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Hyperhomocysteinemia associated skeletal muscle weakness involves mitochondrial dysfunction and epigenetic modifications

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ABSTRACT

HHcy has been implicated in elderly frailty, but the underlying mechanisms are poorly understood. Using C57 and CBS +/− mice and C2C12 cell line, we investigated mechanisms behind HHcy induced skeletal muscle weakness and fatigability. Possible alterations in metabolic capacity (levels of LDH, CS, MM-CK and COX-IV), in structural proteins (levels of dystrophin) and in mitochondrial function (ATP production) were examined. An exercise regimen was employed to reverse HHcy induced changes. CBS +/− mice exhibited more fatigability, and generated less contraction force. No significant changes in muscle morphology were observed. However, there is a corresponding reduction in large muscle fiber number in CBS +/− mice. Excess fatigability was not due to changes in key enzymes involved in metabolism, but was due to reduced ATP levels. A marginal reduction in dystrophin levels along with a decrease in mitochondrial transcription factor A (mtTFA) were observed. There was also an increase in the mir-31, and mir-494 quantities that were implicated in dystrophin and mtTFA regulation respectively. The molecular changes elevated during HHcy, with the exception of dystrophin levels, were reversed after exercise. In addition, the amount of NRF-1, one of the transcriptional regulators of mtTFA, was significantly decreased. Furthermore, there was enhancement in mir-494 levels and a concomitant decline in mtTFA protein quantity in homocysteine treated cells. These changes in C2C12 cells were also accompanied by an increase in DNMT3a and DNMT3b proteins and global DNA methylation levels. Together, these results suggest that HHcy plays a causal role in enhanced fatigability through mitochondrial dysfunction which involves epigenetic changes.

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1. Introduction

Impaired cardiovascular function and derailed energy metabolism (as in diabetes and obesity) are the two major causes for human mortality and morbidity in the 21st century [1–3]. Exercise has been shown to correct both metabolic impairments and cardiovascular deficiencies [4–6]. However, exercise intolerance and fatigue are the leading causes that undermine the benefits of exercise in apparently physically normal individuals [7]. Furthermore, certain factors such as hyperhomocysteinemia aggravate age associated decline in physical function [8]. In this regard, understanding molecular changes associated with the factors that cause fatigue or exercise intolerance not only benefit efforts that raise physical capabilities, but also significantly reduces mortality and morbidity associated with cardiovascular disease and metabolic syndromes such as diabetes and obesity through improved exercise competence.

Fatigue could result from decreased muscle mass and/or structural and metabolic/energy production alterations intrinsic to muscle cells [9]. Structural alterations that lead to progressive muscle weakness are well characterized. Of note are the defects associated with dystrophin complex assembly [10]. Dystrophin anchors the inner cytoskeleton to the outer matrix via a dystrophin complex and is perceived to provide membrane stabilization, especially during rigorous muscle contractions [10–12]. Dystrophin deficiency was proposed to cause muscle membrane injury during exercise and limits exercise capacity [11]. Other deficiencies in structural proteins are also reported [11]. While genetic causes underlying changes in dystrophin and other important structural components, such as truncated protein products and defective splicing, are relatively well characterized, involvement of epigenetic modifications is less well characterized [13].

Apart from structural deficiencies, energy imbalances were also reported to play a crucial role in causation of fatigue [14,15]. As mitochondria play a pivotal role in the production of bulk cellular ATP, the cellular currency, the factors that either compromise mitochondrial function and/or prevent mitochondrial biogenesis and content would

Abbreviations: Mir, microRNA; DNMT, DNA methyltransferase; CBS, cystathionine β-synthase; HHcy, hyperhomocysteinemia; Hcy, homocysteine; mtTFA, mitochondrial transcription factor A; Mfn2, mitofusion 2; H3K18, histone H3 lysine 18; LDH, lactate dehydrogenase; CS, citrate synthase; COX-IV, cytochrome C oxidase subunit IV; 5-mC, 5 methylCytosine; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha; BHMT, betaine-homocysteine *S*-methyltransferase; NRF, nuclear respiratory factor; MM-CK, creatine kinase muscle specific

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limit muscle's ability to contract [16–18]. For example, deficiency of regulators of mitochondrial gene transcription and mitochondrial DNA replication such as mitochondrial transcription factors (mtTF), nuclear encoded mitochondrial localized proteins, have been reported to cause exercise intolerance and fatigue in mouse models [16]. Moreover changes in genes that regulate aerobic and anaerobic metabolism have also been implicated in causation of exercise intolerance [19]. For example, alterations in CS, LDH and COX-IV were reported to limit physical capacity [19–22]. Identification of factors that cause mitochondrial dysfunction and/or declined biogenesis as well as changes in energy metabolism is necessary to enhance physical capacity and to maximize exercise benefits.

Recent evidence suggests that epigenetics can also modulate gene expression levels through changes in DNA methylation, regulation of chromatin structure and gene accessibility and most recently through microRNA expression levels [23–25]. Though involvement of epigenetic modifications in regulation of important gene expression levels associated with skeletal muscle function are characterized relatively well [26], the role of epigenetic modifications in causation of fatigue is largely unknown [13]. Importantly knowledge regarding pathological factors that contribute to fatigue and associated epigenetic modifications is lacking. In the current study we evaluated the influence of hyperhomocysteinemia (HHcy) on skeletal muscle fatigability and associated epigenetic modifications.

HHcy is a metabolic disorder mainly due to improper removal and/or accumulation of the non-protein coding sulfur containing amino acid homocysteine (Hcy) [8]. Genetic, nutritional and disease specific factors have been implicated in causation of HHcy [8]. Though skeletal muscle deformities in HHcy patients have been reported long ago [27], it is now that the association between HHcy and decline in muscle function is increasingly recognized [8]. However, the causes for such a decline in physical function are not well characterized [8]. As Hcy metabolism also generates methyl groups to feed to the cellular methylation reactions and DNA methylation is aberrant in HHcy conditions, HHcy is proposed as an epigenetic modulator [8]. However, the relevance of epigenetic changes during HHcy in causation of muscle weakness is not known. In the current study we have characterized the nature of HHcy medicated changes in skeletal muscle function and found that epigenetic modifications contribute to HHcy associated decline in physical function using genetic mouse models of HHcy. The transsulfuration enzyme CBS, Cystathionine β synthase which performs irreversible conversion of Hcy into cysteine, has been found to be frequently mutated in human beings. Consequently, mouse models that lack CBS had been generated. The homozygous CBS knock out mice not only exhibit severe HHcy, but also die shortly after birth [28]. Hence, the mice heterozygous for CBS gene depletion, exhibit moderate HHcy with normal longevity, and are frequently used to know the systemic effects of HHcy condition [8,28]. In the current study, the CBS +/− mice exhibited more fatigability, and generated less contraction force. No significant changes in muscle morphology were observed. However, there is a corresponding reduction in large muscle fiber number in CBS +/− mice. Excess fatigability was not due to changes in key enzymes involved in metabolism, but was due to reduced ATP levels. A marginal reduction in dystrophin levels along with a decrease in mitochondrial transcription factor A (mtTFA) were observed. There was also an increase in the mir-31, and mir-494 quantities that were implicated in dystrophin and mtTFA regulation respectively. The molecular changes elevated during HHcy, with the exception of dystrophin levels, were reversed in CBS +/− mice after exercise. In addition, the amount of NRF-1, one of the transcriptional regulators of mtTFA, was significantly decreased in CBS +/− mice. Furthermore, after treating C2C12 cells with homocysteine (Hcy), there was enhancement in mir-494 levels and concomitant decline in mtTFA protein amount. These changes in C2C12 cells were also accompanied by an increase in DNMT3a and DNMT3b proteins and global DNA methylation levels. Together, these results suggest that HHcy plays a causal role in reduced physical performance and muscle force generation through mitochondrial dysfunction which involve epigenetic changes.

2. Materials and methods

2.1. Ex-vivo muscle contraction

Myobath studies were conducted using multi-channel isolated tissue bath system as described before [29] (Myobath, World Precision Instruments, Sarasota, FL, USA). All in vivo conditions such as temperature (37 °C), pH, electrolyte strength and proper aeration were supplied. The desired muscles were isolated from tendon to tendon without damage to the muscle bundle. Isolated intact muscles were mounted onto a force transducer and muscle contractions were recorded after determining the appropriate tension. For each experiment initial muscle tension was adjusted to give maximal response. Duration of muscle contraction for a given stimulus and maximal response were calculated after supplying field electric stimulus. As there were no significant measurable differences between weight and length of EDL and soleus from different groups no normalization was done to reflect the weight and length of the muscles. All the muscles were stimulated with 40 V (maximal electric output) for a duration of 30 ms (milliseconds) with a frequency of 0.5 Hz.

2.2. Exercise protocol

All mice in the exercise group were administered a swimming protocol, aerobic endurance exercise, developed from recommendations listed in the "Resource Book for the Design of Animal Exercise Protocols" by the American Physiological Society. The protocol consisted of 4 days of exercise per week for 4 weeks with the duration of swimming starting at 30 min on week 1 and increasing by 15 min each week to a maximum duration of 75 min by the fourth week. Large polymer containers measuring 20 in. × 14 in. × 7 in. were filled with warm water to a depth of approximately 5 in. The water temperature was maintained between 32 and 36 °Celsius. Mice were placed in the water and constantly monitored to ensure safety and physical activity. If the mice discontinued swimming for more than 2 s, they were gently nudged to promote movement. Upon completion of exercise, the mice were placed on a paper towel and gently dried off before being placed back into their cage.

2.3. Swim test

Intact male mice of appropriate ages from WT and CBS −/+ groups were subjected to swim performance and the live recordings were obtained using 'Live animal behavior recoding and analysis system' (Topscan) from CLEVER SYSTEMS (Reston, Virginia, USA) as described before [30].

2.4. Tissue ATP estimation

Desired tissues (entire soleus muscle) were snap frozen and were used later for enumeration of ATP levels. Total ATP levels were measured using calorimetric kit from Bio Vision (Milpitas, CA, USA). Cold homogenized tissues were deproteinized and neutralized as described in the kit. Cleared samples were used to assay for the ATP levels using spectromaxx spectrophotometer with appropriate standards. Tissue ATP levels were derived from standard curve equations.

2.5. Global methyl-C estimation

Genomic DNA was isolated using Quick-gDNA™ MiniPrep kit from Zymo Research (Irvine, CA, USA). After quantification, an equal amount of genomic DNA was used to estimate global levels of 5-methylcytosine