Experimental Gerontology 48 (2013) 1294–1302

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

# Aging-related changes in the iron status of skeletal muscle

Keith C. DeRuisseau [a,*], Young-Min Park [a], Lara R. DeRuisseau [b], Patrick M. Cowley [a], Christopher H. Fazen [c], Robert P. Doyle [c]

[a] Department of Exercise Science, Syracuse University, 820 Comstock Ave., Room 201 WB, Syracuse, NY 13244, USA
[b] Department of Biological Sciences, Le Moyne College, 1419 Salt Springs Road, Syracuse, NY 13214, USA
[c] Department of Chemistry, Syracuse University, 1-014 Center for Science and Technology, Syracuse, NY 13244, USA

ARTICLE INFO

*Article history:*
Received 20 June 2013
Received in revised form 17 July 2013
Accepted 21 August 2013
Available online 29 August 2013

Section Editor: Christiaan Leeuwenburgh

*Keywords:*
Sarcopenia
Muscle aging
Pyridoxal isonicotinoyl hydrazone
Muscle function
Skeletal muscle iron regulation

ABSTRACT

The rise in non-heme iron (NHI) concentration observed in skeletal muscle of aging rodents is thought to contribute to the development of sarcopenia. The source of the NHI has not been identified, nor have the physiological ramifications of elevated iron status in aged muscle been directly examined. Therefore, we assessed plantaris NHI and heme iron (HI) levels in addition to expression of proteins involved in iron uptake (transferrin receptor-1; TfR1), storage (ferritin), export (ferroportin; FPN), and regulation (iron regulatory protein-1 (IRP1) and -2 (IRP2)) of male F344xBN F1 rats (n = 10/group) of various ages (8, 18, 28, 32, and 36 months) to further understand iron regulation in aging muscle. In a separate experiment, iron chelator (pyridoxal isonicotinoyl hydrazone; PIH) or vehicle was administered to male F344xBN F1 rats (n = 8/group) beginning at 30 months of age to assess the impact on plantaris muscle mass and function at ~36 months of age. Principle findings revealed the increased NHI concentration in old age was consistent with concentrating effects of muscle atrophy and reduction in HI levels, with no change in the total iron content of the muscle. The greatest increase in muscle iron content occurred during the period of animal growth and was associated with downregulation of TfR1 and IRP2 expression. Ferritin upregulation did not occur until senescence and the protein remained undetectable during the period of muscle iron content elevation. Lastly, administration of PIH did not significantly ($p > 0.05$) impact NHI or measures of muscle atrophy or contractile function. In summary, this study confirms that the elevated NHI concentration in old age is largely due to the loss in muscle mass. The increased muscle iron content during aging does not appear to associate with cytosolic ferritin storage, but the functional consequences of elevated iron status in old age remains to be determined.



## 1. Introduction

The decline in muscle mass and quality that occurs with aging, termed sarcopenia, is a significant health problem afflicting up to half of the elderly population (von Haehling et al., 2012). In recent years, the role of iron as a causative factor in the development of sarcopenia has generated much interest. Iron possesses redox chemistry and can access several oxidation states. It is this property that enables iron to be a suitable component of many electron transferring systems (Comporti, 2002) and affords iron the ability to produce extremely reactive oxygen radical species (ROS). Notably, iron-induced free radical production is hypothesized to be a pivotal factor in the progression of oxidative injury and dysfunction observed in senescent skeletal muscle (Altun et al., 2007; Hofer et al., 2008; Jung et al., 2008; Xu et al., 2008, 2012b).

The majority of total body iron is incorporated into heme proteins that include hemoglobin, myoglobin, cytochromes, and heme thiolates in addition to NHI that serves as an enzyme co-factor and important iron reserve (Finch and Huebers, 1982). Skeletal muscle NHI comprises approximately 60% of the muscle total iron content (Chen and Thacker, 1986) and skeletal muscle contains roughly 10–15% of the total body iron (Barbieri and Sestili, 2012). Our group is among several to report the occurrence of elevated NHI status in skeletal muscle of aged animals (Altun et al., 2007; Hofer et al., 2008; Jung et al., 2008; Xu et al., 2008, 2012b). Skeletal muscle iron elevation during aging is likely to be of vital importance as it could promote greater release of iron from ferritin and further exacerbate ROS production (Jung et al., 2008). Although heme iron (HI) containing proteins can cause intracellular oxidant production (Gorelik and Kanner, 2001; Kanner and Harel, 1985; Turrens,

*Abbreviations:* BM, body mass; CON, control; DMT1, divalent metal transporter-1; FPN, ferroportin; F344xBN F1, Fischer 344 Brown Norway F1 hybrid rat; HI, heme iron; IRP1, iron regulatory protein-1; IRP2, iron regulatory protein-2; IRE, iron responsive element; MM, muscle mass; NADPH, nicotinomide adenine dinucleotide phosphate; NHI, non-heme iron; OCT, optimal cutting temperature; PIH, pyridoxal isonicotinoyl hydrazone; ROS, reactive oxygen radical species; TfR1, transferrin receptor-1; VEH, vehicle.

* Corresponding author at: Department of Exercise Science, Room 201 WB, Syracuse University, Syracuse, NY, 13244, USA. Tel.: +1 315 443 9698; fax: +1 315 443 9375.
*E-mail address:* kcderuis@syr.edu (K.C. DeRuisseau).

0531-5565/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exger.2013.08.011

2003), studies investigating iron status of aging skeletal muscle examined only the NHI fraction since iron released from ferritin or iron–sulfur clusters can exist as low molecular weight iron complexes capable of generating free radicals via Fenton chemistry (Gutteridge, 1989).

Current evidence suggests that the aging-associated elevation in skeletal muscle NHI concentration may be detrimental, and associations with muscle atrophy and oxidative stress have been reported (Hofer et al., 2008; Jung et al., 2008; Xu et al., 2008, 2012b). Skeletal muscle mitochondria also demonstrate increased NHI concentration in senescence that could be linked to mitochondrial dysfunction and muscle degeneration (Seo et al., 2008). It is still unknown whether elevated muscle iron status during aging plays a causative role in these outcomes.

While it is well documented that skeletal muscle NHI concentration and content are elevated in senescence, the factors responsible for these increases remain unclear. The development of muscle atrophy during aging conceivably causes an iron concentrating effect, but the contribution of other factors to the elevation of muscle iron status is unknown. Declines in heme synthesis and concentration (Atamna et al., 2002; Bitar and Shapiro, 1987; Bitar and Weiner, 1983) and/or dysregulation of iron uptake or export pathways (Jung et al., 2008; Xu et al., 2012a, 2012b) could be contributing factors. To date, only limited emphasis has been placed on unraveling the mechanisms of aging-associated increases in skeletal muscle NHI. Delineation of HI and iron regulation responses over the course of aging would fill important gaps in our current understanding of iron metabolism in aging muscle. Moreover, the direct determination of muscle iron status on measures of muscle atrophy and function in old age has not been reported. Therefore, the purpose of the current experiments was three-fold: 1) to discern the contribution of HI to the muscle total iron content over the course of aging; 2) reveal the impact of aging on key iron regulation mRNA and protein responses; and 3) determine the impact of prolonged iron chelator administration on the development of sarcopenia.

These experiments examined the skeletal muscle of F344xBN F1 rats, which is a well-accepted model for the study of sarcopenia. We selected the plantaris since this muscle undergoes significant atrophy during aging and exhibits increased NHI concentration (Jung et al., 2008). We studied animals ranging in age from young adult to senescent to test the overarching hypotheses that skeletal muscle NHI elevation was partially the result of iron concentrating effects of muscle atrophy and decreased heme iron levels, and NHI accumulation contributed to the development of muscle atrophy and dysfunction in old age.

## 2. Material and methods

### 2.1. Experimental animals

Male F344xBN F1 rats were obtained from the National Institutes of Health, National Institute on Aging, Aged Rodent Colony. The animal use protocol was approved by the Syracuse University Institutional Animal Care and Use Committee and all experimental procedures followed guidelines established by the American Physiological Society for the use of animals in research.

#### 2.1.1. Experiment 1

Five groups of rats ($n = 10$/group) ranging in age from young adult to senescent were utilized to determine the impact of aging on skeletal muscle iron status and regulation. Ages varied slightly within each of the five groups (age range; mean ± SEM): 8 months (7–8; 7.5 ± 0.2), 18 months (18–19; 18.1 ± 0.1), 28 months (28–29; 28.4 ± 0.2), 32 months (31–33; 32.0 ± 0.2), and 36 months (36–37; 36.3 ± 0.2) and are reported as single-month values for clarity. These age groups are roughly equivalent to young adult, 35, 50, 65, and 85 year old humans based on survival curves for male F344xBN F1 rats (Turturro et al., 1999) and human males (Arias, 2006). Upon arrival to the animal facility the rats were provided with water and food (LabDiet 5001; St. Louis, MO) containing 270 ppm iron ad libitum and were housed in a thermo neutral environment on a 12-hour light: dark cycle. On the day of tissue dissection all animals were deeply anesthetized by an intraperitoneal injection of pentobarbital sodium (80–120 mg/kg). Upon reaching a surgical plane of anesthesia the liver and plantaris muscles were quickly excised and trimmed of excess tendon, weighed, then frozen in liquid nitrogen and stored at −80 °C. The animals were euthanized by removal of the heart and diaphragm.

#### 2.1.2. Experiment 2

Upon arrival to the animal facility the rats were provided with water and food (7017 NIH-31; Teklad Diets, Madison, WI) containing 270 ppm iron ad libitum and were housed in a thermo neutral environment on a 12-hour light: dark cycle. Animals were divided into control (CON; $n = 14$), vehicle (VEH; $n = 12$), and iron chelator (pyridoxal isonicotinoyl hydrazone; PIH; $n = 19$) groups to determine the effect of prolonged iron chelator administration on plantaris muscle mass and function. One group of CON animals was obtained at 30 months of age ($n = 10$) and was housed in the animal facility for up to 7 months. In addition, four controls were obtained at 36 months of age and euthanized 1-week following their arrival to the facility. Beginning at 30 months of age, animals in the VEH group received weekly i.p. injections of 10% Cremophor EL (Sigma-Aldrich Corporation, St Louis, MO) for 6–7 months. Animals in the PIH group also received weekly i.p. injections beginning at 30 months of age for 6–7 months. Animals in the PIH group received pyridoxal isonicotinoyl hydrazone at a dose of 50 mg/kg (Adamcova et al., 2002). All animals in the VEH and PIH groups were weighed weekly and visually inspected for tumors and outward signs of ill-health. One to two days following the final injection of vehicle or PIH, the animals (including controls) were anesthetized by an intraperitoneal injection of pentobarbital sodium (80–120 mg/kg). Muscle contractile properties were assessed prior to tissue collection from a subgroup of animals ($n = 5$/group). Plantaris muscles were excised, weighed, and utilized for the determination of NHI levels. The animals were euthanized by removal of the heart and diaphragm.

### 2.2. Non-heme iron (NHI)

Non-heme iron was measured according to the method described by Rebouche et al. (2004) using reagents prepared with ultrapure (18.6 MΩ) water. Muscle samples (30–50 mg) were homogenized (1:10; wt.:vol.) on ice in ultrapure water using an all glass Potter-Elvehjem style homogenizer. Tissue homogenates (100 μL) were mixed with an equal volume of protein precipitation solution containing 1 N HCL and 10% trichloroacetic acid in 1.5 mL polypropylene tubes. Samples were vortexed and placed in a water bath for 1 h at 95 °C; following incubation the samples were removed from the water bath, vortexed, and centrifuged 8200 ×*g* for 10 min. An equal amount of supernatant (30 μL) was mixed with chromagen solution containing 0.508 mM ferrozine, 1.5 M sodium acetate, and 0.1% thioglycolic acid. Samples were also incubated with 1.5 M sodium acetate and 0.1% thioglycolic acid to serve as sample blanks. Following a 30-minute incubation at room temperature the absorbance of the reaction mixture was measured in a plate reader (PowerWave HT, BioTek Instruments) at a wavelength of 562 nm. Non-heme iron concentration was calculated from a standard curve (0, 2, 4, 6, 8, and 10 μg/mL) created by diluting iron atomic absorption standard (Sigma-Aldrich Corporation) with an equal volume of protein precipitation solution.

### 2.3. Heme iron (HI)

Heme concentration was measured in crude tissue homogenates generated from the NHI assay. Two rounds of the assay were performed.