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Estimating relative carbonyl levels in muscle microstructures by fluorescence imaging

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Abstract

The increase in the levels of protein carbonyls, biomarkers of oxidative stress, appears to play an important role in aging skeletal muscle. However, the exact distributions of carbonyls among various skeletal muscle microstructures still remain largely unknown, partly owing to the lack of adequate techniques to carry out these measurements. This report describes an immunohistochemical approach to determine the relative abundance of carbonyls in the intermyofibrillar mitochondria (IFM), the subsarcolemmal mitochondria (SSM), the cytoplasm, and the extracellular space of skeletal muscle. These morphological features were defined by labeling the nucleus, the Z-lines, and mitochondria. Carbonyls were detected by derivatization with dinitrophenylhydrazine followed by labeling with an Alexa 488-labeled anti-dinitrophenyl primary antibody. Alexa 488 fluorescence (green) in different fiber microstructures was used to estimate the relative abundance of carbonyls. On the basis of the samples examined, preliminary results suggest that the most dramatic age-related changes in carbonyl levels occur in the extracellular space, followed in a decreasing order by SSM, IFM, and the cytoplasm. These observations were confirmed in the soleus and semimembranosus muscles composed predominantly of type I and type II fibers, respectively. This approach could easily be extended to the investigation of carbonyl levels in other muscles (composed of mixed skeletal muscle fiber types) or other tissues in which protein carbonyls are present.

Keywords

Protein carbonyls; Aging; Skeletal muscle microstructures; Fluorescence microscopy; Mitochondria

Introduction

Proteins, DNA, and lipids may become carbonylated as a result of oxidative stress [1–5]. This modification occurs in most tissues, but is predominant in postmitotic tissues, including skeletal muscle. However, investigating the causes and effects of carbonylation in complex tissues, such as skeletal muscle, is difficult, because carbonyl levels may differ in different tissue microstructures.

Skeletal muscle is particularly susceptible to carbonylation because of its high rate of conversion of metabolic energy into mechanical energy for muscle contraction. This results in high levels of reactive oxygen species (ROS), which are ultimately believed to lead to the carbonylation of biomolecules in various muscle microstructures. The main sources of metabolic energy in muscle are glycolysis occurring in the cytoplasm and oxidative phosphorylation localized in mitochondria. The latter are believed to be particularly susceptible to carbonylation damage owing to their being the major source of ROS in the cell [1]. ROS are produced by the metabolism over time, but they can also be produced under more acute conditions of cell stress. Previous reports have shown that oxidative stress imposed on cells (e.g., by treatment with hydrogen peroxide) results in increased carbonyl levels [6–9].

There are two types of mitochondria in muscle, intermyofibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM). SSM are clustered underneath the sarcolemmal membrane and are suggested to provide energy for membrane-related events, including cell signaling and substrate and ion transport [10]. In contrast, the IFM reside between myofibrils and are believed to provide most of the energy required for contraction of the muscles that rely on oxidative phosphorylation [11]. SSM generally represent only 25–30% of the total amount of skeletal muscle mitochondria [12]. It is expected that there would be differences in carbonyl patterns between these SSM and IFM because of the differences in their location and function.

Muscle cellular morphology and function change in response to development and aging [13–16] and, as such, carbonyl patterns are expected to be intimately associated with the mitochondria type. Some reports indicate that there is an age-related decrease in the rate of oxidative phosphorylation, and increased carbonyl levels in IFM [17,18]. Others suggest no change in the ROS production [19,20] and the rate of oxidative phosphorylation [21,22] in SSM or in a mixed population of SSM and IFM. These results must be interpreted with caution because SSM and IFM cannot be easily separated from each other [17,23], and the measurements of carbonyl levels in isolated mitochondria may be biased. Approaches based on imaging of fluorescently labeled carbonyls, such as the labeling scheme reported by Smith et al. [24,25], suggest that the fluorescence imaging technique may be suitable to assign carbonyl levels to different microstructures in the muscle, including SSM and IFM.

Here, we describe an immunohistochemical approach useful in comparing carbonyl levels in IFM, SSM, cytosolic domains, and the extracellular space. This approach is based on colocalization of these microstructures in muscle fibers with fluorescently labeled carbonyls. This labeling procedure was based on derivatization with dinitrophenylhydrazine (DNPH) followed by labeling with an Alexa 488-labeled anti-dinitrophenyl (DNP) primary antibody. Examination of serial cross sections of soleus (oxidative-phosphorylation-dependent) and semimembranosus (glycolysis-dependent) muscles of young and old animals demonstrated the feasibility of the use of this approach in determining age- and muscle-type-associated carbonyl patterns. Owing to small sample requirements, this approach may be suitable to investigate carbonyl patterns in tissue biopsies.

Materials and methods

Reagents

A rabbit anti-desmin primary antibody (SC-14026) was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). 4',6-Diamidino-2-phenylindole (DAPI; D1306), mouse anti-COXI primary antibody (A21296), Alexa 568-labeled goat anti-mouse secondary antibody (A11031), Alexa 488-labeled goat anti-rabbit secondary antibody (A11008), Alexa 488-labeled rabbit anti-DNP primary antibody (A11097), and Prolong Gold antifade mounting reagent (P 369341) were obtained from Invitrogen (Carlsbad, CA, USA). Goat serum (50–062) was obtained from Zymed Laboratories (San Francisco, CA, USA). DNPH, hydrogen peroxide, sodium borohydride, and sodium cyanoborohydride were from Sigma-Aldrich (St. Louis, MO, USA). TBS buffer consisted of 50 mM tris(hydroxymethyl) aminomethane hydrochloride and 0.15 mM NaCl at pH 7.6.

Animal and muscle fiber preparation

Skeletal muscle serial cross sections, transverse and longitudinal, were obtained from young (12- 13-month-old) and old (26-month-old) Fischer 344 male rats. The soleus muscle (composed primarily of type I fibers) and semimembranosus muscle (composed primarily of type II fibers) were excised, placed on corks in tissue-embedding medium, and flash-frozen in isopentane over liquid nitrogen [26]. Specimens were stored at -80°C until serial muscle cross sections or longitudinal sections were cut. Serial muscle cross sections were sectioned at $10\text{ }\mu\text{m}$ in a cryostat (Leica Microsystems, Nussloch, Germany) at -25°C , and placed on microscope slides. The slides were stored at -80°C until they were brought to room temperature and then used for imaging. All muscle sections were cut from the muscle mid-belly.

Muscle nuclei, mitochondria, and Z-line labeling

Unless indicated otherwise, all solutions were made in TBS buffer. Muscle serial cross sections were first permeated with 50% methanol in TBS buffer for 10 min, followed by thorough TBS washes and a 30-min incubation in 10% goat serum to block nonspecific protein binding sites. Then the samples were overlaid with an anti-COXI primary antibody (2 $\mu\text{g}/\text{ml}$) and an anti-desmin primary antibody (1:50 dilution in TBS). After a 1-h incubation at room temperature, the slices were thoroughly rinsed in TBS, after which they were incubated with an Alexa 568-labeled goat anti-mouse secondary antibody (1:250 dilution from 2 mg/ml in TBS) and Alexa 488-labeled goat anti-rabbit secondary antibody (1:250 dilution from 2 mg/ml in TBS). After a 1-h incubation at room temperature in the dark, the slices were thoroughly rinsed in TBS, followed by incubation with 300 nM DAPI for 5 min.

Carbonyls and mitochondria labeling

Muscle sections were first permeated with 50% methanol in TBS buffer for 10 min. After having been washed thoroughly with TBS, the samples were covered with 0.1% DNPH in 2 N HCl. After a 1-h incubation at room temperature, the slices were treated with 30 mM sodium cyanoborohydride in 0.1 M phosphate buffer, pH 6 for 1 h at room temperature to stabilize the reaction product [24,25]. After that, slices were thoroughly rinsed in TBS, followed by a 30-min incubation in 10% goat serum to block nonspecific binding sites. Then an Alexa 488-labeled rabbit anti-DNP primary antibody was diluted 1:25 in TBS and incubated with the muscle slices for 30 min in the dark. After having been rinsed in TBS exhaustively, the muscle slices were incubated in a mouse anti-COXI primary antibody (1:250 dilution) for 1 h, followed by three TBS rinses. An Alexa 568-labeled goat anti-

mouse secondary antibody was diluted 1:250 in TBS and incubated with the muscle slices for 1 h in the dark, after which they were rinsed in TBS.

Controls were used to confirm carbonyl-specific labeling. The selectivity of labeling carbonyls with DNPH was tested by incubating muscle slides with 25 mM sodium borohydride in 80% methanol for 30 min at room temperature and subsequent washes before the labeling reaction with DNPH [24,25]. Since sodium borohydride reduces carbonyl groups to alcohols as described by Smith et al. [25], it is reasonable to expect a decrease in the intensity of the Alexa 488 fluorescence when treating muscle slides with sodium borohydride prior to DNPH labeling. As a positive control, muscle slices were treated with 1 mM hydrogen peroxide in TBS for 2 h and washed thoroughly as described by Radak et al. [27]. It is expected that after hydrogen peroxide treatment, the intensity of Alexa 488 fluorescence will increase owing to the generation of more carbonyls in vitro.

Fluorescence microscopy

After muscle sections had been labeled, they were rinsed with TBS and the mounting reagent was applied. Images were collected using an Olympus IX-81 inverted microscope (Melville, NY, USA) equipped with an Olympus IX2-DSU disk scanning unit and a $\times 60$ (numerical aperture 1.45) oil immersion objective. Confocal and epifluorescence images were used for the definition of microstructures and for intensity measurements, respectively. Excitation light was supplied by an Exfo (Addison, TX, USA) XCite 120 metal halide lamp source coupled to the microscope via a liquid light guide. The specifications of the filter cubes used for imaging are summarized in Table 1. The binning factors for epifluorescence and confocal images were 1 and 2, respectively. For evaluation of fluorescence intensities, the exposure time was 10 s. Images were captured with a C9100-01 CCD camera (Hamamatsu, Bridgewater, NJ, USA). CImaging SimplePCI version 5.3 (Compix, Cranberry Township, PA, USA) was used to control the microscope and camera, and to collect and process the images.

All samples were labeled and imaged using the same microscope settings on the same day to avoid sample preparation variation. For each sample, different regions were imaged to ensure a representative fluorescence response of the sample. In addition, imaging a fluorophore solution yields a uniform fluorescence signal throughout the entire field of view (data not shown).

Data analysis with SimplePCI

Image processing and analysis was performed using SimplePCI (Hamamatsu, Sewickley, PA, USA). SimplePCI is capable of identifying a region of interest (ROI) which has a red fluorescence intensity (resulting from labeling the sample with a mouse anti-COXI primary antibody followed by an Alexa 568-labeled goat anti-mouse secondary antibody) higher than a visually determined threshold. This procedure aids in identifying both IFM and SSM. Subsequently, the green fluorescence intensity associated with the carbonyl level (upon treatment with DNPH and subsequent labeling with an Alexa 488-labeled rabbit anti-DNP primary antibody) was measured in both the IFM and SMM regions of muscle fibers. For the measurement of carbonyl content in the cytoplasmic and extracellular regions, the green fluorescence signals were measured in five different areas. Their green fluorescence values are reported as the mean $\pm$ the standard deviation (SD). Four different samples were examined: cross sections of slow-twitch and fast-twitch muscles from young rats, and cross sections of slow-twitch and fast-twitch muscles from old rats.

Results

Defining muscle fiber morphology

Initially, longitudinal muscle cross sections were utilized to confirm the effectiveness of the various labeling schemes. As seen in Fig. 1, Z-lines were identified by the immuno-labeling of desmin (green); myonuclei were visualized by labeling with DAPI (blue), and mitochondria were detected by the immunolabeling of COXI (red). Because myonuclei are proximate to the sarcolemmal membrane, they assist in identifying the regions in which SSM are found. Thus, when mitochondria were between myofibrils and lined up perpendicularly to the Z-lines, they were identified as IFM. In contrast, SSM were found nearby the sarcolemmal membrane.

Imaging carbonyl levels

Carbonyls were detected by first derivatizing them with DNPH and then detecting the DNP moiety by immunolabeling with an Alexa 488-labeled anti-DNP primary antibody. The specificity of DNPH to carbonyls was investigated by partially reducing and increasing carbonyls in muscle cross sections prior to derivatization and labeling. As seen in Fig. 2, borohydride treatment drastically reduced the Alexa 488 (green) fluorescence intensity by 85% (mean $\pm$ SD) (i.e., from 30.5 ± 6.8 to 4.5 ± 0.7 AU, $P < 0.0001$, $n=9$), while peroxide treatment increased it by 261% (i.e., from 30.5 ± 6.8 to 110.2 ± 15.3 AU, $P < 0.0001$, $n=9$). Stated values are corrected for background levels in the fluorescence intensity analysis.

Spatial distribution of protein carbonyls in muscle

In order to better appreciate the colocalization of carbonyl-associated fluorescence (green) with various muscle microstructures, transverse cross sections were utilized (Fig. 3). Carbonyls were visualized in the IFM, SSM, cytoplasmic region, and extracellular region (i.e., connective tissue surrounding a fascicle), demonstrating that carbonyls are present in both mitochondria and nonmitochondrial regions of muscle tissues.

On the basis of colocalization with each of the stains associated with each microstructure, as defined by its respective ROI, we determined the green fluorescence intensity (i.e., carbonyl levels) in IFM, SSM, cytoplasmic, and extracellular domains. Images with higher magnification (i.e., $\times 60$), shown in Fig. 4, were used for the analysis because the improved resolution makes the definition of the various ROI more accurate. The green fluorescence intensities (mean $\pm$ SD) in different muscle microstructures of four cross sections are shown in Fig. 5. Although the goal of these measurements was to demonstrate the feasibility of the technique, this preliminary study (one cross section per sample) suggests that differences in the carbonyl levels between the same microstructures in different muscle types are age-dependent.

Discussion

This work demonstrated that fluorescence labeling of several muscle landmarks (e.g., Z-lines and myonuclei) can be used as a guide to identify microstructures such as SSM, IFM, cytoplasmic, and extracellular domains. Using a carbonyl labeling technique including DNPH derivatization followed by treatment with an Alexa 488-labeled anti-DNP primary antibody, we used the colocalization of the Alexa 488 fluorescence with the microstructures of the same tissue to determine their relative carbonyl levels (Fig. 5).

The specificity of carbonyl labeling with DNPH was demonstrated by the decrease in the fluorescence intensity in muscle slices reduced by sodium borohydride compared with that of a control (30.5 ± 6.8 vs. 4.5 ± 0.7 AU). Similarly, peroxide treatment prior to derivatization

and labeling of carbonyls resulted in increased fluorescence (30.5 ± 6.8 vs. 110.2 ± 15.3 AU). An increase in carbonyls as a result of the peroxide treatment was expected owing to the generation of more carbonyls in vitro [27].

With use of this imaging technique, comparisons of the relative carbonyl levels in different microstructures of the same muscle cross section are highly feasible. Although we cannot rule out that there may be variations in DNP derivatization and immunolabeling in different microstructures, this is unlikely because the muscle cross sections were permeabilized with 50% methanol in TBS buffer, and transverse cross sections eliminate complications associated with the transport of reagents across biological membranes. Instrumental variations (i.e., illumination heterogeneity or variation in pixel-to-pixel response) have not been significant in other experiments using the instrumentation used here. On the other hand, a comparison between different muscle cross sections should only be considered semiquantitative, because parameters such as the reproducibility of the derivatization and labeling procedures cannot be easily assessed in a heterogeneous tissue.

Comparisons within the same muscle cross section clearly indicated that carbonyl levels are consistent for the four sample types examined (cross sections of slow-twitch and fast-twitch muscles from young rats, and cross sections of slow-twitch and fast-twitch muscles from old rats). Their carbonyl levels decrease in the following order: cytoplasmic < IFM < SSM < extracellular. Interestingly, the relative increase in carbonyl levels in SSM and extracellular space with respect to the cytoplasmic and IFM levels is more dramatic in older animals across both muscle types. These observations might indicate more selective damage in the connective tissue surrounding a fascicle in muscle tissue, which is consistent with a 200% increase in carbonyl levels observed in the connective tissue (tendon) of aged rats, based on a spectrometric DNPH assay [28]. Altogether, these findings might suggest the following about the connective tissue surrounding muscle fibers and nearby SSM: (1) that it generates more ROS, or (2) that it has less effective antioxidant systems, or (3) that proteins within the long-lived structural components of the connective tissue matrix have long half-lives compared with other proteins, which would make their carbonyl damage more profound. This speculation is in line with empirical results that the highest levels of advanced glycosylation end products occur in proteins of connective tissue matrix owing to their slow turnover [29–31].

A slightly higher carbonyl level was observed in SSM than in IFM (Fig. 5). These relative levels of carbonyl (mean $\pm$ SD) between SSM and IFM observed here (for young soleus muscle, 36.7 ± 2.5 vs. 31.5 ± 3.5 , $P < 0.0001$; for old soleus muscle, 56.9 ± 6.6 vs. 42.1 ± 2.0 , $P < 0.0001$; for young semimembranosus muscle, 43.6 ± 3.5 vs. 37.6 ± 2.4 , $P < 0.0001$; for old semimembranosus muscle, 50.5 ± 5.2 vs. 40.5 ± 2.5 , $P < 0.0001$) vary from those determined using an enzyme immunoassay, which led us to conclude that IFM exhibit higher levels of protein carbonyls than SSM in aging muscle [23,32]. Judge et al. [23,32] explained their observations by the energetic demands of IFM, which likely provide the primary energy source for muscle contraction [17,23,32,33]; IFM are expected to have higher rates of oxidative phosphorylation and, consequently, ROS production [17]. Our findings, while being preliminary, are not necessarily contradictory to those of these studies, because our work measured total carbonyl levels as opposed to carbonylated proteins [17,23,32]. In addition, it is important to consider that their study required isolation of separate fractions of SSM and IFM, which may have introduced a bias owing to incomplete separation of the two types of mitochondria [17–22]. On the other hand, our imaging approach suggesting the trend in carbonyl level is supported by the following arguments: (1) the proximity of SSM to the extracellular domains, which have the highest relative level of carbonyls—this may be the result of higher levels of ROS in this region; and (2) the relative carbonyl levels of SSM and IFM are the same for all the samples examined regardless of age or muscle type. Our

technique will have the advantage of identifying if the carbonylation occurs throughout the muscle or is selective to muscle–tendon areas or neuro-muscular junction areas.

Indeed, interpretation of the results presented here is preliminary and further sample analyses will be needed to draw more statistically significant conclusions that take into account animal-to-animal variations and longitudinal locations within the muscle (e.g., muscle mid-belly vs. the muscle-tendon junction). For instance, on the basis of power statistical analysis [34], the minimum required sample size was estimated to be 54. This sample size results from setting a *P* value, number of predictors, effect size (f^2), and power level of 0.05, 1, 0.15, and 0.8 respectively.

Conclusion

Here, we have demonstrated the feasibility of estimating the relative abundance of carbonyls in IFM, SSM, cytoplasmic and extracellular regions using immunofluorescence imaging. Because carbonyl formation is mainly the consequence of the ROS-associated damage of biomolecules, the results presented here are consistent with previously reported multiple potential sites for generation of ROS [35,36], including mitochondria [37–39], the transverse tubule-localized NAD(P)H oxidase enzymes [40], and xanthine oxidase in extracellular regions [41]. The extracellular domain (i.e., the connective tissue surrounding the fascicle) shows the highest carbonyl levels of the four muscle microstructures studied. If these observations hold validity in studies with a large number of samples, these preliminary results would indicate that connective tissue accumulates more oxidative damage than other parts of the muscle.

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Fig. 1. Fluorescence microscopy imaging of longitudinal muscle cross sections. The labeling and detection schemes are given in Table 1. Soleus muscle, deconvoluted, z-slicing 0.33 μm, binning 2, age 12 months old, 60×, exposure time 2.5 s (tetramethylrhodamine isothiocyanate filter cube), 4.5 s (fluorescein isothiocyanate filter cube), and 0.7 s (4',6-diamidino-2-phenylindole filter cube). *SSM* subsarcolemmal mitochondria, *IFM* intermyofibrillar mitochondria

Fig. 2. Fluorescence detection of carbonyls in longitudinal muscle sections. **a** Derivatized with dinitrophenylhydrazine and immunolabeled with Alexa 488-labeled rabbit anti-dinitrophenyl primary antibody without treatment **b** Pretreated with sodium borohydride prior to derivatization and labeling (see **a**). **c** Pretreated with hydrogen peroxide prior to derivatization and labeling, (see **a**). Soleus muscle (10- μ m thickness), 13 months old. Magnification $\times 60$, exposure time 10 s. See Table 1 for other detection conditions

Fig. 3. Carbonyls detected in IFM, SSM, cytoplasmic, and extracellular regions of muscle: **a** 13 months old, soleus muscle; **b** 26 months old, soleus muscle; **c** 13 months old, semimembranosus muscle; **d** 26 months old, semimembranosus muscle. Cross-sectional cut (10-µm thickness). Labeling and detection conditions are given in Table 1. Magnification $\times 10$, exposure time 10 s

Fig. 4. Imaging of carbonyls in IFM, SSM, cytoplasmic, and extracellular domains. Except for the use of $\times 60$ magnification, the other conditions are the same as for Fig. 3

Fig. 5.

Relative carbonyl levels in IFM, SSM, cytoplasmic, and extracellular regions of skeletal muscle. The images analyzed are shown in Fig. 4. *Young* 13-month-old Fischer 344 rats; *old* 26-month-old Fischer 344 rats; *semi* semimembranosus muscle (fast twitch); *soleus* soleus muscle (slow twitch). *Error bars* represent one standard deviation. For cytoplasmic and extracellular regions, fluorescence intensities were measured in five different areas

Table 1

Filter cubes and multi-fluorescence labeling systems

	Nuclei	Z-line	Mitochondria	Carbonyl
Primary antibody	DAPI	Rabbit anti-desmin primary antibody	Mouse anti-COXI primary antibody	Dinitrophenylhydrazine
Secondary antibody	NA	Alexa 488-labeled goat anti-rabbit secondary antibody	Alexa 568-labeled goat anti-mouse secondary antibody	Alexa 488-labeled rabbit anti-dinitrophenyl primary antibody
Filter cubes (nm) (excitation; dichroic; emission)	DAPI cube (325–375; 460; 470–750)	FITC cube (460–480; 485; 495–540)	TRITC cube (535–555; 560; 570–620)	FITC cube (460–480; 485; 495–540)

DAPI 4',6-diamidino-2-phenylindole, NA not applicable, FITC fluorescein isothiocyanate, TRITC tetramethylrhodamine isothiocyanate