Published in final edited form as:

Adv Exp Med Biol. 2017 ; 977: 51–57. doi:10.1007/978-3-319-55231-6_8.

Imaging Redox State in Mouse Muscles of Different Ages

Lily Moon,

Molecular Imaging Laboratory, Department of Radiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Britton Chance Laboratory of Redox Imaging, Johnson Research Foundation, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

David W. Frederick,

Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Institute for Diabetes, Obesity and Metabolism, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Joseph A. Baur, and

Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Institute for Diabetes, Obesity and Metabolism, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Lin Z. Li

Molecular Imaging Laboratory, Department of Radiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Britton Chance Laboratory of Redox Imaging, Johnson Research Foundation, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Abstract

Aging is the greatest risk factor for many diseases. Intracellular concentrations of nicotinamide adenine dinucleotide (NAD⁺) and the NAD⁺-coupled redox state have been proposed to moderate many aging-related processes, yet the specific mechanisms remain unclear. The concentration of NAD⁺ falls with age in skeletal muscle, yet there is no consensus on whether aging will increase or decrease the redox potential of NAD⁺/NADH. Oxidized flavin groups (Fp) (e.g. FAD, i.e., flavin adenine dinucleotide, contained in flavoproteins) and NADH are intrinsic fluorescent indicators of oxidation and reduction status of tissue, respectively. The redox ratio, i.e., the ratio of Fp to NADH, may be a surrogate indicator of the NAD⁺/NADH redox potential. In this study we used the Chance redox scanner (NADH/Fp fluorescence imaging at low temperature) to investigate the effect of aging on the redox state of mitochondria in skeletal muscles. The results showed that

there are borderline significant differences in nominal concentrations of Fp and NADH, but not in the redox ratios when comparing 3.5-month and 13-month old muscles of mice ($n = 6$). It may be necessary to increase the number of muscle samples and study mice of more advanced age.

Keywords

Mitochondria; Muscle; NADH; FAD; Oxidized flavoprotein (Fp); Redox ratio, aging

1 Introduction

Imaging tissue redox state can be achieved fluorescently. NADH and FAD are two coenzymes with endogenous fluorescence that play key roles in mitochondrial bioenergetics. NADH and FADH₂ pass their electrons to oxygen to generate ATP and water in the electron transport chain. NADH and FADH₂ are oxidized to NAD⁺ and FAD, respectively. NAD⁺ and FADH₂ are both non-fluorescent. Britton Chance and colleagues demonstrated that the redox ratio, calculated by the fluorescence intensities of NADH and FAD, which originate primarily from the mitochondria, could be used as a surrogate indicator of the metabolic/redox state of the organelles [1]. The tissue Fp/NADH ratio correlates linearly with measurements of NAD⁺/NADH determined biochemically or by mass spectrometry [2, 3]. Note that redox state or potential is the balance between oxidized and reduced forms of specific redox-active molecules and redox state is different from oxidative stress. The redox state of mitochondria may reflect a number of parameters related to aging, including mitochondrial reserve, free radical damage and coupling efficiency.

As aging is the greatest risk factor for a number of diseases, understanding the biological processes underlying aging is important for improving human health and life span. NAD⁺ concentration has been shown to decrease with aging in multiple tissues [4, 5] and the regulation of NAD⁺ can affect health span and life span in laboratory studies [4, 6]. Moreover, the activation of the NAD⁺ salvage pathway enzyme NAMPT (Nicotinamide phosphoribosyltransferase) has been suggested to be neuroprotective [7]. There is not yet a clear consensus on whether the NAD⁺-coupled redox potential (NAD⁺/NADH) decreases or increases with aging [5, 8]; it is unclear whether this discrepancy is due to differences between organs or technical difficulties with existing methodologies.

Previous work demonstrated that redox indices (NADH, Fp, redox ratio) can provide important information about energy-related biological processes [9, 10]. Here we used the Chance redox scanner (a cryogenic NADH/Fp fluorescence imager) to study the effect of aging on the redox state of mitochondria in skeletal muscles.

2 Materials and Methods

Mice bearing a single Cre-inducible (lox-STOP-lox) allele of *Nampt* under the control of the CAGGS promoter were crossed to mice bearing Cre under the control of the muscle creatine kinase promoter to obtain muscle-specific overexpression of *Nampt* as previously described [11]. Twelve male C57BL6 mice (weight ranging 25–35 g) were divided into four groups: (1) elder *Nampt* transgenic, (2) young *Nampt* transgenic, (3) elder *Nampt* control; (4) young

Nampt control. Each group contained three mice. The elder age was 13 months, and the young age was 3.5 months. The mice were sacrificed by cervical dislocation. The hind limb muscles were immediately dissected. The whole quadriceps were removed from the anterior surface of the femur bone and cleaned of residual adipose. The quadriceps muscles were then flash frozen in liquid nitrogen and embedded for subsequent redox scanning.

For embedding tissue samples for redox scanning, previously described methods were followed [12–14]. Briefly, a plastic cap (diameter ~ 2.5 cm) filled with 3–4 ml mounting medium (ethanol:glycerol:water = 1:3:6) was immersed in liquid nitrogen for a moment to make the medium half-frozen like a gel. The tissue sample was then placed in the middle of the mounting medium. Frozen tubes (inner diameter ~ 1.5 mm) of 100 μ M FAD and NADH solution standards were inserted into the medium in the vicinity of the specimen (Fig. 8.1). More embedding medium was added to cover the whole tissue sample, which was then immersed in liquid nitrogen to freeze completely.

We employed the Chance redox scanner to obtain the fluorescence images of Fp and NADH for all muscle samples, each with 2–3 sections and tissue depth ranging 0.4–1.0 mm. Neutral density filters were added to the emission channels to avoid signal saturation. Image data were post-processed by a customized MATLAB program. The nominal concentrations of Fp and NADH in tissue were interpreted based on reference standards and neutral density filter difference between tissue and standards. They were then used to calculate the Fp redox ratio, i.e., $Fp/(NADH + Fp)$, representing the mitochondrial redox state. Data were expressed as mean $\pm$ SD (standard deviation) after first averaging across image sections and then across muscle samples. The comparison between any two groups was analyzed with the t-test, and statistical significance was considered at the level of $p < 0.05$. $0.05 < p < 0.1$ was regarded as borderline significant.

3 Results

The redox scanning results showed no significant difference between *Nampt* transgenic groups and control groups for either elder or young mice (Table 8.1). The differences remain insignificant when we pooled the elder and young mice for comparing the transgenic status ($p > 0.1$).

For the aging effects on redox indices, we did not find significant differences between the transgenic young and transgenic elder groups ($p > 0.3$). Neither did we see any redox indices differences between the control young and control elder groups ($p > 0.1$). Since we did not see significant differences in redox indices between the transgenic and control groups, we pooled the muscle samples from transgenic and control groups in order to enhance the power of statistical analysis of the aging effects on redox state. The redox scanning results showed that there are some borderline significant differences in both Fp and NADH between muscles of young and elder mice (Table 8.2). The Fp redox ratio also tended to be higher in young muscle than elder ones, although this did not reach statistical significance. Figure 8.2 shows typical redox images and histograms of the elder and young muscle samples from the *Nampt* control group.

4 Discussion

The redox imaging techniques employed here may have wide-ranging applications in many fields, such as cancer, diabetes, developmental processes, mitochondrial diseases, neurodegenerative diseases, and aging. In this experiment we used the Chance redox scanner to study the redox status of muscle at different ages through imaging.

Originally we also aimed to observe the effect of *Nampt* overexpression on the redox state of muscles. Since NAD^+ may decrease with aging, previous observation showed that overexpression of *Nampt* preserved muscle NAD^+ levels and exercise capacity in aged mice [15]. Thus, we wondered if *Nampt* overexpression would cause any effect on redox indices (NADH, Fp and redox ratio). However, we found no significant difference between the *Nampt* transgenic group and the control group, consistent with the equilibration of mitochondrial NAD^+/NADH identified in this model using mass spectrometry [11]. Although *Nampt* functions importantly in salvaging NAD^+ , other mechanisms likely exist that resist changes in NAD^+ levels, including enhanced activity of NAD-dependent enzymes and feedback inhibition of *Nampt*. Despite the lack of major changes in middle-aged mice, increased capacity for NAD synthesis may help maintain stable NAD levels and redox status at more advanced ages, when NAD normally falls.

When pooling the transgenic and control data, we obtained a borderline significant difference between young and elder groups both in Fp and NADH. Although the Fp redox ratio tended to be lower in elder than the young muscles, we did not observe a significant difference in the Fp redox ratio.

Since Fp and NADH signals are proportional to mitochondrial density and the redox ratio is not, the borderline changes in Fp and NADH but not in redox ratio probably reflect a minor decrease of mitochondrial density with age. We reasoned that the NAD^+ regulating system might be sufficient enough for mice to sustain redox homeostasis up to 13 months of age, potentially explaining why we did not observe significant change in the redox indices. In the future, we can increase the number of mice to improve the statistical power. We can also study mice at a more advanced age of 24–36 months to investigate whether their redox indices might be significantly different from those of young mice.

Acknowledgments

We appreciate Dr. Yu Wen and Dr. He N. Xu for their assistance with the paper revision. This work was supported by the NIH grant R01CA155348 and R01CA191207 (PI: L.Z. Li), the Center for Magnetic Resonance and Optical Imaging (CMROI) – an NIH supported research resource (P41EB015893, PI: R. Reddy).

References

1. Chance B, Schoener B, Oshino R, et al. Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. NADH and flavoprotein fluorescence signals. J Biol Chem. 1979; 254:4764–4771. [PubMed: 220260]
2. Ozawa K, Chance B, Tanaka A, et al. Linear correlation between acetoacetate beta-hydroxybutyrate in arterial blood and oxidized flavoprotein reduced pyridine-nucleotide in freeze-trapped human liver-tissue. Biochim Biophys Acta. 1992; 1138:350–352. [PubMed: 1562619]

3. Varone A, Xylas J, Quinn KP, et al. Endogenous two-photon fluorescence imaging elucidates metabolic changes related to enhanced glycolysis and glutamine consumption in precancerous epithelial tissues. *Cancer Res.* 2014; 74:3067–3075. [PubMed: 24686167]
4. Gomes AP, Price Nathan L, Ling Alvin JY, et al. Declining NAD⁺ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. *Cell.* 2013; 155:1624–1638. [PubMed: 24360282]
5. Braidly N, Guillemin GJ, Mansour H, et al. Age related changes in NAD⁺ metabolism oxidative stress and Sirt1 activity in wistar rats. *PLoS One.* 2011; 6:e19194. [PubMed: 21541336]
6. Verdin E. NAD(+) in aging, metabolism, and neurodegeneration. *Science.* 2015; 350:1208–1213. [PubMed: 26785480]
7. Wang G, Han T, Nijhawan D, et al. P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. *Cell.* 2014; 158:1324–1334. [PubMed: 25215490]
8. Pugh TD, Conklin MW, Evans TD, et al. A shift in energy metabolism anticipates the onset of sarcopenia in rhesus monkeys. *Aging Cell.* 2013; 12:672–681. [PubMed: 23607901]
9. Xu HN, Li LZ. Quantitative redox imaging biomarkers for studying tissue metabolic state and its heterogeneity. *J Innov Opt Health Sci.* 2014; 07:1430002.
10. Li LZ. Imaging mitochondrial redox potential and its possible link to tumor metastatic potential. *J Bioenerg Biomembr.* 2012; 44:645–653. [PubMed: 22895837]
11. Frederick DW, Davis JG, Davila A Jr, et al. Increasing NAD synthesis in muscle via nicotinamide phosphoribosyltransferase is not sufficient to promote oxidative metabolism. *J Biol Chem.* 2015; 290:1546–1558. [PubMed: 25411251]
12. Moon L, Sun N, Li LZ. Preliminary investigation on the spatial distribution of the metabolic state in tissue by the chance redox scanner. *China Med Devices.* 2013; 28:1–7.
13. Xu HN, Nioka S, Glickson J, et al. Quantitative mitochondrial redox imaging of breast cancer metastatic potential. *J Biomed Opt.* 2010; 15:036010. [PubMed: 20615012]
14. Xu HN, Wu B, Nioka S, et al. Quantitative redox scanning of tissue samples using a calibration procedure. *J Innov Opt Health Sci.* 2009; 2:375–385.
15. Frederick DW, Loro E, Liu L, et al. Loss of NAD homeostasis leads to progressive and reversible degeneration of skeletal muscle. *Cell Metab.* 2016; 24:269–282. [PubMed: 27508874]

Fig. 8.1.

A white-light picture of a mounted mouse quadriceps, showing a flat surface after grinding. The two tubes on the *left side* of the sample were FAD (*above*) and NADH reference standards

Elder control **Young control** **Fig. 8.2.**

The typical redox images and corresponding histograms of elder age (13 months) and young age (3.5 months) mouse quadriceps samples (*Nampt* control). From *left to right*: Fp, NADH, and Fp redox ratio. As shown by the *color bars* on the *right side* of each image, the Fp or NADH nominal concentrations are in μM ; the Fp redox ratio ranges between 0 and 1. The small round spots outside the muscle section are Fp or NADH reference standards. The concentration of reference standard FAD = $46 \mu\text{M}$, NADH = $100 \mu\text{M}$ after the correction of neutral density filters, image matrix 128×64 and pixel size $200 \mu\text{m}$

Table 8.1Mean redox indices for mouse quadriceps (*Nampt* transgenic versus control)

	Mean Fp (μM)	Mean NADH (μM)	Fp redox ratio Fp/(Fp + NADH)
Transgenic elder (n = 3)	10.5 ± 3.6	62 ± 12	0.15 ± 0.04
Control elder (n = 3)	9.9 ± 2.1	66 ± 20	0.14 ± 0.02
p	0.83	0.77	0.79
Transgenic young (n = 3)	14.8 ± 7.2	77 ± 22	0.17 ± 0.02
Control young (n = 3)	16.1 ± 4.9	87 ± 12	0.16 ± 0.02
p	0.81	0.56	0.61

Table 8.2

Mean redox indices of young and elder mouse quadriceps

	Mean Fp (μ M)	Mean NADH (μ M)	Fp redox ratio Fp/(Fp + NADH)
Young (n = 6)	15.4 $\pm$ 5.6	82 $\pm$ 17	0.17 $\pm$ 0.02
Elder (n = 6)	10.2 $\pm$ 2.7	64 $\pm$ 15	0.15 $\pm$ 0.03
p	0.08	0.07	0.2