measured *in vivo* in aged rat muscles. We also showed that mitochondria from gastrocnemius muscle of aged rat manifest an interesting increase in atractyloside-sensitivity, as compared to young ones (Gouspillou et al., 2010). Even though the apparent mitochondrial oxidative phosphorylation affinity for ADP involves several electrogenic—membrane potential sensitive—enzymatic complexes (ATPsynthase, ANT and phosphate transporter), this parameter greatly depends on ANT kinetic properties (Gouspillou et al., 2011). These results prompted us to propose that aging-related changes in mitochondrial oxidative phosphorylation implicate a modification of the functionning of ANT (Gouspillou et al., 2010, 2014). Since we also showed that ANT content was not modified in aged muscles (Gouspillou et al., 2014), we propose that a functional change in ANT characteristics may be at the origin of the aging-related change in atractyloside sensitivity and energetics remodeling in muscle. It has also been shown that ANT carbonylation, indicative of oxidative damage by ROS, was increased during aging in the flight muscle of the housefly (Yan and Sohal, 1998) and in rat skeletal muscle (Feng et al., 2008). Since the increase in the carbonyl content of ANT has been associated with impaired ANT function (Yan and Sohal, 1998), these results strongly suggest that oxidative damage to ANT with aging represents a possible mechanism that may trigger dysfunction of mitochondrial bioenergetics with aging (**Figure 6**).

By contrast with previous studies reporting either a decline (Tonkonogi et al., 2003; Kumaran et al., 2005; Marcinek et al., 2005) or increase (Kerner et al., 2001) in mitochondrial coupling efficiency, we did not observe any aging-related changes in proton leak, since membrane potential and state 4 oxidation rates were unchanged, as well as proton leak curve (Gouspillou et al., 2010). This absence of change in proton leak curve also clearly indicate that the age-related modification of ANT regulation described here do not affect the proton leak capacity of ANT, now considered as the primary route for proton leak in mammalian mitochondria (Parker et al., 2009; Jastroch et al., 2010). We recently added complementary data to confirm the absence of uncoupling by directly measuring the ATP/O ratio (phosphorylation efficiency) for a range of ADP concentrations driving increasing phosphorylation rates. The results confirmed in our hands the total absence of a decrease of coupling efficiency in aged mitochondria as compared to young ones (**Figure 5** and Gouspillou et al., 2014). In this latter paper, we even showed that the decrease in membrane potential for a given ATP demand (phosphorylation rate) results in a tendency to increase coupling efficiency. This result is the consequence of down-regulation of proton leak by the decrease in membrane potential (Brand et al., 1994). We should however stress here that even though the decrease in $\Delta\psi$ by aged ANT has beneficial effect on mitochondrial ROS production,

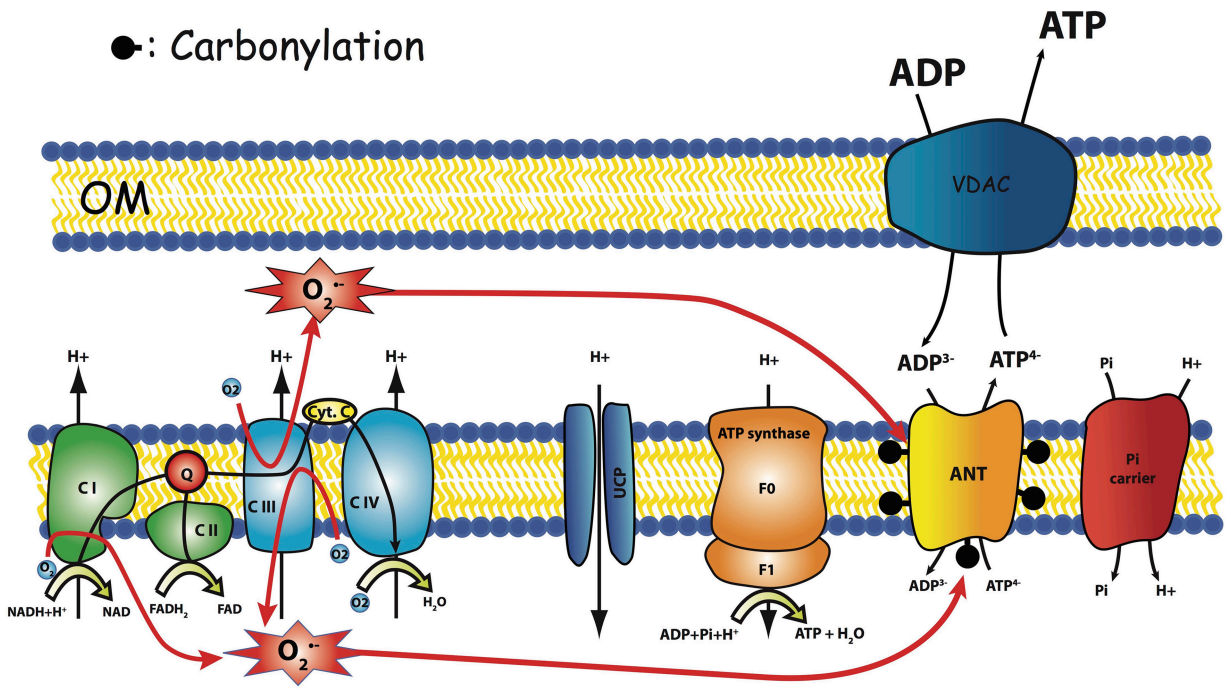

FIGURE 6 | Hypothesis. Carbonylation of ANT as the result of respiratory chain ROS production modify ANT regulation. OM, Outer Membrane.

it may also have harmful consequence for muscle cells since mitochondrial membrane potential is deeply involved in ion (calcium) homeostasis.

In the framework of the radical theory of aging, these important modifications in ANT function may be the result of oxidative damage caused by intra mitochondrial ROS and may appear like a protective remodeling where ROS induce a mechanism that reduces their production, without causing uncoupling. However, further work is now required to define the origin of the impairment of ANT function with aging and to better characterize the importance on mitochondrial bioenergetics defects in sarcopenia. Because of the importance of ROS as therapeutic targets, we believe that this impairment in ANT function might be a central mechanism causing aging-related defects in mitochondrial bioenergetics which deserves further studies.

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Protein Kinase A Governs Oxidative Phosphorylation Kinetics and Oxidant Emitting Potential at Complex I

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The mitochondrial electron transport system (ETS) is responsible for setting and maintaining both the energy and redox charges throughout the cell. Reversible phosphorylation of mitochondrial proteins, particularly via the soluble adenylyl cyclase (sAC)/cyclic AMP (cAMP)/Protein kinase A (PKA) axis, has recently been revealed as a potential mechanism regulating the ETS. However, the governance of cAMP/PKA signaling and its implications on ETS function are incompletely understood. In contrast to prior reports using exogenous bicarbonate, we provide evidence that endogenous CO_2 produced by increased tricarboxylic acid (TCA) cycle flux is insufficient to increase mitochondrial cAMP levels, and that exogenous addition of membrane permeant 8Br-cAMP does not enhance mitochondrial respiratory capacity. We also report important non-specific effects of commonly used inhibitors of sAC which preclude their use in studies of mitochondrial function. In isolated liver mitochondria, inhibition of PKA reduced complex I-, but not complex II-supported respiratory capacity. In permeabilized myofibers, inhibition of PKA lowered both the K_m and V_{max} for complex I-supported respiration as well as succinate-supported H_2O_2 emitting potential. In summary, the data provided here improve our understanding of how mitochondrial cAMP production is regulated, illustrate a need for better tools to examine the impact of sAC activity on mitochondrial biology, and suggest that cAMP/PKA signaling contributes to the governance of electron flow through complex I of the ETS.

Keywords: mitochondria, adenylyl cyclase, cAMP, protein kinase A, complex I, respiration, skeletal muscle, liver

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INTRODUCTION

Mitochondrial function is a key determinant of skeletal muscle metabolic health since it governs both the energetic and redox environments of the myocyte. Under conditions of overnutrition and/or obesity, evidence suggests mitochondria from humans and rodents increase their rate of hydrogen peroxide (H_2O_2) emission (Houstis et al., 2006; Fisher-Wellman et al., 2014), with the resulting increase in oxidative burden impairing skeletal muscle insulin action (Anderson et al., 2009; Hoehn et al., 2009; Lark et al., 2015). Key enzymes within the electron transport system and the matrix regulate the rate of H_2O_2 production and scavenging that ultimately determine the rate of H_2O_2 release. Therefore, a better understanding of how mitochondrial enzymes are regulated may lead to better

treatments for diseases like diabetes that are linked to mitochondrial H_2O_2 emission.

Mounting evidence implicates post-translational modifications to mitochondrial proteins, particularly phosphorylation events mediated by the cyclic adenosine monophosphate (cAMP)/Protein kinase A (PKA) axis, as a key regulator of cellular metabolism (Valsecchi et al., 2013; Di Benedetto et al., 2014). Mitochondrial cAMP/PKA signaling is thought to be initiated by soluble adenylyl cyclase (sAC) (Buck et al., 1999), a bicarbonate (HCO_3^-)- and Ca^{2+}-activated (Chen et al., 2000) enzyme that generates cAMP in various intracellular compartments (e.g., mitochondrial matrix) (Zippin et al., 2003). The implication is that CO_2 generated during accelerated flux through the tricarboxylic acid (TCA) cycle is converted to HCO_3^- via carbonic anhydrase (CA) and activates the mitochondrial cAMP/PKA axis. However, although it is well-established that exogenous HCO_3^- can activate mitochondrial sAC (Chen et al., 2000; Zippin et al., 2003), it is not known whether increased endogenous metabolic CO_2 production increases mitochondrial cAMP.

Analysis of the MitoCarta mitochondrial proteome database (Pagliarini et al., 2008) has revealed approximately 75 different putative targets of PKA-mediated phosphorylation, some of which are altered by dietary manipulation (Grimsrud et al., 2012). Available evidence suggests cAMP/PKA signaling alters oxidative phosphorylation (OXPHOS) by regulating cytochrome C oxidase (Acin-Perez et al., 2009a,b, 2010) or enhancing ATP production in the presence of Ca^{2+} (Di Benedetto et al., 2013). Additionally, several independent groups have identified Complex I of the electron transport system (ETS) as a target of PKA-dependent phosphorylation (Papa, 2002; De Rasmo et al., 2010) with a potential role in a number of human pathologies (Valenti et al., 2011; Papa et al., 2012). Despite the cummulative evidence implicating cAMP/PKA-mediated regulation of the ETS in human disease, the potential functional impact of cAMP/PKA-mediated phosphorylation on mitochondrial bioenergetics is not well understood.

Therefore, the purpose of the present study was to determine: (1) if endogenous CO_2 production from the TCA cycle is sufficient to increase mitochondrial cAMP levels and (2) whether PKA acts on multiple ETS complexes (including Complex I) as a feed-forward mechanism to enhance OXPHOS in response to metabolic demand.

METHODS

Chemicals and Reagents

All chemicals and reagents were obtained from Sigma Aldrich except for Amplex Ultra Red reagent, which was purchased from Molecular Probes Inc.

Animal Use Procedures

All aspects of rodent studies were approved by the East Carolina University Animal Care and Use Committee. Male C57BL6/NJ mice were purchased from Jackson Laboratories and were the only model used in these studies. Mice were housed in a temperature- (22°C) and light-controlled room and given free access to food and water. At the time of experiment, mice were 8–12 weeks of age.

Mitochondrial Isolation

For mitochondrial isolation, mice were anesthetized by inhalation of isoflurane following a 4 h fast and were euthanized via double pneumothorax. Under anesthesia, liver, or hind limb muscles (gastrocnemius, quadriceps, and biceps femoris) were immediately excised and rinsed in ice-cold mitochondrial isolation medium (MIM) containing: 300 mM Sucrose, 10 mM HEPES, and 1 mM EGTA. Tissues were then transferred to a dry dish and minced continuously for 5 min, then transferred to a 50 ml tube containing 10 ml of MIM. For skeletal muscle, trypsin (100 mg/ml) was added for exactly 2 min, then soybean trypsin inhibitor in 10 ml of MIM + 1 mg/ml BSA was added to halt the reaction. Tissue was then gently mixed by inversion and allowed to settle to the bottom of the tube. Supernatant was discarded and tissue re-suspended in MIM+BSA (20 ml/g tissue). Minced liver was not treated with trypsin. Tissues were then homogenized using a tight-fitting Teflon glass homogenizer (∼10 passes) and centrifuged at 800 g for 10 min at 4°C. Supernatant was transferred to Oakridge tubes and centrifuged at 8000 g for 15 min at 4°C. Supernatant was discarded and pellet was washed and re-suspended in 10 ml of MIM+BSA and centrifuged again at 8000 g for 15 min at 4°C. The final pellet was re-suspended in 50 µl of MIM. Mitochondrial protein concentration was determined by spectrophotometry using the bicinchoninic acid method (Pierce). In some experiments, mitochondria were fractured by three freeze-thaw cycles and directly assayed for complex I specific activity (Barrientos et al., 2009).

Preparation of Mouse Permeabilized Myofiber Bundles (PmFBs)

The PmFB technique used was partially adapted from previous methods (Kuznetsov et al., 1996; Tonkonogi et al., 2003) and has been described previously (Anderson and Neufer, 2006). Mice were anesthetized by inhalation of isoflurane and the red (RG) and white (WG) portions of the gastrocnemius muscle were immediately excised. Muscle samples were placed in ice-cold (4°C) Buffer X containing (in mM): 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 20 Taurine, 5.7 ATP, 14.3 Phosphocreatine, 6.56 $MgCl_2$-$6H_2O$, and 50 MES (pH 7.1, 295 mOsm). Under a dissecting microscope (Leica Optics), fat, and connective tissue were removed and muscle samples were separated into small bundles of fibers (<1 mg wet weight/fiber bundle). Fiber bundles were permeabilized in Buffer X supplemented with 40 µg/ml saponin, a mild, cholesterol-specific detergent for 30 min at 4°C as previously described (Anderson and Neufer, 2006). Since the sarcolemmal membrane contains a large amount of cholesterol relative to the mitochondrial membrane, this technique selectively permeabilizes the sarcolemma while leaving mitochondrial membranes and ultra-structure intact (Kuznetsov et al., 2008; Picard et al., 2011). PmFBs were then washed in ice-cold Buffer Z containing (in mM): 105 K-MES, 30 KCl, 5 KH_2PO_4, 5 $MgCl_2$-$6H_2O$, and 0.5 mg/ml Bovine serum albumin (pH 7.1,

295 mOsm) and remained in Buffer Z on a rotator at 4°C until analysis (<4 h).

Mitochondrial Camp Production Assay

Two different sets of experiments were done to measure mitochondrial cAMP production in the current study. First, isolated liver mitochondria were incubated for 10 min at 37°C in 300 µl of MAITE medium containing (in mM): 10 Tris-HCl, 25 sucrose, 75 sorbitol, 100 KCl, 0.5 EDTA, 5 $MgCl_2$, and 1 mg/ml BSA; pH 7.4. MAITE medium was also supplemented with 300 mM HEPES to maintain pH in the presence of HCO_3^- (Acin-Perez et al., 2009b) and 1 mM ATP as substrate for cAMP production. Experiments were performed under three conditions: no additions, 30 mM HCO_3^- and HCO_3^- plus 25 µM KH7, an inhibitor of sAC (Hess et al., 2005). Second, skeletal muscle mitochondria (250 µg/ml) were incubated at 37°C in 300 µl of MAITE medium supplemented with 1 mM ATP, 10 µg/ml oligomycin and in the presence or absence of 25 µM KH7 or 5 µM acetazolamide (AZA), a carbonic anhydrase inhibitor to prevent conversion of CO_2 to HCO_3^- (Maren, 1960). Following an initial 10 min acclimation period, mitochondria were incubated for 5 min in the presence of the following respiratory substrate combinations: 5 pyruvate/2 mM malate, 5 mM succinate, or 25 µM palmitoyl-L-carnitine/2 mM malate. A separate set of control samples did not receive respiratory substrates. In some experiments, 1 µM FCCP was added to uncouple O_2 consumption from ATP synthesis and thereby accelerate TCA cycle flux. Reactions were halted by the addition of 0.1 M HCl, and then samples were flash frozen and stored in liquid N_2 until analysis for cAMP (Complete cAMP ELISA Kit, Enzo Life Sciences).

Mitochondrial Bioenergetics Assays

Mitochondrial respiration experiments in both isolated mitochondria and PmFBs were performed using a high-resolution oxygraph (Oroboros O_2k, Innsbruck Austria). Respirometry experiments using isolated mitochondria were performed in Buffer Z at 25°C while substrate titration experiments in PmFBs were performed at 37°C in Buffer Z supplemented with 20 mM creatine monohydrate to maximize phosphate transfer in PmFBs (Kuznetsov et al., 1996). Blebbistatin (20 µl) was also added during PmFB experiments to mitigate the effects of contraction on respiratory kinetics (Perry et al., 2011).

Mitochondrial H_2O_2 emitting potential, defined as the H_2O_2 that escapes the matrix, was measured via Amplex Ultra Red (Invitrogen) fluorescence detected at 565/600 ex/em at 37°C in a monochromatic spectrofluorometer (Horiba Jobin-Yvon) with Buffer Z as previously described (Anderson and Neufer, 2006). Assays were performed in the presence of 25 U/ml superoxide dismutase to ensure superoxide produced and released on the outer surface of the mitochondrial inner membrane was converted to H_2O_2. Mitochondrial H_2O_2 emitting potential in PmFBs was measured during either reverse electron flow using 5 mM succinate or forward electron flow using 5 mM glutamate and 2 mM malate followed by the addition of rotenone (Lambert and Brand, 2004). Once steady-state rates of H_2O_2 emission were established (< 10 min), 1 µM auranofin, a thioredoxin reductase inhibitor, was added to remove oxidant scavenging as a potentially confounding factor (Fisher-Wellman et al., 2013). In addition to yielding a measure of H_2O_2 production, this allowed for the determination of oxidant scavenging capacity as the difference in H_2O_2 emission before and after the addition of auranofin.

In experiments utilizing 8Br-cAMP or H89, compounds were added to the oxygraph chamber or cuvette with isolated mitochondria or PmFBs for 10 min prior to any subsequent additions.

Statistical Analyses

Comparisons between control and treatment groups were made using One-way ANOVA with Student Newman-Keuls *post-hoc* test where appropriate using Prism statistical software (GraphPad Prism 6). Pair-wise comparisons were made using student's paired two-way *t*-test. In all experiments, data are reported as mean ± SEM unless otherwise noted. Significance level was set at $p < 0.05$.

RESULTS

TCA Cycle Flux Does Not Increase [cAMP] in Isolated Mitochondria

The inner mitochondrial membrane is impermeable to cytosolic cAMP (Di Benedetto et al., 2013) and, therefore, matrix cAMP has been proposed to be generated locally by CO_2-mediated activation of sAC. Evidence in support of this hypothesis comes from data showing that addition of exogenous HCO_3^- induces a small increase (~10%) in mitochondrial cAMP that is prevented by the sAC inhibitor KH7 (Chen et al., 2000; Litvin et al., 2003; Zippin et al., 2003; Acin-Perez et al., 2009b; Di Benedetto et al., 2013). In the present study, addition of HCO_3^- to isolated liver mitochondria generated a small but significant increase in cAMP that, in contrast to previous findings (Acin-Perez et al., 2009b), was not blunted by KH7 (**Figure 1A**).

The TCA cycle has been proposed as the source of CO_2 needed to activate sAC in mitochondria (Acin-Perez et al., 2009b), although this has yet to be demonstrated experimentally. Using isolated mitochondria from skeletal muscle in the presence of 1 mM ATP, we were unable to detect any increase in cAMP during respiration supported by CO_2-generating substrates (pyruvate/malate or palmitoyl-L-carnitine/malate) compared with mitochondria in the absence of substrate or those oxidizing non CO_2-generating substrates (succinate) (**Figure 1B**). Mitochondrial cAMP remained unchanged even when TCA cycle flux was accelerated by the mitochondrial uncoupler FCCP. Finally, consistent with the data from liver mitochondria (**Figure 1A**), cAMP levels were not decreased by the putative sAC inhibitor KH7 or acetazolamide (AZA), a carbonic anhydrase inhibitor. These findings suggest that, at least under the conditions tested, endogenous TCA cycle-derived CO_2 production is not sufficient to increase mitochondrial cAMP in skeletal muscle mitochondria.

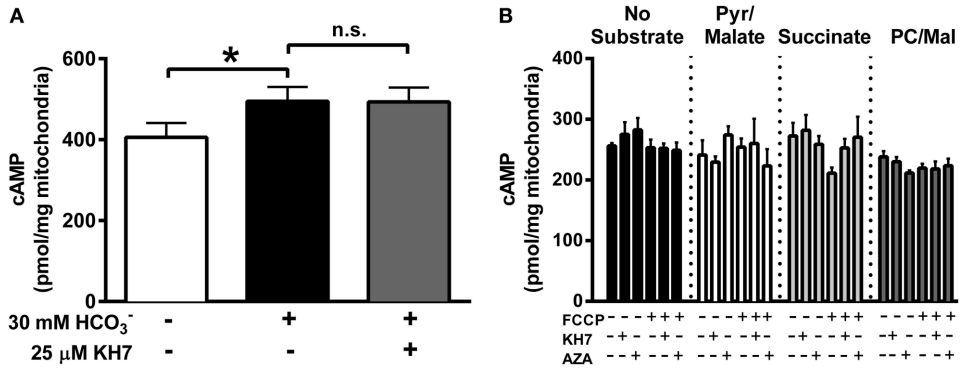

FIGURE 1 | TCA-cycle dependent flux does not increase mitochondrial cAMP. (A) cAMP was measured in isolated liver mitochondria in the presence of 1 mM ATP, and in the absence of inhibitors (white bar), the presence of 30 mM HCO_3^- (black bar) or the presence of both HCO_3^- and 25 μM KH7 (gray bar). $N = 4$/condition. * denotes $p < 0.05$ compared to untreated condition. **(B)** cAMP was measured in isolated skeletal muscle mitochondria in the presence of 1 mM ATP, and in the absence (black bars) of respiratory substrates or in the presence of pyruvate/malate (white bars), succinate (light gray bars) or palmitoyl-L-carnitine/malate (dark gray bars). For each round of experiments, a single mitochondrial preparation was used for all substrate conditions, including parallel experiments with FCCP, KH7, and acetazolamide (AZA). $N = 4$ mitochondrial preparations from individual mice.

Regulation of OXPHOs Function by Mitochondrial cAMP/PKA Signaling

The functional consequence of mitochondrial cAMP/PKA signaling on OXPHOS function is unclear as exogenous activation of PKA has been reported to either increase (Acin-Perez et al., 2009b, 2011) or decrease (Di Benedetto et al., 2013) mitochondrial ATP production. Here, a series of experiments were performed to test the hypothesis that OXPHOS is regulated by mitochondrial cAMP/PKA signaling.

First, rates of oxygen consumption (JO_2) were measured in isolated liver mitochondria in the absence or presence of 1 mM 8Br-cAMP, a membrane-permeable cAMP mimetic (**Figure 2A**). Surprisingly, 8Br-cAMP did not alter basal or maximal ADP-stimulated glutamate/malate-supported respiration. Similar to previous findings (Acin-Perez et al., 2009b), KH7 nearly completely inhibited ADP-stimulated respiration. Curiously however, the effect was not reversed or attenuated by the addition of 8Br-cAMP, which should bypass the inhibition of sAC. Addition of cytochrome c or FCCP also failed to restore respiration in the presence of KH7. Together, these findings suggest that the inhibitory effect of KH7 occurs independent of sAC/cAMP/PKA signaling and is not associated with loss of mitochondrial membrane integrity.

To further define the mechanism by which KH7 acts independently of cAMP signaling (Tian et al., 2011; Di Benedetto et al., 2013), complex I activity was measured in freeze-fractured fragments of isolated skeletal muscle mitochondria in the absence or presence of KH7. Addition of KH7 led to an immediate ablation of complex I activity that was not recovered by the addition of 8Br-cAMP (**Figure 2B**). Dose-response curves for complex I activity as a function of KH7 concentration in PKA-depleted mitochondrial fragments revealed an IC_{50} value of 3.7 μM (**Figure 2C**), comparable to previously reported IC_{50} values of KH7 for sAC (Hess et al., 2005; Bitterman et al., 2013). These findings suggest that the effects of KH7 on mitochondrial respiration are mediated by direct inhibition of complex I.

Besides KH7, the only other known sAC inhibitor with an IC_{50} below 10 μM is the naturally occurring estrogen metabolite 2-hydroxyestradiol (2-HE) (Steegborn et al., 2005). Anecdotal reports have suggested that 2-HE is also capable of generating oxidant species via redox cycling (Fussell et al., 2011), although this has not been demonstrated experimentally. Using a cell/organelle-free based H_2O_2 detection system, we found that 2-HE, but not its metabolite 2-methoxyestradiol (2-ME), generates H_2O_2 spontaneously (**Figure 3A**) in a dose-dependent (**Figure 3B**) and catalase-sensitive (**Figure 3C**) manner. H_2O_2 production was detected with as little as 200 nM 2-HE, a concentration more than 50-fold lower than has been previously used to inhibit sAC in cell-based assays (Tian et al., 2011; Di Benedetto et al., 2013). These findings raise significant concerns regarding the specificity and use of both KH7 and 2-HE as tools to study cAMP-related signaling events.

Inhibition of PKA Decreases Complex I-supported Respiratory Capacity

To further explore the potential impact of kinase activity on mitochondrial function, we focused our attention on PKA. Incubation of HeLa cells for 30 min with 1 μM H89, a PKA inhibitor (Chijiwa et al., 1990), has been shown to decrease mitochondrial respiratory capacity (Acin-Perez et al., 2009b), but the specific site(s) of regulation remains unknown. Using mitochondria isolated from liver, H89 dose-dependently decreased ADP-stimulated respiration supported by complex I (**Figure 4A**), but not complex II (**Figure 4B**) substrates. These findings prompted us to specifically focus on the role of PKA in the regulation of complex I activity.

H89-mediated PKA Inhibition Alters ADP-supported Respiratory Kinetics

To further define the impact of PKA inhibition on complex I-supported respiration, ADP titration experiments were performed on H89-treated PmFBs during respiration supported

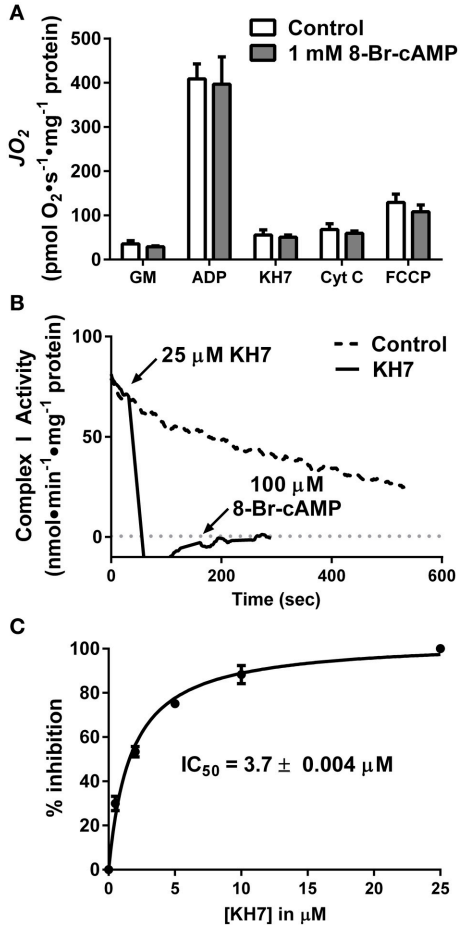

FIGURE 2 | The soluble adenylyl cyclase inhibitor KH7 inhibits complex I activity independent of cAMP/PKA signaling. (A) JO_2 was measured during a step-wise titration protocol in isolated liver mitochondria in the absence (white bars) or presence (gray bars) of 1 mM 8Br-cAMP. Each sequential addition was made after reaching a steady-state rate of O_2 flux. $N = 4$/condition. **(B)** Complex I activity was determined by the oxidation rate of NADH in fragments of isolated skeletal muscle mitochondria following no treatment (dashed line) or sequential addition of 25 μM KH7 and 100 μM 8Br-cAMP (solid line). **(C)** Dose-response inhibition curve generated for inhibition of complex I activity as a function of KH7 concentration. $N = 3$ separate observations.

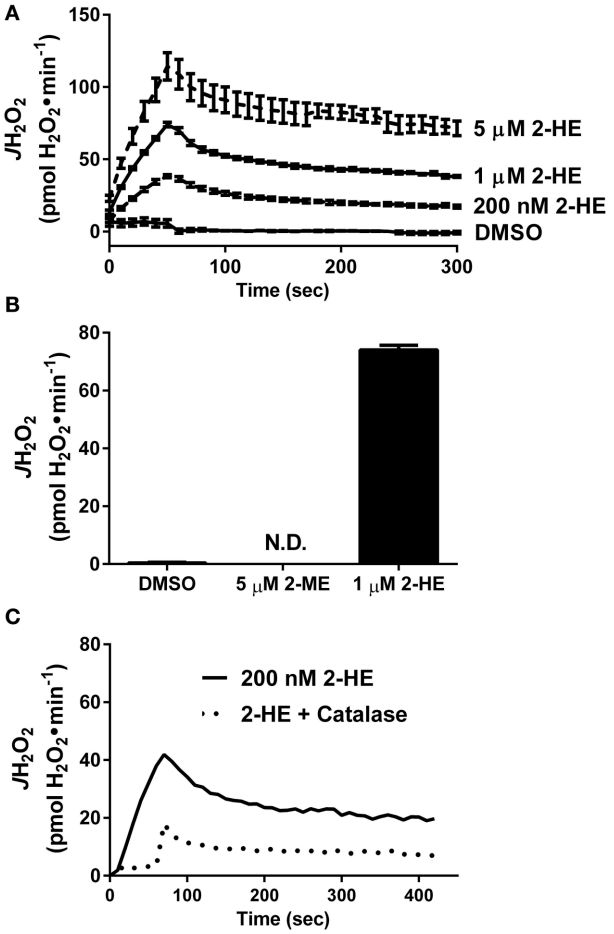

FIGURE 3 | The soluble adenylyl cyclase inhibitor 2-hydroxyestradiol (2-HE) produces H_2O_2 in a cell-free system. (A) Comparison of H_2O_2 production from 2-methoxyestradiol (2-ME) and 2-hydroxyestradiol (2-HE). **(B)** Dose-dependent rate of H_2O_2 production of 2-HE over a 5-min span. Results in **(A,B)** are the product of N=3 separate experiments. **(C)** H_2O_2 production by 200 nM 2-HE in the absence (solid line) or presence (dashed line) of 100 U/ml catalase.

by pyruvate/malate. Both slow-twitch (RG) and fast-twitch (WG) PmFBs were used for these studies because the metabolic phenotype (e.g., oxidative vs. glycolytic) of the muscle governs both respiratory kinetics (Kuznetsov et al., 1996) and H_2O_2 emitting potential (Anderson and Neufer, 2006). Initial examinations revealed a decrease in respiration at and above an ADP concentration of 75 μM in RG (**Figure 5A**) and 200 μM in WG (**Figure 5B**). Applying Michaelis Menten-like kinetic analyses, these data were further dissected to yield maximal respiratory capacity (V_{max}) and sensitivity to ADP (apparent K_m—the ADP concentration required to elicit 50% of V_{max}) (Kuznetsov et al., 1996). H89 treatment decreased both the apparent K_m and V_{max} in RG (**Figure 5C**) and WG (**Figure 5D**), indicating an increased sensitivity to ADP but decreased maximal respiratory capacity. H89 treatment decreased non-ADP stimulated respiration in RG with a similar, but non-significant ($p = 0.08$), trend in WG (**Figure 5E**). Finally, H89 treatment decreased respiratory control ratio (RCR), an index of mitochondrial coupling, in both RG and WG (**Figure 5F**). Altogether, these data suggest that inhibition of PKA decreases respiration supported by complex I, but not complex II, and does so during both proton leak- and ADP-dependent respiration.

H89-mediated PKA Inhibition Alters Complex I Substrate Kinetics

To further explore the possibility that PKA regulates complex I, pyruvate and glutamate titrations were performed under ADP-stimulated conditions in RG and WG PmFBs in the absence or presence of H89. In both RG (**Figure 6A**) and WG (**Figure 6B**), inhibition of PKA decreased respiration at or above

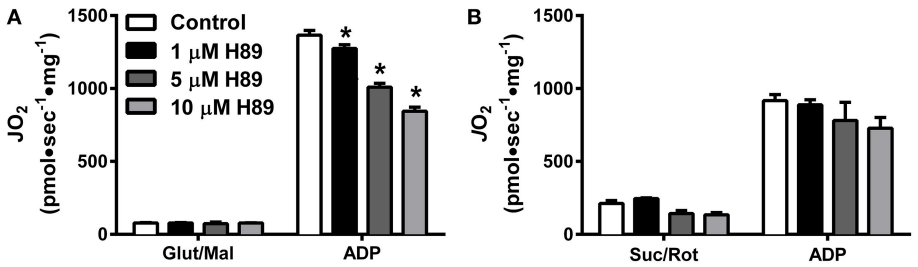

FIGURE 4 | PKA regulates Complex I- but not Complex II-supported respiration in isolated liver mitochondria. JO_2 was measured with complex I **(A)** or complex II **(B)** supported substrates in the absence (white bars) or presence of 1 (black bars), 5 (dark gray bars), or 10 (light gray bars) μM H89. N = 4–6/condition. * denotes $p < 0.05$ compared to control.

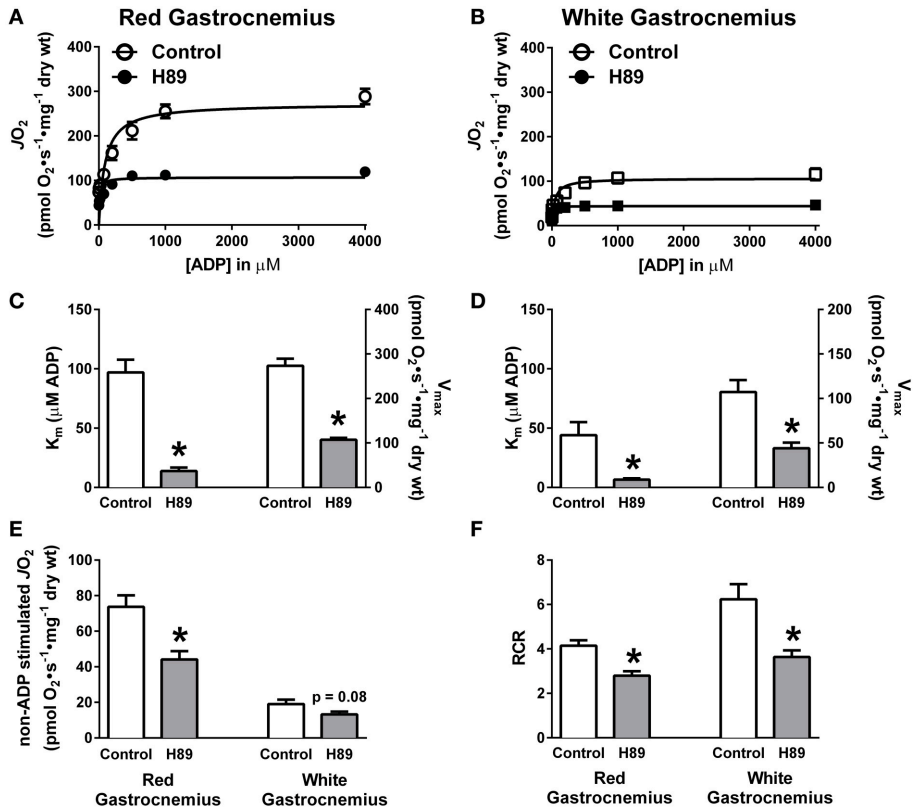

FIGURE 5 | PKA regulates ADP kinetics, proton leak and respiratory control ratio in oxidative and glycolytic mouse PmFBs. ADP titration experiments were performed in RG **(A)** and WG **(B)** PmFBs with pyruvate and malate in the absence (open symbol) or presence (closed symbol) of 10 μM H89 in the assay media. Michaelis Menten-like kinetics generated from ADP titration experiments in RG **(C)** and WG **(D)**. **(E)** Non-ADP stimulated JO_2 was compared from ADP titration experiments in the absence (white bars) or presence (gray bars) of 10 μM H89 in RG (left) and WG (right). **(F)** Respiratory control ratio (ADP-stimulated JO_2/non-ADP stimulated JO_2) was calculated from ADP titration experiments in the absence (white bars) or presence (gray bars) of 10 μM H89 in RG (left) and WG (right). N = 4–6/condition. * denotes $p < 0.05$ compared to Control.

a pyruvate concentration of 100 μM. This was accompanied by an increase in sensitivity to pyruvate and a decrease in respiratory capacity in both tissues (**Figures 6C,D**). Glutamate titration experiments yielded similar data (**Figures 6E–H**). These findings using two distinct NADH-linked substrates provide evidence that PKA-mediated phosphorylation influences complex I-supported respiratory kinetics.

H89-mediated PKA Inhibition Decreases H_2O_2 Production during Reverse Electron Flow

With evidence suggesting a role for PKA in the regulation of respiratory kinetics, particularly at complex I, we next sought to examine whether PKA affects the susceptibility of complex I to electron leak and H_2O_2 production/emission during reverse

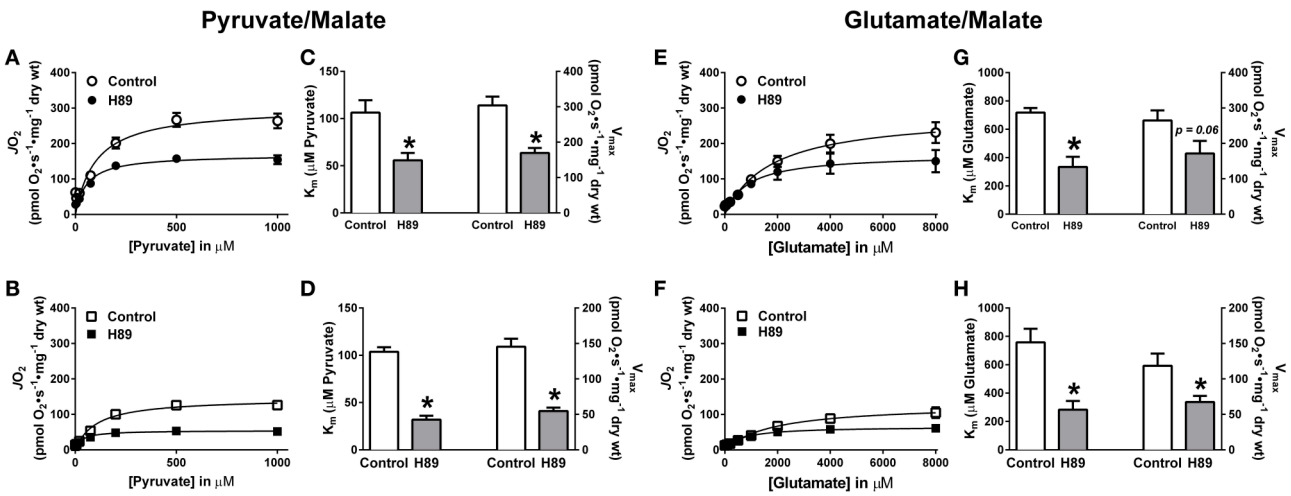

FIGURE 6 | Effects of PKA inhibition on substrate oxidation kinetics in mouse PmFBs. Pyruvate titrations were performed in PmFBs from RG **(A)** and WG **(B)** in the absence (open symbol) or presence (closed symbol) of 10 μM H89 in the assay media. K_m and V_{max} were determined based on Michaelis-Menten like kinetics in RG **(C)** and WG **(D)**. N = 4–8/condition. * denotes $p < 0.05$ compared to Control. Glutamate titrations were performed in RG **(E)** and WG **(F)** in the absence (open symbol) or presence (closed symbol) of 10 μM H89. K_m and V_{max} were determined based on Michaelis-Menten like kinetics in RG **(G)** and WG **(H)**. N = 4–8/condition. * denotes $p < 0.05$ compared to Control.

(i.e., succinate) or forward (glutamate/malate/rotenone) electron flow. In PmFBs from both RG (**Figure 7A**) and WG (**Figure 7B**), inhibition of PKA decreased H_2O_2 emission during reverse, but not forward, electron flow. Addition of auranofin to inhibit mitochondrial H_2O_2 scavenging elicited similar increases in H_2O_2 emission in the absence or presence of H89. Total mitochondrial oxidant scavenging was also not affected by H89. Together these data indicate that PKA inhibition affects H_2O_2 production, not scavenging, and that PKA may mediate its effects on mitochondrial energetics, at least in part, via regulation of electron flow at or near the interface between complex I and the Q-pool.

DISCUSSION

In recent years, starting with the discovery of sAC (Buck et al., 1999), a potential role for cAMP signaling in the mitochondrial matrix has emerged (Valsecchi et al., 2013). Several reports have described a role for sAC within mitochondria (Zippin et al., 2003; Acin-Perez et al., 2009b; Di Benedetto et al., 2013), the existence of mitochondrial cAMP/PKA signaling microenvironments (Papa et al., 1999; Livigni et al., 2006; Di Benedetto et al., 2008; Acin-Perez et al., 2011), and a wide variety of reversibly phosphorylated mitochondrial proteins (Zhao et al., 2011; Grimsrud et al., 2012). In contrast with plasma membrane-bound G-protein-linked forms of AC, sAC is activated by bicarbonate and calcium (Litvin et al., 2003). CO_2 produced by the TCA cycle, and subsequent conversion to HCO_3^- by carbonic anhydrase, has been suggested as a mechanism by which sAC/PKA signaling is activated in mitochondria (Acin-Perez et al., 2009b). In the present study however, evidence is provided that mitochondrial cAMP/PKA signaling is not activated by increased flux through the TCA cycle. In addition, two widely used inhibitors of sAC were found to have distinct non-specific effects that limit their utility in studies of mitochondrial function. Notably however, pharmacological inhibition of PKA was found to alter OXPHOS kinetics during respiration supported by NADH-linked substrates and H_2O_2 emission during reverse electron flow through complex I, providing additional evidence that complex I may be regulated by reversible phosphorylation.

The discovery and subsequent characterization of sAC within specific cellular organelles has led to the concept of compartmentalized cAMP signaling. A seminal finding in this field was that exogenous HCO_3^- can increase cAMP levels via activation of sAC (Chen et al., 2000), a discovery that has been confirmed in multiple subsequent studies (Litvin et al., 2003; Zippin et al., 2003; Di Benedetto et al., 2013), including this report (**Figure 1A**). A central premise of the sAC-cAMP-PKA axis is that endogenous HCO_3^- generated during increased flux through the TCA cycle is responsible for activating sAC. Here we directly tested this hypothesis and found that even during maximal uncoupled respiration, and in the presence of multiple substrate combinations that feed into the TCA cycle, mitochondrial cAMP levels did not change (**Figure 1B**). ATP was included in the assay at a concentration (1 mM) sufficient to provide substrate for sAC without inducing substrate inhibition (>5 mM) (Litvin et al., 2003). These findings therefore suggest that endogenous production of CO_2 from the TCA cycle does not activate sAC in skeletal muscle mitochondria. More recent findings have provided evidence that an increase in the frequency and amplitude of matrix Ca^{2+} oscillations, as would occur during muscle contractions, is likely the more physiologically important regulator of sAC in mitochondria (Di Benedetto et al., 2013).

Defining the role of sAC in the regulation of mitochondrial bioenergetics has also hinged greatly on the use of two

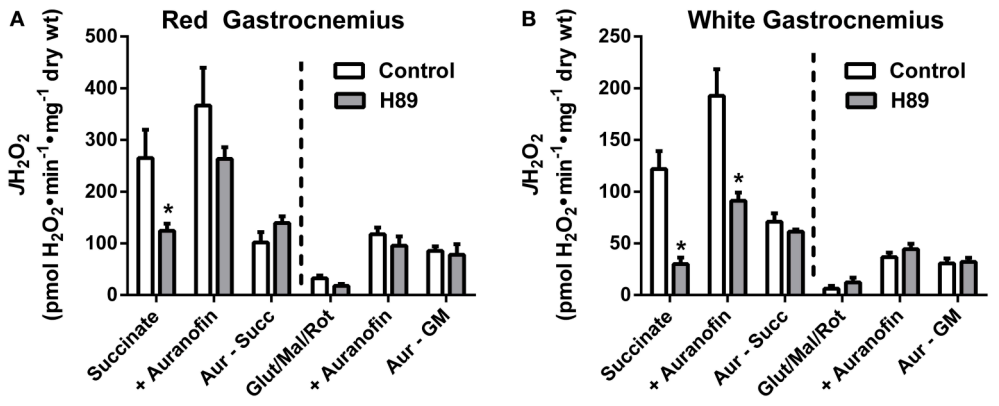

FIGURE 7 | PKA signaling regulates reverse, but not forward electron flow, through complex I in mouse PmFBs. Mitochondrial H_2O_2 emitting potential (mOEP) was measured in RG **(A)** and WG **(B)** with succinate (left) or glutamate/malate/rotenone (right) and in the absence (white bars) or presence (gray bars) of 10 μM H89 in the assay media. Auranofin (1 μM) was added to specifically measure H_2O_2 production, and an "oxidant scavenging index" was determined based on the increase in mOEP following addition of auranofin. $N = 4$–8/condition. * denotes $p < 0.05$ compared to Control.

compounds marketed as sAC inhibitors: KH7 (Hess et al., 2005; Acin-Perez et al., 2009a,b, 2010) and 2-HE (Steegborn et al., 2005; Tian et al., 2011; Di Benedetto et al., 2013). In this report, we provide evidence demonstrating that both KH7 and 2-HE have distinct non-specific effects that preclude their use for studying mitochondrial energetics. First, KH7 directly inhibits mitochondrial respiration independent of cAMP/PKA signaling (**Figure 2A**) (Di Benedetto et al., 2013), and it appears to do so via direct inhibition of complex I (**Figures 2B,C**). Second, 2-HE, a naturally occurring estrogen metabolite, spontaneously generates high rates of H_2O_2 (**Figures 3A–C**), potentially affecting redox buffering systems and thus the oxidation state of mitochondrial proteins. Previous studies (Hess et al., 2005; Steegborn et al., 2005; Acin-Perez et al., 2009a,b, 2010; Tian et al., 2011; Di Benedetto et al., 2013) using one or both of these compounds to examine the link between cAMP/PKA signaling and mitochondrial function should therefore be interpreted with caution. The recently elucidated crystal structure of human sAC during catalysis and activation via HCO_3^- (Kleinboelting et al., 2014) will hopefully facilitate the development of sAC inhibitors with greater specificity.

The impact of membrane permeable cAMP analogs on mitochondrial bioenergetics has also produced conflicting data. In both intact cells and isolated mitochondria, Acin-Perez et al. (2009b) found that 8Br-cAMP induced a slight but statistically significant increase in respiration under both basal and maximally-stimulated respiration conditions. ATP synthesis rate and mitochondrial membrane potential under non-phosphorylating conditions were also increased by 8Br-cAMP (Acin-Perez et al., 2009b). Di Benedetto et al. (2013) however failed to observe any impact of 8Br-cAMP, or several other more permeable analogs, on mitochondrial ATP concentration in intact cells. In the present study, 8Br-cAMP also failed to increase either basal or ADP-stimulated respiration in isolated liver mitochondria (**Figure 2A**).

However, the notion that a mitochondrial matrix sAC-cAMP-PKA axis regulates OXPHOS is supported by multiple lines of direct and indirect evidence (Raha et al., 2002; Acin-Perez et al., 2009b; Valenti et al., 2011; Di Benedetto et al., 2013), including the recent finding that numerous electron transport proteins in mouse liver and skeletal muscle originally identified in the MitoCarta (Pagliarini et al., 2008) have PKA consensus phosphorylation sites (Zhao et al., 2011; Grimsrud et al., 2012). Using cAMP-specific FRET sensors, two groups (Di Benedetto et al., 2013; Lefkimmiatis et al., 2013) have recently provided the most direct evidence that cAMP is produced inside mitochondria by sAC in response to increased matrix Ca^{2+} and, to a lesser extent, HCO_3^-. Additionally, literature spanning over 20 years implicates complex I as a target of PKA-dependent phosphorylation (Technikova-Dobrova et al., 1993; Sardanelli et al., 1995; Papa et al., 1999). In the current study, PKA inhibition was found to dose-dependently decrease complex I, but not complex II, supported respiration (**Figures 4A,B**), thus providing further direct evidence that PKA-mediated phosphorylation plays an important role in the regulation of complex I activity.

In PmFBs, H89-mediated inhibition of PKA elicited effects on mitochondrial respiratory kinetics, respiratory capacity, and oxidant emission that converged on complex I. Increased sensitivity of OXPHOS to both ADP (**Figures 5C,D**) and complex I-supported respiratory substrates (**Figures 6C,D,G,H**), combined with decreased maximal respiratory capacity, suggest a "bottleneck" in the ETS established by PKA inhibition. The effects of PKA inhibition were qualitatively similar in PmFBs from predominantly red oxidative and white glycolytic muscles, suggesting the mitochondrial phospho-regulatory mechanisms are similar in the two fiber types. To elucidate how PKA may regulate complex I-mediated electron flow, two substrate/inhibitor combinations were used to examine forward or reverse electron flow. The flavin (F) site of complex I is responsible for NADH reduction and the majority of electron leak from forward electron flow (Treberg et al., 2011).

H_2O_2 emitting potential at this site was not affected by H89 (**Figures 7A,B**). The quinone (Q) site of complex I is responsible for donating electrons to the Q pool and accounts for the majority of electron leak that can occur from reverse electron flow during respiration supported by the complex II substrate succinate. Inhibition of PKA decreased electron leak from the Q-site during reverse electron flow but not from the F-site during forward electron flow (**Figures 7A,B**), suggesting that PKA regulates complex I activity somewhere between these two sites of electron transfer. This is of particular interest because the nuclear-encoded 18 kDa subunit of complex I physically lies in between these two sites of electron transfer, is exposed to the mitochondrial matrix (Baradaran et al., 2013), and is a physiologically relevant site of PKA-mediated phosphorylation (Sardanelli et al., 1995; Papa et al., 1999; Papa, 2002). Loss of the gene that encodes this subunit (NDUFS4) in mice replicates Leigh syndrome (Quintana et al., 2010; Johnson et al., 2013), a devastating human neurological, mitochondrial-linked disease. Although the NDUFS4 subunit is not thought to be directly involved in electron transfer, it is possible that PKA-mediated phosphorylation within this subunit alters electron transfer and/or (Lochner and Moolman, 2006) complex I function in a currently undefined manner.

The finding that PKA inhibition lowered mitochondrial JH_2O_2 emission in PmFBs is intriguing, as it suggests that activation of PKA signaling may accelerate mitochondrial JH_2O_2 emission. However, in the present studies, addition of 8Br-cAMP to activate PKA signaling failed to alter ADP-stimulated respiratory capacity, and accelerating TCA cycle flux failed to enhance cAMP levels. Along these same lines, calcium has recently been shown to enhance the driving forces of the oxidative phosphorylation system, although the effect is seen only when calcium is depleted from mitochodria prior to calcium stimulation (Glancy et al., 2013). Together, these findings were interpreted to suggest that calcium, and potentially PKA signaling, may be already relatively high/active in isolated mitochondria, and thus experiments to further activate PKA signaling were not pursued.

A caveat to experiments using H89 is that this drug is not entirely specific for PKA (Davies et al., 2000; Lochner and Moolman, 2006). However, the data provided are in agreement with previous reports demonstrating a role for PKA in the regulation of complex I in other tissues (Sardanelli et al., 1995; Papa et al., 1999; Papa, 2002). In the present study, H89 was chosen because a primary objective of this project was to compare findings in muscle PmFBs to previous findings in isolated liver mitochondria (Acin-Perez et al., 2009a,b; 2010; 2011). Interestingly, a recent report in isolated rat liver mitochondria suggests that hydrogen sulfide (H_2S) is capable of regulating mitochondrial respiration (Módis et al., 2013), possibly via inhibition of phosphodiesterase 2A (PDE2A), a mitochondrial PDE isoform (Acin-Perez et al., 2011). Furthermore, in this report (Módis et al., 2013), the authors found that the inhibitory cAMP analog Rp-cAMP decreased complex II-supported respiration, although complex I-supported respiration was not reported. It remains to be seen whether PKA-mediated regulation of skeletal muscle complex I activity occurs with alternative PKA inhibitors like Rp-cAMP.

In conclusion, this report provides a novel collection of studies that: (1) challenge the notion that mitochondrial cAMP is regulated by TCA cycle flux, (2) reveal significant non-specific effects of widely used sAC inhibitors, and (3) provide the first functional evidence of PKA regulation of complex I in mouse muscle and liver mitochondria. These findings are of physiological significance particularly because they suggest that cAMP/PKA signaling not only regulates mitochondrial respiration, but also oxidant production. There is promise in the possibility that the mitochondrial cAMP/PKA axis can be manipulated to improve skeletal muscle metabolic health. Future studies await the development of targeted genetic approaches to more mechanistically examine the physiological role of mitochondrial cAMP-PKA signaling in health and disease.

AUTHOR CONTRIBUTIONS

DL and DN designed the experiments with input from LR, TR, MT, CS, and CL. DL, LR, and TR performed experiments. DL, CL, and DN analyzed data and prepared figures. DL and DN drafted the manuscript. DL, TR, MT, CS, CL, and DN edited the paper to the final version.

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Skeletal Muscle Mitochondrial Bioenergetics and Morphology in High Fat Diet Induced Obesity and Insulin Resistance: Focus on Dietary Fat Source

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It has been suggested that skeletal muscle mitochondria play a key role in high fat (HF) diet induced insulin resistance (IR). Two opposite views are debated on mechanisms by which mitochondrial function could be involved in skeletal muscle IR. In one theory, mitochondrial dysfunction is suggested to cause intramyocellular lipid accumulation leading to IR. In the second theory, excess fuel within mitochondria in the absence of increased energy demand stimulates mitochondrial oxidant production and emission, ultimately leading to the development of IR. Noteworthy, mitochondrial bioenergetics is strictly associated with the maintenance of normal mitochondrial morphology by maintaining the balance between the fusion and fission processes. A shift toward mitochondrial fission with reduction of fusion protein, mainly mitofusin 2, has been associated with reduced insulin sensitivity and inflammation in obesity and IR development. However, dietary fat source during chronic overfeeding differently affects mitochondrial morphology. Saturated fatty acids induce skeletal muscle IR and inflammation associated with fission phenotype, whereas ω-3 polyunsaturated fatty acids improve skeletal muscle insulin sensitivity and inflammation, associated with a shift toward mitochondrial fusion phenotype. The present minireview focuses on mitochondrial bioenergetics and morphology in skeletal muscle IR, with particular attention to the effect of different dietary fat sources on skeletal muscle mitochondria morphology and fusion/fission balance.

Keywords: mitochondrial fusion, mitochondrial fission, lard, fish oil, omega-3 fatty acids

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INTRODUCTION

Skeletal muscle seems to play a central role in whole body insulin resistance (IR) and metabolic syndrome associated with high fat (HF) feeding, obesity and aging (see Corpeleijn et al., 2009; DeFronzo and Tripathy, 2009; Lark et al., 2012).

Some evidence suggested that cytosolic ectopic accumulation of fatty acids (FA) metabolites, such as diacylglycerols (DAG) and/or Ceramides, (Yu et al., 2002; Adams et al., 2004), underlies IR development in skeletal muscle (lipotoxicity theory) (reviewed in Lark et al., 2012). Numerous evidence has also suggested a link between elevated systemic and tissue inflammation with IR

(inflammatory theory) (see Shenk et al., 2008; Lark et al., 2012). The effectors of IR in HF diet-induced inflammation are suggested to involve hyperactivation of stress-sensitive Ser/Thr kinases, such as JNK and IKKβ, which in turn inhibits insulin receptor/IRS1 axis. Several mechanisms were proposed to explain the link between inflammation and IR: endoplasmic reticulum (ER) stress (Ozcan et al., 2004; Lionetti et al., 2009; Mollica et al., 2011), oxidative stress (Lark et al., 2012), signaling through inflammation-associated receptors, such as TLR4 signaling (Uysal et al., 1997; Shi et al., 2006), and partitioning/activation of c-SRC (a key mediator of JNK activation) by saturated FA (Holzer et al., 2011; **Figure 1A**).

In recent years, different reviews focused on mechanism(s) by which mitochondrial bioenergetics (Fisher-Wellman and Neufer, 2012; Lark et al., 2012; Muoio and Neufer, 2012; Holloszy, 2013) and morphology (Liesa and Shirihai, 2013; Montgomery and Turner, 2015) may be linked to the etiology of IR in skeletal muscle. In the present review, the challenging debate on the involvement of mitochondrial dysfunction in IR will be briefly reviewed. Then, the main aim of the review will be to underline the importance of including mitochondrial morphology/dynamics and dietary fat source in the debate on skeletal muscle mitochondria involvement in IR etiology and to highlight the need of further research studies to clarify the involved mechanism(s).

SKELETAL MUSCLE MITOCHONDRIAL BIOENERGETICS AND IR

Two leading theories on mechanisms underlying skeletal muscle IR onset focus on mitochondria, although with opposite views (**Figure 1B**). In one theory, mitochondrial dysfunction is suggested to cause intramyocellular lipid accumulation leading to IR (Kelley et al., 1999; Lowell and Shulman, 2005; reviewed in Civitarese and Ravussin, 2008; Montgomery and Turner, 2015). In this case, the strategies to accelerate flux through β-oxidation should improve insulin sensitivity. In the second theory, excess fuel within mitochondria in the absence of increased energy demand stimulates mitochondrial oxidant production and emission, ultimately leading to the development of IR (Fisher-Wellman and Neufer, 2012; **Figure 1B**). In this case, elevated flux through β-oxidation without added energy demand is viewed as an underlying cause of IR disease (Muoio and Neufer, 2012).

Several studies have revealed a reduction in skeletal mitochondrial mass in obesity and type 2 diabetes (Kelley et al., 2002; Morino et al., 2005; Ritov et al., 2005), decreased ATP synthesis in insulin resistant offspring of patients with type 2 diabetes (Petersen et al., 2004, 2005) and decreased maximal respiration rates in skeletal muscle isolated mitochondria from type 2 diabetics (Mogensen et al., 2007). Moreover, with the limitation that gene expression is not a direct assessment of function itself, HF diet has been shown to coordinately down-regulate genes required for mitochondrial oxidative phosphorylation in human and rodents skeletal muscle (Sparks et al., 2005). Interestingly, in skeletal muscle from obese or diabetic patients, decreased activity of electron transport chain and reduced number of mitochondria have been mainly reported in skeletal muscle mitochondria located beneath the sarcolemmal membrane (SS mitochondria) (Ritov et al., 2005). SS mitochondria also displayed lower respiratory capacities in presence of succinate as substrates in adult rats exhibiting HF diet-induced IR (Lionetti et al., 2007). The two mitochondrial subpopulations (SS and intermyiofibrillar, IMF) are differentiated not only by the different localization but also by the different functions (Cogswell et al., 1993; Mollica et al., 2006): SS mitochondria could be more affected by the impairing effect of saturated FA due to their localization beneath the sarcolemmal membrane.

However, although correlative studies seem to implicate mitochondrial dysfunction and impaired β-oxidation as predisposing risk factors for IR, still uncertain is whether diminished fat oxidation reflects a cause or a late stage consequence of the disease process (reviewed in Muoio, 2010). In fact, obese/diabetic humans never use their mitochondrial capacity for lipid oxidation; therefore, a marginal decline in oxidative potential has little relevance in causing lipotoxicity and IR in sedentary individuals. Moreover, it has been suggested that early stages of obesity and IR are accompanied by increased, rather than reduced β-oxidation (Muoio, 2010). These findings question the concept that mitochondrial dysfunction is a primary cause of IR (Hoeks et al., 2010, 2011), as also underscored by the study of Bonnard et al. (2008), showing mitochondrial dysfunction in skeletal muscle after 16 weeks, but not after 4 weeks HF feeding, while muscle IR was observed after both 4 and 16 weeks of HF feeding. In addition, mitochondrial deficiency, severe enough to impair fat oxidation in resting muscle, cause an increase, not a decrease, in insulin action (reviewed in Holloszy, 2013). Altogether, these studies suggest that deficiency of mitochondria in muscle does not cause IR (reviewed in Holloszy, 2013).

An alternative mechanism to explain the connection between mitochondria and IR focused on reactive oxygen species (ROS) production (reviewed in Muoio and Neufer, 2012; Holloszy, 2013). Lefort et al. (2010) showed normal oxidative capacity, decreased mitochondrial mass and high rates of ROS production in mitochondria isolated from skeletal muscle of obese insulin resistant individuals. Moreover, FA overload within the mitochondria results in the accumulation of partially oxidized acyl-carnitines, increased mitochondrial hydrogen peroxide (H_2O_2) emission and a shift to a more oxidized intracellular redox environment (Anderson et al., 2009; reviewed in Lark et al., 2012). H_2O_2 emission may induce IR by directly targeting protein involved in the glucose uptake process. On the other hand, given the sensitivity of cellular phosphatase to redox state, it has been suggested that elevated mitochondrial H_2O_2 production may decrease phosphatase tone in cells, increasing the inhibition state of insulin signaling proteins by stress-sensitive kinases (reviewed in Lark et al., 2012).

Although the primary role of skeletal muscle mitochondrial dysfunction in the pathogenesis of IR and type 2 diabetes is under debate (Hoeks et al., 2010, 2011; Holloszy,

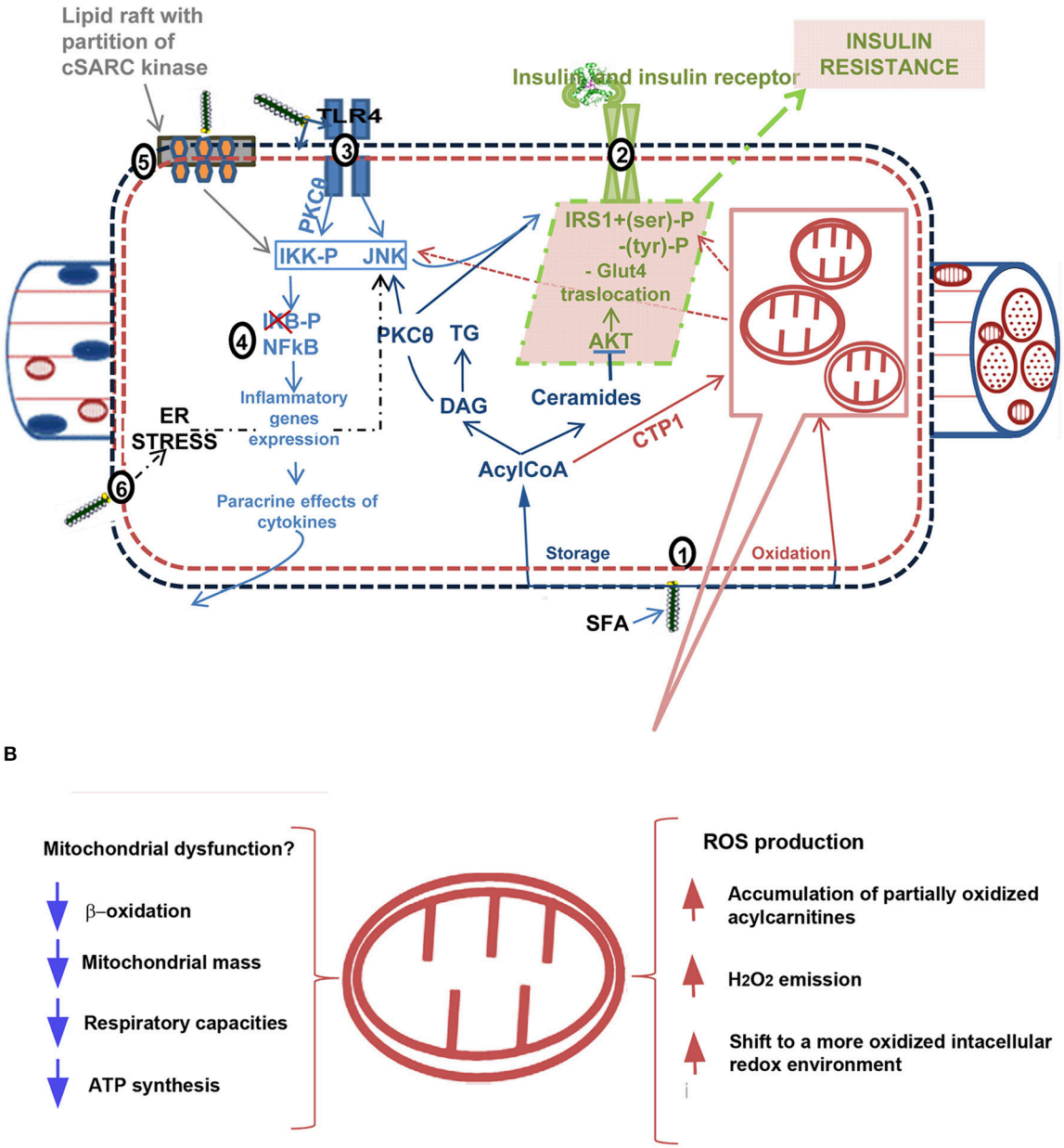

FIGURE 1 | Mechanism linking excess fatty acids to insulin resistance in skeletal muscle. (A): (1) Excess free fatty acids (FFAs) are esterified in AcylCoAs, substrates involved in both synthetic and oxidative pathways. In the synthetic pathway, they are either stored as Triacylglycerols (TG) in lipid droplets or accumulated in metabolites (DAGs, Ceramides) that act as signaling molecules and may interfere with normal cellular signaling. DAGs are associated with membrane translocation and activation of Protein kinase C theta (PKC-θ), increased IRS1 serine/threonine phosphorylation and decreased insulin-stimulated IRS1 tyrosine phosphorylation, whereas Ceramides impair insulin action by inhibiting protein kinase PKB/AKT (dark blue). In the oxidative pathway, AcylCoAs are imported into the mitochondria by carnitine palmitoyltransferase-1 (CPT-1) shuttle and degraded via β-oxidation (in purple). (2) Insulin signaling pathway impaired by excess FFA (in green). Among FFAs, saturated FAs (SFAs) stimulate the activation of several inflammatory pathways. (3) Receptor-mediated mechanisms, as those of Toll like receptors 2/4 (TLR 2/4), activate serine kinases inhibitor kappaB kinase (IKK) and c-JUN NH_2-terminal kinase (JNK). The activation of PKCθ also contributes to IKK and JNK activation. Altogether, these kinases catalyze IRS1 serine phosphorylation and lead to a reduction of insulin-induced IRS1 tyrosine phosphorylation, impairing insulin action. Moreover, IKK/NFkB axis (4) triggers expression of inflammatory genes with cytokines production (e.g., Tumor necrosis factor, TNFα), which in turn activate intracellular pathways promoting insulin resistance development (in light blue). (5) SFAs enter the cellular membranes and incorporate into them reducing membrane fluidity and

(Continued)

FIGURE 1 | Continued
creating or expanding subdomains rich in cholesterol and sphingolipids (lipid raft). They induce clustering and activation of cytosolic cSRC. cSRC activity is required for JNK1 activation and inhibition of insulin signaling (in grey). (6) Endoplasmic reticulum stress (ER stress), induced by lipotoxicity, contributes to activate inflammatory pathways and impair insulin signaling. **(B)**: Putative role of mitochondria in development of IR. Mitochondrial dysfunction in presence of excess FFAs leads to intramyocellular lipid accumulation due to impaired β-oxidation. Decreased mitochondrial mass, respiratory capacities and ATP synthesis have been found in obesity and diabetes. Alternatively, excess FFA within mitochondria in the absence of increased energy demand stimulates oxidative stress with high rates of ROS production and H_2O_2 emission and a shift to a more oxidized intracellular redox environment, ultimately leading to the development of IR.

2013), it is generally accepted that in this disease a mitochondrial defect occurs, possibly secondary to a fat intake increase.

MITOCHONDRIAL MORPHOLOGY AND SKELETAL MUSCLE IR

It is well known that mitochondrial morphology is highly variable, ranging between long tubular mitochondria and short circular ones and it is maintained through a dynamic balance between fusion and fission processes (Westermann, 2010, 2012), which allow mitochondria to redistribute in a cell, exchange contents and repair damaged mitochondria. These two opposing processes are finely regulated by mitochondrial fusion proteins mitofusins 1 and 2 (Mfn1 and Mfn2), and optic atrophy gene 1 (OPA1) (Cipolat et al., 2004; Palmer et al., 2011) and by mitochondrial fission protein dynamin-related protein 1 (DRP1) and fission protein 1 (Fis1) (Nunnari et al., 2002; Liesa et al., 2009).

Several pieces of evidence suggested that mitochondrial dynamic behavior play a key role in mitochondrial health, bioenergetics function, quality control, and cell viability. Notably, disruption of mitochondrial dynamics has been found in IR and type 2 diabetes (Bach et al., 2003, 2005; Yu et al., 2006; Liesa and Shirihai, 2013).

The group of Zorzano showed that decreased expression of Mfn2 and altered expression of OPA1 participated in obesity and type 2 diabetes development in both patients and rodent models (Bach et al., 2003; Zorzano et al., 2009a,b, 2010; Hernández-Alvarez et al., 2010; Quirós et al., 2012). In obese Zucker rats, skeletal muscle mitochondrial network was reduced by 25% associated with a repression of Mfn2 (Bach et al., 2003). In addition, skeletal muscle of obese subjects and type 2 diabetic patients also showed a reduced expression of Mfn2 mRNA and Mfn2 protein compared to lean subjects (Bach et al., 2003, 2005). Mfn2 repression was detected in the skeletal muscles of both obese and non-obese type 2 diabetic patients (Bach et al., 2005). Notably, the expression of one of the mitochondrial proteases involved in OPA1 processing, presenilin-associated rhomboid-like (PARL), was also reduced in diabetic animals. In humans, a positive linear correlation between PARL mRNA levels and insulin sensitivity has been reported (Walder et al., 2005). These data suggest multiple alterations in mitochondrial fusion in IR. However, reduction of Mfn2 expression together with decreased mitochondrial size in skeletal muscle in obesity and type 2 diabetes states allow proposing a relevant role for Mfn2 in IR (Civitarese and Ravussin, 2008; Zorzano et al., 2009a,b). In agreement with this suggestion, a positive correlation between Mfn2 expression in skeletal muscle and insulin sensitivity has been reported (Bach et al., 2005). It is of interest that the involvement of Mfns in diet-induced obesity via the regulation of leptin resistance and systemic energy metabolism was also revealed (Dietrich et al., 2013; Schneeberger et al., 2013; reviewed in Putti et al., 2015). Moreover, it has been suggested that there is an association between increased mitochondrial fission, mitochondrial bioenergetics and fat induced-IR in skeletal muscle (Jheng et al., 2012). Indeed, in differentiated C2C12 muscle cells mitochondrial fragmentation and increased mitochondrion associated-DRP1 and Fis1 was induced by excess palmitate and this fission phenotype was associated with increased oxidative stress, loss of ATP production and reduced insulin-stimulated glucose uptake. These authors also found smaller and shorter mitochondria and increased mitochondrial fission machinery in the skeletal muscle of mice with genetic or diet-induced obesity. Furthermore, inhibition of mitochondrial fission improved muscle insulin signaling and systemic insulin sensitivity in obese mice (Jheng et al., 2012).

A shift toward fission was also found in skeletal muscle of HF diet (HFD)-induced obese mice by Liu et al. (2014). Notably, these authors also faced the question of whether mitochondrial dynamics exists in skeletal muscle *in vivo*. It should be considered that mitochondria in skeletal muscle are rigidly located between bundles of myofilaments in a highly regular "crystal like" pattern (Vendelin et al., 2005) and therefore, their motility and dynamics may be very restricted. Liu et al. (2014) confirmed that mitochondria are dynamic organelles in skeletal muscle *in vivo*, by demonstrating that they exchange contents within the whole mitochondrial population through nanotunneling-mediated mitochondrial fusion. In this way, mitochondria can bypass the restriction of myofilament and exchange mitochondrial matrix contents even if they are distant. This dynamic communication among mitochondria in skeletal muscle may protect from injury by preventing the accumulation of detrimental metabolites. Liu et al. (2014) also showed that this dynamic behavior was inhibited in skeletal muscle of HFD–induced obese mice associated with decreased Mfn2 and increased Fis1 and DRP1 expression compared to normal diet fed mice. This impaired mitochondrial fusion in skeletal muscle of HFD-induced obese mice was accompanied with damaged mitochondrial respiratory function and decreased ATP content. Therefore, the authors suggest that mitochondrial dynamics play an important role in regulating mitochondrial function, including respiration rate and ATP production (Liu et al., 2014).

DIETARY FAT SOURCE DIFFERENTLY AFFECT SKELETAL MUSCLE IR AND MITOCHONDRIAL MORPHOLOGY

It has been suggested that diverse dietary fat sources have different effects on obesity and associated diseases development. Saturated FA are well known to induce both obesity and related disease, whereas omega 3 polyunsaturated FA (ω-3 PUFA) from fish oil have been shown in many studies to protect against these metabolic diseases (Xin et al., 2008; Gonzalez-Periz et al., 2009; Abete et al., 2011). The effect of ω-3 PUFA on metabolic disease has been extensively studied during the past three decades since the first studies such as the one by Storlien et al. (1987) showing that fish oil prevents IR induced by high-fat feeding in rats. Further studies confirmed that ω-3 PUFA had an anti-obesity effect and enhanced insulin sensitivity and glucose homeostasis in rodent models of IR: the replacement of a small proportion of the diet with ω-3 PUFAs from fish oil completely prevents the development of skeletal muscle IR (Storlien et al., 1991; Fryer et al., 1995). Recent studies hypothesized that ω-3 PUFAs protect glucose tolerance, in part by preventing the accumulation of bioactive lipid mediators that interfere with the insulin signaling pathway (Lanza et al., 2013). Lanza et al. (2013) evaluated the influence of dietary ω-3 PUFAs on mitochondrial physiology and muscle lipid metabolites in the context of 10 weeks high-fat feeding in mice. They found a lower content of long-chain Acyl-CoAs and Ceramides in the presence of fish oil, whereas mitochondrial oxidative capacity was similarly increased with or without fish oil. Several studies have also indicated that ω-3 PUFAs possess anti-inflammatory properties that prevent and reverse the development of IR in mice which are fed a high-fat diet in an adiponectin-dependent manner (Kalupahana et al., 2010, 2011). On the other hand, unsaturated FA prevent c-Src membrane partitioning and activation and block JNK activation with consequent beneficial effects on insulin sensitivity (Holzer et al., 2011). Considering the anti-inflammatory properties of ω-3 PUFAs, in a recent study, we compared the effects of chronic high-fish oil and high-lard diets on obesity-related inflammation by evaluating serum and tissue adipokine levels and histological features in insulin-sensitive tissues (white adipose tissue, liver and skeletal muscle) (Lionetti et al., 2014b). We showed that the replacement of lard (saturated FA) with fish oil (ω-3 PUFAs) in chronic high-fat feeding attenuated the development of systemic and tissue inflammation. Indeed, compared with a high-lard diet, a high-fish oil diet resulted in a lower degree of systemic inflammation and IR that were associated with a lower ectopic lipid depot, inflammation degree and IR in the skeletal muscle (Lionetti et al., 2014b). In a further study on the same experimental design, we confirmed that the replacement of lard with fish oil in HF diet had preventive effects on obesity and systemic inflammation and IR development as well as we showed a fusion mitochondrial phenotype in association with the improvement of IR in skeletal muscle (Lionetti et al., 2013). As for preventive effects on obesity, body weight gain after 6 weeks of HF diet was lower in fish oil fed rats compared to lard fed rats. As for skeletal muscle IR, we showed that high-lard diet induced a defect in the skeletal muscle insulin signaling pathway with a lower immune-reactivity to IRS1 and pIRS (Tyr632), in agreement with other authors (Yaspelkis et al., 2009; Yuzefovych et al., 2013). On the other hand, a high fish oil diet elicited IRS1 and pIRS (Tyr632) immune-reactivity similar to a control diet, in agreement with ameliorated systemic insulin sensitivity (Lionetti et al., 2013). We cannot exclude the possibility that the fish oil protective effect was due to indirect effects of differences on adiposity. We also showed that the beneficial effects of fish oil feeding on skeletal muscle IR development was associated with changes in protein involved in mitochondrial dynamic behavior, with a greater number of immunoreactive fibers for Mfn2 and OPA1 proteins, as well as a weaker immunostaining for DRP1 and Fis1 compared to high lard feeding. Skeletal muscle electron microscopy observations also suggested a prominent presence of fission events in high-lard diet fed rats, and fusion events in high-fish oil diet fed rats (Lionetti et al., 2013).

The finding on the effects of different dietary FA on skeletal muscle mitochondrial fusion/fission proteins may be associated with effects on inflammatory processes involved in IR development. Indeed, Bach et al. (2005) suggested that TNF-α inhibits Mfn2 gene expression in cells in culture, suggesting that inflammatory parameters may play a regulatory role on Mfn2. In agreement, we showed that pro-inflammatory dietary saturated FA reduced Mfn2 expression and induced fission phenotype in skeletal muscle (Lionetti et al., 2013). On the other hand, the anti-inflammatory effect of dietary ω-3 PUFAs was associated with no reduction in skeletal muscle Mfn2 content and a tendency to mitochondrial fusion. This shift toward fusion may be an adaptive mechanism to counteract cellular stress induced by chronic HF diet, by allowing functional mitochondria to complement dysfunctional mitochondria. In accordance, myocytes cultured with docosahexaenoic acid exhibited a higher mitochondrial mass with a higher proportion of large and elongated mitochondria with downregulation of fission genes DRP1 and Fis1 (Casanova et al., 2014).

The pro-fusion effect of ω-3 PUFAs dietary fat on skeletal muscle mitochondria is in agreement with the results found in liver mitochondria, where a shift toward mitochondrial fusion phenotype was also suggested (Zhang et al., 2011; Lionetti et al., 2014a).

The mechanism underlying fish oil/ω-3 PUFAs mitochondrial fusion stimulation may involve receptor-mediated signaling and/or lipid membrane composition, among other factors. Indeed, ω-3 PUFAs are incorporated into cellular membranes and may affect lipid-protein interactions as well as membrane fluidity, and therefore the function of embedded proteins. Recently, it has been suggested a role for ω-3 PUFAs in reorganizing the composition of the mitochondrial membrane, while promoting improvements in ADP sensitivity, determined as mitochondrial responses during ADP titration (Herbst et al., 2014). Moreover, it is well known that saturated FA incorporation reduces membrane fluidity, whereas PUFA do not have such effect (Clamp et al., 1997; Stulnig et al., 2001; Holzer et al., 2011). Further studies are needed to elucidate these mechanisms.

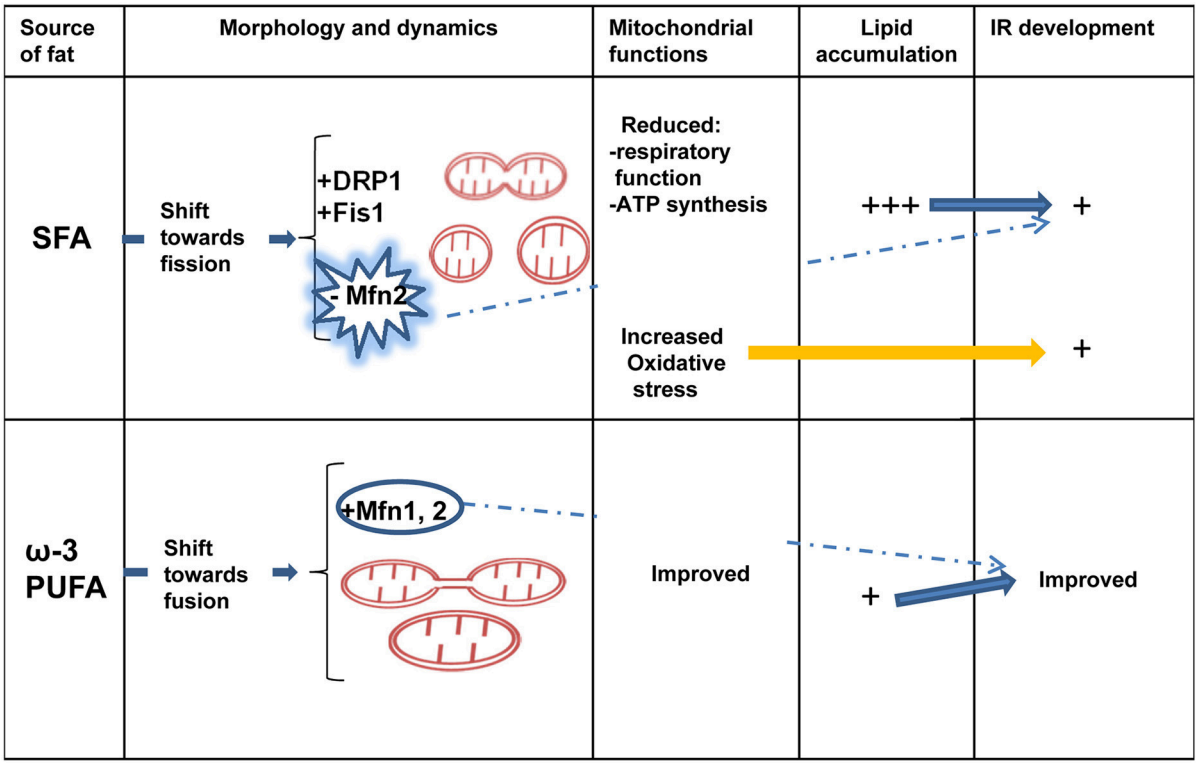

FIGURE 2 | Dietary fat source, mitochondrial dynamics and skeletal muscle IR. Diverse dietary fat sources induce different effects on mitochondrial morphology/dynamics and IR development in skeletal muscle. Saturated fatty acids (SFA) elicit a shift toward fission processes (decreased Mfn2 and increased Fis1 and DRP1 expression) associated with reduced mitochondrial function, increased oxidative stress, lipid accumulation and IR development. Ω-3 polyunsaturated fatty acids (PUFAs) induce Mfn2 expression and fusion processes associated with less lipid accumulation and improved IR. IR, insulin resistance; Mfn2, mitofusin 2; DRP1, dynamin-related protein 1; Fis1, fission protein 1.

CONCLUSIONS

Recent evidence highlighted an association between mitochondrial morphology and IR development in skeletal muscle. Few works on different dietary fat source started to underline the different effect of saturated and ω-3 PUFAs on skeletal muscle IR and mitochondrial protein involved in dynamics behavior, suggesting an association between beneficial protective effect of ω-3 PUFAs toward IR development and mitochondrial fusion phenotype (**Figure 2**). However, it is important to underline that most of the data present in literature on skeletal muscle mitochondrial morphology and IR are from associational studies. Therefore, there is an urgent requirement for *in vivo* mechanistic studies to confirm the associational relationship between mitochondrial morphology/dynamics and IR development.

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REVIEW
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Utilizing small nutrient compounds as enhancers of exercise-induced mitochondrial biogenesis

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Endurance exercise, when performed regularly as part of a training program, leads to increases in whole-body and skeletal muscle-specific oxidative capacity. At the cellular level, this adaptive response is manifested by an increased number of oxidative fibers (Type I and IIA myosin heavy chain), an increase in capillarity and an increase in mitochondrial biogenesis. The increase in mitochondrial biogenesis (increased volume and functional capacity) is fundamentally important as it leads to greater rates of oxidative phosphorylation and an improved capacity to utilize fatty acids during sub-maximal exercise. Given the importance of mitochondrial biogenesis for skeletal muscle performance, considerable attention has been given to understanding the molecular cues stimulated by endurance exercise that culminate in this adaptive response. In turn, this research has led to the identification of pharmaceutical compounds and small nutritional bioactive ingredients that appear able to amplify exercise-responsive signaling pathways in skeletal muscle. The aim of this review is to discuss these purported exercise mimetics and bioactive ingredients in the context of mitochondrial biogenesis in skeletal muscle. We will examine proposed modes of action, discuss evidence of application in skeletal muscle *in vivo* and finally comment on the feasibility of such approaches to support endurance-training applications in humans.

Keywords: mitochondrial biogenesis, skeletal muscle, bioactives, nutraceuticals, exercise mimetics

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INTRODUCTION

Endurance training leads to increased rates of fat oxidation and glycogen synthesis in skeletal muscle, which in turn contributes to enhanced endurance performance (Achten and Jeukendrup, 2004). As such, nutritional and training strategies aimed to maximize these adaptive responses have been an area of intense investigation. Even though traditional nutritional supplementation strategies were primarily aimed at prolonging endurance performance (Maughan, 2002), increased understanding of the molecular regulation of skeletal muscle adaptation during, and in response to exercise, have led to contemporary approaches utilizing pharmacological compounds, functional foods, or small naturally occurring bioactive ingredients to substitute for, or augment the training response. The aim of this review is to (1) discuss the benefits of regular exercise training on whole body and skeletal muscle-specific adaptation, (2) introduce the concept of exercise mimetics and

discuss their feasibility in skeletal muscle *in vivo*, (3) critique the literature detailing the use of small nutritional bioactive ingredients as modulators of mitochondrial function in skeletal muscle *in vitro* and *in vivo*, and finally (4) discuss the efficacy of each approach for use in humans.

ENDURANCE EXERCISE AND AEROBIC ADAPTATIONS

Regular physical activity in the form of endurance training can substantially improve endurance capacity in a range of populations (Ferketich et al., 1998; Gibala et al., 2006; Alemo Munters et al., 2013). This is achieved both by an increased maximal oxygen uptake ($\dot{V}O_2$ max) and the ability to work at a given submaximal intensity with a smaller homeostatic disturbance (Bassett and Howley, 2000). Maximal oxygen uptake is principally governed by the delivery of oxygen to the musculature by the cardiovascular system and, to a lesser degree, the removal of oxygen from the blood at the exercising muscles (Saltin and Strange, 1992; Montero et al., 2015). Following regular bouts of aerobic exercise, left-ventricular hypertrophy, increased myocardial contractility, and increased end-diastolic volume increases stroke volume (Baggish et al., 2008; Bonne et al., 2014), with little to no difference in heart rate at maximal exercise intensities (Baggish et al., 2008; Murias et al., 2010; Bonne et al., 2014). Thus, maximal cardiac output increases as a result of greater stroke volume and is strongly related to the increase in $\dot{V}O_2$ max (Jones and Carter, 2000).

At submaximal exercise intensities and at rest, the required cardiac output remains similar, however the elevated stroke volume results in a compensatory reduction in heart rate, known as bradycardia (Spina, 1999). Additionally, systemic resistance is decreased following aerobic exercise training (Klausen et al., 1982). This has the additional health benefit of decreasing blood pressure (Klausen et al., 1982), thereby reducing the risk of coronary heart disease and stroke (Morris et al., 1980; MacMahon et al., 1990; Lee et al., 2003).

Arterial oxygen carrying capacity is primarily determined by red blood cell and hemoglobin concentration. During aerobic training, initial decreases in hematocrit and hemoglobin concentration have been identified, which can be attributed to the rapid increase in plasma volume and does not reflect a decrease in red blood cell count (Sawka et al., 2000). In fact after several weeks of endurance training hematocrit returns to pre-training levels, despite greater plasma volume, indicating an increased hemoglobin and red blood cell volume and, therefore, oxygen carrying capacity of the blood (Sawka et al., 2000; Bonne et al., 2014). Aerobic training also results in an increased number and density of capillaries per muscle fiber (Ingjer, 1979; Murias et al., 2011), allowing for a more efficient and homogenous distribution of the increased cardiac output with little to no change in transit time through the musculature (Saltin, 1985; Kalliokoski et al., 2001). Increased capillary density results in shorter diffusion distances (Saltin and Rowell, 1980) and together with possible increases in myoglobin concentration within working muscles (Harms and Hickson, 1983) increases the oxygen extraction capacity of the musculature. This results in a greater arteriovenous oxygen difference across the working muscles (Beere et al., 1999; Murias et al., 2010) and therefore increases oxygen delivery for oxidative phosphorylation in skeletal muscle mitochondria at a given blood flow. Together, increased cardiac output and greater oxygen delivery and extraction in the exercising muscles increases $\dot{V}O_2$ max (Spina, 1999).

While the cardiovascular system may limit maximal aerobic capacity, oxygen uptake at a given submaximal intensity is the same in the trained and untrained state (Hagberg et al., 1980). Exercise capacity at submaximal workloads is more closely related to adaptations in skeletal muscle (Bassett and Howley, 2000), which demonstrates considerable plasticity when exposed to different functional demands. Following endurance training, the shift in whole-body substrate oxidation toward greater lipid oxidation (Koivisto et al., 1982) and reduced glycolysis (Green et al., 1995) allows for a greater absolute exercise intensity to be supported predominantly by aerobic energy production. This results in reduced lactate accumulation in blood and muscle (Karlsson et al., 1972; Saltin et al., 1976; Bonen et al., 1998; Philp et al., 2008) and sparing of muscle glycogen stores at a given workload (Green et al., 1995), which play a pivotal role in the increased exercise capacity and performance following endurance training.

Endurance exercise training results in a shift toward a more oxidative, fatigue-resistant, phenotype of the trained muscle. An increased proportion of slow-twitch type I, fast oxidative type IIa, and hybrid fibers is apparent, with a reduction in rapidly fatiguing fast glycolytic type IIx and IIb fibers (Andersen and Henriksson, 1977; Simoneau et al., 1985; Fitts et al., 1989; Coggan et al., 1992). This is caused by hypertrophy of type I and type IIa fibers (Coggan et al., 1992) and a transformation of fibers to a slower phenotype, by an altered expression of myosin heavy chain isoforms (Putman et al., 2004). The shift toward a slower muscular phenotype is of physiological importance to endurance performance given the close relationship between muscle fiber composition and both the oxygen cost of locomotion and lactate threshold (Ivy et al., 1980).

Aerobic exercise promotes a large increase in mitochondrial mass, mitochondrial enzyme activity, and oxidation efficiency (Holloszy, 1967; Molé et al., 1971; Oscai and Holloszy, 1971; Hoppeler et al., 1973; Spina et al., 1996). Holloszy first demonstrated an increased mitochondrial enzyme activity in rats following progressive endurance training (Holloszy, 1967), a finding that has subsequently been replicated in numerous human studies (Gollnick et al., 1973; Hoppeler et al., 1973; Spina et al., 1996; Gibala et al., 2006; Little et al., 2010). The activity of enzymes in the electron transport chain, such as succinate dehydrogenase, NADH dehydrogenase, NADH-cytochrome-c reductase and cytochrome-c oxidase, can increase up to two-fold in response to training (Coggan et al., 1992). Concentrations of cytochrome-c also increase by approximately two-fold, suggesting the increased enzyme activity is due to an increase in mitochondrial enzyme protein content (Holloszy, 1967). Crucially, oxidative phosphorylation was tightly coupled, suggesting that the increase in electron transport capacity was

associated with a proportional increase in the capacity for ATP production by oxidative phosphorylation (Holloszy, 1967). Enzymes involved in the citric acid cycle (Holloszy et al., 1970), fatty acid oxidation (Molé et al., 1971), and ketone oxidation (Winder et al., 1974) also increase. However, mitochondrial enzymes do not respond in a uniform manner to endurance training. In response to the same exercise stimulus in rats, enzymes involved in the oxidation of fatty acids increase by approximately two-fold (Molé et al., 1971), whereas enzymes of the citric acid cycle only increase by up to 50% (Holloszy et al., 1970). Glycolytic enzymes such as creatine phosphokinase, adenylate kinase, and α-glycerophosphate dehydrogenase remain unchanged, or even decrease in activity when expressed per milligram of mitochondrial protein content (Holloszy and Oscai, 1969; Oscai and Holloszy, 1971). Therefore, regular endurance exercise results in an adaptive response to increase the capacity for ATP resynthesis by oxidative phosphorylation, especially from the oxidation of fatty acids, and in doing so reduces the reliance upon glycolysis.

MOLECULAR REGULATION OF SKELETAL MUSCLE MITOCHONDRIAL BIOGENESIS

The driving stimulus by which exercise initiates mitochondrial biogenesis in skeletal muscle has been an area of intense investigation in the past decade. Current opinion is that alterations in the cellular environment, as a consequence of skeletal muscle contraction, are a principal signal driving the adaptive response (**Figure 1**). One of the most defined energy sensors in skeletal muscle is the adenosine monophosphate (AMP)-activated protein kinase (AMPK), an enzyme complex that is allosterically activated through increased AMP:adenosine triphosphate (ATP) ratios and phosphorylated via calcium dependent signaling pathways (Steinberg and Kemp, 2009). The activation of AMPK following exercise is intensity dependent with intensities of 60% VO_2 peak reported to consistently induce activation (Chen et al., 2003). AMPK activity is amplified during exercise in a fasted or glycogen depleted state, following which it acutely stimulates increased rates of fat oxidation (Steinberg and Kemp, 2009). Once activated AMPK increases ATP production via an increase in lipid oxidation, by enhancing fatty acid uptake into skeletal muscle and increasing the transport of fatty acids into the mitochondria. The phosphorylation of acetyl-CoA carboxylase (ACC) by AMPK reduces the concentration of malonyl-CoA, which in turn reduces the allosteric inhibition of carnitine palmitoyltransferase 1 (CPT-1), allowing increased fatty acid transport into the mitochondria (Steinberg and Kemp, 2009).

In addition to the activation of AMPK, exercise also activates a number of other signaling intermediaries including, but not limited to, the nicotinamide adenine dinucleotide $(NAD)^+$ dependent protein deacetylases Sirtuin 1 (SIRT1) and 3 (SIRT3) (White and Schenk, 2012), the tumor suppressor p53 (Bartlett et al., 2014), (p38 MAPK) (Lluis et al., 2006), and calcium calmodulin-dependent protein kinase II (CaMKII) (Wright, 2007). Briefly, exercise alters the cellular redox state in skeletal muscle leading to an increase in the NAD^+:NADH ratio (White and Schenk, 2012). This subsequently results in an increase in the activation of SIRT1. SIRT1-mediated deacetylation of metabolic targets has been linked to transcriptional and post-translational regulation of intermediatory metabolism (White and Schenk, 2012). The tumor suppressor p53 has also been recently implicated in the regulation of mitochondrial function (Bartlett et al., 2014) given that following contraction, p53 has been reported to exhibit post-translational modification and alter its subcellular location (Saleem and Hood, 2013). Increases in the phosphorylation of p53, which is typically associated with an increase in activity and stability, have been reported in both rodent (Saleem and Hood, 2013) and human skeletal muscle (Bartlett et al., 2012) following exercise. The reported changes also act in a time course that could be related to the upstream signaling of either AMPK or p38MAPK (Bartlett et al., 2012). Following exercise, p38MAPK through a proposed calcium sensitive mechanism (Wright et al., 2007), is also activated and this regulates transcriptional events via phosphorylation (Bartlett et al., 2012). Numerous transcription factors and co-activators located in the cytosol and the nucleus are phosphorylated by p38MAPK, and loss of the gamma subunit of p38 blocks endurance training increases in mitochondrial biogenesis in mice (Pogozelski et al., 2009).

These signaling cascades ultimately converge on a host of co-activators and nuclear receptors that mediate the initiation of mitochondrial biogenesis (Perez-Schindler and Philp, 2015). Of note, the peroxisome proliferator-activated receptor-gamma co-activator (PGC-1α), a transcriptional co-activator that interacts with transcription factors at target gene promoters to increase transcriptional activity and promote mitochondrial remodeling (Puigserver et al., 1998) has received considerable attention in skeletal muscle. Transgenic activation of PCG-1α has been shown to mimic endurance training-induced adaptations including increased oxidative fiber content, mitochondrial biogenesis and angiogenesis (Yan et al., 2011), suggesting that activation of PGC-1α is a key driver of mitochondrial biogenesis is skeletal muscle. Further, following exercise the expression of PGC-1α shifts to an alternate promoter producing a smaller but more active form of the protein (Martínez-Redondo et al., 2015). The phosphorylation of PGC-1α by both AMPK and p38MAPK and deacetylation by SIRT1 are thought to increase its activity and translocation to the nucleus (Cantó and Auwerx, 2009; Dominy et al., 2010) (**Figure 1**). It is here that PGC-1α activates a number of transcription factors associated with mitochondrial biogenesis. These include nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2), peroxisome proliferator-activated receptor (PPARδ), estrogen-related receptor (EERα), and myocyte enhancer factor 2 (MEF2) (**Figure 1**). In addition, PGC-1α translocation to the nucleus enhances the transcription of mitochondrial-encoded proteins via increased expression of mitochondrial transcription factor A (Yan et al., 2011) (**Figure 1**). It is the cumulative effect of these signaling cascades that result in long term adaptations in skeletal muscle including the increased abundance of proteins involved in mitochondrial ATP production, the tricarboxylic acid (TCA) cycle and the transport and oxidation of fatty acids.

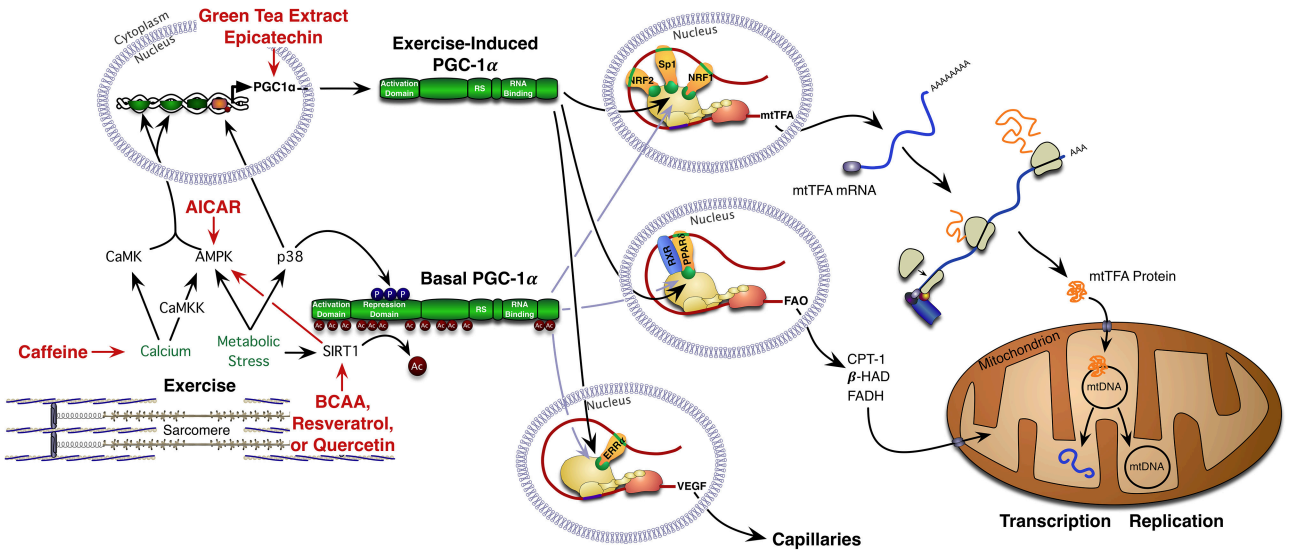

FIGURE 1 | Exercise-mediated mitochondrial biogenesis: exercise triggers mitochondrial biogenesis in skeletal muscle via the activation of numerous signaling pathways that ultimately converge on the transcriptional co-activator PGC-1α. Once activated, PGC-1α translocates to the nucleus to activate numerous transcription factors and nuclear receptors. Bioactive compounds have the capacity to enhance exercise-mediated mitochondrial biogenesis through contraction-dependent signaling cascades. AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, 5′ AMP-activated protein kinase; βHAD, beta-hydroxyacyl-CoA dehydrogenase; BCAA, branch chain amino acids; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKK, Ca(2+)/calmodulin-dependent protein kinase kinase; CPT-1, carnitine palmitoyltransferase I; ERRα, estrogen-related receptor alpha; FADH, flavin adenine dinucleotide; FAO, fatty acid oxidation; mRNA, messenger ribonucleic acid; mtDNA, mitochondrial deoxyribonucleic acid; mtTFA, mitochondrial transcription factor A; NRF1, nuclear respiratory factor-1; NRF2, nuclear respiratory factor-2; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARδ, peroxisome proliferator-activated receptor delta; RS, RS domain; RXR, retinoid X receptor; SIRT1, sirtuin-1; Sp1, specificity protein 1 transcription factor; VEGF, vascular endothelial growth factor.

THE SCIENTIFIC BASIS OF EXERCISE MIMETICS

Based on the premise that energy-sensing proteins such as AMPK and PPAR-δ are key signaling proteins modulating mitochondrial biogenesis in skeletal muscle, Narkar and coworkers examined the effect of administrating the PPAR-δ agonist GW501516 and the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) to mice (Narkar et al., 2008). The authors reported that GW501516 administration resulted in an increase in the mRNA expression of oxidative related genes such as Uncoupling Protein 3, CPT1, and Pyruvate Dehydrogenase Kinase 4, which was similar to the metabolic remodeling previously reported in PPAR-δ transgenic mice (Wang et al., 2004). However, despite the potent effects of GW501516 on oxidative gene expression, GW501516 treatment did not increase endurance capacity in supplemented mice (as was observed following exercise training), suggesting incomplete metabolic remodeling. Interestingly, GW501516 in combination with exercise training had synergistic effects on gene expression and endurance performance. In light of this observation, Narkar and colleagues substituted the AMPK agonist AICAR for exercise and reported a synergistic effect of the dual treatment strategy compared to either of the compounds in isolation. Unfortunately the authors did not compare this combined approach to endurance exercise, or the synergistic effect of the treatment with exercise. Based on this data, the authors proposed that AICAR and GW501516 are exercise mimetics and could be used as a strategy to increases skeletal muscle metabolism in the absence of an exercise stimulus. This concept received widespread attention and led to a boom in "exercise mimetic" therapy research (Matsakas and Narkar, 2010; Fan et al., 2013).

It should be noted that the use of AICAR to modulate skeletal muscle metabolism was not novel, in fact Will Winder's group had demonstrated the metabolic remodeling capacity of AICAR *in vivo* 11 years earlier (Merrill et al., 1997). It should also be highlighted that GW501516 was only effective in increasing endurance capacity when combined with exercise, and so cannot be regarded as an exercise mimetic, rather at best an exercise enhancer. To date, the work from Narkar and colleagues has failed to translate into human studies, mainly due the poor bioavailability of AICAR *in vivo* (Cuthbertson et al., 2007; Boon et al., 2008; Bosselaar et al., 2011). In addition, given that AICAR inhibits oxygen consumption in isolated muscle fibers (Spangenburg et al., 2013), the suitability of using of this compound *in vivo* is questionable. The efficacy of long-term GW501516 treatment has also been questioned due to links to cancer progression in number of tissues following chronic PPAR-δ activation (Sahebkar et al., 2014). The feasibility of an exercise mimetic has also raised considerable opposition in the literature (Goodyear, 2008; Richter et al., 2008; Carey and Kingwell, 2009), mainly due to the widespread, multi-organ health benefits of exercise (Hawley and Holloszy, 2009) that cannot be recapitulated with single-protein targeted therapeutics (Goodyear, 2008; Richter et al., 2008; Carey and Kingwell, 2009).

BEYOND EXERCISE MIMETICS, CAN SMALL BIOACTIVE INGREDIENTS ENHANCE EXERCISE-INDUCED MITOCHONDRIAL BIOGENESIS?

Whilst the concept of an exercise mimetic, as proposed by Narkar and colleagues would appear to have a number of inherent flaws when it comes to *in vivo* application in humans (Goodyear, 2008; Richter et al., 2008), the use of functional foods or small bioactive ingredients to target exercise-responsive signaling networks does appear to hold promise (Crowe et al., 2013). Typically, bioactive ingredients are viewed as both essential and non-essential compounds (e.g., vitamins or polyphenols) that occur in nature and can be shown to have an effect on human health. Whilst bioactive ingredients are already known to have far-reaching health benefits (Crowe et al., 2015), there is limited information with specific regard to skeletal muscle mitochondrial biogenesis. In the following sections we will briefly highlight a selection of bioactives and when appropriate discuss their proposed mode of action and efficacy/feasibility for translating this research into human-based investigation.

Green Tea Extracts (GTEs)

GTEs are a class of polyphenolic flavonoids which are suggested to play a role in fatty acids (FA) mobilization and oxidation (Shimotoyodome et al., 2005). The polyphenolic compounds in GTEs are epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and gallocatechin gallate (GCG). EGCG is suggested to be the most pharmacologically active; between 210 and 760 times potent as the others (Zhu et al., 2008). GTEs have been suggested to modulate fat oxidation via altered catecholamine release, with Dulloo et al. (1999) demonstrating greater 24-h basal energy expenditure (EE) following GTE supplementation compared to caffeine or a placebo. In addition, they observed a higher percentage of fat-derived 24-h EE compared to the other groups (Dulloo et al., 1999). In support, Venables et al. (2008) demonstrated an increased FA oxidation rate in GTE treated participants vs. a placebo group during exercise, indicated by increased circulating free fatty acids (FFAs) and glycerol (Venables et al., 2008). In this study plasma glucose and insulin concentrations were concurrently lower in the GTE group, indicating a significant metabolic shift toward lipid oxidation (Venables et al., 2008). More recently, Hodgson et al. (2013) and Randell et al. (2013) demonstrated that 7 days GTE supplementation altered global metabolite profiles and increased lipolysis (Randell et al., 2013).

In contrast, (Randell et al., 2013) recently failed to fully reproduce the data from Venables et al. (2008), demonstrating no effect of GTE supplementation on fat oxidation during exercise. A follow up study by the same group (Randell et al., 2014) also demonstrated that de-caffeinated GTE supplementation over 1, 7, and 28 days had no effect on whole-body fat oxidation or fat metabolism-related metabolites during exercise (Randell et al., 2014). Thus, it is currently unclear whether GTE supplementation alone is enough to alter fat oxidation. A small number of studies have been performed to investigate the chronic effects of GTE supplementation on fat oxidation. Based on acute study data, it could be suggested that longer-term GTE supplementation may result in greater increases in fat oxidation. In mice, GTE ingestion during 15 weeks of regular exercise significantly lowered respiratory exchange ratio (RER) and also increased fat utilization compared to an exercise-only group (Shimotoyodome et al., 2005). In humans, Ota et al. (2005) demonstrated that combined GTE supplementation and endurance exercise elicited 24% higher FA oxidation compared to a placebo supplement (Ota et al., 2005). Combined with findings of acute studies, these data lend support to the argument that GTE supplementation with exercise may be efficacious in improving fat oxidation. However, an optimal supplementation strategy is yet to be defined.

Regarding mechanisms of action, Murase et al. (2006) observed that GTE supplementation led to increased activation of PGC-1α mRNA in skeletal muscle and parallel increases in treadmill running time in mice (Murase et al., 2006). As such, it is hypothesized that GTEs may increase mitochondrial biogenesis and skeletal muscle FA oxidation through a PGC-1α-dependent pathway however this has yet to be directly tested.

Caffeine

Caffeine has been shown to stimulate sympathetic nervous system (SNS) activity (Graham et al., 2000) and increase norepinephrine (NE) at the synaptic junction (Dulloo et al., 1992). Caffeine also harnesses the potential to inhibit phosphodiesterase, a cyclic AMP (cAMP) degrading enzyme (Dulloo et al., 1999). Thus, intracellular cAMP may increase with caffeine ingestion promoting subsequent rises in catecholamine concentrations (Dulloo et al., 1999). In addition, caffeine has been reported to stimulate intracellular calcium release (Youn et al., 1991) to a similar extent to contracted skeletal muscle (Baar, 2006). This increased calcium flux activates an upstream kinase to AMPK, calmodulin kinase kinase (Egawa et al., 2011). The caffeine-induced activation of AMPK has also been shown to increase insulin-dependent uptake of glucose similarly to exercise-induced activation (Egawa et al., 2009). It is therefore suggested that caffeine may promote fat oxidation through increased thermogenesis (via AMPK) possibly via increased SNS activity.

Graham et al. (2000) demonstrated that following steady-state exercise at 70% $\dot{V}O_2$ max and caffeine supplementation, serum FA and glycerol concentration was increased compared to a placebo, but that no differences in RER or FA uptake were found (Graham et al., 2000). Acheson et al. (2004) showed that caffeine supplementation increased FA disposal and EE during steady-state cycling exercise. They demonstrated that lipid turnover was markedly increased, but only small increments in FA oxidation were observed (Acheson et al., 2004). In both cases, the results suggest that caffeine supplementation stimulated the SNS, but had minimal "downstream" effects on FA oxidation; despite increased FA utilization. To our knowledge, there have been no studies directly examining the intra-muscular signaling related to caffeine supplementation, or whether caffeine can alter mitochondrial biogenesis in skeletal muscle.

Epicatechins

Cocoa-derived epicatechins, specifically (−)-epicatechin, have been shown to activate mitochondrial biogenesis and capillary proliferation in murine skeletal muscle, in addition to having multiple health benefits in humans (Buijsse et al., 2010). Nogueira et al. (2011) were the first to demonstrate that 15 days (−)-epicatechin supplementation in mice increased treadmill running performance, fatigue resistance, mitochondrial volume, and muscle capillarity in mice compared to activity-matched control groups (Nogueira et al., 2011). These results suggest that (−)-epicatechin supplementation independently resulted in a response similar to endurance exercise as well as augmented the endurance exercise training response, therefore epicatechin has the potential to promote metabolic changes within skeletal muscle resulting in mitochondrial biogenesis. A second study by the same group (Huttemann et al., 2012) investigated the influence of (−)-epicatechin supplementation in mice undergoing 5 weeks of endurance training and then 2 weeks of de-training. Of interest, the epicatechin treated group maintained capillary-fiber ratio and cytochrome-c oxidase activity after the de-training period suggesting that (−)-epicatechin may maintain the endurance-training effect through specific up regulation of angiogenesis and mitochondrial biogenesis pathways (Huttemann et al., 2012).

The first translation of these rodent studies into human investigation was recently performed by Gutiérrez-Salmeán et al. (2014), who investigated the effects of epicatechin supplementation on post-prandial fat metabolism in normal and overweight adults (Gutiérrez-Salmeán et al., 2014). Following supplementation of (−)-epicatechin (1 mg/kg), participants displayed a lower RER, indicative of increased lipid oxidation. In addition, lower plasma glucose concentrations were observed following the supplementation (Gutiérrez-Salmeán et al., 2014). From the available data, it is suggested the cocoa-derived epicatechin is a promising ergogenic aid for increasing mitochondrial biogenesis and lipid oxidation, with the nitric-oxide (NO)/vascular endothelial growth factor (VEGF) pathway suggested to be the primary molecular mechanisms linking (−)-epicatechin supplementation to enhanced muscle adaptation. It will be interesting to see whether the work from Nogueira et al. (2011) can be recapitulated in humans and enhance mitochondrial adaptation to endurance exercise training.

Polyphenols

Polyphenol compounds are found in a variety of herbal medicines commonly used as ethnopharmaceutical agents. These compounds have attracted the attention of researchers owing to their cardio-protective qualities (Chong et al., 2010). Two compounds in particular (resveratrol and quercetin) have been recently studied, and their relevance to fat oxidation during endurance exercise and mitochondrial adaptation to training is discussed here:

Resveratrol

Resveratrol is a stilbenoid polyphenol, a molecule belonging to the phenylpropanoid family commonly found in red wine. During the past decade, resveratrol has emerged as a potent cardio-protective compound. It is also associated with reduction in ischemic injuries and incidence of cancer. In 1992, resveratrol was first isolated from red wine (Siemann and Creasy, 1992) and subsequently attracted significant attention from researchers. It has since been demonstrated that it targets various signaling molecules that work to promote fat metabolism (Lopez-Lluch et al., 2008), and thus could be considered as a potential ergogenic aid.

Howitz et al. (2003) suggested that resveratrol may mimic the effect of caloric restriction by stimulating SIRT1 (Howitz et al., 2003). The mechanism by which resveratrol is able to increase fat oxidation is considered to be via SIRT1-dependent activation of AMPK (Lin et al., 2010), although SIRT1/AMPK independent pathways of resveratrol action have also been reported (Park et al., 2012). Smith et al. (2009) demonstrated that formulated resveratrol (SIRT501 and SRT1720) induced a signaling profile mirroring a reduction in type 2 diabetes pathology, which includes mitochondrial biogenesis and an improved metabolic signaling pathway (Smith et al., 2009).

Resveratrol has been shown to promote fat oxidation and enhance endurance performance in mice (Murase et al., 2009). However, Scribbans et al. (2014) recently reported that resveratrol supplementation during exercise training in healthy individuals led to a maladaptive response in exercise-stimulated gene expression (Scribbans et al., 2014). In agreement with this observation, Gliemann et al. (2013) showed that resveratrol supplementation in combination with high-intensity training in older men not only blunted the increase in maximal oxygen uptake observed in the placebo group, but also eradicated the effects of the exercise on low-density lipoprotein, total cholesterol, and triglyceride concentrations in the blood (Gliemann et al., 2013). Using a similar protocol, Olesen et al. (2014) recently showed that resveratrol supplementation also blunted training-induced decreases in protein carbonylation and tumor necrosis factor α (TNFα) mRNA within older individuals' skeletal muscle (Olesen et al., 2014). Thus, there are clear discrepancies between cell, rodent, and human studies investigating resveratrol supplementation. Given these discrepancies further studies are clearly warranted to understand the interaction between resveratrol and exercise in skeletal muscle, and begin to develop optimal supplementation strategies for improving mitochondrial biogenesis and fat oxidation during endurance exercise.

Quercetin

Quercetin is the most ubiquitous class of flavonoids; found in apples, onions, berries, leafy green vegetables, hot peppers, red grapes, and black tea. It has a similar structure and function to resveratrol, and numerous positive effects on skeletal muscle have been reported (Davis et al., 2009a). Though quercetin has a similar structure and function to resveratrol, it is classified as a different polyphenol owing to its ketone-containing structure (Bravo, 1998).

Quercetin supplementation has been reported to increase mitochondrial biogenesis and exercise tolerance in mice, through

a proposed SIRT1/AMPK/PGC-1α mode of action (Davis et al., 2009b). Quercetin is suggested to act as an ergogenic aid by mimicking the effects of caffeine and its ability to activate Adenosine (A1) receptor (Alexander, 2006), by increasing AMPK activation (Hawley et al., 2010) and also by increasing SIRT1 gene expression and activation (Howitz et al., 2003). By decreasing ATP concentration, quercetin may activate AMPK by increasing the AMP:ATP ratio. This has been observed in isolated mitochondria (Dorta et al., 2005), and is thought to induce this in a manner similar to resveratrol (Hawley et al., 2010).

Quercetin increases β-oxidation in hepatocytes (Suchankova et al., 2009), C2C12 muscle cells (Eid et al., 2010), and Hela cells (Jung et al., 2010). However, short-term quercetin supplementation (1000 mg/d) in humans does not affect $\dot{V}O_2$ max (Ganio et al., 2010), whereas prolonged oral supplementation (3 weeks of 1000 mg/d) elicited no change in RER or fat oxidation (Dumke et al., 2009). Similarly, Cureton et al. (2009) observed no difference in exercise performance or substrate shifts in recreationally active participants (Cureton et al., 2009). In contrast, Nieman et al. (2010) reported small improvements in endurance performance and PGC-1α, SIRT1, citrate synthase and cytochrome-c oxidase mRNA in untrained participants supplemented with quercetin (Nieman et al., 2010).

From the available evidence, it is suggested that quercetin supplementation has little or no effect on mitochondrial biogenesis/fat oxidation in human skeletal muscle performing endurance exercise. However, similar to resveratrol, there are only a paucity of studies that have investigated this and the apparent discrepancies between the rodent and human studies have yet to be fully elucidated.

Amino Acids

A substantial body of evidence now supports the use of protein and amino acids for skeletal muscle training adaptation to resistance training programs aimed at increasing muscle mass (Atherton and Smith, 2012). However, in comparison, the role of amino acids in mitochondrial biogenesis/fat oxidation is under-represented in the literature.

Branch chain amino acid (BCAA) supplementation (particularly leucine) has become popular amongst athletes and recreational exercisers owing to its established role in promoting muscle protein synthesis and positive changes in body composition. A high-protein diet elicits greater levels of resting fat oxidation compared with an iso-energetic high-carbohydrate or high-fat meal (Raben et al., 2003). In addition, Labayen et al. (2004) showed that a single high-protein meal could induce an increase in post-prandial fat over 6 h compared to a standard mixed meal in both lean and obese women. Furthermore, post-prandial fat oxidation, protein and leucine oxidation were greater following the high-protein meal (Labayen et al., 2004). This increased fat oxidation rate may be explained by BCAA (specifically, leucine) oxidation.

BCAA transaminase activation has been shown to occur concurrently with exercise-induced glycogen depletion (Gualano et al., 2011), suggesting a possible role for BCAA in lipid oxidation. Accordingly, a study performed by Gualano et al. (2011) investigated the influence of BCAAs on fat oxidation and exercise capacity during endurance exercise. They demonstrated that BCAA supplementation induced a lower RER during the exhaustive exercise test, and promoted a greater resistance to fatigue (Gualano et al., 2011). These results lend further support to the argument that BCAA supplementation may promote increased fat oxidation during exercise.

Regarding BCAA action at the cellular level, Sun and Zemel (2009) reported that leucine administration to C2C12 cells exhibited increased mitochondrial mass, stimulated PGC-1α and SIRT1 gene expression and increased cell respiration (Sun and Zemel, 2009). These data suggest that leucine modulation of muscle energy metabolism may be mediated by mitochondrial biogenesis. Similarly, muscle cells treated with serum from overweight subjects fed a high-dairy diet for 28 days resulted in increased SIRT1 and PGC-1α expression *in vitro* (Bruckbauer and Zemel, 2011). These data suggest that high dairy consumption (thus, high leucine and BCAA consumption) may promote mitochondrial biogenesis within skeletal muscle. In mice, it has been shown that BCAA ingestion increased mitochondrial biogenesis and SIRT1 expression in skeletal muscle, which consequently increased lifespan in middle-aged mice (D'Antona et al., 2010). It was since hypothesized that leucine-induced activation of SIRT1 was a central event that linked the mitochondrial biogenesis and fat oxidation within skeletal muscle (Liang et al., 2014). To test this, Liang et al. (2014) treated C2C12 myotubes with leucine, and observed significantly increased mitochondrial content, fat oxidation and SIRT1 activity following this treatment compared to control treatments. In addition, time-dependent increases in NAD^+ and SIRT1 activity were observed after 24-h leucine treatment (Liang et al., 2014). Beyond *in vitro* approaches, there are few studies examining the direct effect of amino acids on mitochondrial biogenesis in skeletal muscle. Given the efficacy of AAs for human supplementation (Moore et al., 2014), and the known benefits of AAs in recovery from endurance exercise (Moore et al., 2014), extending this research into the regulation of mitochondrial biogenesis seems the next logical progression in this research area (Moore and Stellingwerff, 2012).

CONCLUSIONS

Endurance exercise is a potent stimulus to induce mitochondrial biogenesis in skeletal muscle (Holloszy, 1967; Molé et al., 1971; Oscai and Holloszy, 1971; Hoppeler et al., 1973; Spina et al., 1996). The nutritional approaches described herein could provide a framework to support endurance training via enhancing mitochondrial biogenesis. In this context, we propose that these small molecules should be viewed as exercise enhancers, not mimetics, as they have minimal effect in basal conditions. In the future, it will be interesting to explore the efficacy of using these nutrients in human studies *in vivo,* to identify the exercise setting in which they may have the most benefit as well as developing optimal supplementation strategies. In this regard, future studies could examine the effect of bioactives during and in recovery from exercise across

a variety of intensities, and also examine supplementation during periods of tapering or detraining to shed light on the practical implications of bioactives as regulators of mitochondrial biogenesis in skeletal muscle. In order to achieve this, researchers should perform randomized, placebo-controlled, intervention trials in human subjects (Hasler, 2002), and examine the extent to which the bioactive ingredient in question is absorbed and bioavailable in skeletal muscle (Crowe et al., 2013). Once achieved, it is hoped that bioactives such as those discussed, and derivatives/associated bioactive ingredients yet to be identified may lead to the next-generation of nutritional supplements to specifically enhance mitochondrial adaptations to endurance training.

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MINI REVIEW
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On the mechanism by which dietary nitrate improves human skeletal muscle function

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Inorganic nitrate is present at high levels in beetroot and celery, and in green leafy vegetables such as spinach and lettuce. Though long believed inert, nitrate can be reduced to nitrite in the human mouth and, further, under hypoxia and/or low pH, to nitric oxide. Dietary nitrate has thus been associated favorably with nitric-oxide-regulated processes including blood flow and energy metabolism. Indeed, the therapeutic potential of dietary nitrate in cardiovascular disease and metabolic syndrome—both aging-related medical disorders—has attracted considerable recent research interest. We and others have shown that dietary nitrate supplementation lowers the oxygen cost of human exercise, as less respiratory activity appears to be required for a set rate of skeletal muscle work. This striking observation predicts that nitrate benefits the energy metabolism of human muscle, increasing the efficiency of either mitochondrial ATP synthesis and/or of cellular ATP-consuming processes. In this mini-review, we evaluate experimental support for the dietary nitrate effects on muscle bioenergetics and we critically discuss the likelihood of nitric oxide as the molecular mediator of such effects.

Keywords: dietary nitrate, nitrite, nitric oxide, oxygen cost of human exercise, cellular bioenergetics, skeletal muscle mitochondria, coupling efficiency of oxidative phosphorylation, ATP turnover

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Introduction

Inorganic nitrate (NO_3^-) has long been considered an undesirable food component and pollutant of drinking water as nitrosation of secondary amines may produce carcinogenic *N*-nitrosamines (Magee and Barnes, 1956). However, the evidence that NO_3^- causes human cancers is weak and dietary NO_3^-, as e.g. found in beetroot and spinach, has instead been linked to many physiological benefits (Gilchrist et al., 2010). Humans do not only get NO_3^- from their diet as it is also generated endogenously (Tannenbaum et al., 1978) by oxidation of nitric oxide (NO) formed canonically via the L-arginine/NO synthase pathway (Moncada and Higgs, 1993). Importantly, inorganic NO_3^- can be reduced to nitrite (NO_2^-) and then NO, which offers an additional path of mammalian NO production that, unlike the canonical route, is independent of oxygen (O_2) (Lundberg et al., 2008). NO is widely believed to mediate the benefits of NO_3^- (Lundberg et al., 2009) including protection against cardiovascular

Abbreviations: AMPK, AMP-activated kinase; PCr, phosphocreatine; PKA, protein kinase A; P_{O2}, partial oxygen tension; RNS, reactive nitrogen species; $\dot{V}O_2$, pulmonary oxygen uptake.

disease (Omar and Webb, 2014) and the metabolic syndrome (Carlström et al., 2010). It has recently been found that dietary NO_3^- lowers the O_2 cost of human exercise as less respiratory activity is required for a set rate of skeletal muscle work (Larsen et al., 2007, 2010; Bailey et al., 2009, 2010). This finding is interesting as it challenges exercise physiology dogma that the steady-state O_2 consumption for any individual is immutable at a given sub-maximal workload irrespective of age, fitness, diet, or pharmacological intervention (Poole and Richardson, 1997). This mini-review aims to evaluate the mechanistic understanding of NO_3^- effects on skeletal muscle function.

O_2 Cost of Human Exercise

In a seminal publication, Larsen and colleagues reported that a 3-day supplementation with 0.1 mmol·kg^{-1} $NaNO_3$· day^{-1} lowered pulmonary O_2 uptake ($\dot{V}O_2$) by ∼3–5% in humans completing sub-maximal cycling exercise (Larsen et al., 2007). Bailey and co-workers subsequently observed a 5% lower $\dot{V}O_2$ during low-intensity cycling exercise and a 16% improvement in the tolerable duration of high-intensity exercise over days 3–6 of a 6-day supplementation period with 5.6 mmol NO_3^-· day^{-1}, administered as 500 mL NO_3^--rich beetroot juice· day^{-1} (Bailey et al., 2009). Importantly, NO_3^--depleted beetroot juice does not improve exercise economy and performance (Lansley et al., 2011) eliminating antioxidants and polyphenols (Wootton-Beard and Ryan, 2011) as exclusive "active ingredients". NO_3^--induced improvements have been observed in humans completing walking, running, cycling, rowing and kayaking exercise, and positive responses arise both acutely, i.e., 1–3 h after NO_3^- ingestion, and after prolonged NO_3^- supplementation over 3–15 days (**Table 1**). Acute (2.5 h post-ingestion) lowering of $\dot{V}O_2$ during low-intensity exercise is progressively greater at 4.2, 8.4, and 16.8 mmol NO_3^-, whereas high-intensity exercise tolerance is unaffected by 4.2 mmol NO_3^-, but acutely improved to a similar extent by 8.4 and 16.8 mmol (Wylie et al., 2013). Therefore, short-term supplementation (≥3 days) with at least 5 mmol NO_3^-· day^{-1}, or acute ingestion of at least 8.4 mmol NO_3^-, might represent an effective dietary intervention to improve the economy and performance of human locomotion, at least in healthy, moderately fit adults (Porcelli et al., 2014). Since effects on resting $\dot{V}O_2$ are equivocal (Bailey et al., 2010; Kelly et al., 2013b; Larsen et al., 2014), NO_3^- benefits may be exclusive to contracting skeletal muscles.

TABLE 1 | Dietary nitrate improves the economy and/or performance of human locomotion.

Exercise	References[a]	Exposure period	
		1–3 h	3–15 d
Cycling	Larsen et al., 2007—1st study reporting dietary NO_3^- benefit		✓
	Larsen et al., 2010	✓	
	Larsen et al., 2011		✓
	Bailey et al., 2009—1st study using beetroot juice as NO_3^- source		✓
	Vanhatalo et al., 2010	✓	✓
	Cermak et al., 2012		✓
	Wylie et al., 2013—Study reports dose-dependency of NO_3^-	✓	
Running	Lansley et al., 2011—1st study using NO_3^--depleted beetroot juice as placebo		✓
	Porcelli et al., 2014		✓
Kayaking	Muggeridge et al., 2013	✓	
	Peeling et al., 2014	✓	
Walking	Lansley et al., 2011		✓
Rowing	Bond et al., 2012		✓

[a] *These studies are cited as examples—the list is not a comprehensive account of all available literature.*

Physiological NO_3^- effects appear muscle-fiber-type-specific as evidenced by improved perfusion (Ferguson et al., 2014) and calcium handling (Hernández et al., 2012) of murine fast-twitch type II but not slow-twitch type I muscle. Consistent with this, NO_3^- benefit on $\dot{V}O_2$ adjustment following the onset of exercise and on tolerance to high-intensity exercise is relatively large when the contribution of type II muscle fibers to force production is increased in human skeletal muscle (Breese et al., 2013; Bailey et al., 2015). These preferential physiological effects may relate to the comparably low microvascular P_{O2} in resting and contracting type II muscle (McDonough et al., 2005). Indeed, NO_3^- improves exercise economy and performance in hypoxia (Masschelein et al., 2012; Muggeridge et al., 2014) more markedly than in normoxia (Kelly et al., 2014). Importantly, NO_3^- attenuates the degree of exercise intolerance and the slowing of PCr recovery kinetics in hypoxia to the levels seen in normoxia (Vanhatalo et al., 2011). It thus appears that exercise economy and performance benefit most from NO_3^- when muscle O_2 availability is low.

Although the majority of studies in healthy adults observe NO_3^--improved exercise economy and/or performance, the effects are attenuated in well-trained endurance athletes (Bescós et al., 2012; Peacock et al., 2012; Christensen et al., 2013; Boorsma et al., 2014; Hoon et al., 2014; Lane et al., 2014), inconsistent in diseased populations (Berry et al., 2014; Kerley et al., 2015; Leong et al., 2015; Shepherd et al., 2015; Zamani et al., 2015), and possibly different in aging humans (Kelly et al., 2013b). More generally, there is evidence of distinct NO_3^- responders and non-responders in many studies. The relative efficacy of dietary NO_3^- effects on skeletal muscle thus appears variable, which underscores the need for detailed mechanistic understanding. To aid such understanding, it is important to ascertain how the human body processes dietary NO_3^-.

Molecular Fate of Dietary NO_3^-

When humans eat NO_3^--rich food, NO_3^- is converted to NO_2^- by nitrate reductases in commensal bacteria that reside in the posterior part of the tongue (Duncan et al., 1995). Salivary NO_2^- is rapidly protonated in the acidic environment of the stomach resulting in the formation of NO and other reactive nitrogen species (RNS) including nitrogen dioxide (NO_2), nitrous acid (HNO_2), and dinitrogen trioxide (N_2O_3) (Benjamin et al., 1994; Lundberg et al., 1994; Lundberg and Weitzberg, 2013). NO_3^-

ingestion increases plasma NO_2^- levels in human subjects (e.g., Lundberg and Govoni, 2004; Webb et al., 2008; Bailey et al., 2009; Vanhatalo et al., 2010; Kelly et al., 2013a). Possibly catalyzed by xanthine oxidase (Zhang et al., 1998; Li et al., 2003) and/or deoxyhaemoglobin (Cosby et al., 2003; Gladwin et al., 2004; Gladwin and Kim-Shapiro, 2008), NO_2^- is reduced to NO under conditions of low oxygen tension (**Figure 1**). Other sites of NO_2^- reductase activity include cytochrome *c* (Basu et al., 2008) and mitochondrial respiratory complexes III (Kozlov et al., 1999) and IV (Castello et al., 2006). However, mammalian NO_2^- reductase activity has only been shown *in vitro* and in animal models (Feelisch et al., 2008; Jansson et al., 2008), and under low P_{O2} (Li et al., 2001; Feelisch et al., 2008) and low pH (Modin et al., 2001). Generally, the low pKa of NO_2^- (3.34, Oxtoby and Nachtrieb, 1996) limits its physiological reduction, which is an inefficient process *per se* (Li et al., 2008) and thus requires high NO_2^- concentrations. Indeed, at physiological NO_2^- levels (see below), even *hypoxic* red blood cells do not liberate significant NO (Bryan et al., 2004). O_2 competitively inhibits NO_2^- reduction by xanthine oxidase (Li et al., 2004) and oxygenated haem effectively scavenges free NO (Feelisch et al., 2008). NO may be converted to N_2O_3 that in turn may react with free thiols to generate S-nitrosothiols (Hess et al., 2005) via an S-nitrosation reaction (**Figure 1**). NO is also able to modify proteins through nitrosylation, e.g., via reaction with the haem of myoglobin (Ignarro, 1991). NO furthermore binds, in a reversible and O_2-competitive manner, to the haem of cytochrome *c* oxidase, and in an O_2-independent way, to the enzyme's active site copper (Giulivi et al., 2006; Brown and Borutaite, 2007; Cooper and Giulivi, 2007). Peroxynitrite ($ONOO^-$) arising from the reaction of NO with the superoxide anion radical may undergo a nitration reaction with tyrosine residues to form 3-nitrotyrosine (**Figure 1**—Radi, 2004). Importantly, tyrosine-containing proteins are also nitrated in a myeloperoxidase-catalyzed reaction using NO_2^- and hydrogen peroxide (Marquez and Dunford, 1995).

Physiological NO_2^- levels range from 50 to 500 nM in human plasma (Bryan et al., 2004; Dejam et al., 2005; Feelisch et al., 2008) and a mean concentration of 12 μM has been measured in human skin (Mowbray et al., 2009). In rodents, NO_2^- concentration varies substantially between tissues, from below quantifiable limit in the liver, heart and lung to as high as 2 μM in kidney and 3.7 μM in lymph nodes; NO_3^- varies from 1 μM in the kidney to 50 μM in the aorta (Garcia-Saura et al., 2010). In humans, the ingestion of 10 mg $NaNO_3 \cdot kg^{-1}$ has been shown to increase mean plasma NO_3^- within 90 min from 30 to 432 μM and mean plasma NO_2^- from 123 to 392 nM (Lundberg and Govoni, 2004). Similarly, 500 mL beetroot juice containing 45 mM NO_3^- on average raises mean plasma NO_3^- to 380 μM and NO_2^- to 600 nM within 30 min and 3 h of ingestion, respectively (Webb et al., 2008). Plasma NO_3^- and NO_2^- reach peak concentrations, respectively, 1–2 and 2–3 h post-ingestion, and NO_3^- gradually returns to its base level after about 24 h (Ender et al., 1964; McKnight et al., 1997; Webb et al., 2008; Wylie et al., 2013).

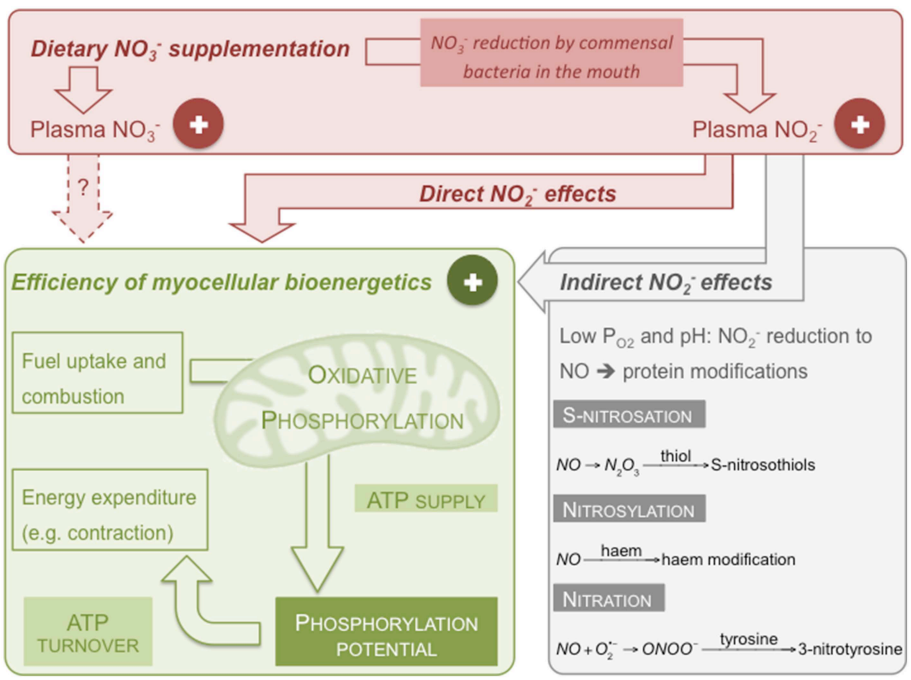

FIGURE 1 | Putative mechanism by which dietary NO_3^- may lower the O_2 cost of human exercise. Dietary NO_3^- increases plasma NO_3^- and NO_2^- levels thus improving efficiency of skeletal muscle ATP supply by oxidative phosphorylation and/or of ATP turnover. Effects on the bioenergetics of skeletal muscle cells may be direct or indirect through formation of NO. Shown reactions are examples of RNS-induced protein modifications (see text for details).

Mammalian tissue NO_2^- and NO_3^- both have *in vivo* half-lives of tens of minutes (Bryan et al., 2005). The half-life of NO_2^- in whole human blood is only about 110 sec (Kelm, 1999) as it is rapidly oxidized to NO_3^-; the half-life of NO_3^- in blood is 5–8 h (Wagner et al., 1983; McKnight et al., 1997). About 60% of ingested NO_3^- is excreted by the kidneys (Green et al., 1981; Wagner et al., 1983).

The reliability of commonly reported NO_3^- and NO_2^- values very much depends on the assays used to measure these inorganic anions. The modified Griess reaction using sulfanilamide and N-1-napthylethylenediamine dihydrochloride is a frequently used assay for measuring NO_2^- (Tsikas, 2007). Plasma NO_3^- concentrations are readily determined using this spectrophotometric assay following NO_3^- reduction to NO_2^- by cadmium (Green et al., 1982) or vanadium salts (Miranda et al., 2001). However, the Griess test lacks the sensitivity to probe the nanomolar NO_2^- levels present in human plasma. Ozone-based chemiluminescence is a preferred method of detection, which often involves deproteinisation of plasma samples by zinc sulfate precipitation before analysis (Higuchi and Motomizu, 1999). NO_2^- measurement by chemiluminescence usually involves acetic acid/sodium iodide-mediated reduction to NO, which then reacts with ozone to produce a chemiluminescence signal (Bateman et al., 2002). NO_3^- can also be measured this way by reduction to NO via reflux of the sample in vanadium chloride at 95°C. Confounding the NO_3^-/NO_2^- literature, in some assays NO_3^- is reduced to NO_2^- by bacterial nitrate reductases (Sun et al., 2003) whose activity varies from batch to batch. Confusing matters further, mere "NO_x" values are reported to denote the sum of NO_2^- and NO_3^- levels. A last analytical note concerns the use of NO_3^--depleted beetroot juice as placebo control in *in vivo* studies (see Section O_2 Cost of Human Exercise). It is important for experiments involving human participants to use a placebo juice that looks, tastes, and smells the same as the "real thing". A placebo that meets these criteria can be prepared by passing beetroot juice through a Purolite a520e anion exchange column, which effectively and selectively removes NO_3^- (Gilchrist et al., 2013).

Skeletal Muscle Bioenergetics

Dietary NO_3^- benefits on the O_2 cost of exercise likely arise from increased efficiency of ATP synthesis and/or of skeletal muscle work (**Figure 1**). Indeed, NO_3^- increases the rate of human skeletal muscle PCr recovery after exercise in hypoxia suggesting an augmented maximum rate of oxidative ATP synthesis (Vanhatalo et al., 2011), and lowers the ATP cost of contractile force production (Bailey et al., 2010). These *in vivo* studies confirm that NO_3^- indeed affects skeletal muscle bioenergetics, but they do not disclose the underlying molecular mechanism. *In vitro* experiments with C2C12 myocytes show that beetroot juice provokes mitochondrial biogenesis and modestly increases basal cellular respiration without affecting respiratory capacity and proton leak (Vaughan et al., 2014). These observations indicate improved mitochondrial coupling efficiency as beetroot juice has increased the proportion of total O_2 consumption coupled to ATP synthesis. Although the C2C12 experiments lack an appropriate NO_3^--depleted beetroot juice control (see above), increased coupling efficiency of oxidative phosphorylation agrees with data reported by Larsen et al. (2011), who show that skeletal muscle mitochondria isolated from NO_3^--supplemented subjects exhibit higher respiratory control and P/O ratios (defined in Brand and Nicholls, 2011) than mitochondria from non-supplemented controls, and that increases in P/O ratio correlate with NO_3^--induced decreases in whole-body O_2 uptake during exercise. This increased efficiency of ATP synthesis in isolated mitochondria, however, emerges from decreased respiration linked to mitochondrial proton leak, not from stimulated O_2 uptake coupled to phosphorylation (Larsen et al., 2011). NO_3^--lowered proton leak coincides with decreases in adenine nucleotide translocase protein and, to a lesser extent, uncoupling protein-3 (Larsen et al., 2011). It should be emphasized that these mitochondrial carriers do not necessarily contribute to proton leak (Nedergaard and Cannon, 2003; Vozza et al., 2014) and that leak is expected to account for little skeletal muscle respiration at low protonmotive force (Affourtit and Brand, 2006), i.e., the bioenergetic state attained during exercise. Dietary NO_3^- also lowers the apparent affinity of mitochondrial respiration for O_2, an effect that is reproduced *in vitro*—acutely and pH-dependently—by NO_2^- (Larsen et al., 2011). Lowered affinity is attributed to an NO-induced rise in the apparent K_m of cytochrome *c* oxidase for O_2 (Larsen et al., 2011) but, inconsistently, NO_2^- does not affect mitochondrial respiration or efficiency (Larsen et al., 2011) like NO is expected to (Brown and Borutaite, 2007). Apparent mitochondrial respiratory affinity for O_2 depends strongly on the extent to which respiration is controlled by the enzyme reacting with O_2 (Affourtit et al., 2001)—control of cytochrome *c* oxidase over O_2 consumption may well have been affected by NO_2^- and pH, and also by dietary NO_3^--induced mitochondrial changes.

It remains to be demonstrated convincingly whether or not dietary NO_3^- effects in skeletal muscle are mediated by NO. Nitrite reductase activity requires high NO_2^- levels and exceptionally low P_{O2} and pH (see Section Molecular Fate of Dietary NO_3^-) that may indeed manifest in the ischaemic heart (Brown and Borutaite, 2007; Hendgen-Cotta et al., 2010), but are unlikely in healthy muscle. In contracting muscle, myoglobin O_2 saturation remains as high as 50% (Takakura et al., 2015) and although globins indeed exhibit nitrite reductase activity at this saturation (Huang et al., 2005), cytoplasmic NO will likely be scavenged by oxymyoglobin (Hendgen-Cotta et al., 2010). Even if O_2 were sufficiently low for NO_2^- reduction in exercising muscle, we stress that dietary NO_3^- intake remodels skeletal muscle bioenergetics in the hours to days *before* exercise (see Section O_2 Cost of Human Exercise), i.e., when the muscles are at rest. Importantly, NO_2^- also modulates cell signaling independently of NO in hypoxia and normoxia (Bryan et al., 2005). NO_2^- activates AMPK in rat aortic smooth muscle cells thus stimulating mitochondrial biogenesis, and increasing coupling efficiency and cellular respiratory control (Mo et al., 2012). NO_2^- activates PKA in cultured cardiomyocytes, stimulating mitochondrial fusion and again increasing cellular respiratory control (Pride et al., 2013). In both systems, NO_2^- improves efficiency of oxidative ATP synthesis without apparent effect on proton leak, which agrees with the beetroot juice effects on C2C12 respiration

(Vaughan et al., 2014). NO_2^- also activates PKA in cultured adipocytes, increasing mitochondrial fusion, and stimulating glucose uptake (Khoo et al., 2014). Moreover, NO_2^- increases proliferation of muscle (Totzeck et al., 2014) and epithelial cells (Wang et al., 2012).

RNS can modify proteins (see Section Molecular fate of dietary NO_3^-) and may thus improve mitochondrial coupling efficiency in various ways, e.g., by increasing proton translocation to electron transfer stoichiometries of respiratory complexes (*cf.* Clerc et al., 2007). By definition (Brand and Nicholls, 2011), coupling efficiency benefits from decreased proton leak and increased phosphorylation-linked respiration, as indeed reported by (Larsen et al., 2011) and (Vaughan et al., 2014), respectively. System-kinetic modeling furthermore suggests that substrate oxidation capacity, which is dependent on fuel and O_2 availability, correlates positively with coupling efficiency (Affourtit and Brand, 2009). Dietary NO_3^- may thus improve efficiency of muscle ATP synthesis, at least in part, by increasing expression of glucose transporters (Jiang et al., 2014) and/or by raising insulin availability (Nyström et al., 2012).

Dietary NO_3^- increases the contractile force of fast-twitch mouse muscle by improving calcium handling (Hernández et al., 2012) suggesting the efficiency of ATP-demanding contraction may have increased. To our knowledge, no other data are available on the mechanism by which dietary NO_3^- affects ATP turnover. However, NO_3^- supplementation may also alter efficiency of other ATP-consumers and, importantly, the relative importance of dietary NO_3^- effects on skeletal muscle ATP supply and ATP turnover remains unclear. A systems-level functional analysis of cellular energy metabolism (*cf.* Brand, 1998) may shed light on these issues. Using myocytes isolated from human muscle biopsies (Nisr and Affourtit, 2014) the relative effects of RNS on ATP-generating and ATP-consuming fluxes—linked through the cell's phosphorylation potential (**Figure 1**)—may be identified and quantified in an unbiased manner. A challenge of such *in vitro* analysis will be the approximation of physiologically meaningful conditions, in particular the O_2 tensions and energy demands that prevail during the development of dietary NO_3^- benefits.

Conclusion

The striking benefit of dietary NO_3^- on the O_2 cost of exercise is of obvious interest to athletes (Jones, 2014), but may also well impact on the quality of life of aging people suffering from muscle weakness and exercise intolerance. To rationally evaluate translational potential, our mechanistic understanding of dietary NO_3^- benefits on human skeletal muscle needs to be improved. By integrating biochemistry and physiology, and studying subjects at different age, it will be important to demonstrate which reactive nitrogen species mediate dietary NO_3^- effects at the cellular level, disclose all effects of nitrogen species on myocellular bioenergetics, confirm if they are direct or indirect via action on other tissues, and quantify the relative importance of these effects.

Author Contributions

CA wrote Sections Introduction, Skeletal Muscle Bioenergetics, and Conclusion, and produced the table and figure; SB and AJ wrote Section O_2 Cost of Human Exercise; MS and PW wrote Section Molecular Fate of Dietary NO_3^-. All authors edited and approved the entire manuscript.

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In utero Undernutrition Programs Skeletal and Cardiac Muscle Metabolism

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In utero undernutrition is associated with increased risk for insulin resistance, obesity, and cardiovascular disease during adult life. A common phenotype associated with low birth weight is reduced skeletal muscle mass. Given the central role of skeletal muscle in whole body metabolism, alterations in its mass as well as its metabolic characteristics may contribute to disease risk. This review highlights the metabolic alterations in cardiac and skeletal muscle associated with *in utero* undernutrition and low birth weight. These tissues have high metabolic demands and are known to be sites of major metabolic dysfunction in obesity, type 2 diabetes, and cardiovascular disease. Recent research demonstrates that mitochondrial energetics are decreased in skeletal and cardiac muscles of adult offspring from undernourished mothers. These effects apparently lead to the development of a thrifty phenotype, which may represent overall a compensatory mechanism programmed *in utero* to handle times of limited nutrient availability. However, in an environment characterized by food abundance, the effects are maladaptive and increase adulthood risks of metabolic disease.

Keywords: intrauterine growth restriction, metabolic programming, mitochondria, oxidative phosphorylation, uncoupling, epigenetics

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Early life environmental factors, such as maternal food restriction, contribute to the development of metabolic diseases in offspring (Gluckman et al., 2008). Intrauterine growth restriction (IUGR) is one environmental perturbation that has been linked to the development of obesity and type 2 diabetes mellitus (T2DM). The idea that prenatal events may be important in determining risk for adult disease was first reported by David Barker who made a landmark observation that birth weight is inversely correlated with the risk of coronary heart disease in adulthood (Barker et al., 1989). The birth records of 16,000 men and women who were born in Hertfordshire between 1911 and 1930 were examined. Death from coronary heart disease was associated with low birth weight, with the rates falling progressively between individuals with a birth weight less than 2500 g and individuals with a birth weight of 4310 g.

Low birth weight is defined by the World Health Organization as weight at birth <2500 g (World Health Organization United Nations Children's Fund, 2004). 15.5% of all babies are born with low birth weight, representing over 20 million infants worldwide (World Health Organization United Nations Children's Fund, 2004). While the incidence of low birth weight is greater in developing countries, it remains a significant problem in developed countries as well. In North America, 7.7% of infants are low birth weight (World Health Organization United Nations Children's Fund, 2004). Low birth weight may be a result of preterm birth or poor fetal growth. Poor fetal substrate supply can be due to poor maternal energy intake (insufficient intake of a specific micro- or macronutrient,

or reduced total calories), placental insufficiency, maternal smoking, pregnancy at high altitude, or high maternal levels of stress hormones (e.g., cortisol). Interestingly, infants who are born with a high birth weight are also susceptible to metabolic disease (Boney et al., 2005). Studies have shown that there is a U-shaped correlation between birth weight and obesity with a higher prevalence of obesity for low birth weight and high birth weight (McCance et al., 1994; Wei et al., 2003). Here the focus will be on low birth weight as a result of poor fetal substrate supply and its impact on skeletal and cardiac muscle. These tissues have high metabolic demands and are known to be sites of major metabolic dysfunction in chronic diseases such as T2DM and cardiovascular disease.

After Barker's initial observation, subsequent epidemiological studies showed a strong correlation between *in utero* undernutrition, low birth weight, and risk of adult cardiovascular disease, impaired glucose tolerance, T2DM, and obesity (**Figure 1**) (Hales et al., 1991; Barker et al., 1993; Ravelli et al., 1999; Roseboom et al., 2000; Painter et al., 2005). The well-studied epidemiological data from the Dutch Hunger Winter show the importance of adequate fetal nutrition. During this short-term famine in 1944–1945, the daily nutritional intake was reduced to ∼400–1000 kcal. Adults whose mothers were exposed to the famine during pregnancy had low birth weight and had impaired glucose tolerance and predisposition to T2DM (Hales et al., 1991). These studies gave rise to the "developmental origins of adult disease" hypothesis, which states that adverse influences early in development result in physiological adaptations that increase susceptibility to adult disease. The increased risk of obesity, insulin resistance, and T2DM has been suggested to be due to a thrifty phenotype programmed *in utero* that endows offspring with an increased capacity to store fuels rather than burning them (Hales and Barker, 1992). This apparent adaptive response by the fetus involves metabolic alterations that could altogether conserve energy expenditure to allow growth of key organs such as the brain, at the expense of other tissues such as muscle. Thus, when the nutrients provided to a fetus are limited, the fetus adapts to this environment through physiological changes that enhance its survival under these conditions. However, if the fetus is born into an environment in which nutrients are abundant, the adaptations made *in utero* may become a disadvantage (Gluckman and Hanson, 2004). Thus, disparities between the predicted environment and the actual environment into which the child is born may result in an increased disease risk.

Research based on animal models of IUGR has provided extensive support for the findings from human epidemiological studies and has substantially advanced our understanding of the negative impact of a suboptimal *in utero* environment. The most commonly used animal models of IUGR are maternal caloric or protein restriction and induction of uteroplacental insufficiency. These models have shown that a suboptimal *in utero* environment has deleterious consequences for adult health, with effects in many organs and tissues including skeletal muscle, heart, pancreas, liver, blood, and the brain (Snoeck et al., 1990; Woodall et al., 1996a,b; Park et al., 2003, 2004; Peterside et al., 2003; Qiu et al., 2004; Jimenez-Chillaron et al., 2005, 2009; Bubb et al., 2007; Schober et al., 2009; Woo et al., 2011; Fung et al., 2012; Thorn et al., 2013; Tare et al., 2014; Beauchamp et al., 2015a,b). We are only just beginning to understand the profound impact of suboptimal *in utero* nutrition on adult metabolic health.

A common phenotype in IUGR humans and animals is reduced lean mass (Hediger et al., 1998; Jimenez-Chillaron et al., 2005; Kensara et al., 2005; Wells et al., 2007). Lean body mass, primarily skeletal muscle, is known to be the best predictor of basal metabolic rate (Zurlo et al., 1990; Rolfe and Brown, 1997). Skeletal muscle comprises ∼40% of the body mass in an adult human and although its metabolic rate per gram of tissue is relatively low, it greatly contributes to metabolic rate due to its high fractional contribution to body mass (Zurlo et al., 1990; Rolfe and Brown, 1997). Therefore, differences in muscle metabolism have potentially substantial implications in determining one's susceptibility to obesity and related metabolic disease, such as T2DM. Indeed, skeletal muscle is the largest insulin-sensitive tissue in the body and is the primary site for insulin-stimulated glucose utilization (Defronzo et al., 1985). Thus, blood glucose homeostasis, particularly in the post-prandial state is greatly impacted by insulin resistance in muscle. As muscle is a key determinant of whole body metabolism and insulin sensitivity, reductions in muscle mass and/or function may be especially important to the increased metabolic disease risk (Defronzo et al., 1985; Zurlo et al., 1990; Rolfe and Brown, 1997).

In addition to reduced lean mass, low birth weight is associated with altered skeletal muscle fiber composition, and oxidative capacity (**Figure 2**). Human studies have documented a shift toward more type II glycolytic fibers, which accompanied skeletal muscle insulin resistance (Jensen et al., 2007). When challenged with a hyperinsulinemic-euglycemic clamp, a measure of tissue insulin sensitivity, those who had a low birth weight had decreased glucose uptake, consistent with impaired insulin sensitivity (Jaquet et al., 2000). People with low birth weight have also been shown to have reduced muscle glucose uptake after local insulin infusions and decreased expression of insulin signaling proteins and glucose transporter 4 (GLUT4) in skeletal muscle (Hermann et al., 2003; Ozanne et al., 2005; Jensen et al., 2008). In more rigorously controlled animal models of low birth weight, many of these same skeletal muscle alterations have also been observed. In IUGR animal models, skeletal muscle has reduced mass, decreased GLUT4 expression, decreased glycogen content, decreased insulin-stimulated glucose uptake, decreased oxidative capacity, and increased lipid accumulation (Selak et al., 2003; Jimenez-Chillaron et al., 2005; Zhu et al., 2006; Raychaudhuri et al., 2008; Huber et al., 2009; Dai et al., 2012; Beauchamp et al., 2015a).

Recently, we have used a mouse model system of maternal undernutrition during late pregnancy to examine offspring from undernourished dams. Consistent with previous studies, these low birth weight offspring had increased adiposity and decreased glucose tolerance in adulthood compared to controls (Beauchamp et al., 2015a). Our studies focused on female offspring, as our pilot studies indicated a more pronounced metabolic phenotype than in male offspring. In permeabilized

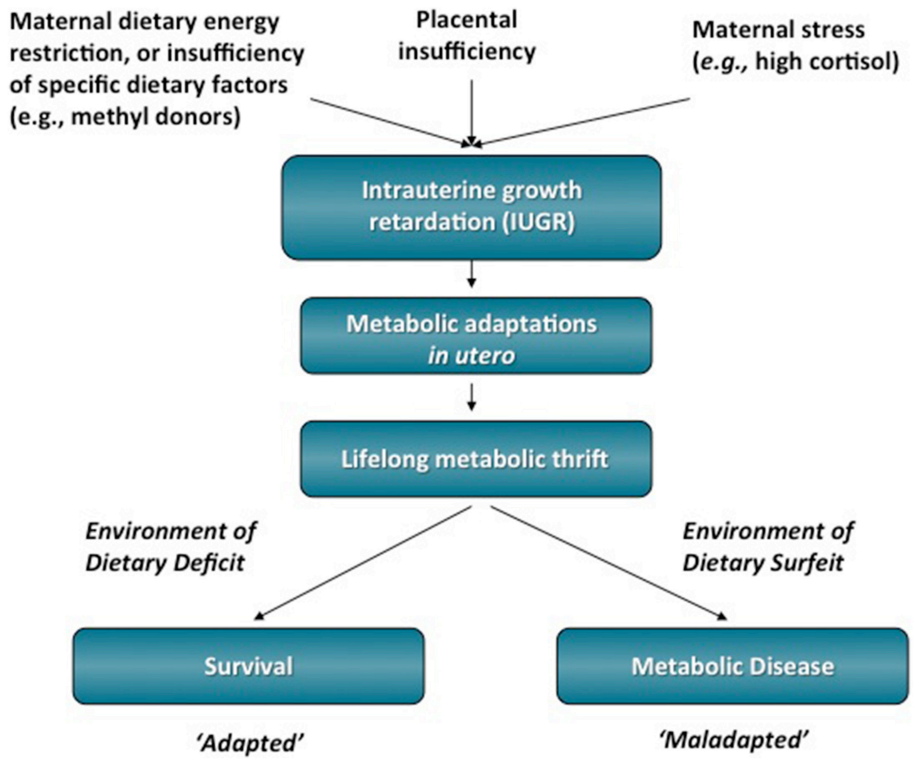

FIGURE 1 | IUGR is one environmental perturbation that has been linked to the development of T2DM and obesity in adulthood. It is hypothesized that the early life stressor of dietary energy restriction may program metabolic adaptations that favor survival initially, but are ultimately detrimental to adult health in an environment of dietary energy surfeit. Therefore, what was an advantage *in utero* in which energy substrates was scarce can become a disadvantage by increasing the person's susceptibility to metabolic diseases in adulthood.

Studies in adult humans with low birth weight:

Skeletal Muscle	Heart
↓ Muscle mass	↓ Cardiac function
↓ Oxidative capacity	↑ Blood pressure
↑ Glycolytic capacity	↑ Stiffening of carotid arteries
↓ Proportion of type I fibers	↑ Risk of cardiovascular disease

FIGURE 2 | Numerous studies have demonstrated that adult humans who were born with low birth weight have abnormalities in characteristics of skeletal muscle and heart, two tissues that have high metabolic demands. These abnormalities increase the risk for metabolic diseases including obesity, type 2 diabetes, and cardiovascular diseases. Please refer to text for specific references.

fiber preparations from mixed fiber type muscle of adult females, *in utero* undernourished mice had decreased mitochondrial content and decreased mitochondrial proton leak respiration, fatty acid oxidative capacity, and state three respiratory capacity through complex I (Beauchamp et al., 2015a). The findings have implications for obesity risk. Obesity is a result of an energy imbalance, in which energy intake exceeds energy expenditure over a sustained period of time. In the long-term this results in energy storage in the form of triglycerides in adipose tissue. Therefore, our findings of decreased mitochondrial content and decreased capacity for fuel oxidation in muscle, an indicator of tissue energy expenditure, may in part explain the increased susceptibility to obesity in IUGR offspring. Furthermore, we have shown that IUGR offspring lose less weight after a 4 week 40% calorie restriction diet (Beauchamp et al., 2015a). We have suggested that this resistance to weight loss may be due to the thrifty metabolic mechanisms programmed in skeletal muscle *in utero*, and may have implications for diet-resistant obesity, which we investigate in human clinical populations (Harper et al., 2002; Gerrits et al., 2010; Thrush et al., 2014). Thus, it seems that *in utero* undernutrition not only increases susceptibility to obesity but may also make weight loss more difficult.

It has been hypothesized that mitochondrial programming may be a key adaptation made by an IUGR fetus to promote survival in a nutrient-restricted environment (Lee et al., 2005). Mitochondria play a key metabolic role and are responsible for oxidizing energy substrates to support ATP synthesis, which can then be used to drive a very wide range of energy demanding reactions in cells. Mitochondrial dysfunction is implicated in many disease states, including obesity and T2DM and thus, mitochondrial dysfunction may be a link between *in utero* nutrition and health and disease in adult life. IUGR has been

associated with decreased skeletal muscle mitochondria DNA content and decreased expression levels of genes involved in mitochondrial biogenesis and function (Lane et al., 1998; Park et al., 2004; Liu et al., 2012). Consistent with these findings, we have shown that *in utero* undernourished offspring have decreased skeletal muscle mitochondrial content and impaired mitochondrial function (Beauchamp et al., 2015a). Moreover, we assessed energetics in isolated mitochondria and found that mitochondria from *in utero* undernourished offspring have decreased coupled and uncoupled respiration compared to mitochondria from control mice (Beauchamp et al., 2015a). Therefore, we have shown that not only do IUGR offspring have decreased skeletal muscle mitochondrial content but respiration per mitochondrion is also decreased. These skeletal muscle adaptations are consistent with a programmed thrifty phenotype, which would set the stage for the development of adult metabolic disease in an environment with abundant nutrition.

Given the high energy requirements of the heart, IUGR may be associated with cardiac metabolic alterations that have negative effects in adulthood. Many cardiac diseases and heart failure are associated with altered metabolism in the heart, including a general decrease in oxidative capacity and the down-regulation of enzymes of fatty acid oxidation (Sack et al., 1996; Sharov et al., 2000; Razeghi et al., 2001; Stanley et al., 2005; Boudina et al., 2007; Anderson et al., 2009). In humans, IUGR is associated with changes in cardiac morphology, premature stiffening of carotid arteries, impaired cardiac function, and elevated blood pressure (Martin et al., 2000; Bahtiyar and Copel, 2008; Crispi et al., 2010). In animal models, IUGR is associated with the development of adult hypertension, vascular dysfunction, and increased myocardial lipid content (Battista et al., 2002; Cheema et al., 2005; Zohdi et al., 2012). IUGR rats have an increased susceptibility to ischemia/reperfusion injury that is associated with a mismatch between myocardial glycolysis and glucose oxidation rates (Rueda-Clausen et al., 2011). In this study, IUGR offspring during reperfusion had decreased cardiac performance and significant increased amount of glucose that underwent glycolysis relative to the amount that was oxidized (Rueda-Clausen et al., 2011). Recently, we assessed energetics in a cardiac muscle homogenate and found that *in utero* undernourished mice in adulthood have decreased mitochondrial proton leak respiration (adenylate-free, and oligomycin-induced rates), fatty acid oxidative capacity, and maximum oxidative phosphorylation capacity (Beauchamp et al., 2015b). These findings are consistent with the decreased respiration in cardiac tissue reported in adults with obesity and T2DM and the decreased cardiac energy transduction associated with heart failure (Sharov et al., 1998, 2000; Boudina et al., 2007; Anderson et al., 2009; Doenst et al., 2010). Therefore, our results demonstrated that maternal undernutrition alters mitochondrial metabolism in the heart, which may contribute to the increased risk of cardiovascular and other metabolic diseases in the offspring. However, studies examining the metabolic effects of IUGR on cardiac muscle are very limited.

Skeletal muscle has a remarkable ability to adapt and respond to its environment and physiological challenges by changing its phenotype in terms of size, composition, and aerobic capacity, outcomes that are brought about by changes in gene expression, biochemical, and metabolic properties (Flück and Hoppeler, 2003; Luquet et al., 2003; Hénique et al., 2015). As such, skeletal muscle can modify its functional characteristics to adapt to metabolic need. The fetal adaptations to undernutrition that produce the long-term outcomes of IUGR are not fully understood. Intriguingly, some of these effects are transmissible across generations, suggesting that heritable changes in gene expression occur with *in utero* undernutrition. Experimental studies have shown intergenerational transmission of obesity and altered glucose metabolism associated with low birth weight (Benyshek et al., 2006; Harrison and Langley-Evans, 2009; Jimenez-Chillaron et al., 2009). The increased susceptibility to metabolic disease in adulthood may arise, at least in part, from epigenetic mediated alterations in gene expression. Epigenetic modification refers to modifications of DNA and chromatin that result in differential gene expression without altering the DNA sequence itself. These modifications include DNA methylation, genomic imprinting, and chromatin modifications such as post-translational modification of histones. These epigenetic modifications alter the binding of transcription factors to specific promoters and/or alter chromatin conformation, which in turn modulate gene expression. Thus, epigenetic modifications of the fetal genome based on maternal environmental cues may reset the metabolic state of the fetus to produce phenotypes in the offspring that are best suited for the predicted environment and that are maintained into adulthood. Evidence indicates that environmental factors acting during critical developmental periods can alter the epigenome. For example, in the mouse, the level of methyl donors, such as methionine, folate, and choline in the maternal diet has been shown to alter DNA methylation in the offspring (Wolff et al., 1998).

Human data that link maternal undernutrition to epigenetic changes are limited. In one study, whole blood genomic DNA was analyzed in adults who were *in utero* during the Dutch Hunger Winter, a period of famine, compared to their unexposed same-sex sibling. Adults who were *in utero* during the famine, and thus were undernourished, showed hypomethylation of the insulin-like growth factor II gene, a maternally imprinted gene that is a key factor in mammalian growth (Heijmans et al., 2008). Modifications to the methylation status of genes produce stable alterations in gene expression and represent a potential mechanism by which early life nutrition may influence susceptibility to metabolic disease in adulthood (Waterland and Jirtle, 2004). However, to date, epigenetic modification of muscle in IUGR offspring has not been described in humans.

Animal models are increasing our understanding of the mechanisms that cause the deleterious effects of IUGR. Epigenetic modifications that affect glucose metabolism have been described in IUGR pancreas, liver, and muscle. Pancreatic and duodenal homeobox 1 (Pdx-1) is a transcription factor that plays an important role in β-cell development and function. Expression of the *Pdx-1* promoter is decreased in IUGR and promotes the development of T2DM in adulthood. It has been shown that islets isolated from IUGR fetuses have decreased histone acetylation at the proximal promoter of *Pdx-1,* which is associated with decreased *Pdx-1* expression and defective

glucose homeostasis (Pinney et al., 2011). In another study, maternal protein restriction in rats led to decreased methylation of genes for the glucocorticoid receptor (GR) and peroxisome proliferator-activated receptor alpha (PPARα) in the liver of the offspring after weaning (Jing-Bo et al., 2013). This was associated with greater mRNA expression of GR and PPARα, both of which are involved in glucose and lipid metabolism (Jing-Bo et al., 2013). In IUGR, skeletal muscle becomes insulin resistant and glucose uptake is reduced. It has been shown that IUGR is associated with alterations in transcription factor binding to the GLUT4 promoter, and this was associated with silencing histone modifications and reduced *glut4* gene expression (Raychaudhuri et al., 2008). In skeletal muscle, it has been shown that IUGR rats have increased methylation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis (Xie et al., 2015). Accordingly, this was associated with a reduction in PGC-1α transcription activity, mitochondrial content, and protein level of components of the insulin signaling pathway (Xie et al., 2015). Taken together, these results support the idea that alterations in the maternal diet can induce epigenetic changes in muscle that are associated with altered gene expression.

While there is growing evidence for the role of epigenetics in metabolic programming in the development of chronic diseases, the detailed molecular mechanisms mediating the effects of *in utero* undernutrition remain unknown. In the future, epigenetic markers such as DNA methylation in blood and tissue samples may be able to serve as biomarkers to identify individuals at increased risk. Ultimately, this may allow prevention of disease by nutritional or pharmacological interventions.

In conclusion, *in utero* undernutrition is associated with skeletal and cardiac muscle alterations such as decreased mass, mitochondrial content, and metabolism. The adaptations in skeletal muscle are consistent with the idea that low birth weight offspring may develop a protective mechanism *in utero* for species survival in times when energy supply is restricted. However, in an environment characterized by the abundant availability of highly palatable food and a decreased need for physical activity, such adaptive mechanisms become detrimental, increasing the risk for metabolic diseases including obesity and T2DM.

AUTHOR CONTRIBUTIONS

BB and MH wrote the manuscript, approved the final version, and agree to be accountable for all aspects of the work.

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REVIEW
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Mitochondrial Quality Control and Muscle Mass Maintenance

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Loss of muscle mass and force occurs in many diseases such as disuse/inactivity, diabetes, cancer, renal, and cardiac failure and in aging-sarcopenia. In these catabolic conditions the mitochondrial content, morphology and function are greatly affected. The changes of mitochondrial network influence the production of reactive oxygen species (ROS) that play an important role in muscle function. Moreover, dysfunctional mitochondria trigger catabolic signaling pathways which feed-forward to the nucleus to promote the activation of muscle atrophy. Exercise, on the other hand, improves mitochondrial function by activating mitochondrial biogenesis and mitophagy, possibly playing an important part in the beneficial effects of physical activity in several diseases. Optimized mitochondrial function is strictly maintained by the coordinated activation of different mitochondrial quality control pathways. In this review we outline the current knowledge linking mitochondria-dependent signaling pathways to muscle homeostasis in aging and disease and the resulting implications for the development of novel therapeutic approaches to prevent muscle loss.

Keywords: atrophy, mitochondria, fission, fusion, biogenesis, autophagy, muscle, sarcopenia

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INTRODUCTION

Nearly 40–50% of total body mass in non-obese mammals is composed by skeletal muscles. Striated muscles are plastic tissues that can undergo adaptive changes in their structure or functional properties to meet new challenges. For example, muscle mass can either increase or decrease in response to metabolic demands like exercise or inactivity. Moreover, muscle strength or resistance to fatigue can be modulated by training or detraining. The regulation of muscle size, due to its limited proliferative capacity, is determined by the coordinated balance between protein synthesis and protein degradation. Mechanical overload or anabolic hormonal stimulation shifts the balance toward protein synthesis with consequent increases in fiber size, a process called hypertrophy. Conversely, in catabolic conditions protein degradation exceeds protein synthesis leading to muscle weakness and muscle atrophy. The decrease in cell size is mainly due to loss of organelles, cytoplasm and proteins. Skeletal muscle atrophy occurs in several pathological conditions like disuse, denervation, immobilization, sepsis, burn injury, cancer, AIDS, diabetes, heart and renal failure and during aging. Importantly, the decrease of muscle mass increases morbity, impairs the efficacy of many therapeutic treatments and contributes to mortality. Muscle mass is mainly controlled by two major signaling pathways: TGFβ/Smads and IGF1-AKT-mTOR-FoxO. TGFβ superfamily of ligands regulate the size of muscles via the Smad transcription factors (Sandri, 2013). Smad2/3 control catabolic genes while Smad1/5/8 regulate anabolic genes (Sartori et al., 2013, 2014). The IGF1-AKT- mTOR axis increases protein synthesis

by stimulating the translational machinery while simultaneously blocking FoxOs transcription factors and protein degradation pathways (Sandri, 2013). Two main ATP-dependent proteolytic systems are activated during muscle atrophy in order to contribute to muscle loss. The ubiquitin-proteasome system degrades predominantly myofibrillar proteins, whereas the autophagy-lysosome system removes dysfunctional organelles, protein aggregates as well as unfolded and toxic proteins. Muscle atrophy requires the activation of gene transcription programs that regulate the expression of a subset of genes that are named atrophy-related genes or atrogenes (Bodine et al., 2001; Gomes et al., 2001; Lecker et al., 2004; Sandri et al., 2004; Sacheck et al., 2007). These atrogenes belong to several fundamental biological processes such as the ubiquitin-proteasome and autophagy-lysosome systems, protein syntesis, ROS detoxification, DNA repair, unfolding protein response (UPR), mitochondria function and energy balance. FoxO family of transcription factors (FoxO1, FoxO3, and FoxO4), which are targets of AKT, are key mediators of the catabolic response during atrophy (Sandri et al., 2004; Mammucari et al., 2007; Milan et al., 2015). Indeed, specific inhibition of FoxOs in muscle protects from cancer cachexia-, fasting- or denervation-induced atrophy (Judge et al., 2014; Milan et al., 2015) as they are critical for the regulation of several atrogenes. Moreover, at least half of the atrogenes require FoxOs for their up or downregulation (Milan et al., 2015). FoxO-dependent atrogenes include the E3 Ubiquitin ligases Atrogin-1, MuRF-1, MUSA1, SMART, and several autophagy-related genes such as LC3, GABARAPL1, BNIP3, CATHEPSIN L (Bodine et al., 2001; Sandri et al., 2004; Mammucari et al., 2007; Milan et al., 2015). Therefore, FoxOs coordinate both major proteolytic systems of the cell, the autophagy-lysosome and the ubiquitin-proteasome.

Interestingly, in addition to genes linked with proteolytic pathways, more than 10% of the atrophy-related genes are directly involved in energy production. Furthermore, several genes coding for enzymes important in glycolysis and oxidative phosphorylation are coordinately suppressed in atrophying muscles (Lecker et al., 2004). These interesting findings suggest that alterations in mitochondria and the mitochondrial network morphology can have potential deleterious consequences for the maintenance of muscle mass and function. Recent data shows that the mitochondrial network communicates with myonuclei to adapt muscle function to the physiological or pathological demands. Here we will review the principal pathways that control mitochondrial quality and the importance of mitochondrial network in the regulation of muscle mass and metabolism.

OPTIMIZATION OF MITOCHONDRIAL FUNCTION: A QUALITY CONTROL ISSUE

Mitochondria are continuously challenged by reactive oxygen species (ROS), an inexorable by-product of oxidative phosphorylation. In order to prevent an excessive production of ROS but also the release of dangerous factors such as cytochrome c, AIF or endonuclease G, mammalian cells contain several systems that maintain mitochondrial integrity and function. This mitochondrial quality control includes pathways related to protein folding and degradation as well as systems involved in organelle shape, movement and turnover (**Figure 1**). The activation of each specific quality control system depends on the degree of mitochondrial damage.

Mitochondrial Proteolysis Controls Both Mitochondrial Protein Turnover and Mitochondrial Function

Proteomic studies identified in human mitochondria approximately 1200 proteins (Calvo et al., 2015). However, only 1% of these proteins is encoded by mitochondrial DNA (mtDNA) (13 proteins in humans). Therefore, the remaining 99% is encoded by nuclear DNA (nDNA) genes, which are synthesized by ribosomes in cytoplasm and are imported into mitochondria (Harbauer et al., 2014). The incorporation of these precursors into specific mitochondria subcompartments such as the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM) and the mitochondrial matrix requires a specific and highly regulated import machineries (Harbauer et al., 2014). To maintain the proteostasis in each mitochondrial compartment, specific proteases (mitoproteases) will rapidly degrade misfolded or oxidized proteins. Mitoproteases-mediated quality control is the first line of defense against a mild mitochondrial damage. In the mitochondrial matrix, protein turnover is controlled by 3 AAA proteases: the soluble Lon and ClpP and the membrane-bound m-AAA. Protein degradation in the IMS is controlled by the membrane-bound i-AAA Yme1L1, the soluble HtrA2/Omi, the metallopeptidases OMA1 and the rhomboid protease PARL. Different reports showed that these mitoproteases do not only monitor mitochondrial protein quality but they can also decide mitochondrial fate. For example, m -AAA, Yme1L1, HtrA2, OMA1, and PARL l cleave the profusion protein Opa1 affecting mitochondrial morphology and function (Alavi and Fuhrmann, 2013). In addition, PARL modulates mitophagy by degrading the mitophagy protein PINK1 (Jin et al., 2010). For a review on the roles and the pathological relevance of these mitoproteases please refer to Quiros et al. (2015). Beside subcompartment-specific proteases, mitochondrial protein turnover is regulated also by the cytosolic ubiquitin-proteasome system (UPS). The UPS marks proteins for proteasomal degradation by the covalent linkage of a chain of ubiquitin proteins. A proteomic study in mouse cardiac muscle identified numerous OMM as well as IMS, IMM, and matrix proteins to be ubiquitinated (Jeon et al., 2007). A still unanswered question is how the cytosolic proteasome can degrade integral mitochondrial membrane proteins. During ER stress, misfolded proteins accumulate into the ER lumen and need to be retrotranslocated into the cytosol where they are flagged with ubiquitin and degraded by the proteasome in a process called ER-associated protein degradation (ERAD). Mitochondria have an ERAD-like mechanism, the mitochondria-associated degradation (MAD) pathway (Heo et al., 2010). ERAD and MAD pathways share some key components like the AAA ATPase p97 and the cofactor Npl4 (Ye et al., 2003; Heo et al., 2010). p97/Npl4 complex regulates the retrotranslocation of

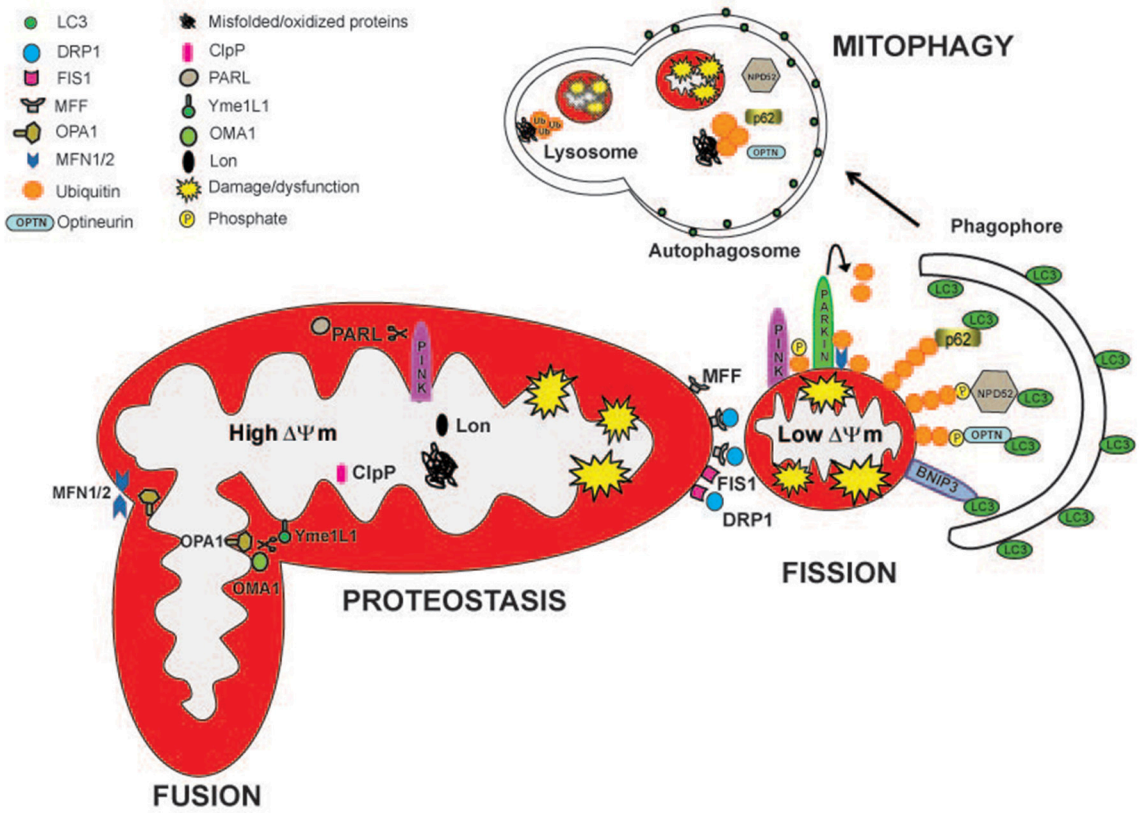

FIGURE 1 | Mitochondrial quality control pathways are depicted. The actions of specific mitoproteases, Lon, ClpP, Oma1, Yme1L1, and PARL, maintain mitochondrial proteostasis and regulate mitochondrial function. PARL, Oma1, and Yme1L1 control mitochondrial dynamics by proteollytically processing Opa1 protein, which is important for mitochondrial fusion and cristae remodeling. PARL degrades PINK1, regulating mitophagy. Drp1, Fis, and Mff are the major proteins involved in mitochondrial fission. Partially damaged mitochondrial network divides into two fragments with different $\Delta\Psi m$. The fragment with higher $\Delta\Psi m$ rejoins the functional mitochondrial network through mitochondrial fusion. Depolarized organelles will be removed by mitophagy. Bnip3 acts as a mitophagic receptor, binding to LC3 to tether mitochondria to the autophagosome. PINK1 accumulates on the surface of depolarized mitochondria where it phosphorylates ubiquitinated OMM proteins and the Parkin UBL domain. Parkin will further promote the ubiquitination of the outer mitochondrial membrane proteins, like MFN1/2. Then, the ubiquitinated proteins can be recognized by the adaptors p62/SQSTM1, Optineurin (OPTN), and NPD52 to initiate mitophagy.

proteins from both the ER (Ye et al., 2001, 2003) and OMM (Heo et al., 2010; Tanaka et al., 2010; Xu et al., 2011). In mitochondria, p97 provides the driving force to extract Mfn1, Mfn2 and the anti-apoptotic protein Mcl1 from the OMM and chaperoned them for their degradation by the proteasome in the cytosol (Tanaka et al., 2010; Xu et al., 2011). Several components of the UPS are recruited to mitochondria such as the E3 ligases Parkin, March V/Mitol, Huwe, and Mulan (Livnat-Levanon and Glickman, 2011).These mitochondrial E3 ligases are a good example of the synergistic crosstalk between different mitochondria quality control systems. For instance, the activity of these ubiquitin ligases impacts on mitochondrial morphology and half-life. Indeed, Parkin-mediated ubiquitination of Mfn1, Mfn2, and VDAC1 blocks/reduces mitochondrial fusion and promotes mitophagy (Narendra et al., 2008; Gegg et al., 2010; Geisler et al., 2010; Tanaka et al., 2010). On the other hand, in physiological conditions Parkin avoid mitochondrial fragmentation by repressing Drp1 levels (Wang et al., 2011). Finally, MarchV/Mitol ubiquitinates Drp1, Fis1, and mitofusins 1 and 2 (Nakamura et al., 2006; Yonashiro et al., 2006; Karbowski et al., 2007). One intriguing issue is whether intramitochondrial proteins that do not face the cytosol can be substrates of the MAD pathway. Even though a retrotranslocation machinery has not been identified, some evidence indicates that the UPS controls IMM proteins. In fact, as a consequence of proximity to the respiratory chain, IMM proteins are exposed to ROS generated by mitochondrial respiration and therefore, oxidized. For example, the mitochondrial uncoupling protein 2 (UCP2) (Azzu and Brand, 2010; Bragoszewski et al., 2013) and the subunit of the OXPHOS complex V OSCP have been found to be retrotranslocated to the OMM for ubiquitination and proteasome degradation (Margineantu et al., 2007). Indeed, proteasome inhibition leads to accumulation of the IMM proteins UCP2, COXI, III, IV, OSCP (Margineantu et al., 2007; Azzu and Brand, 2010). Although the role of the UPS in different cellular processes has been well characterized, studies concerning the interaction with mitochondria are only at the beginning. Several critical issues related to how substrates are identified and how they are transported out for degradation need to be solved in the future.

Under stress conditions, when the degradation pathways are not sufficient to blunt the damage and restore a normal mitochondrial function, a retrograde signal is activated which coordinates nuclear gene expression. This mitochondria-to-nucleus response is named mitochondrial unfolding protein response (UPR^{mt}) (Zhao et al., 2002). The ultimate purpose of UPR^{mt} is to maintain proteostasis by promoting the expression of chaperones to improve protein folding, inhibit protein synthesis to alleviate ER stress and to induce expression of m-AAA proteases like ClpP and Yme1 to remove damaged proteins. For reviews on this topic see (Jovaisaite and Auwerx, 2015; Schulz and Haynes, 2015).

Mitochondrial Dynamics: Not Only a Matter of Shape

Mitochondrial dynamics is defined by the capacity of the organelle to rapidly change its size, shape and distribution by continuously alternating fusion and fission events. Fusion leads to elongated mitochondria with increased interconnectivity into a tubular network. On the contrary, fission fragments the network into unconnected shorter organelles. In physiological conditions, most mammalian cells show a continuous filamentous mitochondrial network with the exception of cardiomyocytes that display fragmented mitochondria that do not form a network (Song and Dorn, 2015). However, the mitochondrial pool rapidly changes its morphology according to the cellular needs. Increased mitochondrial fusion facilitates the redistribution of metabolites, proteins and mtDNA within the network. Moreover, fusion between healthy and damaged organelles allows to dilute the damaged material into the healthy network, avoids the accumulation of dysfunctional mitochondria and maintains their overall fitness (function) (Twig et al., 2008). On the other hand, mitochondrial fragmentation is a mechanism that segregates dysfunctional or damaged components of the mitochondrial network, allowing their removal via mitophagy (Otera and Mihara, 2011).

However, excessive fission generates isolated mitochondria which are less efficient in ATP production and are dysfunctional because they consume ATP to maintain their membrane potential (Benard et al., 2007).

Fusion Machinery

Mitochondrial structure is optimized to ensure respiration and ATP production with a minimal release of ROS. This architecture must be preserved during fusion and fission events. Therefore, when mitochondria fuse they follow a scheme which starts with mitochondrial tethering, continues with fusion of OMM and concludes with fusion of IMM. In mammals, the membrane-bound GTPases mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy protein 1 (Opa1) are required for fusion of the OMM and IMM respectively.

OMM profusion proteins

Mfn1 and Mfn2 are integrated into the OMM and have two cytosolic heptad repeat domains (HR1 and HR2) which interact to form a dimeric anti parallel coiled-coil structure. Both mitofusins can consequently interact homotypically (Mfn1-Mfn1, Mfn2-Mfn2) or heterotypically (Mfn1-Mfn2) to promote the tethering of different adjacent mitochondria and fusion of the OMMs (Koshiba et al., 2004).These proteins have a high degree of homology but they do not have the same functions. Mfn1 has higher GTPase and organelle fusion activity while Mfn2 has greater affinity for GTP (Ishihara et al., 2004). Additionally, Mfn2 and not Mfn1 is expressed on the mitochondria-associated endoplasmic reticulum membranes (MAM) and to a lesser extent to the endoplasmic/sarcoplasmic reticulum (ER/SR) (de Brito and Scorrano, 2009). This unique distribution allows for the close communication between the two organelles. Indeed, Mfn2 bridges mitochondria to ER/SR facilitating important processes linked to ER-mitochondria interactions like calcium homeostasis and signaling (de Brito and Scorrano, 2009; Ngoh et al., 2012; Sebastian et al., 2012; Munoz et al., 2013; Ainbinder et al., 2015). Recent data indicates Mfn2 as a modulator of the UPR during ER stress. In fact, Mfn2 deficiency leads to fragmented ER network and ER stress (Ngoh et al., 2012; Sebastian et al., 2012; Munoz et al., 2013; Bhandari et al., 2015) by chronic activation of PERK (Munoz et al., 2013). Mfn2, like the other mitochondria-shaping proteins is not free from post-translational modifications. For instance, when Mfn2 is phosphorylated by PINK1, it becomes a receptor for the ubiquitin ligase Parkin (Chen and Dorn, 2013). In addition, the E3 ligases HUWE1 and Mul1 also promote Mfn2 ubiquitination and proteasomal degradation (Leboucher et al., 2012; Lokireddy et al., 2012). Conversely, the formation of a Lys63- polyubiquitin chain by the E3 ligase MARCHV/Mitol does not induce Mfn2 degradation but increases its activity as well as MAM formation and function (Sugiura et al., 2013). Altogether, Mfn2 promotes mitochondrial fusion, enhances ER-mitochondria and activates mitophagy via PINK1/Parkin pathway.

Opa1: The pleiotropic IMM fusion protein

The profusion protein Opa1 is regulated by proteolytic processing. There are several splicing variants of Opa1 (8 in humans and 4 in mice) which are expressed in a tissue specific manner. Some of them are cleaved to generate soluble short Opa1 (Opa1S) from long Opa1 isoforms (Opa1L) (Ishihara et al., 2006). Opa1L is anchored to the IMM by a transmembrane domain at the N-terminus and can be further cleaved in exon 5 (site S1) by OMA1, a process that is mitochondrial membrane potential($\Delta\Psi m$)-dependent (Ehses et al., 2009; Head et al., 2009). The mitoprotease Yme1 cut the site S2 in exon 5b of a subset of Opa1L belonging to the splicing variants 4, 6, 7, and 8 (Anand et al., 2014). As aforementioned, other mitoproteases such as m -AAA, HtrA2, and PARL l are also involved in Opa1 processing (Alavi and Fuhrmann, 2013). Furthermore, Opa1 is cleaved in its C-terminal region by an unknown cysteine protease in the post-prandial liver, in a process dependent on Mfn2 and independent of OMA1 (Sood et al., 2014). Opa1-dependent mitochondrial fusion needs Mfn1 (Cipolat et al., 2004) as well as Opa1S and Opa1L forms (Frezza et al., 2006; Song et al., 2007). However, under stress conditions fusion can rely only on Opa1L while Opa1S forms are dispensable (Tondera et al., 2009; Anand et al., 2014). In addition, oligomerization of

soluble and membrane-bound OPA1 isoforms controls cristae remodeling and the assembly of respiratory chain complexes into supercomplexes, a structure that enhances mitochondrial respiration (Cogliati et al., 2013). On the contrary, complete conversion of Opa1L into Opa1S inhibits fusion and increases mitochondrial fission (Ishihara et al., 2006; Anand et al., 2014).

Fission Machinery

Mitochondrial fission depends on the cytosolic GTPase dynamin-related protein 1 (Drp1). Drp1 is recruited to the OMM where it assembles into multimeric ring complexes that form active GTP-dependent mitochondrial fission sites (Smirnova et al., 2001). Drp1 lacks hydrophobic membrane-binding domains and for this reason its recruitment on OMM is dependent on mitochondrial membrane proteins that act as receptors. Accordingly, Drp1 interacts with the integral OMM proteins: Fis1, Mff, mitochondrial elongation factor 2/mitochondrial dynamics protein 49 (MIEF2/MiD49) and MIEF1/MiD51. Fis1 is the major Drp1 receptor in yeasts (Karren et al., 2005). However, increasing evidence suggests that in mammals Fis1 is not the only OMM-anchor receptor of Drp1. For example, downregulation of Fis1 leads to mitochondrial elongation, even if the recruitment of Drp1 to mitochondria is not reduced (Lee et al., 2004). Furthermore, conditional deletion of Fis1 in a carcinoma model did not lead to defects in mitochondrial fission, indicating that Fis1 is not essential for fission (Otera et al., 2010). Recently, new components of the mammalian fission machinery were identified (Otera et al., 2010; Palmer et al., 2011; Zhao et al., 2011). Mff, the OMM-anchored mitochondrial fission factor, colocalizes with Drp1 on mammalian mitochondria. Its overexpression promotes mitochondrial fission with increased Drp1 recruitment to mitochondria. On the contrary, silencing Mff generates elongated mitochondria and Drp1 cytosolic distribution. Moreover, Drp1 affinity for Mff is higher than for Fis, suggesting that Mff preferentially functions as Drp1 receptor (Otera et al., 2010). MIEF1/MiD51 and the variant MIEF2/MiD49 recruit Drp1 to mitochondria independently of Fis1or Mff. Moreover, overexpression of MiD51 does not induce mitochondrial fragmentation but instead prevents mitochondrial fission by sequestering and inhibiting Drp1 (Palmer et al., 2011, 2013; Zhao et al., 2011; Loson et al., 2013). MIEF1/MiD51 preferentially binds Fis1 but can also interact with DRP1 and consequently, depending from the levels of Fis1, two different protein complexes are generated. When the level of Fis1 is high, it sequesters MIEF1/MiD51 that cannot anymore interact and inhibit DRP1 allowing mitochondrial fission. In contrast, the downregulation of Fis1 releases MIEF1/MiD51 that can now block Drp1 resulting in mitochondrial fusion (Zhao et al., 2011). Finally, in yeast Drp1 has been suggested to act as a regulatory factor not only of fission but also of fusion (Huang et al., 2011). In fact, it has been reported that mitochondria-localized Drp1 can interact with the HR1 domain of MFN2 to promote mitochondrial fusion (Huang et al., 2011).

DRP1 regulation

The complexity of Drp1 regulation has been recently reviewed (Elgass et al., 2013; Otera et al., 2013), here we will briefly summarize some critical steps. Drp1-dependent mitochondrial fission is regulated by post-translational modifications like ubiquitination, phosphorylation and SUMOylation to ensure a rapid adaptation to cellular needs (Otera et al., 2013). For example, Drp1 is targeted for proteasomal degradation by Parkin-mediated ubiquitination (Wang et al., 2011) while the ubiquitination mediated by MARCHV/Mitol is controversial with different outcomes depending on cell context (Nakamura et al., 2006; Karbowski et al., 2007). Sumoylation of Drp1 by the SUMO E3 ligase mitochondria-anchored protein ligase (MAPL) stimulates mitochondrial fission (Braschi et al., 2009). Phosphorylation of different residues of DRP1 cause opposing effects. During mitosis, when organelles are inherited by daughter cells, mitochondrial fission activity is promoted by Cdk1-cyclin B-dependent Drp1 phosphorylation at Ser616 (in humans) in the GTPase effector domain (GED) (Taguchi et al., 2007). However, Drp1 activity is also induced by the posphorylation of Ser637 in the GED by the calcium/calmodulin-dependent protein kinase I alpha (CaMK1 alpha). As a result, this phosphorylation increases mitochondrial translocation of Drp1 because it facilitates the interaction with Fis1 (Han et al., 2008). However, phosphorylation of the same Ser637 by the protein kinase A (PKA) has an opposite effect causing DRP1 retention in the cytosol (Cribbs and Strack, 2007). This inhibition is counteracted by the calcium dependent phosphatase, calcineurin (Cribbs and Strack, 2007; Cereghetti et al., 2008). An interesting model of how post-translational modulation of fission can impinge on both, fusion and autophagy is what happens during early stages of starvation. Under this condition, mitochondrial fission is impaired due to the inhibition of Drp1. Two different reports indicate that starvation in cells leads to increased phosphorylation of Ser637 due to PKA activity as well as to decreased phosphorylation of Ser616. As a consequence, Drp1 is retained in the cytosol and mitochondria are elongated and spared from autophagic degradation. The resulting tubular mitochondrial network displays an increased number and density of cristae and presents more dimers of the ATP synthase (Gomes et al., 2011; Rambold et al., 2011). In conclusion, the shaping machinery adapts mitochondrial morphology to the bioenergetic requirements of the cell determining the homeostasis and the fate of the cell. Even though, our knowledge of the mechanisms controlling mitochondria dynamics is growing, many aspects of this process are still unclear.

Lysosomal-Dependent Degradation of Mitochondria: Mitochondria-Derived Vescicles (MDV) vs. Mitophagy

Depending from the type of injury, mitochondria can adopt two different routes for delivering the damaged components to lysosome for degradation. When mitochondria deterioration is mild without global mitochondrial depolarization, local removal of mitochondrial content can be done by the generation of vesicles that bud-off from mitochondria and contain matrix components (Soubannier et al., 2012a,b). These mitochondria-derived vescicles (MDV) are 70–150 nm in size and do not require the fission machinery for their biogenesis. MDVs

carry oxidized proteins coming from different mitochondrial compartments and are directed to lysosomes (Soubannier et al., 2012b). Therefore, mitochondrial oxidative stress induces MDVs via Pink-Parkin system (McLelland et al., 2014) independently of the autophagy-related proteins Atg5, Rab9, or Beclin (McLelland et al., 2014). Since Drp1 fission activity is not required for MDV budding (Soubannier et al., 2012a,b), the identification of the machinery necessary for MDV biogenesis is still unknown. This system works in conjunction with other mitochondria quality control pathways like mitochondrial proteolysis and dynamics and precedes mitophagy. In fact, when mitochondria are irreversibly damaged, they are excised from the mitochondrial network by the fission machinery and sequestered into autophagic vesicles for their degradation in the lysosome. This selective mitochondrial degradation via autophagy is called mitophagy. Apart from removal of dysfunctional mitochondria, mitophagy is essential for mitochondria turnover in the basal state and during the development of specialized cells such as red blood cells (Sandoval et al., 2008). The loss of mitochondrial membrane potential is a major trigger for mitophagy (Elmore et al., 2001). The selectivity of mitophagy is controlled by mitochondria dynamics and by the proteins PINK1, Parkin, Bnip3L/Nix, and Bnip3.

Recessively inherited forms of Parkinson's disease are associated with loss-of-function mutations of the PTEN-induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin. Under basal conditions Pink1 is imported into the IMM where it is cleaved by PARL in a voltage dependent manner. The resulting fragments will be further degraded by the cytosolic UPS (Jin et al., 2010). Therefore, healthy mitochondria have undetectable levels of PINK1. However, when $\Delta\Psi m$ is lost, full length PINK1 is not further imported and accumulates on OMM. Here, PINK1 phosphorylates ubiquitin at Ser65 of ubiquitinated OMM proteins and the ubiquitin-like domain of Parkin (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Once phosphorylated, Parkin enhances the mitophagy signal by generating more ubiquitin chains on OMM proteins that can be further substrates for PINK1 (Lazarou et al., 2015). Subsequently, the autophagic adaptors optineurin, NDP52 (Lazarou et al., 2015) bind the phosphorylated ubiquitins tagging the OMM proteins with LC3 to initiate mitophagy. The contribution of the autophagy adaptor p62/SQSTM1 has been found to be dispensable for mitophagy but important for perinuclear clustering of depolarized mitochondria (Narendra et al., 2010; Lazarou et al., 2015). However, these data were obtained *in vitro* and thus the physiological relevance *in vivo* needs to be validated. For instance, while p62 and NBR1 are well expressed in adult muscles, optineurin and NDP52 proteins are barely detectable (Nicot et al., 2014; Lazarou et al., 2015). Bnip3 and Nix (also called Bnip3L) belong to the BH3-only proteins and are implicated in both apoptosis and mitophagy. These proteins translocate to mitochondria, form homodimers and disrupt mitochondrial membrane potential (Sandoval et al., 2008). They contain two evolutionary conserved LIR (LC3 Interacting Region) domains to bind LC3 and GABARAP. Nix preferentially interacts with GABARAP whereas Bnip3 binds LC3 (Novak et al., 2010; Hanna et al., 2012). These results indicate that Bnip3 and Nix have an important role in mitochondrial turnover, acting as autophagy receptors which tether the mitochondria to the autophagosome. Moreover, the induction of autophagy by the overexpression of Bnip3 in cardiomyocytes requires Drp1 translocation to mitochondria to mediate mitochondrial fission (Lee et al., 2011).

UNRAVELING THE MECHANISMS LINKING MITOCHONDRIAL FITNESS TO MUSCLE HEALTH

Energy requirements during contraction in skeletal muscles increase instantaneously up to 100-fold the consumption of ATP (Blei et al., 1993; Gaitanos et al., 1993). Therefore, to support this high demand of ATP, skeletal and cardiac muscles rely on oxidative phosphorylation for energy production. Indeed these tissues contain the greatest amount of mitochondria. Several reports identified within the myofibers two distinct mitochondrial populations that differ in their subcellular localization, morphology as well as biochemical and functional properties (Howald et al., 1985; Hoppeler et al., 1987; Romanello and Sandri, 2013). Subsarcolemmal (SS) mitochondria are located just underneath the sarcolemma and have a large, lamellar shape. In contrast, the intermyofibrillar (IMF) mitochondria are smaller, more compact, and located between the contractile filaments. However, the existence of two separated mitochondrial pools was challenged by recent findings on high-resolution, three-dimensional electron microscopy (FIB-SEM). Dahl and collaborators showed that SS and IMF are physically connected in human muscles (Dahl et al., 2015). Moreover, Ballaban's lab identified 4 different mitochondrial morphologies: the paravascular mitochondria (PVM), I-band mitochondria (IBM), fiber parallel mitochondria (FPM), and cross fiber connection mitochondria (CFCM) (Glancy et al., 2015). All these mitochondria are highly interconnected forming a conductive pathway that can rapidly distribute energy in the form of electrical conduction throughout myofibers, enabling muscles to respond immediately to changes in energy requirements.

Exercise and Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 (PGC-1) Family Members

There is compelling evidence that regular exercise has many beneficial effects for human health and life span (Neufer et al., 2015). The effects of exercise are sensed by most organs including cardiovascular, neuroendocrine, respiratory and musculoskeletal systems. Importantly, some forms of exercise training counteract skeletal muscle loss (Hourde et al., 2013; Cohen et al., 2015). On the other hand, physical inactivity has been identified as the fourth risk factor of morbidity/disability and mortality (Neufer et al., 2015). Beneficial adaptations to endurance exercise training (moderate intensity with prolonged duration) in skeletal muscle include the activation of transcriptional dependent pathways that impinge on mitochondrial biogenesis (Little et al., 2011), mitochondrial quality and mitophagy (Grumati et al., 2011; He et al., 2012a; Lo Verso et al., 2014).

PGC-1α and PGC-1β

Mitochondrial biogenesis requires a coordinated action on nuclear and mitochondrial genomes that is regulated by the coactivators PGC-1α and PGC-1β (peroxisome proliferator-activated receptor-γ coactivator-1α and β) PGC-1 family members are preferentially expressed in tissues enriched in mitochondria like heart, adipose tissue and slow-twitch skeletal muscle. Exercise, fasting and cold exposure highly regulate their expression. Since PGC-1α and PGC-1β are coactivators that lack DNA binding domains, they elicit their function by modulating the activity of several transcription factors including PPARs, nuclear respiratory factors (NRFs), myocyte enhancing factors (MEFs), estrogen-related receptor (ERR), forkhead box (FoxOs), and yin-yang (YY1) (Lin et al., 2005; Olesen et al., 2010; Zechner et al., 2010). Exercise greatly stimulates PGC-1α via a transcription-dependent upregulation and by several post-translational modifications like phosphorylation and deacetylation (Wright et al., 2007; Cantó et al., 2009; Egan et al., 2010). Moreover, acute endurance exercise promotes the translocation of PGC-1α from the cytosol to the nucleus (Wright et al., 2007) and mitochondria (Safdar et al., 2011). This subcellular relocalization helps nuclear and mitochondrial crosstalk to promote mitochondrial biogenesis (Safdar et al., 2011). Indeed, both Tfam and nuclear gene products are imported into mitochondria where they regulate the expression of mitochondrial proteins required for ATP synthesis. Interestingly, deletion of PGC-1α or PGC-1β in mice showed mild phenotypes (Leone et al., 2005; Lelliott et al., 2006; Vianna et al., 2006; Sonoda et al., 2007). Although, exercise capacity was found reduced in PGC-1α knockout mice (Leone et al., 2005; Handschin et al., 2007; Wende et al., 2007), exercise-induced mitochondrial adaptations in PGC-1α knockout mice were similar to wild-type animals (Leick et al., 2008; Geng et al., 2010). These confilicting results can be probably explained because these two coactivators are redundant and partially compensate each other. In fact, when PGC-1β is deleted on a generalized PGC-1α-deficient background (Zechner et al., 2010) it was shown that these factors share a subset of target genes and display overlapping functions. Accordingly, exercise performance, in this model, is greatly decreased compared to single PGC-1α or PGC-1β knockout animals (Zechner et al., 2010). PGC-1α and PGC-1β are necessary for the maintenance of mitochondrial function (Zechner et al., 2010). Therefore, the deletion of both, PGC-1α and PGC-1β induce severe mitochondrial dysfunction that leads to rapid depletion of glycogen stores and to premature fatigue during exercise (Zechner et al., 2010). Moreover, PGC-1α and PGC-1β control mitochondrial dynamics by stimulating Mfn1 and Mfn2 gene expression in a ERRα-dependent manner (Soriano et al., 2006; Russell et al., 2013). Accordingly, Mfn1, Mfn2, and Drp1 are severely downregulated in PGC-1α and PGC-1β muscle-specific knockout mice (Zechner et al., 2010). Moreover, PGC-1α fine tunes autophagy regulation during some forms of disuse atrophy (Vainshtein et al., 2015). Therefore, PGC-1 coactivators not only increase mitochondrial content but also mitochondrial quality by modulating fusion/fission processes and mitophagy. Surprisingly, PGC-1α and PGC-1β muscle-specific knockout mice do not exhibit defects in muscle fiber type formation in terms of myosin content (Zechner et al., 2010). This is in contrast with a previous study that showed an increase of type I fibers in transgenic mice that specifically express PGC-1α in fast glycolytic muscles (Lin et al., 2002). PGC-1α mRNA levels drop in different atrophying conditions like denervation (Sandri et al., 2006), hind limb suspension (Cannavino et al., 2015) aging (Chabi et al., 2008) or type 2 diabetic patients (Patti et al., 2003). Importantly, maintaining PGC-1α levels high during catabolic conditions, either by the use of transgenic mice or by transfecting adult muscle fibers, spares muscle mass during hind limb suspension, sarcopenia, cardiac cachexia, denervation, fasting, or expression of constitutively active FoxO3 (Sandri et al., 2006; Wenz et al., 2009; Geng et al., 2011; Cannavino et al., 2015). Similar beneficial effects have been recently obtained by overexpression of PGC-1β, an homolog of PGC-1α (Brault et al., 2010). The positive action on muscle mass of these cofactors is caused by the inhibition of the autophagic-lysosomal and the ubiquitin proteasome degradation pathways. PGC-1α and β reduce protein breakdown by inhibiting the transcriptional activity of FoxO3 and NF-κB, but they do not affect protein synthesis (Brault et al., 2010). Thus, these cofactors can prevent the excessive activation of proteolytic systems by inhibiting the action of the pro-atrophy transcription factors without perturbing the translational machinery.

PGC-1α4

A new splicing variant transcript from PGC-1α gene, PGC-1α4, was identified and shown to be involved in the regulation of muscle mass (Ruas et al., 2012). PGC-1α4 expression is induced with resistance exercise protocols (fewer repetitions at high muscle loads aimed at increasing muscle mass) and not with endurance exercise. Skeletal muscle-specific transgenic overexpression of PGC-1α4 results into hypertrophic and stronger mice (Ruas et al., 2012). PGC-1α4 controls muscle mass by inducing IGF1 and repressing the myostatin pathway. Importantly, PGC-1α4 overexpression counteract muscle loss induced by hindlimb suspension and cancer-cachexia (Ruas et al., 2012). However, the results obtained by Ruas and collegues are in contrast with different reports analyzing exercise-mediated adaptations in human muscles (Ydfors et al., 2013; Lundberg et al., 2014; Silvennoinen et al., 2015). In humans, PGC-1α4 is regulated transiently with exercise regardless of mode (resistance or endurance) (Ydfors et al., 2013; Lundberg et al., 2014; Silvennoinen et al., 2015). Moreover, chronic training adaptations, such as increases in muscle mass and force do not correlate with changes in the expression of PGC-1α4 after resistance exercise or after the combination of aerobic and resistance exercise (Lundberg et al., 2014).

Calcium and AMPK Signaling Pathways

The beneficial effects of exercise can be also mediated by contraction-induced changes in calcium homeostasis, ATP consumption and ROS production. The alteration in calcium levels activates calcium-sensitive signaling such as calcium/calmodulin-dependent protein kinases (CaMK) and calcineurin/NFAT systems that are important for fiber type

regulation and muscle plasticity (Wu et al., 2002; Blaauw et al., 2013).

ER-mitochondria crosstalk is essential for excitation-calcium coupling during muscle contraction. Indeed a great amount of ATP produced by mitochondria is consumed by SERCA pumps. Moreover, the positioning of intermyofibrillar mitochondria nearby to SR makes them affected by the calcium wave of contraction. In this context, the role of mitochondrial calcium uptake in the maintenance of skeletal muscle mass was recently investigated (Mammucari et al., 2015). *In vivo* gain- and loss-of-function experiments in skeletal muscle showed that modulation of mitochondrial calcium uniporter (MCU) controls mitochondrial volume. Overexpression of MCU in muscles leads to increased protein synthesis with a resulting hypertrophic phenotype which impinge on IGF1/AKT and PGC-1α4 pathways. Moreover, MCU overexpression protects from denervation-induced atrophy. Supporting the importance of MCU in skeletal muscle is the identification of mutations of MICU1, an MCU regulator, in patients with proximal muscle weakness, learning difficulties and extrapiramidal motor disorder (Logan et al., 2014). In addition, different reports linked calcium homeostasis with the control of muscle mass (Blaauw et al., 2013). Therefore, the investigation of calcium entry pathways in skeletal muscle mitochondria open the possibility to better understand the role of calcium in ER-mitochondria communication and its impacts on muscle mass regulation.

On the other side ATP depletion modifies the AMP/ATP ratio, activating the energy sensor AMP-activated protein kinase (AMPK). The activation of this signal transducer is crucial for the metabolic adaptations in response to energy stress. AMPK inhibition of anabolic pathways together with stimulation of catabolic pathways are aimed at conserving/restoring ATP (Ha et al., 2015). In skeletal muscle AMPK activation is sufficient to increase glucose uptake (by increasing GLUT4 translocation), fatty acid oxidation and mitochondrial biogenesis. In fact, AMPK-dependent phosphorylation of PGC-1α is required for the induction of PGC-1α promoter and for the transcription of many AMPK target genes (Jäger et al., 2007). Summarizing, both calcium-dependent pathways and AMPK modulate the activity and the expression of PGC-1α (Wu et al., 2002; Jäger et al., 2007). Therefore, many of the beneficial effects of exercise converge on the activation of PGC-1 signaling which is central for the mitochondrial and metabolic adaptation to exercise.

Autophagy and ROS Production

Different reports demonstrated that acute as well chronic exercise stimulates autophagy in different tissues (Grumati et al., 2011; He et al., 2012a,b; Lo Verso et al., 2014). Experiments conducted in inducible Atg7-deficient mice unraveled the mechanisms behind autophagy induction following exercise (Lo Verso et al., 2014). Interestingly, autophagy impairment does not affect exercise performance suggesting that autophagy is not required to sustain muscle contraction during exercise training (Lira et al., 2013; Lo Verso et al., 2014). However, autophagy is critical for muscle homeostasis after eccentric contractions. In fact, autophagy is necessary to remove dysfunctional mitochondria that have been altered by exercise in order to prevent excessive ROS production (Lo Verso et al., 2014). It is well known that exercise training and muscle contraction cause an increase in ROS production and a transient oxidative stress (Davies et al., 1982; Powers and Jackson, 2008). Indeed, oxidative stress has an important role in muscle signaling (Finkel, 1998; Rhee et al., 2000; Sena and Chandel, 2012), but can also induce damage to contractile proteins and organelles (Carnio et al., 2014). Although counterintuitive, antioxidant treatment leads to exercise intolerance in wild-type animals and do not ameliorate the physical performance of autophagy-deficient mice (Lo Verso et al., 2014). Moreover, control animals treated with antioxidants display mitochondrial dysfunction and autophagy impairment. Different reports support a crucial role of oxidative stress signaling during exercise in the maintenance of mitochondrial fitness (Gomez-Cabrera et al., 2005, 2008) and in the induction of molecular regulators of insulin sensitivity and antioxidant defense (Ristow et al., 2009). All together these results are consistent with the mitohormesis concept (Ristow and Schmeisser, 2014). Mitohormesis, the non-linear response to mitochondrial ROS formation, exhibits a dual dose-response (Ristow and Schmeisser, 2014). Accordingly, high ROS levels are detrimental due to the induction of oxidative stress with cellular damage. On the contrary, low ROS levels are essential for maintenance of cellular function as well as for improving oxidative stress for the promotion of health and lifespan (Ristow and Schmeisser, 2011; Owusu-Ansah et al., 2013). Therefore, understanding how mitohormesis is regulated could promise a potential application in the promotion of cellular homeostasis and longevity.

IGF1/PI3K/AKT/mTOR Pathway

Although both endurance as well as resistance exercise can promote significant health benefits, the molecular response signature will be different according to the frequency, intensity, duration and the type of exercise (resistance or endurance). The objectives of resistance exercise are an increase in muscle mass, strength and power. The resistance exercise-induced adaptive hypertrophic response is mediated by the anabolic factor insulin growth factor 1 (IGF1) via the IGF1/PI3K/AKT/mTOR pathway. Indeed, increased IGF1 levels precedes muscle hypertrophy achieved by functional overload (Adams and Haddad, 1996). Moreover, IGF1 stimulates fatty acid uptake and insuling sensitivity (Clemmons, 2012) explaining in part some of the beneficial effects of exercise. ATP citrate lyase (ACL) is a cytosolic enzyme that catalyzes the conversion of mitochondria-derived citrate into oxaloacetate and Acetyl-CoA important for lipid synthesis and acetylation. ACL activity is controlled by a post-translational modification via the IGF1/PI3K/AKT pathway. Overexpression of ACL in muscles improved mitochondrial function via stabilization of respiratory chain supercomplexes formation due to increases in cardiolipin synthesis (Das et al., 2015). Thus, IGF1 treatment of myotubes significantly increases mitochondrial respiration due to IGF1-dependent ACL activation suggesting that the activation of the IGF1-ACL pathway increases protein synthesis as well as stimulating the formation of the ATP necessary for this anabolic process (Das et al., 2015). The correlation of mitochondrial fitness

to muscle homeostasis is confirmed by the fact that ACL overexpression in human myotubes is sufficient to induce hypertrophy while ACL activity is downregulated in sarcopenic and in dexamethasone-induced atrophic muscles (Das et al., 2015). All together, these different models offer a useful platform to further investigate the molecular mechanisms explaining how improvements in mitochondrial function can be translated into an increase in protein synthesis and therefore in hypertrophy.

HOW DOES MITOCHONDRIAL DYSFUNCTION ACTIVATE MUSCLE PROTEOLYTIC PATHWAYS?

Muscle atrophy is an active process, controlled by specific signaling pathways and transcriptional programs (Lecker et al., 2004; Sandri, 2013). Of note, the muscle wasting transcriptome signature is characterized by the coordinated reduction of genes encoding key enzymes for ATP production and glycolysis (Lecker et al., 2004). Accordingly, alterations in mitochondrial morphology and function are frequently associated with atrophying muscles in aging (see Section "The Role of Disrupted Mitochondria Quality Control Pathways in Aging Sarcopenia") as well as in several wasting conditions such as burn injury (Porter et al., 2013), intensive care unit-acquired weakness (Friedrich et al., 2015), insulin resistance (Crescenzo et al., 2014), chronic obstructive pulmonary disease (COPD) (Mathur et al., 2014), cancer cachexia (Antunes et al., 2014), and in different neuromuscular disorders (Katsetos et al., 2013). Over the last years, several *in vivo* genetic perturbation models indicate a key role of mitochondrial morphology and mitochondrial dysfunction signaling in the activation of nuclear programs controlling muscle loss. The maintenance of a functional mitochondrial network is indeed particularly important for tissues that are highly structured and metabolically active such as neurons, cardiac and skeletal muscles. These tissues are constituted by post-mitotic cells that do not divide and consequently, cannot dilute damaged/dysfunctional mitochondria through cellular division. Therefore, post-mitotic tissues depend on the activation of mitochondria quality control pathways to preserve or restore mitochondrial function.

Mitochondrial damage is attenuated with different defensive strategies including the activation of mitochondrial proteolytic systems, mitochondrial dynamics and the lysosomal degradation of mitochondrial cargo. For this reason, a failure in any of these systems predisposes to tissue dysfunction and degeneration (**Figure 2**). The importance of the actions of mitochondrial proteases, which remove misfolded or damaged mitochondrial proteins, in the control of muscle mass was confirmed by data obtained with several knockout animal models. Loss in the activities of the proteases PARL

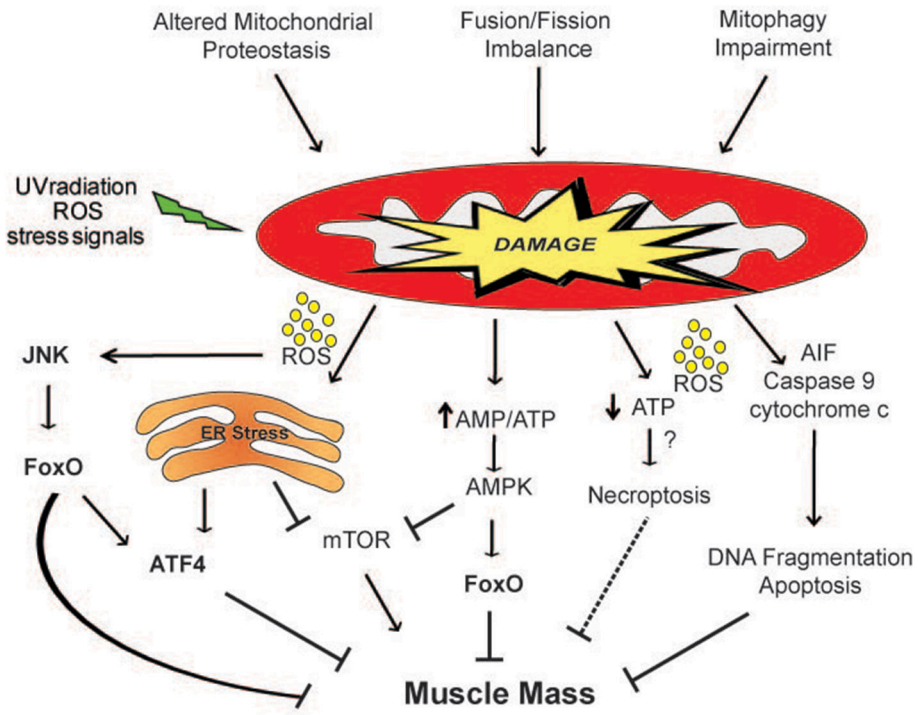

FIGURE 2 | Mitochondria-derived signaling pathways controlling muscle mass. Mitochondrial network accumulates damage when the mitochondrial quality control mechanisms are impaired. ROS produced by the defective organelles induces muscle atrophy through the activation of the JNK-FoxO signaling pathway and of ER stress. Moreover, the release of dangerous factors such as AIF, cytochrome c and ROS can induce apoptosis and/or necroptosis. The activation of the energy sensor AMPK by an increase in the AMP/ATP ratio inhibits mTOR and directly phosphorylates FoxO3 (Ser413 and Ser588) increasing its transcriptional activity, affecting muscle mass. The dashed line indicates a mechanism that needs more studies.

(Cipolat et al., 2006), Omi/HtrA2 (Jones et al., 2003; Martins et al., 2004), and Hax1 (Chao et al., 2008) lead to a similar phenotype, characterized by mitochondrial dysfunction, increased apoptosis, neurodegeneration and atrophy. A fine equilibrium of mitochondrial fusion/fission processes is needed to preserve muscle mass and prevent muscle wasting. This balance is regulated according to the physiological needs of cells.

Fusion Machinery and Atrophy

Fused mitochondria are preferred when optimal mitochondrial function is needed. Indeed intermixing of mitochondrial content by mitochondrial fusion increases the bioenergetic capacity of the cell and is essential for the excitacion-contraction coupling in skeletal muscles (Eisner et al., 2014). Moreover, under conditions of high energy demand, mitochondrial fusion has a survival function, by protecting mitochondria against mitophagy, optimizing ATP production and leading to a sustained cell viability during nutrient deprivation (Gomes et al., 2011; Rambold et al., 2011). The biological importance of mitochondrial fusion in early development is revealed by the embryonic lethality of mouse knockouts for Opa1, Mfn1, and Mfn2 (Chen et al., 2003; Davies et al., 2007). However, few conditional tissue-specific knockout mice have been generated (Chen et al., 2011; Zhang et al., 2011) and some of them show a lethal phenotype therefore, limiting the studies of the role of these factors in mitochondrial function in adulthood. Therefore, little is known about the contribution of fusion machinery to mitochondrial function and tissue homeostasis in most tissues *in vivo*, including skeletal muscle fibers. The neurodegenerative disorders dominant optic atrophy (DOA) (Amati-Bonneau et al., 2008) and Charcot-Marie-Tooth type 2A (CMT2A) (Zuchner et al., 2004), are associated with loss of function mutations of Opa1 and Mfn2 genes, respectively. Noteworthy, patients affected with both DOA and CMT2A develop myopathies (Yu-Wai-Man et al., 2010; Feely et al., 2011). Likewise, neuromuscular defects related to precocious age-dependent axonal and myelin degeneration are present in mouse models resembling the Opa1 mutations most frequently associated with DOA patients (Alavi et al., 2009; Sarzi et al., 2012). Skeletal muscle from these models have alterations at the ultrastructural level with increased smaller mitochondria. In addition, mitochondria display disorganized cristae and accumulate lipid droplets (Sarzi et al., 2012).

Opa1 and Muscle Mass

Further support in the role of Opa1 in the homeostatic control of muscle mass comes from the observations obtained with a genetic model of controlled Opa1overexpression (Civiletto et al., 2015; Varanita et al., 2015). Opa1 transgenic mice are protected from acute muscle loss induced by denervation (Varanita et al., 2015) as well as from chronic muscle loss in a model of myopathy caused by muscle-specific deletion of the mitochondrial subunit COX15 (Civiletto et al., 2015). The analysis of the expression levels of the major atrogenes belonging to the ubiquitin-proteasome and of the autophagy-lysosome systems revealed a reduction of MuRF1 and LC3 expression levels in denervated Opa1 transgenic muscles. Furthermore, increased levels of Opa1 during denervation were sufficient to blunt denervation-induced mitochondrial dysfunction (Varanita et al., 2015).

Mfn2 and Muscle Loss

In line with the relevance of mitochondrial fusion proteins in the normal physiology of skeletal muscles, Mfn2 downregulation induces changes in ER-mitochondria communication that alters mitochondrial calcium buffering (Sebastian et al., 2012; Ainbinder et al., 2015). Moreover, Mfn2 protein levels are reduced in several catabolic conditions (Bach et al., 2005; Hernandez-Alvarez et al., 2010; Lokireddy et al., 2012). Mul1 is a FoxO-dependent ubiquitin-ligase involved in the ubiquitination and further degradation of Mfn2 during muscle atrophy. Accordingly, the maintenance of high Mfn2 proteins levels in starved muscles achieved by the downregulation of Mul1 partially protected from starvation-induced muscle loss (Lokireddy et al., 2012). On the contrary, the ablation of Mfn1 and Mfn2 in muscle-specific knockout mice induces a profound muscle atrophy. These models display mitochondrial dysfunction and compensatory mitochondrial proliferation, reduction of mtDNA as well as accumulation of point mutations and deletions of the mitochondrial genome (Chen et al., 2010). Mechanistically, Mfn2 deficiency in muscles induces a remodeling of the mitochondrial network leading to mitochondrial dysfunction and ROS production (Sebastian et al., 2012). Oxidative stress signaling is sufficient to activate an UPR that blunts insulin signaling via JNK and leads to insulin resistance (Sebastian et al., 2012). Mitochondrial fission is an essential component of the mitochondrial quality control mechanism. When mitochondria divide, two organelles are generated, one with high membrane potential that might undergo fusion and another fragment with low mitochondrial potential and reduced levels of OPA1 that will not rejoin the mitochondrial network and will be likely removed by autophagy (Twig et al., 2008).

Fission Machinery and Atrophy

Impairment of fission leads to the disruption of the selective mitochondrial autophagic degradation followed by accumulation of dysfunctional organelles (Twig et al., 2008; Dagda et al., 2009; Kageyama et al., 2014; Ikeda et al., 2015; Song et al., 2015). In fact, mutations in mitochondrial fission components can be lethal. An infant girl born with a dominant-negative mutation of Drp1 presented alterations in brain development and in metabolism that cause neonatal lethality (Waterham et al., 2007). Moreover, genetic perturbations leading to the ablation of Drp1 in heart or brain are lethal (Ishihara et al., 2009; Wakabayashi et al., 2009; Kageyama et al., 2014; Ikeda et al., 2015; Song et al., 2015). Three different reports investigate Drp1 role in heart physiology. Disrupted mitochondrial fission, obtained with cardiac-specific ablation of Drp1, induces accumulation of defective mitochondria due to impaired autophagy/mitophagy, that over-time promotes cardiomyocyte death (Kageyama et al., 2014; Ikeda et al., 2015; Song et al., 2015). In addition, abnormalities in mitochondrial fission has also been implicated in neurodegenerative disorders such as Alzheimer's, Huntington, and Parkinson (Chaturvedi and Flint Beal, 2013). In skeletal

muscle, atrophying conditions such as fasting, denervation or the overexpression of a constitutively active form of FoxO3 are characterized by alterations of the mitochondrial network (Romanello et al., 2010). Furthermore, the disruption of the mitochondrial network is a crucial amplificatory loop for the muscle atrophy program. The acute overexpression in muscles of the fission machinery *per se* promotes changes of mitochondrial morphology which are followed by mitochondrial dysfunction, energy stress and AMPK activation. As a consequence, AMPK modulates FoxO3 transcriptional activity in order to induce muscle atrophy via the activation of ubiquitin-proteasome and autophagy pathways (Romanello et al., 2010). Accordingly, inhibition of fission machineries or downregulation of AMPK is sufficient to protect against muscle loss in atrophying conditions (Romanello et al., 2010). These findings pave the way to further investigate the causal link between changes in mitochondria morphology and alterations in muscle homeostasis. A recent report investigates the consequences of mitochondrial network remodeling on muscle growth. The constitutive muscle-specific overexpression of Drp1 (Touvier et al., 2015) leads to muscle mass loss and decreased exercise performance (Touvier et al., 2015). In this mouse model, stress-induced mitochondria-dependent signals activate both, the UPR^{mt} and the eIF2α-ATF4-Fgf21 axis causing a reduction in protein synthesis and a blockade of growth hormones actions that prevent muscle growth (Touvier et al., 2015).

Autophagy and Atrophy

Mitophagy is a housekeeping mechanism important to keep under control the turnover of mitochondria in both, the basal state as well as under stress conditions. A finely tuned autophagic system is required for muscle maintenance. Notably, genetic manipulations to activate or inhibit autophagy results into muscle atrophy (Mammucari et al., 2007; Masiero et al., 2009; Romanello et al., 2010). Therefore, autophagy needs to be properly regulated, otherwise it can become detrimental instead of being protective. Several lines of evidence demonstrate that constitutive basal autophagy, which preserves mitochondrial function, is crucial for skeletal muscle homeostasis. Indeed, dysregulation of autophagic flux is detrimental for myofiber health and is a common feature of a group of muscle disorders with alterations of lysosomal proteins such as Danon or Pompe Disease as well as in the Vici Syndrome (Sandri et al., 2013). Moreover, defective autophagy plays a role in congenital muscular dystrophies caused by defects in collagen VI production (Grumati et al., 2010), laminin A/C (Ramos et al., 2012), or dystrophin (De Palma et al., 2012). These dystrophic models have in common a hyperactivation of Akt/mTOR signaling pathway that inhibits autophagy. Dystrophic muscles present accumulation of structurally altered mitochondria together with myofiber degeneration. Noteworthy, reactivation of autophagy flux by dietary or pharmacological tools, like rapamycin, cyclosporine A, or AICAR rescues the dystrophic phenotype by clearing the abnormal mitochondria (Grumati et al., 2010; De Palma et al., 2012; Pauly et al., 2012; Ramos et al., 2012). In agreement, suppression of autophagy by muscle-specific ablation of Atg5 and Atg7, the E3 ubiquitin ligases necessary for autophagosome formation, exacerbates fasting- and denervation- induced atrophy (Masiero et al., 2009). Moreover, impairment of autophagy induces accumulation of abnormal mitochondria, induction of oxidative stress, apoptosis, muscle atrophy, weakness and several features of myopathy (Raben et al., 2008; Masiero et al., 2009). Likewise, reduction of mitophagy caused by ablation of Pink and Parkin induces mitochondrial dysfunction and increased sensitivity to oxidative stress followed by muscle degeneration (Clark et al., 2006; Park et al., 2006; Billia et al., 2011). On the contrary, autophagic flux is increased in muscles in both exercise (Grumati et al., 2011; Lo Verso et al., 2014) and catabolic conditions (Sandri, 2013). For example, in skeletal muscle, mitochondrial dysfunction caused by the transient overexpression of FoxO3, Mul1 (Lokireddy et al., 2012), Bnip3, or Nix (Romanello et al., 2010) triggers autophagy and induces muscle atrophy (Mammucari et al., 2007; Romanello et al., 2010). Naf-1, the nutrient-deprivation autophagy- factor 1, is a ER-Bcl2 interacting protein identified as a novel autophagy regulator. Its downregulation is accompanied by the presence of abnormal mitochondria and increased autophagy that leads to muscle weakness and atrophy (Chang et al., 2012).

Mitophagy Impairment and ROS Production

Oxidative stress is increased when autophagy is dysregulated. ROS has detrimental effects both within and outside mitochondria. Indeed, autophagy deficient muscle show an increase of myosin and actin oxidation that reduces their sliding properties and causes muscle weakness. Anti-oxidant treatment restores a normal force generation and sliding properties even if it does not rescue muscle atrophy (Carnio et al., 2014). Mutations of the Superoxide Dismutase (SOD1) gene is behind 20% of cases of inherited amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative disease characterized by motoneuron degeneration and muscle atrophy. Aberrant ROS production affects mitochondrial function producing a feedback loop that in turn exacerbates mitochondrial damage. Transgenic muscle-specific mice expressing the SOD1 G93A mutation, an ALS-associated human SOD1 mutation display muscle atrophy which is mainly through autophagy activation (Dobrowolny et al., 2008).

Autophagy and Muscle Metabolism

Skeletal muscle plays a significant role in the metabolic control of glucose, lipids, and energy. Importantly, these processes are coordinated with changes in mitochondrial content, shape, and/or function. Therefore, the question now arises as to how modulation of the mitochondrial quality pathways and specifically of mitophagy can affect muscle metabolism. Despite conflicting results, two different studies investigate the connection between autophagy/mitophagy and metabolic regulation in muscles. The first one, uses transgenic mice with knock-in mutations on Bcl2 phosphorylation sites that cannot trigger autophagy by fasting or exercise. These mice show decreased exercise endurance, impaired exercise-induced glucose uptake and are more sensitive to high fat diet-induced glucose intolerance (He et al., 2012a). Since the transgene is expressed in all cell types, it cannot be ruled out whether the observed

effects are resulting from the combination of the actions exerted by different metabolically active tissues (liver, heart, muscle, brain). In the second study, muscle-specific autophagy deficient mice (Atg7 knockout) are protected from high-fat diet induced obesity and insulin resistance. The protective effects depend on dysfunctional mitochondrial signals sufficient to activate an UPR via ATF4, which induces Fgf21 expression (Kim et al., 2013). Therefore, it is not clear how autophagy can fine-tune the metabolic pathways in response to different cellular cues. An important future goal is to establish the metabolic impact of mitochondrial quality control pathways in skeletal muscle.

Oxidative Stress and Atrophy

As already discussed, ROS act in a hormetic fashion (Ristow and Schmeisser, 2014). The beneficial or detrimental action will depend on the level and persistance of ROS flow, as well as on the antioxidant capacity of target cells (Barbieri and Sestili, 2012). While low levels of ROS are associated with positive effects on muscle physiology, on the other hand, excessive production of free radicals accelerates muscle proteolysis (Powers et al., 2012; Reid et al., 2014). Accordingly, the exposure of oxidants to myotubes results in muscle atrophy (McClung et al., 2009; Gilliam et al., 2012). In addition, several models of disuse atrophy like mechanical ventilation, limb immobilization, hindlimb unloading or bed rest are associated with increased ROS production (Pellegrino et al., 2011). Recent evidence indicates mitochondrial ROS production as a required signaling step to induce mitochondrial dysfunction and muscle disuse atrophy (Min et al., 2011; Powers et al., 2011; Talbert et al., 2013b). Mechanistically, oxidative stress can impinge on proteolytic pathways in multiple ways; First, ROS can regulate NF-kB and FoxO transcriptional factors (Dodd et al., 2010) activating the autophagy-lysosome and the ubiquitin-proteasome systems (Taillandier et al., 1996; Levine et al., 2008, 2011; Andrianjafiniony et al., 2010; Hussain et al., 2010; Brocca et al., 2012). Second, the muscle proteases calpain (Taillandier et al., 1996; McClung et al., 2009; Andrianjafiniony et al., 2010; Nelson et al., 2012; Talbert et al., 2013a) and caspase-3 (Levine et al., 2008; Nelson et al., 2012; Talbert et al., 2013a) are activated during disuse atrophy. Third, oxidized proteins are more prone to proteolysis because: (a) their susceptibility to be degraded by caspase-3 and calpain is enhanced, in part because of unfolding of the proteins (Smuder et al., 2010), (b) they can be directly degraded by the proteasome without being ubiquitinated (Grune et al., 2003), and (c) ubiquitin conjugation activity is increased by oxidative stress (Shang et al., 1997).

Summarizing, all these data strongly support the involvement of mitochondrial morphology and/or function in the activation of signaling pathways that control muscle mass.

THE ROLE OF DISRUPTED MITOCHONDRIA QUALITY CONTROL PATHWAYS IN AGING SARCOPENIA

Sarcopenia is defined as the progressive age-related decline in muscle mass and force. It is considered a primary risk for developing major human pathologies that can result into disability and increased vulnerability to death. It has been estimated that roughly, 1.1 and 1.9/kg of muscle mass are lost per decade in women and men, respectively (Janssen et al., 2000). Of note, muscle strength precedes muscle loss, declining three-times faster than muscle loss (Goodpaster et al., 2006). Age-associated muscle loss begins at a slow rate around 30 years of age, then it accelerates and starts to be noticeable after 45 years of age (Janssen et al., 2000). Beyond the sixth decade of life more severe alterations such as spinal motor neurons alterations can be observed (Booth et al., 1994) while the most affected age is over 80 years old (Cruz-Jentoft et al., 2010). The average life expectancy of human beings is rapidly increasing. According to the National Institute on Aging (NIH), in 2010 8% of the world's population were people over 65 years old and this number is expected to double by 2050. Although the resulting clinical, economical and social relevance of sarcopenia, the mechanisms that drive the age-related loss of muscle mass and function are not completely understood. Sarcopenia is a complex multifactorial process. Recently, nine primary hallmarks of aging were specified (Lopez-Otin et al., 2013). Among these, mitochondrial dysfunction has been long suggested as one of the mechanisms of aging sarcopenia (Trounce et al., 1989; Melov et al., 2007; Gouspillou et al., 2010, 2014a,b; Picard et al., 2011; Carnio et al., 2014).

Decreased Mitochondrial Biogenesis

It has been shown that muscle mitochondrial content declines with age (Conley et al., 2000; Short et al., 2005; Chabi et al., 2008), however, there is no clear consensus (Mathieu-Costello et al., 2005; Callahan et al., 2014; Gouspillou et al., 2014b). Mitochondrial biogenesis is important for determining mitochondrial content and function. It depends on the transcription, translation and import of new proteins into pre-existing organelles. In agreement to the observed age-associated mitochondrial content reduction and dysfunction, PGC1-α mRNA and protein levels are reduced in aged muscles (Short et al., 2005; Chabi et al., 2008; Safdar et al., 2010; Ghosh et al., 2011; Joseph et al., 2012; Ibebunjo et al., 2013). On the other hand, muscle-specific PGC1-α transgenic mice are protected from the reduction in mitochondrial function and content observed during aging (Wenz et al., 2009). Moreover, overexpression of PGC1-α is sufficient to prevent age-related muscle loss (Wenz et al., 2009). Blunted mitochondrial biogenesis in aging is supported also by the fact that nascent mitochondrial precursors are more prone to be degraded by cytosolic factors (Huang et al., 2010).

Age-Associated Mitochondrial Oxidative Damage

Several mechanisms necessary for mitochondrial homeostasis are dysregulated in sarcopenia. In fact, during aging, ROS production increases over a certain threshold, overcoming cellular antioxidant defense. Therefore, the initial homeostatic response of ROS signaling is subverted and accelerates the age-associated damage (Lopez-Otin et al., 2013). The resulting oxidative stress, induces post-translational modifications which

compromise protein function (Baraibar et al., 2013). The target proteins modified by oxidative stress (the oxy-proteome components) in aged muscles have been identified recently, by the resolution of carbonylated proteins (an indicator of oxidized damaged proteins) by two-dimensional gel electrophoresis (Lourenco Dos Santos et al., 2015). Many of them are mitochondrial proteins which affect mitochondrial proteostasis (Lourenco Dos Santos et al., 2015; Ross et al., 2015).

Mitochondrial Dynamics Changes

Despite certain discrepancy between reports, the balance between fusion and fission is altered in sarcopenic muscles. Some studies reported that muscle mitochondria in old rats are smaller than young controls (Ljubicic et al., 2009; Iqbal et al., 2013). On the other hand, giant mitochondria were observed in aged muscles of houseflies (Rockstein and Bhatnagar, 1965), muscle cells of humans (Beregi et al., 1988), mice (Beregi et al., 1988), and rats (Navratil et al., 2008). Analysis of the longitudinal vs. transversal mitochondrial orientation with transmission electron microscopy reveals that muscle mitochondria from aged mice are much more enlarged and branched than young muscles (Leduc-Gaudet et al., 2015). Even though the authors did not find any difference in the expression of the dynamic machineries between young and old mice, they suggest that the alteration of mitochondrial morphology in old muscles is the result of a fusion/fission imbalance toward mitochondrial fusion (Bori et al., 2012; Leduc-Gaudet et al., 2015). In contrast, other groups find a downregulation only of Opa1 (Navratil et al., 2008; Joseph et al., 2012) or an upregulation of the fission machinery (Iqbal et al., 2013). Interestingly, a transcriptomic and proteomic study found that reduced expression of all the components of the mitochondria dynamic machineries correlates with age-related muscle loss in rats (Ibebunjo et al., 2013). The employment of new tools, like FIB-SEM, would be useful to correlate mitochondrial dynamics alterations with mitochondrial function in sarcopenic muscles.

The Role of Mitophagy in Sarcopenia

Several evidences indicate an alteration in mitochondrial turnover during sarcopenia. Considering the decline of mitochondrial biogenesis together with the progressive accumulation of macromolecules and dysfunctional organelles it seems to create a likely basis for impaired mitochondrial function and removal during aging. In agreement, some autophagic regulators like LC3 lipidation, Atg7, p62, beclin1, Bnip3, Parkin, and LAMP2 decrease with age (Russ et al., 2012; Joseph et al., 2013; Carnio et al., 2014; Gouspillou et al., 2014b). Moreover, a genetic muscle-specific model of autophagy deletion (Atg7 knockout mice) displays precocious aging characterized by increased oxidative stress, mitochondrial dysfunction, muscle loss and weakness and degeneration of neuromuscular junctions. All together, these results put mitophagy central in age-related mitochondrial dysfunction and critical to prevent age-related denervation and weakness. In contrast, others found increased levels of lipidated LC3 (Wenz et al., 2009), Atg7, beclin1 (Fry et al., 2013), parkin, and p62 (O'Leary et al., 2013). The divergence in these results highlight the necessity of complementing the analysis of the expression of single autophagic regulators with assays to evaluate the complete autophagic process by flux measurements for the correct interpretation of the results (Klionsky et al., 2012).

Interventions to Attenuate Mitochondrial Dysfunction in Aging

Even though, aging sarcopenia is an unavoidable consequence of getting old, it can be reduced with regular exercise and with nutritional interventions. Notably, the benefits of both strategies are mediated by activation of autophagy. Exercise training increases mitochondrial content and turnover by activating both, mitochondrial biogenesis (Little et al., 2011) and autophagy (Vainshtein et al., 2014). As mitochondrial turnover and function are both altered in sarcopenic muscles, exercise seems to be a good countermeasure to improve mitochondrial function (Jubrias et al., 2001; Short et al., 2003; Menshikova et al., 2006; Melov et al., 2007) and therefore, to reduce the sarcopenic process (Jubrias et al., 2001; Melov et al., 2007; Kern et al., 2014; Zampieri et al., 2015). Interestingly, Melov et al. (2007) defined the aging transcriptome signature. The sarcopenic muscle profile was enriched with genes associated with mitochondrial dysfunction compared with young muscles (Melov et al., 2007).Genes important for energy production and mitochondrial function decreased with age. Examples of these genes are the genes encoding the ubiquinol-cytochrome C reductase hinge protein (UQCRH), the β subunit of succinyl-CoA synthase (SUCLA2), and the C subunit of succinate dehydrogenase (SDH). Six months of resistance training were sufficient to revert the transcriptome expression profile of the genes associated with mitochondrial metabolism and electron transport to the expression levels characteristic of young people. However, at the functional/phenotypical levels resistance exercise partially improved muscle strength (Melov et al., 2007). Importantly, the benefits of exercise in reverting the sarcopenic phenotype do not have the same effect on advanced age (over 80 years of age) which have limited muscle plasticity (Slivka et al., 2008; Raue et al., 2009), with the oldest ones being the most affected by the consequences of aging muscle atrophy (Cruz-Jentoft et al., 2010). Caloric restriction (CR) is a powerful nutritional intervention that has been reported to increase lifespan, to maintain metabolism at a more youthful-like state and to prevent chronic diseases (Fontana and Partridge, 2015). The investigation of the effects of lifelong CR in rats muscles demonstrated that CR increases the expression of some autophagic proteins like LC3, Atg7, and 9 and LAMP2 attenuating the age-dependent decline in autophagy. Therefore, the chronic activation of autophagy decreases oxidative damage as well as apopoptotic DNA fragmentation and is sufficient to reduce age-related myocyte degeneration maintaining skeletal muscle homeostasis (Wohlgemuth et al., 2010). Chronic moderate CR adaptation in muscles from humans (30% lower energy intake) and rats is mediated by three major pathways: IGF1/insulin/FoxO, mitochondrial biogenesis, and inflammation (Mercken et al., 2013). CR-mediated responses lead to reduction of IGF1/AKT pathway with the consequent increase of FoxO3 and 4 transcript as well as an upregulation of FoxO-dependent antioxidant

system and autophagy-related genes (Mercken et al., 2013). Moreover, CR induces a transcriptional reprogramming profile of molecular pathways that become more similar to the profile of young individuals (Mercken et al., 2013). The involvement of age-dependent mitochondrial decline in sarcopenia as well as the physiological significance of this decline clearly needs more studies. In years to come it will be exciting to see if manipulations aimed at enhancing mitochondria quality control pathways will be sufficient to promote a healthy aging of muscles.

FINAL CONSIDERATIONS

In the last years our understanding of the different mechanisms controlling mitochondrial morphology/function and the impact of these pathways in the homeostatic control of muscle mass has experienced an unprecedented advance. However, there are still many challenges that should be addressed. First, since the components of the mitochondria quality control system are closely interconnected, the alteration in one system can impinge on the activation/inhibition of the other repair mechanism. Therefore, this interplay, which is of fundamental importance for the integration of mitochondrial function within the network, should be considered in future studies. Second, mitochondria are social organelles that establish direct or indirect (via mitochondria-derived vesicles) contacts with other cellular components. Indeed, mitochondrial communication with the endoplasmic reticulum, peroxisomes, and lysosomes/vacuoles are hot topics of study at the moment. Then, it will be of great interest to investigate the functional consequences of these interactions in the physiology of muscle in the future. Finally, mitochondria-derived metabolites can shuttle within the cell and within the whole organism. Thus, exploring the interrelation between mitochondria quality control pathways and the metabolic regulation of muscle mass as well as the muscle-interorgans crosstalk should give additional insights to understand the way these relationships can be manipulated to treat human diseases.

AUTHOR CONTRIBUTIONS

VR and MS wrote the manuscript.

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MINI REVIEW
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Mitochondrial Regulation of the Muscle Microenvironment in Critical Limb Ischemia

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Critical limb ischemia (CLI) is the most severe clinical presentation of peripheral arterial disease and manifests as chronic limb pain at rest and/or tissue necrosis. Current clinical interventions are largely ineffective and therapeutic angiogenesis based trials have shown little efficacy, highlighting the dire need for new ideas and novel therapeutic approaches. Despite a decade of research related to skeletal muscle as a determinant of morbidity and mortality outcomes in CLI, very little progress has been made toward an effective therapy aimed directly at the muscle myopathies of this disease. Within the muscle cell, mitochondria are well positioned to modulate the ischemic cellular response, as they are the principal sites of cellular energy production and the major regulators of cellular redox charge and cell death. In this mini review, we update the crucial importance of skeletal muscle to CLI pathology and examine the evolving influence of muscle and endothelial cell mitochondria in the complex ischemic microenvironment. Finally, we discuss the novelty of muscle mitochondria as a therapeutic target for ischemic pathology in the context of the complex co-morbidities often associated with CLI.

Keywords: skeletal muscle, vascular diseases, mitochondria, ischemia, peripheral arterial disease, angiogenesis

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INTRODUCTION

Peripheral artery disease (PAD) presents as either symptom-free, intermittent claudication (IC, pain with exertion that is relieved with rest) or critical limb ischemia (CLI, pain at rest with or without tissue necrosis or gangrene). CLI carries alarmingly high morbidity and mortality rates and patients have a risk of major amputation or death that approaches 40% in 1 year (Dormandy et al., 1999; Hirsch et al., 2001; Taylor et al., 2009). A common misconception is that CLI represents the natural progression of IC in patients; however the same degree of stenosis can present as symptom-free, IC, or CLI, implying that factors other than limb blood flow contribute to pathology. Despite recent advances in stem cell biology and genetics (Matzke and Lepantalo, 2001; Chalothorn et al., 2007; Dokun et al., 2008; Chalothorn and Faber, 2010; Wang et al., 2010, 2012; Katwal and Dokun, 2011), surprisingly little progress has been made toward effective therapeutic options for CLI, warranting the consideration of alternative and novel treatment approaches. Limb skeletal muscle is uniquely positioned to alter the clinical course of CLI due to its inherent plasticity, role as a paracrine signaling organ, and reservoir of endogenous pluripotent progenitor cells (Seale et al., 2001; Chargé and Rudnicki, 2004; Abou-Khalil et al., 2010). Currently, PAD research is overwhelmingly focused on limb collateral vessel development and nascent conduit promotion

and survival (Annex, 2013), while potential alternative therapies directed at limb muscle in CLI have been slow to develop. In this mini-review we highlight the importance of skeletal muscle in the manifestation of CLI and discuss the potential influence of muscle and endothelial cell mitochondria on the ischemic limb.

SKELETAL MUSCLE PATHOLOGY IN THE ISCHEMIC LIMB

Variations in the clinical course of CLI raise the intriguing possibility that disease manifestation is in part dependent on genetic determinants of susceptibility to ischemia (Matzke and Lepantalo, 2001; Chalothorn et al., 2007; Dokun et al., 2008; Chalothorn and Faber, 2010; Wang et al., 2010, 2012; Katwal and Dokun, 2011). The genetics of PAD are not well understood (Gudmundsson et al., 2002; Knowles et al., 2007; Messina, 2008; Katwal and Dokun, 2011; Leeper et al., 2012; Murabito et al., 2012) but present a complicated paradigm whereby differential determinants could direct the ischemic responses of multiple cell types (endothelial, muscle, fibroblast, etc.) in the affected limb. In this regard, inbred mouse strains have dramatically different responses to a murine model of PAD, analogous to the range of responses seen in humans. For example, limb perfusion recovers rapidly and without tissue loss in C57BL/6J (BL6) mice while BALB/cJ mice display significant tissue necrosis and poor perfusion recovery (Chalothorn et al., 2007; Dokun et al., 2008; Chalothorn and Faber, 2010; Wang et al., 2010). Inherent genetic differences in muscle regeneration are known to occur in BALB/cJ mice (Grounds, 1987; Grounds and McGeachie, 1989; McGeachie and Grounds, 1995; Mitchell et al., 1995; Roberts et al., 1997; Lagrota-Candido et al., 2010), and includes temporal alterations in the expression of traditional vascular growth factors and their receptors (McClung et al., 2012) that coincide with the strain-dependent segregation of limb blood flow. Differentiating muscle cells secrete traditional vascular growth factors that act as both autocrine and paracrine factors to stimulate maturation in both endothelial and muscle cells (McClung et al., 2012, 2015; Mofarrahi et al., 2015) and represent a unique source of regenerative signals that could potentially be harnessed to improve the local ischemic microenvironment. Because a large proportion of murine pre-clinical limb ischemia work is performed in mice on either a mixed or largely BL6 background, regeneration from ischemic muscle myopathy is often masked or ignored.

In a clinical CLI scenario, focusing on solely the vascular response is predicated on the idea that the ischemic muscle tissue is dispensable, at least short-term. Treatments that induce revascularization and/or nascent collateral vessel formation have proven ineffective to date (Annex, 2013; Hammer and Steiner, 2013; Cooke and Losordo, 2015) and indicate that a "restoration of flow approach" is not independently sufficient to rescue the limb. It is likely that myopathy and vasculopathy are interrelated components of a coordinated tissue response to CLI. Recent insights into the skeletal muscle response indicate that while the background genetics of an individual contributes to the density of pre-existing collateral vessels and the endogenous ability to generate nascent collateral vessels and capillaries, this simply isn't the sole determinant of pathology. The plasticity of the skeletal muscle facilitates temporal ischemic degeneration/regeneration in this environment, whereby genetically pre-determined deficits in muscle regenerative processes would result in cellular apoptosis and tissue necrosis that could negatively impact both endogenous neovascularization and/or the survival of a vessel graft. Simply put, limb muscle tissue that is already necrotic or beyond repair by endogenous regenerative mechanisms is representative of a local ischemic environment that is unable to sustain or promote neovascularization (**Figure 1**). Intricate coordination of therapies targeting muscle plasticity may be required to allow tissue survival and facilitate recovery until blood flow can be fully restored by surgical intervention and/or collateral vessel formation.

The importance of striated muscle to ischemic outcomes is readily accepted in cardiac ischemia/reperfusion, and there are numerous clinical trials involving therapeutic targeting of the cardiomyocyte (*clinicaltrials.gov*: NCT01502774, NCT01374321,

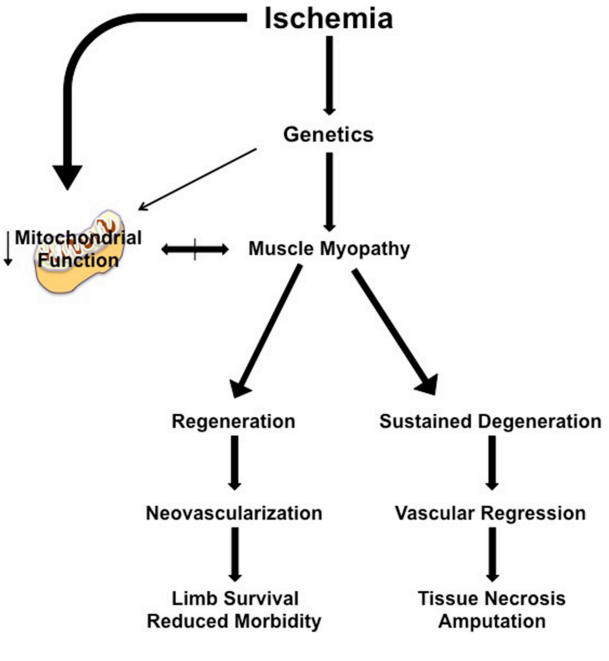

FIGURE 1 | Simplified model of the proposed role of muscle myopathy in the progression of limb pathology in critical limb ischemia. Individual genetics play a role in determining the severity of the ischemic manifestation of limb pathology. Clinical interventions (endovascular or revascularization in nature) occur after the patient clinically presents with identifiable symptoms/manifestation of PAD, at a time when muscle myopathy is initiated or ongoing. Ischemic muscle myopathy involves muscle degeneration/regeneration cycles that: (1) function properly and result in a limb tissue microenvironment that is supportive of neovascularization and/or the clinical intervention, reducing morbidity and increasing the likelihood of limb survival, or (2) improperly function, resulting in a microenvironment that promotes continued degenerative myopathy and vascular regression that ultimately leads to tissue necrosis, morbidity, and secondary amputation. The role of mitochondrial function in ischemic limb muscle myopathy is not currently understood, but represents an exciting area for therapeutic exploration.

NCT01172171, NCT00966563, NCT01572909). Therapies aimed at the skeletal muscle represent an untapped arena with great potential to advance the field of CLI research. **Table 1** highlights the clinical work verifying limb skeletal muscle's role in PAD mortality over the last 10-years. Documented histochemical evidence of skeletal muscle myopathies and necrosis in PAD patients exists (Rissanen et al., 2002; Pipinos et al., 2007, 2008a); however the majority of the field operates under the assumption that myopathy is not important (Sealock et al., 2014). In stark contrast, muscle biologists studying neuromuscular diseases, dystrophy, or myofibrillar myopathies have embraced the contributions of the vasculature to the pathologic manifestations of their respective diseases. Abnormal skeletal muscle perfusion and resultant ischemia are believed to contribute to the pathology of Duchenne and Becker muscular dystrophies, and have spawned the "two hit (ischemia-metabolic stress) hypothesis" for muscle injury in these diseases (Asai et al., 2007). This hypothesis has driven approaches to treat these muscular dystrophies with phosphodiesterase-5 inhibitors like tadalafil, which improves blood flow, in an attempt to circumvent the ischemic component and improve muscle bioenergetics (Martin et al., 2012). Given the similarities between dystrophic myopathies and those identified with CLI, therapies aimed at skeletal muscle could be effective treatments for tissue degeneration and dysfunction during ischemia while also providing benefits to the vascular compartment of the affected limb. Interestingly, a common pathology linking neuromuscular disorders involving degeneration/regeneration is mitochondrial dysfunction (Katsetos et al., 2013). Mitochondria have recently garnered attention in the PAD literature (Brass, 2013; Hiatt et al., 2015) and may represent an evolutionarily conserved "starting point" for investigation into CLI myopathy.

SKELETAL MUSCLE MITOCHONDRIA IN THE ISCHEMIC LIMB

Mitochondria have numerous roles in the muscle cell, including the generation and maintenance of energy and redox charge, gatekeeping the mortality of ischemic cells (Karch and Molkentin, 2015; Shirihai et al., 2015), and the production of reactive oxygen species (ROS). Mitochondria also communicate with the rest of the cell through "signals" such as metabolites, cytochrome c release, and via redox-dependent cascades. Decreased muscle metabolism, impaired mitochondrial respiration, decreased expression of mitochondrial enzymes, increased oxidative stress, and somatic mutations in mitochondrial genes have been reported in limb muscle of patients' with PAD (Keller et al., 1985; Hands et al., 1986; Zatina et al., 1986; Bhat et al., 1999; Brass and Hiatt, 2000; Pipinos et al., 2000a, 2006, 2008b; Brass et al., 2004; Isbell et al., 2006; Schocke et al., 2008; Wurdeman et al., 2012; Weiss et al., 2013; Koutakis et al., 2014). Using non-invasive magnetic resonance spectroscopy, several labs have demonstrated that limb muscle from PAD patients' displays slower phosphocreatine (PCr) recovery, indicative of a lower muscle/mitochondrial oxidative capacity (Keller et al., 1985; Hands et al., 1986; Pipinos et al., 2000a,b; Isbell et al., 2006; Schocke et al., 2008). Interpretation of these data can be complicated by the influence of an intact but poorly functioning vascular system, perpetuating the idea that the reduced PCr recovery rates are more related to poor perfusion during the recovery period. Gastrocnemius muscle biopsies from PAD patients, however, also demonstrate reduced mitochondrial content and enzyme activity *ex vivo* (where oxygen delivery is not a limitation; Pipinos et al., 2003, 2006, 2007), and pre-clinical studies have recapitulated these findings (Pipinos et al., 2008b; Lejay et al., 2015). It is not currently known whether alterations in mitochondrial content or function cause ischemic muscle myopathy, but a recent report linked muscle mitochondrial content (reported as citrate synthase protein abundance) to PAD mortality (Thompson et al., 2014).

A lack of oxygen delivery to limb muscle tissue induces a progressive accumulation of ischemic injury that manifests as declining muscle function (Pipinos et al., 2007, 2008a; McDermott et al., 2012; Cluff et al., 2013; Weiss et al., 2013; Koutakis et al., 2014). A potential source for this tissue injury may be mitochondrial-derived ROS and the resulting oxidative stress with chronically elevated ROS. Pipinos et al. reported the first indirect evidence for skeletal muscle "oxidative stress" in patients with PAD (Pipinos et al., 2006). Recent work from this group

TABLE 1 | Clinical studies implicating skeletal muscle function with mortality.

Study	*n*	Patient population	Skeletal muscle factors associated with mortality
Gardner et al., 2008	434	PAD	6-min walk test, speed, and stair climbing scores
de Liefde et al., 2009	2191	PAD	Total treadmill walking distance
Singh et al., 2010	410	PAD	Attenuated knee extensor/flexion and hip extension strength in men, but not women
McDermott et al., 2011	440	PAD	Decline in 6-min walk test, and fast- and usual-paced 4-m walk test
McDermott et al., 2012	434	PAD	Lower calf muscle density and strength
Raval et al., 2012	425	PAD	Obesity associated with lower calf muscle density and greater declines in muscle density over time.
Jain et al., 2013	442	PAD	Walking speed and strain climbing scores from walking impairment questionnaire
Leeper et al., 2013	725	PAD	Symptom limited walking time on ramped treadmill test
Thompson et al., 2014	187	PAD	Calf muscle citrate synthase activity (marker of mitochondrial content)
Matsubara et al., 2015	64	CLI	5-year survival rate significantly lower in patients with sarcopenia (total body)

A brief literature search using PUBMED, MEDLINE, and SCOPUS was conducted. Studies assessing the association between skeletal muscle health/function and cardiovascular/all-cause mortality are shown above with abbreviated summary of findings. PAD, peripheral arterial disease; CLI, critical limb ischemia; n, number of patients.

suggests that these same indirect markers of oxidative stress may be related to disease severity (Fontaine Stage and ABI;Weiss et al., 2013). The potential also exists for repeated ischemia-reperfusion events in skeletal muscle from CLI patients (Lejay et al., 2014). When blood flow and pressure is low, arterial blockages may result in low oxygen tensions in muscle tissue that could be severe enough to inhibit mitochondrial complex IV (cytochrome c oxidase) and consequently electron flow in the electron transport system. This would result in the accumulation of metabolites and reducing equivalents (NADH and $FADH_2$) that, upon re-oxygenation by surgical intervention or endogenous collateral flow with activity or mechanical loading, would be rapidly metabolized. These ischemia-reperfusion events have been well documented to produce large amounts of ROS in cardiac, brain, liver and renal tissues (Chouchani et al., 2014) and could be intermittently triggered by small amounts of physical activity or mechanical loading. For additional details on oxidative stress with PAD, we would recommend other excellent reviews (Brass, 1996; Pipinos et al., 2007, 2008a).

Because mitochondria are a major source of both reductive power (e.g., NADPH) and oxidants (superoxide anion and hydrogen peroxide), they serve as a metabolic rheostat controlling cellular redox homeostasis. Flux through both the reductive and oxidative arms contributes to redox signaling through redox modifications to cysteine residues that regulate the structure/function of target proteins (Go and Jones, 2013). Post-translational modifications such as S-nitrosylation, glutathionylation, sulfenylation, and disulfide bond formation are also considered mechanisms of redox signaling. Although the redox signaling field is at an early stage, recent studies suggest regulation of several cellular pathways relevant to the ischemic microenvironment including: muscle autophagy (Rahman et al., 2014), contractile dysfunction (reviewed in Powers et al., 2011), atrophy (Lawler et al., 2003), mitochondrial fission and fusion (reviewed in Willems et al., 2015), vascular growth and remodeling (reviewed in Bir et al., 2012), gene stability (Mikhed et al., 2015), and cellular proliferation and death (Wang et al., 2013; L'honoré et al., 2014). An oxidative shift with elevated ROS production in one cell type may have a direct and/or indirect effect on other resident cell types. Although it is difficult to imagine that charged, highly reactive oxygen/nitrogen species arising within subcellular organelles (e.g., mitochondria) or from cytosolic enzymes (e.g., xanthine oxidase) could escape the oxidant buffering systems (e.g., glutathione peroxidases, peroxiredoxins, superoxide dismutase, catalase) and travel to neighboring cells, ROS species, particularly those not carrying a charge (e.g., H_2O_2), produced by membrane bound enzymes (e.g., NADPH oxidase) may be capable of directly affecting nearby cells. It is likely that altered redox homeostasis in one cell would dramatically alter the local microenvironment through paracrine signaling. For example, skeletal muscle redox alterations have been shown to decrease endothelial cell angiogenic properties via the HIF-1α signaling cascade (Dromparis et al., 2014). Further, HIF-1α is a known transcriptional regulator for vascular endothelial growth factor (VEGF), which plays a vital role in angiogenesis (Rhoads et al., 2009).

ENDOTHELIAL MITOCHONDRIA IN THE ISCHEMIC LIMB

Early research on the cellular bioenergetics of endothelial cells (ECs) suggested a heavy cellular reliance on glycolytic metabolism for the energy requirements of normal processes (Dobrina and Rossi, 1983; Leighton et al., 1987; Krützfeldt et al., 1990; Laing et al., 1992). These studies reported high activities of key enzymes in glycolytic metabolism (phosphofructokinase, hexokinase), high rates of lactate production in aerobic conditions, and low rates of glucose oxidation especially when high levels of glucose are present (Crabtree Effect). Additionally, ECs have a relatively low mitochondrial content, [less than 5% of the cell volume vs. 5–20% in skeletal muscle (Hoppeler et al., 1981; Groschner et al., 2012; Dahl et al., 2015; Jacobs et al., 2015)]. Some studies suggest mitochondrial ATP production is dispensable in ECs (Quintero et al., 2006) and there appears to be supportive evidence in that limb ECs are resistant to ischemic insult in CLI patients (Mertens et al., 1990; Noll et al., 1990). There is also a distinct body of research, however, indicating that mitochondria are critical organelles to the viability and function of ECs (Quintero et al., 2006; Goveia et al., 2014; Eelen et al., 2015). Mesenchymal stem cells form tunneling nanotubes that transfer mitochondria to ECs to rescue cellular aerobic respiration and stave off apoptosis induced by ischemia/reperfusion (Liu et al., 2014), a response that could be particularly important in stroke patients (Chan, 2005; Li et al., 2012; Lejay et al., 2014; Mishiro et al., 2014). Capillary EC mitochondrial cytopathies decrease angiogenesis and precede myofiber injury in early infants with mitochondrial diseases, a finding termed "mitochondrial angiopathy" (Sarnat et al., 2012). Overexpression of mitochondrial Thioredoxin-2 (Trx2) improves EC proliferation and arteriogenesis in the ischemic limb (Dai et al., 2009) and cancer researchers now utilize mitochondrial uncouplers in attempts to reduce tumor size due to their effects on neovascularization (Coutelle et al., 2014). These recent findings support an integral role for the mitochondria in the regulation of EC function and indicate this organelle's potential as a therapeutic target for CLI.

MITOCHONDRIAL DYNAMICS

Mitochondria are dynamic organelles that rely on complex signals orchestrating dynamic fission and fusion events believed to be responsible for regulating mitochondrial quality control. Fission and fusion are involved in the elimination of damaged/dysfunctional mitochondria (Song et al., 2015) which may serve as major sources of ROS. A cell's decision to remove dysfunctional mitochondria plays a vital role in limiting cellular damage/apoptosis while maintaining cell function. Damaged and depolarized mitochondria are targeted by PTEN-induced putative kinase 1 (PINK1), which drives Parkin-mediated mitophagic engulfment by autophagosomes (termed "mitophagy," for detailed reviews see Dorn and Kitsis, 2015; Shirihai et al., 2015). Recent preclinical evidence suggests that defects in mitophagy exacerbate cardiomyocyte injury and decrease survival following ischemia/reperfusion (Song

et al., 2014), indicating an increased cellular sensitivity to ischemic stress (Kubli et al., 2013). Mitophagy is critically important to the plasticity of skeletal muscle (Liesa and Shirihai, 2013) and is a unique process that could be similarly important to the health of the vasculature in the ischemic limb. Ischemia/reperfusion-induced impairments in EC mitochondrial respiratory capacity have been intricately linked to accelerated fission caused by excessive oxidative and nitrosative stress (Giedt et al., 2012). Moreover, siRNA-knockdown of mitofusin-1 or mitofusin-2 impairs EC angiogenic function *in vitro* and increases markers of apoptosis under stress (serum-deprivation; Lugus et al., 2011). There are no current investigations into the potential role of mitophagy in limb muscle pathology with CLI, although mitochondrial dynamics provide an attractive candidate for exploration. The accumulation of damaged mitochondria is likely to lead to increased ROS, an oxidative shift in the redox environment, and impaired energy production; all factors contributing to a pathologic ischemic microenvironment.

PRIMARY CLI RISK FACTORS ASSOCIATED WITH ALTERED MITOCHONDRIAL FUNCTION

There are numerous risk factors linked to the CLI manifestation in PAD patients (Fowkes et al., 2013; Nehler et al., 2014). The two strongest risk factors for CLI, smoking and diabetes, are particularly provocative in terms of the subject matter of this review due to the ability of both to impair mitochondrial function in multiple cellular compartments of the ischemic limb.

Smoking

From a physiologic perspective, smoking impairs microvascular reactivity (Ijzerman et al., 2003), increases intima-media carotid wall thickness (Howard et al., 1994), decreases flow-mediated dilation in the brachial artery (Langham et al., 2015) and increases the likelihood of atherosclerotic lesion formation (Yanbaeva et al., 2007). Pre-clinically, chronic cigarette smoke exposure severely alters vascular structure and function, including facilitating oxidative and nitrosative stress (Talukder et al., 2011). ECs exposed to cigarette smoke extract *in vitro* have lower mitochondrial integrity, rapid loss of mitochondrial membrane potential, and arrest of cell cycle progression (Henderson et al., 2008). Interference of the respiratory chain by either hydroquinone or carbon monoxide is believed to be a key component of smoking induced mitochondrial dysfunction in skeletal muscles (Degens et al., 2015), as well as impaired oxygen delivery due to carbon monoxide binding with hemoglobin/myoglobin. Interestingly, the combination of high-fat diet and nicotine results in increased oxidative stress and substantial lipid accumulation adjacent to swollen intramyofibrillar mitochondria in peripheral skeletal muscle (Sinha-Hikim et al., 2014). Taken together, the global cellular response to smoking demonstrates the potential for smoking to alter not only physiologic vessel function and the time-course of atherosclerotic lesion formation, but also the health of peripheral muscle cells. Interestingly, only a small series of research studies examine the muscle regenerative aspect, several of which are linked with healing rates after orthopedic surgery (Karim et al., 2006; Lundgreen et al., 2014; Mall et al., 2014). There are, however, a number of studies demonstrating ultrastructural and functional alterations in cardiomyocyte mitochondria after exposure to cigarette smoke (Yamada et al., 2009; Hu et al., 2013; Tippetts et al., 2014). Further work is necessitated to directly examine the effects of smoking on the mitochondria of the ischemic limb muscle, but this area represents an exciting arena with the potential to result in singular therapies for multiple co-morbidities associated with smoking and cardiovascular disease.

Diabetes

Type II Diabetic patients with PAD are five times more likely to present clinically with CLI accompanied by tissue loss (Jude et al., 2001) and do not respond well to revascularization or endovascular interventions (Derubertis et al., 2008; Malmstedt et al., 2008). While diabetes may exacerbate the development of plaque blockages in the arteries, the impact of metabolic syndrome/diabetes on other tissue compartments has not been investigated in the context of CLI. As a common risk-factor, one explanation for the diabetic increase in PAD susceptibility could conceivably be exacerbated muscle damage originating from compromised mitochondrial function prior to the onset of ischemia. Diabetes both reduces skeletal muscle mitochondrial function (Kelley et al., 2002; Petersen et al., 2004; Bonnard et al., 2008) and increases mitochondrial fission, fragmentation, and ROS production in human venous ECs (Shenouda et al., 2011), indicating the potential for exacerbated ischemic responses in multiple cellular compartments of the ischemic limb. Chronic oxidative stress caused by nutrient oversupply to muscle mitochondria is implicated in reduced diabetic mitochondrial respiratory function (Bonnard et al., 2008; Anderson et al., 2009a,b), whereas mitochondrial-targeted antioxidants confer protection against diet-induced dysfunction (Hoehn et al., 2009; Anderson et al., 2009b; Lee et al., 2010). Taken together, these findings suggest the possibility that compromised muscle and endothelial mitochondrial function may be pre-conditioning the limb tissue to respond poorly to the ischemic insult in diabetic CLI patients, resulting in greater myopathy and sustained tissue degeneration regardless of genetic susceptibility.

CONCLUSIONS AND FUTURE DIRECTIONS

A critical barrier to developing therapeutic strategies to PAD has been a lack of understanding of the mechanisms underlying the etiology and pathology of PAD. While the cause of PAD is unquestionably occlusive arterial disease, the limited success of surgical and angiogenic treatments suggest that factors other than blood flow may significantly contribute to patient outcomes. Physiologically, angiogenesis and neovascularization are directed by the metabolic demand of the resident tissue. Simply put, the return of blood flow will have little effect if the limb tissue

is beyond repair. In this review, we have highlighted recent trends in CLI research that suggest limb musculature may be a viable and potentially parallel therapeutic option for both the myopathy and vasculopathy of CLI. Furthermore, limb muscle and EC mitochondria provide attractive specific targets for novel therapeutic intervention.

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MINI REVIEW
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Complex Coordination of Cell Plasticity by a PGC-1α-controlled Transcriptional Network in Skeletal Muscle

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Skeletal muscle cells exhibit an enormous plastic capacity in order to adapt to external stimuli. Even though our overall understanding of the molecular mechanisms that underlie phenotypic changes in skeletal muscle cells remains poor, several factors involved in the regulation and coordination of relevant transcriptional programs have been identified in recent years. For example, the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a central regulatory nexus in the adaptation of muscle to endurance training. Intriguingly, PGC-1α integrates numerous signaling pathways and translates their activity into various transcriptional programs. This selectivity is in part controlled by differential expression of PGC-1α variants and post-translational modifications of the PGC-1α protein. PGC-1α-controlled activation of transcriptional networks subsequently enables a spatio-temporal specification and hence allows a complex coordination of changes in metabolic and contractile properties, protein synthesis and degradation rates and other features of trained muscle. In this review, we discuss recent advances in our understanding of PGC-1α-regulated skeletal muscle cell plasticity in health and disease.

Keywords: skeletal muscle, transcriptional regulation, PGC-1α, exercise, metabolism, co-regulator

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INTRODUCTION

Cell plasticity is often controlled by complex transcriptional networks. While traditionally, much of the research on such networks was focused on transcription factors, the important role of co-regulators has been increasingly appreciated in recent years (Dasgupta et al., 2014; Mouchiroud et al., 2014). Co-regulator proteins have no intrinsic DNA-binding domain and thus rely on transcription factors to be recruited to regulatory elements. The ability of co-regulators to bind to various partners enables the regulation of broad, complex transcriptional programs. The peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) is a prototypical member of this class of proteins. Initially discovered in a screen comparing white and brown adipose tissue, expression of PGC-1α has subsequently been documented in all tissues with a high energetic demand, including brain, kidney, skeletal, and cardiac muscle, liver, pancreas, or the retina (Martínez-Redondo et al., 2015). The core function of PGC-1α centers on the induction of mitochondrial biogenesis and oxidative metabolism. PGC-1α likewise controls highly tissue-specific programs, such as hepatic gluconeogenesis or mitochondrial uncoupling in brown adipose tissue. Since the phenotype of global PGC-1α transgenic and

knockout mice is complex (Lin et al., 2004; Liang et al., 2009), many insights into organ-specific function and regulation of PGC-1α, including those discussed in this review unless otherwise stated, have mostly been obtained from murine tissue-specific gain- and loss-of-function models (Handschin et al., 2005). In skeletal muscle, specific overexpression of PGC-1α at physiological levels is sufficient to induce an endurance-trained phenotype (Lin et al., 2002) while super-physiological overexpression promotes fiber damage and impaired muscle function (Lin et al., 2002; Miura et al., 2006). Inversely, ablation of PGC-1α gene expression in this tissue promotes several typical signs of pathological inactivity, including a local and systemic chronic inflammation (Handschin et al., 2007a,b). A reduction in PGC-1α gene expression in human skeletal muscle has been associated with insulin resistance and type 2 diabetes, at least in some patient cohorts (Patti et al., 2003). Of note, mice with a heterozygous deletion of PGC-1α in skeletal muscle also exhibit a dysregulation of glucose homeostasis (Handschin et al., 2007b).

PGC-1α integrates the activity of the major signaling pathways that are important in a contracting muscle fiber and accordingly, PGC-1α transcript and protein levels are elevated after a training bout (Pérez-Schindler and Handschin, 2013). As consequence, PGC-1α then controls the biological program encompassing all plastic changes of the muscle cell to endurance exercise. For example, mice with elevated muscle PGC-1α levels exhibit a higher number of mitochondria, a switch toward oxidative, slow-twitch muscle fiber types, altered substrate synthesis and metabolism, and a reduction in muscle protein breakdown (Handschin, 2010). Importantly, the effects of PGC-1α extend beyond muscle cells: elevation of PGC-1α in muscle leads to higher tissue vascularization (Arany et al., 2008) and a remodeling of the post- and presynaptic side of the neuromuscular junction (Handschin et al., 2007c; Arnold et al., 2014). Furthermore, PGC-1α-regulated endocrine mediators, members of the so-called myokine protein family, promote beige fat cell differentiation and activation in white adipose tissue or neurogenesis in the hippocampus and thereby dramatically extend the reach of muscle PGC-1α (Schnyder and Handschin, 2015). Surprisingly, while overexpression of PGC-1α is sufficient to induce an endurance-trained muscle phenotype, several aspects of training adaptation seem to be retained even in mice with a global or muscle-specific knockout of this coactivator, respectively (Leick et al., 2008; Rowe et al., 2012). Discrepancies in such studies however indicate that the specific animal model, training type, timing, intensity, and other aspects of the exercise protocol are important for the assessment of the requirement of PGC-1α in exercise adaptation (Geng et al., 2010). Furthermore, these studies imply a redundant regulation of these evolutionarily extremely important plastic changes in skeletal muscle in which PGC-1α can be partially replaced by other, so far unknown regulators. Thus, in a physiological setting, PGC-1α controls a highly complex transcriptional network that requires spatial and temporal specification, coordination of anabolic and catabolic pathways as well as precise activation and termination. In this mini review, we highlight some of the recent mechanistic findings that contribute to the ability of PGC-1α to regulate transcription in such a broad and precise manner.

INTEGRATION OF CONTRACTION-INDUCED SIGNALING PATHWAYS BY PGC-1α IN SKELETAL MUSCLE

Muscle fiber contraction is linked to activation by the motor neuron, mechanical stress, relative tissue physoxia, an altered neuroendocrine milieu, changes in metabolic demand and other stimuli that engage various signaling pathways. All of these signals converge on PGC-1α and promote a transcriptional induction of the gene or induce posttranslational modifications (PTM) of the protein (**Figure 1A**), including PGC-1α protein phosphorylation by various kinases on different phosphorylation sites, acetylation, methylation, sumoylation, ubiquitination, and acetylglucosamination (**Figure 1B**; Fernandez-Marcos and Auwerx, 2011). The effects of most of these modifications on PGC-1α function are still poorly understood. Some of the PTMs can alter the stability of the PGC-1α protein or modulate the interaction with transcription factors or other co-regulators. For example, phosphorylation by the p38 mitogen-activated protein kinase (p38 MAPK) results in a prolongation of the normally very short half-life of the PGC-1α protein of ∼2.5 h (Puigserver et al., 2001), at least in part by preventing ubiquitination of PGC-1α and therefore stabilizing the protein (Olson et al., 2008). The AMP-dependent protein kinase (AMPK) likewise phosphorylates the PGC-1α protein in addition to its positive effect on PGC-1α gene transcription and predominantly triggers catabolic pathways to rectify a relative energy deficit, e.g., in exercise (Jager et al., 2007). Such a temporal specification is extremely important to avoid futile cycles of PGC-1α-controlled anabolic and catabolic pathways, e.g., fatty acid β-oxidation and *de novo* lipogenesis (Summermatter et al., 2010). Moreover, PTMs could also determine spatial differentiation of PGC-1α function. For example, the interaction between PGC-1α and the GA-binding protein (GABP, also called nuclear respiratory factor 2 or NRF2) not only requires the presence of host cell factor (HCF) as an additional adaptor protein, but also specific phosphorylation events both on PGC-1α as well as the GABPB1 subunit of the GABP complex (Handschin et al., 2007c). These PTMs can be triggered by motor neuron-evoked neuregulin stimulation of the muscle fiber and thereby control a specific transcriptional activation of post-synaptic neuromuscular junction genes by PGC-1α and GABP exclusively in sub-synaptic nuclei (Handschin et al., 2007c). Thus, in addition to the modulation of protein stability, PTMs might alter the activity and stability of PGC-1α as well as the ability to interact with transcription factors and thereby regulate specific transcriptional programs (Handschin and Spiegelman, 2006). For most modifications of the PGC-1α protein, a "PTM code" (Lonard and O'malley, 2007) that determines transcription factor interaction specificity has not been elucidated. A prototypical example for a PGC-1α PTM code however is provided by the S6 kinase (S6K)-mediated phosphorylation that selectively

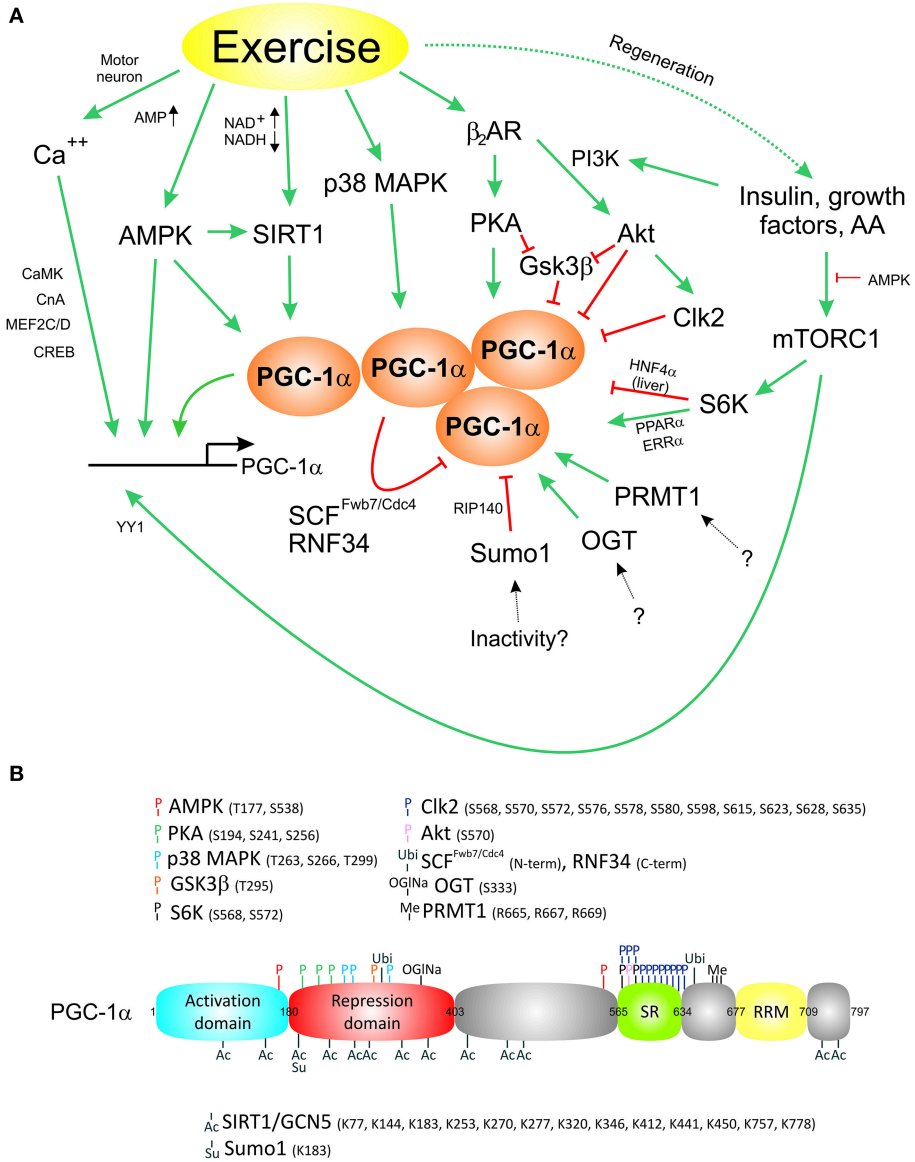

FIGURE 1 | Complex activation of the transcriptional co-activator PGC-1α by different signaling pathways. (A) Exercise triggers a complex transcriptional activation as well as various posttranslational modifications to control PGC-1α levels and activity. **(B)**, Posttranslational modifications of the PGC-1α protein. AA, amino acids; Ac, acetylation; Akt, protein kinase B; AMPK, AMP-activated protein kinase; β2AR, β2 adrenergic receptor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CnA, calcineurin A; Clk2, Cdc2-like kinase 2; CREB, cAMP response element binding protein; ERRα, estrogen-related receptor α; Gsk3β, glycogen synthase kinase 3 beta; HNF4 α, hepatic nuclear factor 4 α; MEF2C/2D, myocyte enhancer factors 2C/D; Me, methylation; mTORC1, mammalian target of rapamycin complex 1; OGlNa, O-linked β-N-acetylglucosamination; OGT, O-linked β-N-acetylglucosamine transferase; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3 kinase; PKA, protein kinase A; P, phosphorylation; PPARα, peroxisome proliferator-activated receptor α; PRMT1, protein arginine methyltransferase 1; RIP140, receptor interacting protein 140; RNF34, ring finger protein 34; S6K, S6-kinase; $SCF^{Fwb7/Cdc4}$, SCF ubiquitin ligase complex subunit Fwb7/Cdc4; SIRT1, sirtuin-1; Su, sumoylation; Sumo1, small ubiquitin related modifier 1; Ubi, ubiquitination; YY1, yin yang 1.

retains the ability of PGC-1α to boost fatty acid oxidation and mitochondrial function while attenuating its effect on gluconeogenesis in the liver (Lustig et al., 2011). Mechanistically, these PTMs reduce the interaction between PGC-1α and the hepatic nuclear factor 4 α (HNF4α), but not co-activation of the estrogen-related receptor α (ERRα) or PPARα. In addition to binding to transcription factors, PTMs of the PGC-1α protein can also affect the interaction with other co-regulators. For example, the co-repressors p160 myb binding protein (p160MBP) and receptor interacting protein 140 (RIP140) are recruited to PGC-1α in a PTM-dependent manner: p160MBP inhibits the ability of PGC-1α to regulate mitochondrial gene expression in the absence of p38 MAPK-mediated phosphorylation (Fan et al., 2004) while RIP140 associates with and represses sumoylated

PGC-1α (Rytinki and Palvimo, 2009). Other co-repressors reduce PGC-1α activity by competing for binding to transcription factors, for example modulation of ERRα co-activation by the nuclear receptor co-repressor 1 (NCoR1; Pérez-Schindler et al., 2012) or of the glucocorticoid receptor by the small heterodimer partner (SHP; Borgius et al., 2002). PTM-dependent binding events are also observed for co-activators as exemplified by the interaction of PGC-1α with the Mediator 1 (MED1) subunit of the TRAP/DRIP/mediator complex that is disrupted after phosphorylation of PGC-1α by the Cdc2-like kinase 2 (Clk2; Tabata et al., 2014). Ubiquitination and subsequent proteasomal degradation of the PGC-1α protein form a negative feedback loop to ensure timely termination of the PGC-1α response (Sano et al., 2007). This process might be triggered by PGC-1α protein self-aggregation upon reaching a critical threshold (Sano et al., 2007). Thus, PGC-1α serves as recipient of a multitude of PTMs, thereby integrates the activity of the respective signaling pathways and subsequently triggers a transcriptional response that is adapted to the specific cellular context.

REGULATORY AND FUNCTIONAL DIVERSITY BASED ON THE PGC-1α GENE STRUCTURE AND TRANSCRIPT VARIANTS

PGC-1α gene expression is rapidly and robustly increased in response to external stimuli that increase the energy demand such as cold in brown fat, fasting in the liver, or contraction in skeletal muscle (Lin et al., 2005). The cAMP response element binding protein (CREB) and the activating transcription factor-2 (ATF-2), both of which bind to cAMP response elements (CRE), are common regulators of PGC-1α transcription in most tissues. In addition, tissue-specific transcription factors provide an additional layer of control, e.g., myocyte enhancer factors 2C/D (MEF2C and -2D) in muscle cells (Pérez-Schindler and Handschin, 2013). A positive autoregulatory loop of MEF2C/D co-activation by PGC-1α on its own promoter furthermore contributes to adequate and controlled induction of PGC-1α transcription in this tissue (Handschin et al., 2003). Intriguingly, this theme of using cross- and autoregulatory loops, thus forming biological switches, is also observed in early downstream target gene regulation, for example in the induction of ERRα and GABPA by PGC-1α (Mootha et al., 2004).

PGC-1α transcription can be initiated from three start sites on two alternative promoters. Moreover, alternative RNA processing further increases the number of PGC-1α transcripts and, as a consequence, protein variants (Martínez-Redondo et al., 2015). Even though the regulatory elements of the two promoters have not yet been studied in detail, the proximal promoter seems to provide a more robust basal expression while the distal promoter that is approximately 13 kb upstream exhibits a higher dynamic range in gene expression, at least in skeletal muscle (Martínez-Redondo et al., 2015). It is still unclear whether alternative promoter usage is closely linked to the transcription of PGC-1α isoforms. Moreover, the functional consequence of the selective expression of most PGC-1α transcript variants is unknown. Surprisingly however, the PGC-1α4 variant seems to regulate a highly distinct transcriptional program with very little overlap compared to that of the other variants (Ruas et al., 2012). PGC-1α 4 contributes to skeletal muscle adaptation to resistance training and the ensuing fiber hypertrophy (Ruas et al., 2012), at least in certain contexts (Pérez-Schindler et al., 2013), diametrically opposite to the endurance exercise-like phenotype triggered by the other PGC-1α isoforms. In humans however, some studies questioned an exclusive correlation between PGC-1α4 expression and resistance training (Lundberg et al., 2014) warranting further studies. In any case however, the gene structure and transcript processing of PGC-1α thus provide an additional layer of regulatory and functional specification (Handschin and Spiegelman, 2006).

PGC-1α-CONTROLLED TRANSCRIPTIONAL NETWORK REGULATION ALLOWS CONTEXT-DEPENDENT CONTROL AND SPECIFICATION

Originally, PGC-1α has been discovered as a coactivator of PPARγ and was hence named accordingly (Puigserver et al., 1998). It however became clear that PGC-1α not only interacts with this, but also a number of other nuclear receptors and non-nuclear receptor transcription factors. Intriguingly, these interactions are mediated by different structural domains within the PGC-1α protein, with a more N-terminal preference for nuclear receptor co-activation while others, for example MEF2 or forkhead box protein 1 (Foxo1) bind PGC-1α closer to the C-terminus (Lin et al., 2005). Therefore, the functional specificity of PGC-1α isoforms that lack certain domains of the full-length protein could stem from the specific ablation and enhancement of binding to transcription factor partners (Handschin and Spiegelman, 2006; Martínez-Redondo et al., 2015). Despite the identification of various interaction partners, PGC-1α seems to have a special relationship with ERRα, at least in the regulation of mitochondrial genes (Mootha et al., 2004). ERRα is an orphan nuclear receptor that is mainly activated by co-activator binding in a ligand-independent manner (Kallen et al., 2004). Moreover, PGC-1α rapidly induces the transcription of ERRα as an early response target gene (Mootha et al., 2004). It thus is not surprising that pharmacological inhibition of the interaction between PGC-1α and ERRα or knockdown of ERRα has a potent effect on many PGC-1α target genes in muscle cells (Mootha et al., 2004; Schreiber et al., 2004). A global analysis of PGC-1α recruitment to regulatory elements in the mouse genome combined with an expression analysis however revealed a so-far largely underestimated number of putative transcription factor partners beyond ERRα to be involved in PGC-1α-mediated target gene regulation in muscle cells (Baresic et al., 2014). Furthermore, transcription factor motif activity response analysis not only predicts ERRα to be involved in the regulation of primary, but also secondary PGC-1α target genes, thus both in the co-activated state but also working without PGC-1α in this context. These data indicate a much higher complexity of transcription factor engagement by PGC-1α than previously

suggested. Second, the combination of PGC-1α ChIPseq and gene expression data revealed that of the high number of PGC-1α repressed genes, only a small minority, ∼5%, have a PGC-1α DNA recruitment peak within a distance of ±10 kb of their promoter. Accordingly, the findings imply that the effect of PGC-1α on gene repression is predominantly indirect and that PGC-1α lacks an intrinsic inhibitory function (Baresic et al., 2014). While a systematic analysis of gene repression by PGC-1α remains to be done, PGC-1α-dependent reduction of the activating phosphorylation of the p65 subunit of the nuclear factor κB (NF-κB; Eisele et al., 2013) could account for at least some of the indirect inhibitory effect of PGC-1α on pro-inflammatory muscle gene expression (Eisele and Handschin, 2014). Third, this systematic study revealed novel insights into the mechanisms that ensure functional redundancy and complementation of PGC-1α-mediated transcriptional control. Principal component analysis of the transcription factor binding motifs within the regions of PGC-1α DNA recruitment implied an important role for the activator protein-1 (AP-1) transcription factor complex in PGC-1α-controlled gene expression (Baresic et al., 2014). AP-1 is a well-studied stress response gene in various cellular contexts, but has so far never been associated with PGC-1α function in muscle. Interestingly, the group of direct targets for AP-1 and PGC-1α was significantly enriched in genes associated with the cellular response to hypoxia, including several regulators of vascularization. This regulation complements the previously discovered control of the expression of the vascular endothelial growth factor (VEGF) by PGC-1α and ERRα (Arany et al., 2008). These findings imply that this seemingly redundant usage of different transcription factors to regulate the same biological program ensures adequate regulation of this critical process. Alternatively, the ability of PGC-1α to enhance the transcriptional activity of different partners might also indicate that PGC-1α is able to regulate the respective target genes in different cellular contexts, e.g., by binding to ERRα in a metabolically stressed muscle cell and acting together with AP-1 upon reduced physoxic conditions (**Figure 2**). Exercise triggers a hypoxic response including an activation of the hypoxia-inducible factor-1 (HIF-1) in muscle cells by a lowering of the relative tissue oxygen availability due to a dysbalance between oxygen consumption and supply, exacerbated by contraction-mediated constriction of blood vessels (Lindholm and Rundqvist, 2015). Together, the current data highlight the vast complexity of diverse mechanisms by which PGC-1α exerts a pleiotropic response in muscle cells.

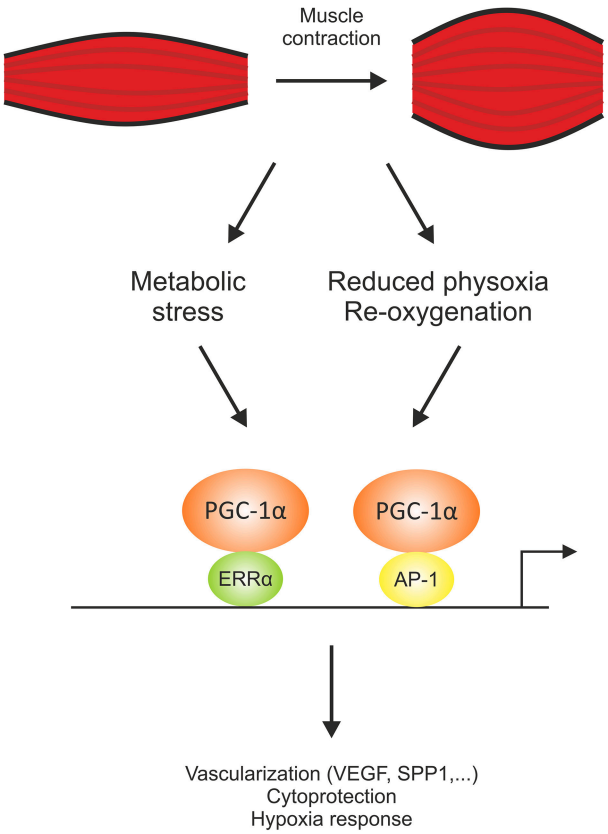

FIGURE 2 | PGC-1α engages different transcription factors to robustly regulate biological programs. Co-activation of the estrogen-related receptor α (ERRα) and the activator protein-1 transcription factor complex (AP-1) allow a differential regulation of the gene program for vascularization, cytoprotection, and hypoxia response triggered by different stimuli, e.g., a change in the metabolic demand and the ensuing angiogenesis to improve substrate availability or neovascularization as a response to a contraction-mediated reduction in tissue physoxia and an ensuing re-oxygenation for adequate oxygen supply. SPP1, secreted phosphoprotein 1; VEGF, vascular endothelial growth factor.

CONCLUSIONS AND OUTLOOK

In essence, PGC-1α is a protein docking platform that on one side is recruited to transcription factors bound to their target gene promoters and enhancers, and on the other side interacts with components of the histone acetyltransferase (Puigserver et al., 1999), TRAP/DRIP/mediator (Wallberg et al., 2003), and SWI/SNF (Li et al., 2008) co-regulator protein complexes. Thereby, PGC-1α greatly boosts transcription even though PGC-1α lacks any discernable intrinsic enzymatic activity. Despite this ostensible simplicity in function, PGC-1α can control highly complex transcriptional programs in various tissues with a significant impact on organ plasticity. The ability of PGC-1α to integrate different signaling pathways through a multitude of PTMs, selective activation of alternative promoters and expression of transcript variants could provide a mechanistic explanation for the key regulatory function of PGC-1α in the regulation of tissue phenotypes. However, more studies will be required to obtain a better understanding and overview on the different aspects of the regulation of a co-activator-controlled transcriptional network, which not only is of high interest to understand the basic biology, but could also have a significant clinical impact. In a physiological and pathophysiological context, elevation of PGC-1α in muscle promotes a high endurance phenotype and ameliorates various muscle diseases with different etiologies, including Duchenne muscular dystrophy (Handschin et al., 2007c), denervation-induced fiber atrophy (Sandri et al., 2006), or sarcopenia (Wenz et al., 2014), respectively. To date, it is not clear which functions

of muscle PGC-1α are responsible for such a broad therapeutic effect (Handschin, 2009). Similarly, pharmacological agents that robustly, specifically and safely elevate PGC-1α in skeletal muscle in the desired therapeutic window remain elusive (Svensson and Handschin, 2014). Finally, based on studies with muscle-specific PGC-1α transgenic animals that have an accelerated development of insulin resistance on a high fat diet (Choi et al., 2008), which can only be rectified by *bona fide* physical activity (Summermatter et al., 2013), the application of so-called "exercise mimetics," compounds that elicit exercise-like effects in muscle and other tissues, might be problematic. Therefore, to design partial exercise mimetics or new compounds that specifically activate certain functions of PGC-1α, better knowledge about upstream regulators and downstream effects on the transcriptional network are needed. In particular, even though a strong correlation between muscle PGC-1α expression, exercise, and diseases states has been repeatedly documented in humans (e.g., see Silvennoinen et al., 2015), information about the regulation and function of human muscle PGC-1α so far remains largely descriptive. Until further insights are obtained, physical activity thus remains a cheap and effective way for the prevention and treatment of many chronic diseases (Handschin and Spiegelman, 2008), at least in exercise-tolerant patients.

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Regulation of skeletal muscle mitochondrial activity by thyroid hormones: focus on the "old" triiodothyronine and the "emerging" 3,5-diiodothyronine

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3,5,3′-Triiodo-L-thyronine (T3) plays a crucial role in regulating metabolic rate and fuel oxidation; however, the mechanisms by which it affects whole-body energy metabolism are still not completely understood. Skeletal muscle (SKM) plays a relevant role in energy metabolism and responds to thyroid state by remodeling the metabolic characteristics and cytoarchitecture of myocytes. These processes are coordinated with changes in mitochondrial content, bioenergetics, substrate oxidation rate, and oxidative phosphorylation efficiency. Recent data indicate that "emerging" iodothyronines have biological activity. Among these, 3,5-diiodo-L-thyronine (T2) affects energy metabolism, SKM substrate utilization, and mitochondrial functionality. The effects it exerts on SKM mitochondria involve more aspects of mitochondrial bioenergetics; among these, respiratory chain activity, mitochondrial thermogenesis, and lipid-handling are stimulated rapidly. This mini review focuses on signaling and biochemical pathways activated by T3 and T2 in SKM that influence the above processes. These novel aspects of thyroid physiology could reveal new perspectives for understanding the involvement of SKM mitochondria in hypo- and hyper-thyroidism.

Keywords: mitochondria, lipid metabolism, uncoupling, thyroid hormones, diiodothyronines

Introduction

Skeletal muscle (SKM) is a metabolically active tissue representing about 40% of total body mass. It significantly affects energy expenditure and plays a significant role in glucose, lipid, and energy homeostasis. SKM shows a remarkable plasticity in functional adaptation and remodeling in response to physiological stimuli, such as exercise, fasting, and hormonal signals. Among the hormones able to influence SKM development, metabolism, and structure, thyroid hormone (T3) plays a key role (Salvatore et al., 2014). SKM responds to variations in thyroid state by coordinately remodeling its cytoarchitecture and metabolic characteristics, with mitochondria playing a significant role. Concerning metabolic adaptations, T3 enhances the use of lipids and carbohydrates as fuel substrates (de Lange et al., 2008; Lombardi et al., 2012), as well as alteration of mitochondrial number and functionality.

Growing evidence indicates 3,5-diiodo-L-thyronine (T2) as a biologically active thyroid hormone derivative able to affect energy metabolism (Goglia, 2015 and references within). T2 increases resting metabolic rate, enhances lipid utilization as a fuel substrate, and prevents the occurrence of diet-induced obesity and associated diseases, including liver steatosis, hypertriglyceridemia, hypercholesterolemia (Lanni et al., 2005; de Lange et al., 2011), and insulin resistance (de Lange et al., 2011; Moreno et al., 2011). In SKM, T2 ameliorates the tissue's response to insulin that is impaired by a high fat diet (Moreno et al., 2011). Importantly, previous studies have shown that, contrary to T3, T2 does not induce thyrotoxicity or undesirable side effects at the cardiovascular level at the doses used (25 μg/100 g rat body weight, Lanni et al., 2005; de Lange et al., 2011).

The present mini review provides an overview of the involvement of SKM mitochondria in T3 and/or T2 effects exerted on modulation of SKM metabolism/plasticity. In particular, it focuses on signaling and biochemical pathways activated by the two iodothyronines in SKM, promoting variations in substrate metabolism, lipid handling, and thermogenesis at the mitochondrial level.

Effect of Thyroid Hormones on SKM Mitochondrial Biogenesis

T3 influences mitochondrial activity and biogenesis by modulating, in a coordinate fashion, expression of proteins encoded by both the nuclear and mitochondrial genome.

Nuclear Events

T3 acts through nuclear receptors (TRs), namely TRα and TRβ, ligand-dependent transcription factors that are constitutively bound to thyroid hormone response elements (TREs). The binding of T3 to TRs leads to stimulation or inhibition of nuclear gene transcription (Brent, 2012). T3 regulates the transcription of a series of genes harboring TREs (direct T3 target genes), some of which serve as intermediate factors (e.g., transcriptional factors and coactivators) needed to regulate a second series of genes (indirect T3 target genes). In SKM, T3-modulated transcription is primarily mediated by the TRα1 isoform and involves a wide array of genes influencing SKM contractile and metabolic properties, as well as those coding components of the tricarboxylic acid cycle and mitochondrial respiratory chain (Wiesner et al., 1992; Short et al., 2001; Clement et al., 2002). In SKM, T3 also influences the transcription of genes controlling mRNA maturation and protein translation. Indeed, T3 up-regulates transcripts encoding ribunucleoproteins and splicing factors as well as ribosomal proteins and translation initiation factors (eIF1A, Clement et al., 2002).

T3 positively regulates the expression of intermediate factors, such as nuclear respiratory factors (NRF)-1 and -2, which enhance the expression of mitochondrial transcription factor-A, a nuclear-encoded transcription factor essential for replication, maintenance, and transcription of mitochondrial DNA. T3 also controls the expression of coactivator of peroxisome proliferator activated receptor γ (PPARγ) PGC-1α (Weitzel et al., 2001), a central regulator of mitochondrial gene expression and biogenesis (Puigserver, 2005). PGC-1α regulates gene expression through its interactions with DNA-bound transcription factors, including TR, PPAR, and NRF-1 (Knutti and Kralli, 2001, **Figure 1**).

In rats, the effect induced by T3 on mitochondrial content and activity is amplified in slow oxidative compared to fast glycolytic muscles (Bahi et al., 2005). This could be explained by higher expression of TRα1 and PGC1α observed in slow oxidative muscle (Garnier et al., 2003; Bahi et al., 2005) associated with opposite regulation of TRα transcription by T3 in the two distinct muscle types (activation in slow oxidative and reduction in fast glycolytic muscle). Conversely, in humans, T3 does not influence NRF-1 or PGC1α levels in SKM (Barbe et al., 2001). Thus, the effect of T3 on SKM mitochondrial biogenesis seems to be species-specific and dependent on SKM metabolism.

In SKM, AMP-activated kinase (AMPK) regulates the expression of genes related to mitochondrial biogenesis, energy production, and oxidative protection. AMPK phosphorylates and activates PGC1α (Jäger et al., 2007; Cantó et al., 2009), and both chronic and acute administration of T3 to euthyroid (Irrcher et al., 2008) and hypothyroid rats (Branvold et al., 2008; de Lange et al., 2008) induces AMPK activation, a putative mediator of the effect of T3 on SKM mitochondrial biogenesis (**Figure 1**).

Mitochondrial Events

Mitochondrial gene expression is also directly activated by T3 through its binding to mitochondrial matrix-localized specific receptors (p43). p43 is a truncated form of TRα1 and is synthesized by the use of an internal initiation site of translation (Wrutniak et al., 1995; Wrutniak-Cabello et al., 2001). The T3–p43 complex binds to TREs of the mitochondrial genome and induces transcription (Casas et al., 1999) in parallel to the transcription of nuclear genes involved in oxidative phosphorylation, thus ensuring complementary signaling between nuclear and mitochondrial pathways (**Figure 1**). p43 regulates SKM phenotypes, contractile features, and metabolism. In mice, p43 deletion leads to muscle hypertrophy and a shift in the direction of more rapid muscle fiber types coordinated with a reduction in mitochondrial content (Pessemesse et al., 2012). p43 overexpression leads to muscle wasting with aging (Casas et al., 2009), suggesting a possible oxidative stress-associated toxic effect due to prolonged stimulation of mitochondrial activity, leading to a deficit of new skeletal muscle fiber replacement and differentiation over time.

P28 is another truncated form of TR-α1 that is localized in the inner mitochondrial membrane and shows a binding affinity for T3 higher than nuclear receptors. Despite it has been shown that p28 regulates mitochondrial functionality in fibroblast, its specific function has not been elucidated yet (Pessemesse et al., 2014).

Effect of T2 on Mitochondrial Biogenesis

Studies supporting the possibility of T2's effects on mitochondrial biogenesis have been focused on liver and brown adipose tissue (de Lange et al., 2011; Lombardi et al., 2015). In SKM, it is possible that activation of AMPK by T2 (Lombardi et al., 2009a) could trigger transcriptional processes leading to mitochondrial

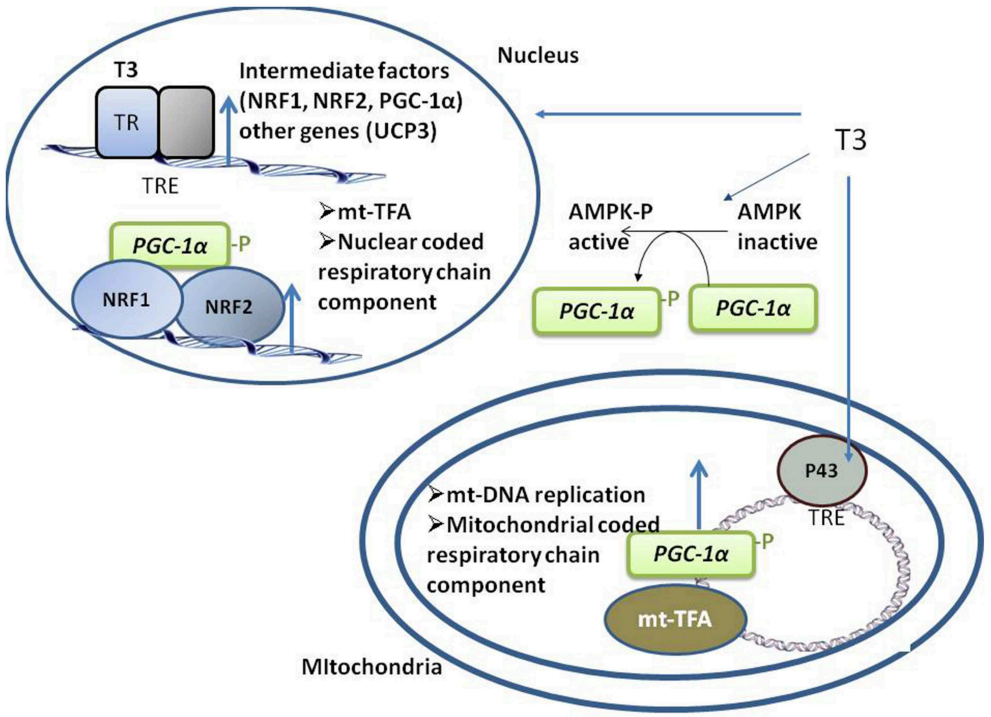

FIGURE 1 | Schematic representation of the coordinated events activated by T3 that take place in both nuclei and mitochondria, promoting mitochondrial biogenesis. T3 directly activates the transcription of nuclear- and mitochondrial-genes coding for component of respiratory chain by binding to its nuclear TRs and mitochondrial p43. T3 also indirectly activates respiratory genes transcription by up-regulating the transcription of intermediate factors (such as NRF-1 and -2 and PGC1a). AMPK activation also mediates an indirect effect of T3 on mitochondrial biogenesis.

biogenesis (see above). Direct evidence regarding the ability of T2 to influence mitochondrial biogenesis in SKM is currently lacking and this aspect needs further investigation.

Whether or not the effects of T2 are mediated by TRs is still under investigation. A recent study in mice showed that T2 evokes TR-mediated effects only when administered at very high doses (250 μg/100 g bw administered daily for 4 week, Jonas et al., 2015). When used at high doses an interaction of T2 with TRs could take place despite the much lower affinity of T2 for TRs when compared to T3 (Ball et al., 1997; Cioffi et al., 2010; de Lange et al., 2011; Mendoza et al., 2013). Interestingly, in a teleost fish species (Tilapia) T2 interacts with a TRβ receptor isoform and activates gene transcription *ex vivo* in a cell- and promoter-specific manner (Mendoza et al., 2013). Thus, further experiments are needed to elucidate whether and how T2 can modulate gene transcription.

Thyroid Hormones Influence SKM Mitochondrial Functionality and Thermogenesis

Mitochondrial functionality is profoundly affected by thyroid state. In SKM, in the transition between hypo- and hyper-thyroidism, a progressive increase in mitochondrial substrate oxidation is detected regardless of substrate [i.e., glycolytic- (Venditti et al., 2003, 2007) or lipid-associated substrates (Silvestri et al., 2005; Lombardi et al., 2012)].

Mitochondrial Uncoupling

Mitochondrial respiration is not fully coupled to ATP synthesis since part of the energy contained in the reduced substrate is lost as heat. Most of the uncoupling is due to a leak of protons across the mitochondrial inner membrane (proton-leak); a failure in proton pumping during electron transport (redox slip) also induces mitochondrial uncoupling. In SKM, proton-leak accounts for a significant portion of the cellular metabolic rate, either when muscle is at rest (Rolfe and Brand, 1996) or in the contracting state (Rolfe et al., 1999). Proton-leak is the sum of two processes: basal and inducible proton-leak (Brand and Esteves, 2005). Basal proton-leak is not acutely regulated. It depends on the fatty-acyl composition of the mitochondrial inner membrane and on the presence of adenine nucleotide translocase (ANT). Inducible proton-leak is acutely controlled by activation of specific proteins, with uncoupling protein (UCP3 in SKM) and ANT (ANT1 in SKM) playing a crucial role (Divakaruni and Brand, 2011).

T3 Induces SKM Mitochondrial Uncoupling

SKM mitochondrial uncoupling induced by T3 has been reported *in vivo* (Lebon et al., 2001) and *ex vivo* (Lanni et al., 1999; de Lange et al., 2001; Lombardi et al., 2002, 2012). Interestingly,

despite uncoupling activation by T3, no variation or increase in SKM ATP levels takes place (Jucker et al., 2000; Lebon et al., 2001; Short et al., 2001). The uncoupling associated with T3-induced mitochondrial biogenesis could counteract possible ATP variations. In addition, an increase in the ability of SKM mitochondria to produce ATP could also take place, as already observed in liver (Harper and Brand, 1993; Nogueira et al., 2002).

The existence of a positive correlation between T3 and SKM mitochondrial proton-leak is also evident during aging. In fact, aging is associated with a decrease in circulating T3 that is evident in 24 month-old rats (Iossa et al., 2002; Silvestri et al., 2008; Valle et al., 2008); therefore, aging represents a condition of physiological hypothyroidism. Concomitantly, SKM mitochondria from old rats exhibited a significant decrease in proton-leak (Lombardi et al., 2009a; Crescenzo et al., 2014), suggesting that with increasing age, the efficiency of oxidative phosphorylation increases in SKM mitochondria. Similar results have been obtained *in vivo* in aged rat SKM, where a trend of higher coupling efficiency was found (Gouspillou et al., 2014). When mitochondria are more efficient, fewer substrates are oxidized to obtain ATP. Therefore, increased mitochondrial coupling in SKM could contribute to the decreased energy expenditure that characterizes the progression of aging and hypothyroidism since SKM energy metabolism accounts for about 30% of whole-body energy expenditure under resting conditions (Rolfe and Brown, 1997).

Factors Involved in T3 Induced- SKM Mitochondrial Uncoupling

T3 affects both basal (Lombardi et al., 2012) and inducible SKM proton-leak (Lanni et al., 1999; Silvestri et al., 2005; Lombardi et al., 2012), with UCP3 and ANT being involved in the effects on free fatty acid (FA)-inducible proton-leak (**Figure 2**). In the transition between hypo- and hyper-thyroidism, the contribution of ANT to FA-induced uncoupling becomes progressively more relevant despite there being no variation in ANT-1 mRNA levels detected (Dümmler et al., 1996; Lombardi et al., 2002). This could be attributed to the gradual increase in mitochondrial SKM FA levels (Lombardi et al., 2002), known activators of ANT-mediated uncoupling (Skulachev, 1991). Concerning UCP3, T3 increases its transcription (Lanni et al., 1999; Barbe et al., 2001), the effect being observed within 8 h of T3 administration to hypothyroid rats (de Lange et al., 2001, 2007). The mechanism of UCP3 promoter stimulation by T3 seems to be species-specific since it involves FA and their target receptors (PPARδ) in humans and rats but not mice (de Lange et al., 2007).

Besides regulating UCP3 expression, T3 also promotes UCP3-mediated uncoupling by synergistically stimulating biochemical pathways underlying activation of this protein (Silvestri et al., 2005). Indeed, T3 enhances reactive oxygen species formation (Venditti et al., 2003, 2007; Silvestri et al., 2005) and mitochondrial FA availability (Lombardi et al., 2002; Silvestri et al., 2005) that have been shown to act in combination to induce UCP3-mediated uncoupling (Echtay et al., 2002; Lombardi et al., 2008, 2010). A single administration of T3 to hypothyroid rats induces parallel increases in (i) whole animal resting metabolic rate, (ii) SKM mitochondrial UCP3 content, and (iii) SKM mitochondrial uncoupling, thus indicating the importance of UCP3 in the regulation of rat resting metabolic rate by T3 (de Lange et al., 2001; Flandin et al., 2009). UCP3 is also involved in mitigation of reactive oxygen species production (Brand and Esteves, 2005) and counteracting lipotoxicity induced by accumulation of FA and lipid hydroperoxides in the mitochondrial matrix (Goglia and Skulachev, 2005; Schrauwen et al., 2006; Lombardi et al., 2010). Thus, the upregulation of UCP3 by T3 would alleviate mitochondrial damage resulting from chronic mitochondrial activation associated with hyperthyroidism.

3,5-T2 Affects Mitochondrial Oxidative Phosphorylation in SKM

The administration of T2 to hypothyroid rats rapidly enhances both coupled and uncoupled respiration with mechanisms that are independent of de novo transcription and translation (Lombardi et al., 2007). Indeed, T2 promotes activation of the kinetics of the reactions involved in the oxidation of substrates (among these respiratory chain), while not primarily influencing reactions involved in the synthesis and export of ATP (Lombardi et al., 2007, **Figure 2**).

The mechanism by which T2 affects uncoupled mitochondrial respiration mainly involves proton leak, since T2 does not affect redox slip nor induce any significant change in the overall respiratory chain H^+/O ratio (Lombardi et al., 2007). Contrary to what is observed for T3, T2 does not activate basal proton-leak, rather its effect is totally dependent on FA presence (Lombardi et al., 2009a, 2012). Although, it is clear that T2 promotes FA-inducible proton-leak, the molecular component involved have not been individuated yet (**Figure 2**).

Thyroid Hormones Influence SKM Mitochondrial Lipid Handling

Alterations in thyroid state are associated with changes in energy demand, with SKM adapting its metabolism by modulating substrate utilization. In the hypothyroid state, SKM enhances FA import into myocytes, a process associated with a decrease in the ability of mitochondria to use FA as fuel and enhancement of oxidative phosphorylation efficiency. Thus, the imbalance between FA supply and oxidation leads to accumulation of intra-myocyte triglycerides (Lombardi et al., 2012). On the other hand, in the hyperthyroid condition, an increase in FA uptake into myocytes is associated with a rise in FA oxidation, which becomes less efficient because of proton-leak activation. Consequently, FAs are not deposited as triglycerides (Lombardi et al., 2012, **Figure 2**).

T3 Affects Mitochondrial Fatty Acid Oxidation

More processes are crucial for SKM mitochondrial FA oxidation, which include FA availability to mitochondria, import of acyl-CoA into the mitochondrion, mitochondrial oxidative capacity, and feedback inhibition by intermediates present in the FA oxidation pathway. T3 influences all the cited processes. Indeed, it promotes mitochondrial localization of FAT/CD36 (Lombardi et al., 2012), known to increase FA supply to the mitochondria (Holloway et al., 2009). This event is coordinated with the import of acyl-CoA into the mitochondria, obtained by activation of

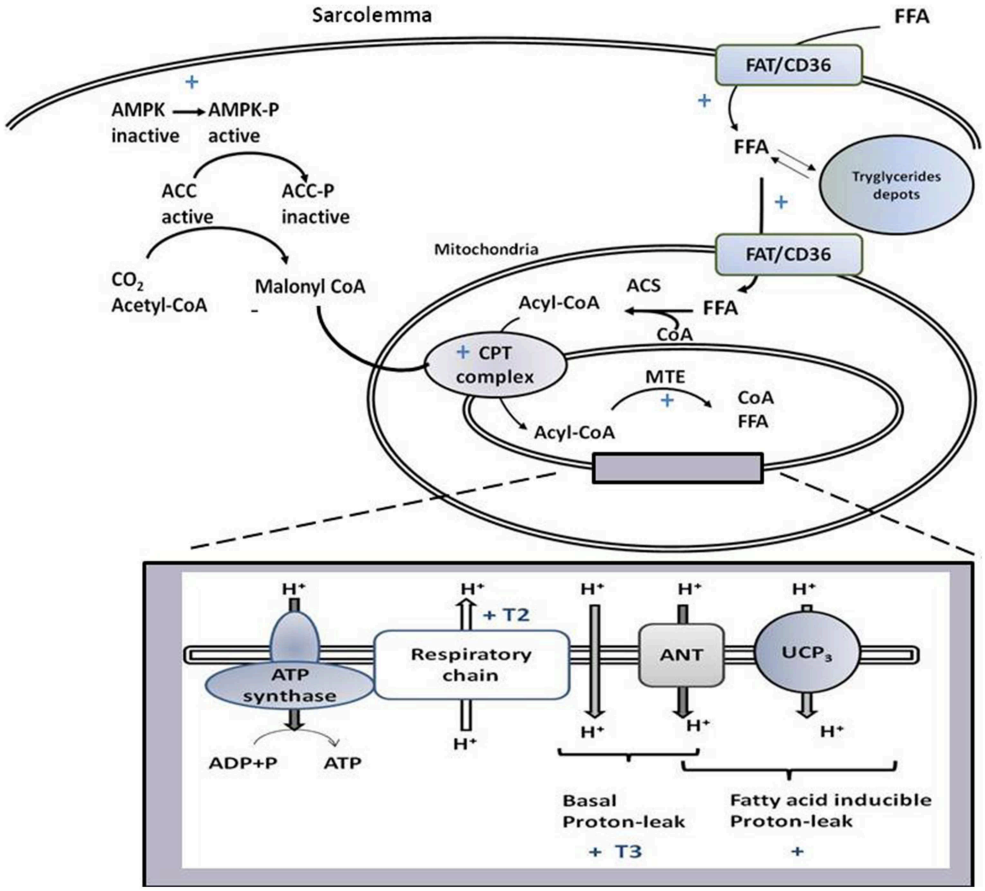

FIGURE 2 | Schematic representation of signaling and biochemical pathways activated by T3 and/or T2 in skeletal muscle that promote variations in substrate metabolism, lipid handling, and thermogenesis at the mitochondrial level. If not accompanied by T3 or T2, the symbol + indicates that the pathway is stimulated by both iodothyronines. T3 and T2 activate processes leading to the import of FFA and their oxidation at the mitochondrial level, with FAT/CD36 playing a role. Through activation of AMPK-ACC signaling pathway, T2 and T3 relieves the inhibition of CPT1 by malonyl-CoA, and thus promote the entrance of fatty acids into mitochondria and their oxidation. The rise in MTE activity, would contribute to maintain a high level of fatty acid oxidation rate. The box represents processes, occurring at the level of mitochondrial inner membrane, underlying coupled and uncoupled respiration affected by T3 and T2.

the carnitine-palmitoyl-transferase (CPT) complex (considered a rate-limiting step for FA uptake into mitochondrion). T3 modulates transcription of CPT complex components (e.g., CPT-1 and -2, Silvestri et al., 2005). In addition, in SKM, T3 promotes AMPK activation and inhibition of its downstream target, acetyl-CoA carboxylase (ACC, Branvold et al., 2008; de Lange et al., 2008; Irrcher et al., 2008). ACC inhibition leads to a reduction in malonyl-CoA levels that inhibits CPT-1 activity. Thus, the activation of AMPK-ACC-malonyl-CoA signaling leads to sequential enhancement of CPT-1 activity, mitochondrial acyl-CoA uptake, and oxidation (de Lange et al., 2008, **Figure 2**).

Mitochondrial Lipid Handling and Uncoupling: Interrelated Role in Mediating the Effect of T3 on SKM Mitochondria

Inside mitochondria, a rise in NADH/NAD^+ and CoA-SH/acetyl-CoA ratios, as well as accumulation of β-oxidation intermediate metabolites, can cause feedback inhibition of the β-oxidation pathway (Koves et al., 2008). The T3-induced uncoupling effect contributes to maintaining the above ratios at low levels and thus, functioning to sustain an elevated mitochondrial FA oxidation rate. Furthermore, the activation of SKM intra-mitochondrial thioesterase (MTE; catalyzes cleavage of acyl-CoA to CoA and free FA) by T3 (Silvestri et al., 2005) contributes to sustaining a high FA oxidation rate, since it maintains a high CoA/acetyl-CoA ratio and supplies CoA, whose pool is limited, for β-oxidation. Intra-mitochondrial production of FA, catalyzed by MTE, could play a role in FA-induced proton leak activated by T3. Thus, an interlink between lipid-handling and mitochondrial uncoupling coexists: the activation of uncoupling could facilitate the FA oxidation rate and, at the same time, the increase availability of FA to mitochondria, associated with lipid handling, would promote FA-induced mitochondrial uncoupling.

T2 Affects SKM Fatty Acid Oxidation

The rapid stimulatory effect of T2 on mitochondrial respiration seems to be specific to FA metabolism, since T2 does not influence mitochondrial ability to use pyruvate as a substrate (Lombardi et al., 2009a). T2 has an effect similar to that induced by T3 in increasing SKM FA uptake and channeling FAs to mitochondria and increasing their oxidation. Indeed, T2 and T3 activate translocation of FAT/CD36 from cellular depots to the sarcolemma and mitochondria, each in a very rapid fashion. In this aspect, the two iodothyronines seem to mimic the effect of physical exercise, which influences FAT/CD36-mediated transport of lipids across the sarcolemmal membrane and into the mitochondria (Holloway et al., 2009).

Although both T2 and T3 increase the SKM mitochondrial FA oxidation rate in hypothyroid rats, the onset of CPT and mitochondrial respiratory pathway activation differ since the two processes were already activated 1 h after T2 administration, whereas T3 was ineffective at that time point (Lombardi et al., 2012). Within 1 h, T2 rapidly activated the AMPK-CPT-malonyl-CoA signaling pathway that leads to enhancement of FA uptake in mitochondria via increased CPT-1 activity (**Figure 2**). Rapid activation of MTE-1 and proton-leak by T2 would contribute to maintaining high FA oxidation rates (Lombardi et al., 2009b).

Conclusions

SKM mitochondrial physiology is profoundly affected by the thyroid state and underlies a significant part of the metabolic effects induced by T3. The recent discovery of T2 as a metabolically active thyroid hormone derivatives indicates that thyroid physiology is continually evolving. These novel aspects of thyroid physiology could reveal new perspectives for understanding the contribution of SKM mitochondria to different thyroid states.

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