Subcellular localization-dependent decrements in skeletal muscle glycogen and mitochondria content following short-term disuse in young and old men

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1Institute of Sports Science and Clinical Biomechanics and 2Clinical Institute, University of Southern Denmark, Odense; 3Institute of Sports Medicine and Centre of Healthy Aging, Bispebjerg Hospital, University of Copenhagen, Copenhagen; and 4Department of Clinical Physiology, Bispebjerg Hospital, Denmark

Submitted 27 May 2010; accepted in final form 20 September 2010

Nielsen J, Suetta C, Hvid LG, Schrøder HD, Aagaard P, Ørtenblad N. Subcellular localization-dependent decrements in skeletal muscle glycogen and mitochondria content following short-term disuse in young and old men. Am J Physiol Endocrinol Metab 299:E1053–E1060, 2010. First published September 21, 2010; doi:10.1152/ajpendo.00324.2010.—Previous studies have shown that skeletal muscle glycogen and mitochondria are distributed in distinct subcellular localizations, but the role and regulation of these subcellular localizations are unclear. In the present study, we used transmission electron microscopy to investigate the effect of disuse and aging on human skeletal muscle glycogen and mitochondria content in subsarcolemmal (SS), intermyofibrillar (IMF), and intramyofibrillar (intra) localizations. Five young (23 yr) and five old (66 yr) recreationally active men had their quadriceps muscle immobilized for 2 wk by whole leg casting. Biopsies were obtained from m. vastus lateralis before and after the immobilization period. Immobilization induced a decrement of intra glycogen content by 54% (P < 0.001) in both age groups and in two ultrastructurally distinct fiber types, whereas the content of IMF and SS glycogen remained unchanged. A localization-dependent decrease (P = 0.03) in mitochondria content following immobilization was found in both age groups, where SS mitochondria decreased by 33% (P = 0.02), superficial IMF mitochondria decreased by 20% (P = 0.05), and central IMF mitochondria remained unchanged. In conclusion, our findings demonstrate a localization-dependent adaptation to immobilization in glycogen and mitochondria content of skeletal muscles of both young and old individuals. Specifically, this suggests that short-term disuse preferentially affects glycogen particles located inside the myofibrils and that mitochondria volume plasticity can be dependent on the distance to the fiber border.

transmission electron microscopy; cell compartmentation; immobilization; aging; metabolism

SKELETAL MUSCLE GLYCOGEN AND MITOCHONDRIA play key roles in metabolism and have in recent years also been implicated in diverse signaling pathways (19, 31, 50), which is accompanied by the recognition of the glycogen particle as a dynamic organelle interacting with several proteins (16, 49, 53) and mitochondria as much more than just a powerhouse, since it interacts with several proteins (16, 49, 53) and organelle interacting with several proteins (16, 49, 53) and organelle interacting with several proteins (16, 49, 53) and therefore take place at distinct subcellular localizations. Examples of this are found in excitation-contraction coupling involving membranes of the sarcolemma, t-system, and sarcoplasmic reticulum (41) and in GLUT4 translocation, where differential properties between sarcolemma and t-system exist (36). Interestingly, in this sarcomeric and membrane organization of skeletal muscle, glycogen and mitochondria are distributed in an inhomogeneous manner, with both found clustering in the subsarcolemmal (SS) space and in the intermyofibrillar (IMF) space, and furthermore, glycogen is also found in the intramyofibrillar (intra) space (22, 24, 38, 43, 52, 55). Thus, the role of glycogen and mitochondria in muscle metabolism and signaling may depend on the site of intracellular localization per se.

Determinants of the subcellular localization of mitochondria have been investigated in animal studies (1, 3, 4, 21, 23, 32, 35, 37) and in human studies (6, 10, 12, 24, 25, 33, 34). Particularly, the effect of disuse on lower limb subcellular localization of mitochondria in humans has been investigated in only one study (12). Ferretti et al. (12) found a localization-dependent decrease in mitochondria volume density following long-term bed rest, where IMF mitochondria decreased by 17% and SS mitochondria remained unchanged. Intuitively, this is not in line with endurance training studies showing relatively higher increments in SS mitochondria compared with IMF mitochondria (25, 43); however, the regulation of mitochondria content and localization in humans is still not fully understood.

Regarding the role and regulation of subcellular glycogen localization, even less is known. On the basis of qualitative studies conducted in the 1980s (13, 14, 52), Marchand et al. (39) estimated the localization of glycogen quantitatively in the recovery period after prolonged glycogen-depleting exercise and found a preferential restoration of intra glycogen, indicating a role of repeated muscle contractions on glycogen localization. Recently, we have shown that 10 wk of endurance training of obese type 2 diabetic patients and weight-matched controls mediated an increase in SS glycogen of 91%, whereas as IMF and intra glycogen increased by only 14 and 15%, respectively (43). This indicates that in obese sedentary subjects, a short-term (10 wk) upregulation of glycogen content is favoring SS glycogen. Thus, the effect of both acute exercise and short-term training on glycogen storage is localization dependent, which emphasizes the necessity to consider cell compartmentalization when investigating the role and regulation of glycogen in skeletal muscle.

As opposed to acute exercise and training, the effect of short-term muscle disuse on glycogen and mitochondria sub-
cellular localization has not been investigated. With the known effects of disuse on muscle remodeling observed by decreased postabsorptive protein synthesis (15), fiber phenotypic shift from slow to fast contractile speed (28), and decreased content of t-system Na⁺-K⁺-pumps (7), which are known to preferentially use glycolytic-derived ATP (11, 30), immobilization is an experimental model that can provide new insights into the understanding of the role and regulation of subcellular glycogen and mitochondria localization. In the present study, we investigated the effect of 2 wk of immobilization on SS, IMF, and intra subfractions of glycogen and mitochondria in young and old men. On the basis of previous studies on exercise training (25, 43), we hypothesized that the SS fractions of glycogen and mitochondria would be preferentially downregulated following immobilization in both young and old men.

**MATERIALS AND METHODS**

**Subjects.** Five young [23 ± 2 yr, 75 ± 8 kg, 184 ± 3 cm; body mass index (BMI): 22.3 ± 0.7] and five old (66 ± 3 yr, 83 ± 6 kg, 177 ± 4 cm; BMI: 26.4 ± 0.8) males that were recreationally active (3–10 h/wk) volunteered to participate in the present study, which was part of a larger immobilization study (54). None of the subjects had previously participated in systematic strength training. The BMI of the old subjects was significantly higher compared with the value of the young subjects (P = 0.004). Furthermore, as reported previously, the old subjects also had higher body fat, lower muscle mass, and lower muscle strength compared with the young subjects (54). Before inclusion, subjects were screened by a physician to exclude subjects with cardiovascular disease, diabetes, neural or musculoskeletal disease, inflammatory or pulmonary disorders, and any known predisposition to deep venous thrombosis. Only nonmedicated individuals were included in the study. One of each of the young and old subjects was a smoker (5–8 cigarettes/day and 1 pipe once/wk, respectively). The two smokers did not have any abnormal values in any of the parameters investigated in the present study. All subjects were instructed to maintain their normal composition of the diet and to refrain from strenuous muscle work during the immobilization period. The local ethics committee approved the conditions of the study (KF01-322606), and all experimental procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants before inclusion in the study.

**Immobilization protocol.** Immobilization was accomplished by 2 wk of randomized unilateral whole leg casting using a lightweight fiber cast applied from just above the malleoli to just below the groin in 30° of knee joint flexion, as described in detail elsewhere (54). This induced substantial muscle atrophy and decreased maximal quadriceps strength (54), in accord with previous short-term immobilization studies in young individuals (8, 20). During the 2-wk muscle immobilization period, the subjects were contacted on a regular basis and carefully instructed to routinely contract the muscles at the ankle joint (venous pump exercises) to prevent potential formation of deep venous thrombosis. Muscle biopsies were obtained from m. vastus lateralis using a modified Bergström needle with suction under local anesthesia (10 ml of lidocaine 2% injected sc) immediately before and after the immobilization period. All biopsies were taken by the same investigator to minimize variation between time points regarding muscle depth and proximity.

**Transmission electron microscopy.** Muscle biopsy specimens were prepared for enhanced glycogen visualization by transmission electron microscopy, as described previously (43). In brief, specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24 h and subsequently rinsed four times in 0.1 M sodium cacodylate buffer. Following rinsing, muscle specimens were postfixed with 1% osmium tetroxide (OsO₄) and 1.5% potassium ferrocyanide [K₄Fe(CN)₆] in 0.1 M sodium cacodylate buffer for 90 min at 4°C. The use of reduced osmium tetroxide containing potassium ferrocyanide favors a high electron density of glycogen particles (9). After postfixation, the muscle specimens were rinsed twice in 0.1 M sodium cacodylate buffer at 4°C, dehydrated through graded series of alcohol at 4–20°C, infiltrated with graded mixtures of propylene oxide and Epon at 20°C, and embedded in 100% Epon at 30°C. To obtain as many fibers as possible, the ultrathin sections were cut (using a Leica Ultracut UCT ultramicrotome) in three depths separated by 150 nm. The sections were contrasted with uranyl acetate and lead citrate and examined and photographed in a precalibrated Philips EM 208 electron microscope and a Megaview III FW camera. In the sections from the three depths of each biopsy, all the longitudinally oriented fibers were included, obtaining a mean of nine fibers per biopsy (range: 6–11). From each fiber, 24 images were obtained at ×40,000 magnification in a randomized systematic order, including 12 from the SS region and six from both the superficial and central regions of the myofibrillar space (Fig. 1). The variation in the parameters between images was used to estimate a coefficient of error (ε_CE), as proposed for stereological ratioestimates by Howard and Reed (26). Fiber type differences were evaluated by classifying myofibers as type I or II on the basis of a combination of IMF mitochondrial volume and Z-line width (51). IMF mitochondrial volume fraction was plotted against Z-line width for all of the fibers (n = 6–11) obtained from each biopsy. The fibers with the highest IMF mitochondrial volume fraction and thickest Z-line width were classified as type I fibers and vice versa for type II fibers. To identify the two main fiber types, all intermediate fibers were discarded, and only distinct type I and II fibers were included (n = 2–3 fibers of each type per biopsy). All of the analyses were conducted by two blinded investigators. Inter- and intrainvestigator variability tests showed no signs of bias and low coefficients of variation (<8%) evaluated as proposed by Bland and Altman (5).

**Glycogen.** Three spatial localizations of glycogen were defined in 1) the IMF space, 2) the intra space (inside the myofibrils, between the contractile filaments), and 3) the SS space (Fig. 1). The glycogen volume fraction (V_V) in each location was estimated as proposed by Weibel (56), taking the effect of section thickness into account: V_V = A_X - (1/π)B_X - N_A[(t[H] + t[H])], where A_X is glycogen area fraction, t is section thickness (60 nm), B_X is glycogen boundary length fraction, N_A is the number of particles per area, and H is the average glycogen profile diameter. It was assumed that the particles were spherical (40). The glycogen area fraction was estimated by point counting, and the average glycogen profile diameter was measured directly using iTEM (FEI). IMF glycogen content was expressed relative to the myofibrillar space (estimated by point counting). The myofibrillar space consists mainly of the myofibrils (intra space), mitochondria, sarcoplasmatic reticulum (SR), t-system, and lipids. Since the intra space occupied >70% of the myofibrillar space and was unaffected by the immobilization period (Table 1), any changes in the other organelles of the myofibrillar space following immobilization did not have any significant effect on the IMF glycogen estimates. The intra glycogen was expressed relative to the intra space (estimated by point counting), which was unaffected by the immobilization period (Table 1). The SS glycogen was expressed relative to the fiber surface (estimated by direct length measurements), assuring that the results were unbiased from changes in other SS organelles and inclusions (e.g., mitochondria, nuclei, and lipids). The ε_CE were 0.10, 0.11, and 0.18 in IMF, intra, and SS glycogen, respectively.

**Mitochondria.** The volume of mitochondria was estimated by point counting. Three localizations were defined: central IMF, superficial IMF, and SS. The IMF mitochondria were expressed relative to the myofibrillar space and the SS mitochondria relative to the fiber surface. The ε_CE were 0.23, 0.21, and 0.28 for central IMF, superficial IMF, and SS mitochondria, respectively.
The combined volume of SR and t-system (SR/Tsys) and the volume of myofibrils were estimated by point counting and expressed relative to the myofibrillar space obtaining meanCEs of 0.11 and 0.23, respectively.

Table 1. Effect of 2 wk of immobilization on volume densities of total glycogen, mitochondria, SR and t-system, and myofibrils

<table>
<thead>
<tr>
<th></th>
<th>Preimmobilization</th>
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<tr>
<td></td>
<td>Young</td>
<td>Old</td>
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<tr>
<td>Mix of fibers</td>
<td>0.086 (0.064–0.088)</td>
<td>0.073 (0.067–0.075)</td>
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<tr>
<td>Type I fibers</td>
<td>0.026 (0.025–0.034)</td>
<td>0.031 (0.022–0.033)</td>
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<tr>
<td>Type II fibers</td>
<td>0.030 (0.025–0.038)</td>
<td>0.039 (0.019–0.040)</td>
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SR and t-system, μm³/μm³ myofibrillar space

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<th>Preimmobilization</th>
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<tr>
<td></td>
<td>Young</td>
<td>Old</td>
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<tr>
<td>Mix of fibers</td>
<td>0.027 ± 0.003</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>Type I fibers</td>
<td>0.030 ± 0.004†</td>
<td>0.027 ± 0.001†</td>
</tr>
<tr>
<td>Type II fibers</td>
<td>0.741 ± 0.007</td>
<td>0.777 ± 0.014</td>
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<tr>
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<td>0.789 ± 0.020†</td>
<td>0.791 ± 0.011†</td>
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Values are medians and interquartile range (in parentheses) or means ± SE (n = 5). SR, sarcoplasmatic reticulum. #Main effect of immobilization, P = 0.007; *main effect of immobilization, P = 0.04 (Wilcoxon signed-rank test); †main effect of fiber type, P < 0.001.

SR, t-system, and myofibrils. The combined volume of SR and t-system (SR + Tsys) and the volume of myofibrils were estimated by point counting and expressed relative to the myofibrillar space obtaining meanCEs of 0.11 and 0.23, respectively.

Total volumes and superficial vs. central differences. The subfractions of IMF and intra are expressed as volume densities, and the SS subfraction is expressed as volume per fiber surface area. Therefore, total volume densities were obtained by recalculating the SS subfractions relative to myofibrillar volume density. Assuming fibers of cylindrical shape, the volume beneath the surface area of fiber (Vb) is: 

\[ V_b = \pi R^2 \cdot 0.5 \cdot A \]

where R is fiber radius and A is the fiber surface area. Fiber radius was assumed to be 40 μm to yield a volume per fiber surface of 20 μm³/μm². Furthermore, the superficial estimates of IMF and intra were weighted three times higher than the central estimates, because in a cylindrical-shaped fiber, the superficial region (outermost half of the diameter) occupies three-quarters of the volume. However, in the volume densities of IMF glycogen, intra glycogen, and SR/Tsys, there were no differences between the central and superficial regions in any age group or fiber type at any time point. The median [interquartile range (IQR)] ratio of superficial-to-central localized IMF glycogen was 0.99 (0.81:1.28), whereas values of 1.03 (0.82:1.27) for intra glycogen and 1.01 (0.94:1.19) for SR/Tsys were found (n = 107 fibers). Thus, for simplicity the results of glycogen and SR/Tsys are presented as weighted means of data from the superficial and central regions.

Fig. 1. Transmission electron microscopy images illustrating 3 distinct subcellular localizations of muscle glycogen. A: an overview of a longitudinal section of 1 muscle fiber is shown (×3,200 original magnification; scale bar, 5 μm). Twelve images of the SS region and 6 images of the superficial and central parts of the myofibrillar region were obtained in a systematically randomized order. B: the subsarcolemmal (SS) volume of glycogen (G), seen as black dots, and mitochondria (M) was estimated (×40,000 original magnification; scale bar, 500 nm). Arrow indicates sarcolemma. C: the volume of intermyofibrillar (IMF) glycogen (white G), IMF mitochondria (white M), and intramyofibrillar (intra) glycogen (black G) was estimated (×40,000 original magnification; scale bar, 500 nm). Z, Z-line; black M, M-band.

Statistics. Statistical analyses were performed using Stata 10.1 (Stata Statistical Software, Release 10; Statacorp, College Station, TX). All interactions or main effects were tested using a linear
mixed-effects model with subjects as random effect and with immobilization, group, fiber type, and location as fixed effects. Variables with skewed distributions were log transformed before analysis. Nonparametric tests were used as stated. Values are presented as medians and IQR, unless stated otherwise. Significance level was accepted at \( P \leq 0.05 \).

RESULTS

Glycogen. The total glycogen volume percentage per fiber remained unchanged (\( P = 0.93 \)) following the immobilization period [pre: 3.1% (2.2:3.8) vs. post: 2.9% (2.4:3.5)]. No significant differences were observed between age groups (\( P = 0.27 \)) and fiber types (\( P = 0.11 \)) or by interaction of age group and fiber type (\( P = 0.10 \)) (Table 1).

In this study, the content of three subcellular fractions of glycogen (IMF, intra, and SS) was estimated quantitatively. Before immobilization with fiber types weighted equally, the relative distribution of glycogen was 79% (76:84) in the IMF region, 15% (11:17) in the intra region, and 6% (5:7) in the SS region. Thus, glycogen appears to be distributed in one major and two minor fractions in lower limb skeletal muscle of recreationally active humans.

Interestingly, the effect of 2 wk of immobilization on muscle glycogen volume fraction was found to be highly dependent on localization (\( P < 0.0001 \); Fig. 2). Whereas IMF and SS glycogen were not significantly changed [\(-9 (-17;+32, P = 0.77)\) and \(+35\% (-15:+84, P = 0.33)\), respectively], a decrease in intra glycogen was observed [\(-54\% (-63;-34) P = 0.0001 \)]. This localization dependency did not differ between young and old individuals (\( P = 0.38 \)) or between type I and II fibers (\( P = 0.44 \)) or both combined (\( P = 0.63 \)) (Fig. 2).

Intriguingly, after immobilization, the maximum value of intra glycogen volume was lower than the median value before immobilization (Fig. 2), indicating that most if not all of the fibers were affected.

Glycogen volume is a product of the number of particles and particle volume. The particle volume was found to depend on localization in the order: intra > IMF > SS (\( P < 0.0001 \); Fig. 3A). Compared with SS particles, the volume of IMF and intra particles was 11 and 16% greater (both \( P < 0.001 \)), respectively. The distribution of particle diameters (relative frequency histograms) showed that the variation in particle diameters followed a normal distribution in all three subfractions pre- and postimmobilization (Fig. 3B).

Intriguingly, the mean particle volume in 28 fibers pre- and postimmobilization revealed an unchanged mean particle volume of all three fractions following immobilization (\( P = 0.28 \); Fig. 3A), indicating that the decrement in intra glycogen content following immobilization was due to a loss of particles. Representative transmission electron microscopy images of the IMF and intra fractions of glycogen pre- and postimmobilization are shown in Fig. 4.

The localization-dependent decrease in myofiber glycogen content following the 2 wk of immobilization demonstrates that subfractions of glycogen are differentially regulated. To further investigate this heterogeneity of subfractions of glycogen, the glycogen content of the three subfractions of all of the single fibers from the biopsies obtained before immobilization is shown in Fig. 5. This shows that high heterogeneity is observed when glycogen levels increase above the mean value, and in the same fiber the glycogen level can be high in one fraction and low in another fraction.

Mitochondria. The total fiber volume percentage of mitochondria was decreased by 19% (\( P = 0.0007 \)) from 7.4 (6.4:8.8) to 6.0% (5.4:6.3) following immobilization. This decrement was not different (\( P = 0.81 \)) between young and old individuals (Table 1). However, the decrease in mitochondria volume percentage was dependent on subcellular localization (\( P = 0.0001 \); Fig. 3B).
DISCUSSION

The main findings of the present study were that 1) a subfraction of muscle glycogen localized inside the myofibrils (intramyofibrillar glycogen) decreased by ~50% following 2 wk of immobilization, 2) mitochondrial adaptation to immobilization was region dependent, with a preferential decrease in SS and superficial IMF mitochondria only, and 3) the old recreationally active men (65 yr) had glycogen and mitochondria content and localization similar to that of the young men (25 yr) and, as evaluated by these parameters, showed comparable adaptations to immobilization. Importantly, the time-consuming nature of transmission electron microscopy analysis limited the sample size to five in each group, and with the given intermyofiber and intersubject variation the detection limit for age differences was ~25% in the present study.

Recently, a study quantified for the first time subfraction usage of glycogen in response to acute exercise and showed that intramyofibrillar glycogen was preferentially replenished in the recovery period after prolonged glycogen-depleting exercise (39). The present data demonstrated a loss of intramyofibrillar glycogen following 2 wk of immobilization, which together with the above findings strongly indicates a role of muscle contraction in the spatial storage distribution of glycogen inside the myofibrils. Interestingly, after exercise, the enzyme responsible for glycogen storage, glycogen synthase, has been shown to be translocated mainly to intramyofibrillar glycogen particles (45, 47), providing an explanation for the preferential replenishment of intramyofibrillar glycogen particles after exercise (39). It remains to be established whether glycogen synthase conversely is translocated away from the intramyofibrillar glycogen particles in response to immobilization, which would explain the findings of the present study. Intriguingly, the remaining intramyofibrillar glycogen particles were not found to be of smaller diameters following immobilization, indicating that the potential relationship with glycogen synthase translocation may affect mainly the number of particles. In this regard, it is important to note that the remaining intramyofibrillar glycogen indeed represents a metabolically active subfraction, since approximately four times lower (almost empty) values of intramyofibrillar glycogen have been reported after glycogen-depleting exercise (39).

Glycogen is known to be associated with proteins, including enzymes (glycogen synthase, glycogen phosphorylase, phosphorylase kinase, glycogenin, phosphatases, and AMP-acti-
Immobilization induces a decrease in Na\textsuperscript{+}-ATPases (17). However, whether the decrease in intramyofibrillar glycogen found in the present study is functionally related to Na\textsuperscript{+}-ATPase content and activity remains to be investigated.

The present data confirm previous study findings showing that skeletal muscle mitochondria content can be maintained in recreationally active individuals of 60–70 yr of age (34, 46, 48) and further add that the subcellular distribution of mitochondria can be maintained. However, it is important to keep in mind that similar mitochondrial content does not necessarily reflect similar mitochondrial function.

The finding of a graded effect of immobilization on mitochondria content from no change in central IMF mitochondria to 20% decrease in superficial IMF mitochondria and to further 33% decrease in SS mitochondria strongly indicates a spatial relationship to the fiber border. This is in contrast to a previous study (12) in young individuals investigating the effect of 37 days of bed rest on subfractions of mitochondria content in m. vastus lateralis showing a similar decrease (17%) in IMF mitochondria content but no significant decrease (11%) in SS mitochondria content. This difference cannot be explained by any difference in preimmobilization/bed rest level of IMF and SS mitochondria content, since they were highly similar. It could be speculated, however, if the difference can be explained by differences in mode of disuse (bed rest vs. immobilization) or duration of disuse (37 days vs. 14 days). Nevertheless, the present study strongly confirms the recognition of SS mitochondria as being more susceptible to environmental alterations than IMF mitochondria (27) found in human studies (25, 42, 43) as well as animal studies (1, 23, 35). However, to this understanding we add here that there are also spatial differences in the regulation of IMF mitochondria content. This

Fig. 6. Effect of immobilization on subfractions of mitochondria in young and old men. IMF mitochondria (central and superficial) and SS mitochondria are shown on the left and right ordinate, respectively. Each point represents 1 fiber from young (circles) and old (triangle) individuals pre- (closed symbols) and postimmobilization (open symbols). Each dot represents individual fibers obtained from the biopsies (4–6 fibers/biopsy, n = 5). Horizontal lines indicate median values. \(P = 0.02\) vs. pre; \(\bar{z}P = 0.05\) vs. pre.
spatial differential effect on mitochondria volume indicates that the explanation is not related to distinct properties of SS and IMF mitochondria but is more likely related to the spatial localization in relation to the fiber border.

In conclusion, the present study demonstrates that immobilization of m. vastus lateralis induces an ~50% decrease in intramyofibrillar glycogen content with no difference between young and old individuals of comparable activity level. This suggests that immobilization preferentially affects glycogen particles located inside the myofibrils. Furthermore, we found a localization-dependent decrease in mitochondria content, indicating that a spatial gradient in the myofibrers from the core to the border is of significance in regulating mitochondria content.

ACKNOWLEDGMENTS

We thank Bente Jørgensen, Kirsten Hansen, and Karin Trampedach for excellent skillful technical assistance, and we express our gratitude to the experimental subjects who participated in this study for their efforts and contribution to this work. Preliminary parts of this work were accepted at the 14th European College of Sport Science Congress, Oslo, Norway, June 24–27, 2009.

GRANTS

The study was supported by grants from the Danish National Research Council (Council for Independent Research, Medical Sciences), the Lundbeck Foundation, the Danish Rheumatology Association, the Faculty of Health Sciences, the University of Copenhagen, Team Denmark Elite Association, and the Ministry of Culture Committee on Sports Research.

DISCLOSURES

No conflict of interest, financial or otherwise, are declared by the authors.

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