**Short-term muscle disuse atrophy is not associated with lipid accumulation or impaired oxidative enzyme activity within skeletal muscle of young or elderly men**

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**Running title**: Short-term disuse and muscle lipid accumulation

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**Abstract**

Aging is generally accompanied by a progressive loss of skeletal muscle mass and impairments in metabolic function. A short period of muscle disuse (such as during injury or illness) also brings about muscle atrophy. The accumulation of brief periods of muscle disuse atrophy through the lifespan has been proposed as a key factor in the age-related loss of muscle mass. However, it remains unknown whether such brief periods of disuse also induce impairments in skeletal muscle metabolic function. Here, we investigated the effects of a five day period of muscle disuse on intramyocellular triacylglycerol (IMTG) content, the maximal activity of selected mitochondrial enzymes and the mRNA expression of genes associated with mitochondrial metabolism/biogenesis in healthy young and elderly men. Muscle biopsies were collected from young (*n*=12; 23±1 y) and elderly (*n*=12; 70±1 y) healthy men prior to and immediately after a five day period of one-legged knee immobilization by way of a full leg cast. At baseline, elderly men had a greater IMTG content when compared with the young (56.3±6.8 and 34.8±7.3 µmol.g-1, respectively; *P*<0.05) but no changes were observed following 5 days of immobilization. In line, 5 days of disuse did not reduce citrate synthase, β-HAD or cytochrome C oxidase activity in skeletal muscle tissue. The muscle activation status of the pyruvate dehydrogenase increased following immobilization in the older subjects only, from 0.39±0.06 to 0.55 0.05 µmol.g-1.min-1 (71±33 %; *P*<0.01). The skeletal muscle mRNA expression of PGC1α and citrate synthase both declined following immobilization in both the young and elderly subjects. We conclude that five days of muscle disuse is not accompanied by a net increase in muscle lipid deposition or a concomitant decline in muscle oxidative capacity in young or elderly men.

**Introduction**

Normal human aging is accompanied by a progressive loss of skeletal muscle mass (sarcopenia) which leads to a loss of strength and functional capacity, increased risk of falls and, ultimately, loss of independence [[1](#_ENREF_1)]. Aside from the loss of muscle mass, another hallmark of aging is increased lipid deposition within skeletal muscle, and the associated declines in muscle oxidative capacity and whole body insulin sensitivity [[2](#_ENREF_2)]. These effects are strongly related to the increasing risk in elderly individuals of developing chronic metabolic diseases, such as obesity and type-2 diabetes [[1-3](#_ENREF_1)].

Prolonged muscle disuse (such as periods of bed-rest or limb immobilization mandated by illness or injury) lead to rapid skeletal muscle atrophy and declines in functional strength in both young and older individuals [[4](#_ENREF_4)]. We [[4](#_ENREF_4), [5](#_ENREF_5)], and others [[6](#_ENREF_6), [7](#_ENREF_7)], have previously hypothesized that much of the loss of muscle mass associated with aging can be attributed to the accumulation of successive bouts of short-term muscle disuse atrophy that occur across the lifespan. Moreover, prolonged disuse protocols have also been shown to cause intramyocellular triacylglycerol (IMTG) accumulation [[8](#_ENREF_8), [9](#_ENREF_9)], reduced muscle oxidative capacity [[8](#_ENREF_8), [10](#_ENREF_10), [11](#_ENREF_11)] and impaired whole body insulin sensitivity [[12-15](#_ENREF_12)] in young and older persons. Based on much of this data, it has been suggested that, rather than aging *per se*, a reduced physical activity status is primarily responsible for declines in metabolic health with advancing age [[16-18](#_ENREF_16)]. Accordingly, experimental models of muscle disuse have emerged as an important tool for investigating the age-related declines in skeletal muscle mass and function.

The majority of studies investigating muscle disuse and its association with age-related declines in muscle mass and metabolic health have utilized inactive periods ranging from two to as long as 12 weeks [e.g. [19](#_ENREF_19), [20](#_ENREF_20), [21](#_ENREF_21)]. However, it is becoming apparent that the majority of periods of disuse or reduced physical activity that occur throughout the lifespan likely last less than one week [[5](#_ENREF_5), [22](#_ENREF_22)]. Accordingly, we have recently argued that the prevalence of short-term (i.e. < one week) periods of disuse are also of important clinical significance when considering age-related declines in muscle mass [[5](#_ENREF_5), [23](#_ENREF_23)]. In support we have recently shown that only five days of muscle disuse already leads to a substantial loss of muscle mass and strength in both young [[23](#_ENREF_23)] and elderly men [[24](#_ENREF_24)]. However, it remains un-investigated whether such a brief period of disuse already leads to impairments in skeletal muscle metabolic health.

In the present study, we investigated the impact of five days of one-legged knee immobilization on IMTG content, the maximal activities of various mitochondrial enzymes and the mRNA expression of key genes involved in the regulation of mitochondrial metabolism in both young and elderly men. In line with previous suggestions that the elderly are more susceptible to muscle loss during a short period of disuse compared to the young [[25](#_ENREF_25)], we hypothesized that disuse would lead to IMTG accumulation and declines in oxidative capacity to a greater extent in older subjects compared to their younger counterparts.

**Methods**

*Subjects*

Twelve young (23±1 y) and twelve elderly (70±1 y), healthy men volunteered to participate in the present study. All subjects were fully informed of the nature and possible risks of the experimental procedures, before their written informed consent was obtained. Subjects were screened to exclude any person with lower limb and/or back injuries sustained within a year prior to beginning the study, a (family) history of thrombosis/cardiovascular disease, use of anti-coagulants, musculoskeletal/orthopaedic/haemostatic disorders, or participation in any regular resistance training program within 6 months of beginning the study. During screening, all subjects were instructed and familiarized with safe lifting technique for the leg extension exercise. Maximum strength was assessed using the multiple repetitions testing procedure [[26](#_ENREF_26)] for each leg separately. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre+, Maastricht, the Netherlands. The present study is part of a greater project investigating muscle disuse atrophy in humans.

*Experimental design*

In the present study a parallel design was applied (young vs old) with each subject cohort undergoing a 5 day period of one-legged knee immobilization by means of a full leg cast. Before and after the immobilization period, computed tomography (CT) scans, dual-energy x-ray absorptiometry (DEXA) scans and 1 repetition max (1-RM) tests were performed to determine changes in muscle mass and strength, and muscle biopsies were collected to assess muscle lipid content, the maximal activities of various mitochondrial enzymes and the mRNA expression of key genes associated with the regulation of muscle mass and mitochondrial metabolism.

*Diet and physical activity*

All subjects received the same standardized meal the evening prior to the experimental test days (33±2 kJ.kg-1 body weight, providing 44 energy% (En%) carbohydrate, 22 En% protein, and 34 En% fat). All volunteers were instructed to refrain from strenuous physical activity, avoid alcohol intake and to keep their diet as constant as possible for 2 days prior to the experimental test day.

*Experimental visits*

Subjects participated in two identical experimental test days, before and immediately after the immobilization period. Approximately two days prior to the immobilization period, subjects participated in the first test day. During the visit, subjects arrived at the laboratory at 08.00 h in the fasted state and body weight was measured with a digital balance with an accuracy of 0.1 kg (SECA GmbH, Hamburg, Germany). Thereafter, a single slice CT-scan (Philips Brilliance 64, Philips Medical Systems, Best, the Netherlands) was performed to assess upper leg muscle cross-sectional area (CSA). The scanning characteristics were as follows: 120 kV, 300 mA, rotation time of 0.75 s, and a field of view of 500 mm. With subjects lying supine with their legs extended and feet secured, a 3 mm thick axial image was taken 15 cm proximal to the top of the patella. The precise scan position was marked with semi-permanent ink for the duration of the experimental protocol to ensure accurate repeat measurements. Muscle area of the right leg was selected between 0 and 100 Hounsfield units [[27](#_ENREF_27)], after which the *quadriceps* muscle was selected by manual tracing using ImageJ software (version 1.45d, National Institute of Health, Maryland, USA) [[28-30](#_ENREF_28)]. After CT scanning, body composition (fat, fat-free mass and bone mineral content) was determined by DXA scan (Hologic Inc., Bedford, USA). Lean mass and percent body fat were determined on a whole body level and for specific regions (e.g. legs). Thereafter, a single venous blood sample was drawn and a muscle biopsy was collected from the *vastus lateralis* muscle of the leg identified as the leg to become immobilized (or the previously immobilized leg in the case of the second visit). Muscle biopsy samples were obtained from the middle region of the *vastus lateralis*, ~1-3 cm below the level that the CT scan was performed, and ~3 cm below entry through the fascia, by using the percutaneous needle biopsy technique [[31](#_ENREF_31)]. Muscle samples were dissected carefully and freed from any visible non-muscle material and were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Thereafter, subjects single leg one repetition maximum (1-RM) was assessed [[32](#_ENREF_32)]. After warming up, the load was set at 97.5% of the estimated 1-RM from the screening visit, and increased after each successful lift until failure. Three min rest periods were allowed between lifts. A repetition was considered valid when the subject used proper form and was able to complete the entire lift in a controlled manner without assistance. Finally, subjects were instructed on, and familiarized with, the use of crutches. On the day of cast removal, all measures of muscle mass and strength were repeated in the same manner, and a muscle biopsy was collected prior to any weight bearing activity from a site ~2 cm from the position of the first biopsy.

*Limb immobilization*

Approximately 48 h after the first test day, subjects reported at 8.00 h at the Casting Room at Maastricht University Medical Centre, to have a full leg cast fitted. The application of the cast signified the first day of the immobilization period. The circular leg cast extended from 10 cm above the ankle to approximately 25 cm above the patella. The knee was casted at a 30o angle of flexion to prevent subjects performing any weight bearing activities with the casted leg. Subjects were provided with crutches for proper ambulation. All subjects were instructed to perform a series of simple ankle exercises (i.e. plantar and dorsal flexion, and circular movements of the entire foot) to keep the calf muscle pump activated in the immobilized leg, thereby minimizing the risk of developing deep vein thrombosis. At the end of the immobilization period, subjects were collected from their home by car, and brought into the laboratory for the second test day at 08.00 h. Prior to the start of the second test day, subjects visited the Casting Room to have the cast removed. Thereafter, subjects were taken by wheelchair to the laboratory so the muscle biopsies could be collected prior to any weight bearing exercise being performed. Following biopsy collection, subjects were able to bear weight on the immobilized leg for approximately 1-2 h prior to strength testing to minimize deficits due to initial joint stiffness.

*Plasma and muscle analyses*

Plasma glucose concentrations were analyzed with an automatic analyzer (ABX Pentra 400; Horiba ABX Diagnostics) using an ABX Pentra Glucose HK CP Kit (A11A01667, Horiba ABX Diagnostics). Insulin was analyzed by radioimmunoassay using a commercially available kit (Human Insulin RIA kit, HI-14K, Millipore).

One portion of each muscle biopsy sample was freeze dried whilst the remainder was left ‘wet' and both were stored at −80°C. Freeze-dried muscle was dissected free of any further visible blood and connective tissue, powdered and used for the biochemical determination of intramuscular triglyceride (IMTG) content [[33](#_ENREF_33)]. Briefly, a 5-10 mg aliquot of muscle powder was used for IMTG extraction as originally described by Folch et al. [[34](#_ENREF_34)] and the chloroform phase was evaporated. After reconstitution, the phospholipids were removed with the addition of silicic acid. The IMTGs were saponified, and the free glycerol was assayed fluorometrically in duplicates [[35](#_ENREF_35)].

Wet muscle was used for the determination of skeletal muscle oxidative capacity in approximately 30 mgs of tissue via the measurement of maximal activities of citrate synthase, β-hydroxyacyl coenzyme A dehydrogenase (β-HAD) and cytochrome C oxidase according to methods published previously [[36](#_ENREF_36)]. As an indicator of metabolic substrate flux, muscle pyruvate dehydrogenase complex activity (PDCa) was measured in approximately 10 mg of wet muscle according to methodology described previously [[37](#_ENREF_37)]. All enzyme activities are expressed as micromoles of product (citrate, 3-ketoacyl CoA, reduced cytochrome C and acetyl-CoA for A, B, C and D, respectively) generated per gram of wet muscle tissue per minute during the assay (µmol.g-1.min-1). Muscle tissue for IMTG and enzyme analyses was not available from two of the elderly subjects, so these analyses represent an *n* of 12 and 10 for young and elderly volunteers, respectively.

Total RNA was isolated from 10-20 mg of frozen muscle tissue using TRIzol® Reagent (Life Technologies, Invitrogen), according to the manufacturer’s protocol. Total RNA quantification was carried out spectrophotometrically at 260 nm (NanoDrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, USA), and RNA purity was determined as the ratio of readings at 260/280 nm. Thereafter, first strand cDNA was synthesized from 1 µg RNA sample using iScriptTM cDNA synthesis kit (BioRad; cat. 170-8891) in a reaction volume of 20 µL. Taqman PCR was carried out using a 7300 Real Time PCR System (AppliedBiosystems, USA), with 2 µL of cDNA, 12.5 µl TaqmanTM master mix, 1.25 µl TaqmanTM probe and 9.25 µl H2O in a 25 µL final well volume. Each sample was run in duplicate, together with a serial dilution standard curve. The housekeeping gene 18S was used as an internal control as this gene has been used previously in similar human immobilization studies by ourselves [[23](#_ENREF_23), [38](#_ENREF_38)] and others [[19](#_ENREF_19), [39](#_ENREF_39), [40](#_ENREF_40)] and was unaffected by immobilization. Taqman primer/probe sets were obtained from Applied Biosystems (Foster City, USA) for citrate synthase, peroxisome proliferator-activated receptor c co-activator 1 alpha isoform (PGC1α), Muscle Atrophy F-Box/atrogin-1 (MAFbx), Muscle-Specific RING-finger protein 1 (MuRF1) and 18S. The thermal cycling conditions used were: 2 min at 50oC, 10 min at 95oC, followed by 40 cycles at 95oC for 15 s and 60oC for 1 min. Ctvalues of the target genes were normalized to Ct values of the internal control and final results were calculated as relative expression against the standard curve.

*Statistics*

All data are expressed as means±SEM. A two-way ANOVA with time (pre and post) and treatment (young and old) as factors was used to compare differences in all time dependent parameters. When a significant main effect was detected, a Bonferonni post-hoc test was applied to locate these differences. For non-time dependent variables, a paired *t* test was used to compare means. For correlational analyses, a one-tailed Pearson’s Correlation Coefficient test was used. Statistical significance was set at *P*<0.05. All calculations were performed by using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

**Results**

*Indices of whole body insulin sensitivity* *and assessments of skeletal muscle mass*

Subjects’ characteristics at baseline and throughout the study are reported in **Table 1**. Compared to the young, elderly individuals demonstrated an impaired whole body insulin sensitivity and glycemic control according to the Homeostatic Model Assessment of insulin resistance (HOMA-IR) [[41](#_ENREF_41)], blood glycated hemoglobin (HbA1c) levels, and fasting concentrations of plasma glucose and insulin. However, none of these variables were affected by immobilization in either group. Compared to the young, the elderly had a higher BMI (*P*<0.05) and a greater amount of fat tissue, both at the whole body level (*P*<0.01) and within the immobilized leg (*P*<0.05); but again, there were no effects of immobilization in either group. Quadriceps muscle cross sectional area (CSA), leg lean mass and strength have been presented elsewhere for young [[23](#_ENREF_23)] and elderly [[24](#_ENREF_24)] subjects separately. When compared together in the present study, quadriceps muscle cross sectional area (CSA) and leg lean mass were similar between old and young men at baseline and decreased following immobilization in both groups (*P*<0.001). Absolute quadriceps muscle CSA decreased to a greater extent in the young compared to elderly subjects (interaction effect; *P*<0.05). The same was also true when looking at relative loss of quadriceps CSA (3.5±0.6 and 1.4±0.8 % loss in young and elderly subjects, respectively; *P*<0.05). **Figure 5** depicts the relationship between initial quadriceps CSA and absolute (**A**) and relative (**B**) loss of quadriceps CSA during immobilization. There was a weak but significant correlation between initial quadriceps CSA and loss of absolute mass only (*P*<0.05, R2=0.13). Young men had a greater level of strength in the immobilized leg at baseline compared with the elderly, and both groups showed a similar significant decline with immobilization (*P*<0.001).

*Skeletal muscle lipid content*

**Figure 1** displays intramyocellular triacylglycerol (IMTG) content quantified by way of biochemical triglyceride extraction analyses. There was a significant effect of age such that the elderly had a greater IMTG content compared to the young (baseline values of 56.3±6.8 and 34.8 ±7.3 µmol.g-1; *P*<0.05); however, IMTG content was not affected by immobilization in either group (*P*=0.82).

*Skeletal muscle enzyme activities*

As markers of skeletal muscle oxidative capacity and/or mitochondrial content, we determined the maximal activities of several mitochondrial enzymes in homogenized muscle samples (**Figure 2**). The maximal activity of citrate synthase (**A**), β-hydroxyacyl coenzyme A dehydrogenase (β-HAD; **B**) and cytochrome C oxidase (**C**) were not different between groups and were unaffected by immobilization. As a marker of resting metabolic fuel selection, we determined the skeletal muscle activation status of the pyruvate dehydrogenase complex (PDC; **Figure 2D**). Muscle PDC activation did not differ between groups at baseline, but there was a significant immobilization x age interaction (*P*<0.05) such that immobilization increased PDC activation status in the elderly subjects, from 0.39±0.06 to 0.55 0.05 µmol.g-1.min-1 (71±33 %; *P*<0.01) but no changes occurred in the young.

*Skeletal muscle* *mRNA expression*

**Figure 3** displays the skeletal muscle mRNA expression of peroxisome proliferator-activated receptor c co-activator 1 alpha isoform (PGC1α; **A**) and citrate synthase (**B**). Muscle PGC1α mRNA was not differentially expressed between groups but decreased with immobilization to a similar extent in both groups (effect of immobilization, *P*<0.001; 54 and 35 % declines in young and elderly, respectively); although a trend for an immobilization x age interaction (*P*=0.059) was observed. Muscle citrate synthase mRNA expression was significantly greater in the elderly compared to young (main effect of age; *P*<0.01) and showed similar significant declines (27 and 19 % in young and elderly, respectively) with immobilization in both groups (main effect of immobilization; *P*<0.01). The skeletal muscle mRNA expression of Muscle Atrophy F-Box/atrogin-1 (MAFbx) and Muscle-Specific RING-finger protein 1 (MuRF1) arepresented in **Figure 4**. Muscle mRNA expression of MAFbx was more highly expressed in the younger subjects (main effect of age; *P*<0.001) and significantly increased with immobilization (86 and 36 % in young and elderly, respectively, main effect of immobilization; *P*<0.001) but in the young to a greater extent (significant interaction; *P*<0.05). Muscle MuRF1 mRNA was more heavily expressed in the young subjects compared to the elderly, but showed no changes with immobilization in either group.

**Discussion**

The present study demonstrates that the loss of muscle mass and strength that occurs during short-term (i.e. five days) disuse is not necessarily accompanied by increased skeletal muscle lipid deposition or impaired muscle oxidative capacity in young or elderly men.

Aging is accompanied by increased ectopic lipid storage in multiple peripheral tissues [[2](#_ENREF_2), [42](#_ENREF_42)], of which skeletal muscle has received particular attention [[43](#_ENREF_43)]. It has been proposed that increased intramyocellular triacylglycerol (IMTG) accumulation in elderly individuals may impact upon muscle oxidative capacity (i.e. mitochondrial content/function) and, ultimately, be a key factor leading to the progression of whole body insulin resistance and chronic metabolic disease [[1-3](#_ENREF_1), [43](#_ENREF_43)]. In agreement, here we report that elderly individuals had considerably more ectopic fat storage at the whole body level (Table 1) which was characterized by a considerably greater (62%) intramyocellular triacylglycerol (IMTG; **Figure 1**) content compared with the young. In line, we also observed that indices of whole body insulin sensitivity and glycemic control were impaired in the elderly compared with the young (Table 1). To assess muscle oxidative capacity we determined the maximal activities of key mitochondrial enzymes; citrate synthase, β-hydroxyacyl coenzyme A dehydrogenase and cytochrome C oxidase in our muscle biopsy samples (**Figure 2**). It has recently been demonstrated that skeletal muscle citrate synthase activity is a strong biomarker of mitochondrial content, and that cytochrome C oxidase activity correlates well with mitochondrial oxidative phosphorylation [[44](#_ENREF_44)]. We did not detect any differences between old and young volunteers in any of the mitochondrial enzyme activities we determined, thus providing evidence that neither mitochondrial content nor function differed with age in our study. This likely reflects that the habitual activity levels of our subject groups were well matched. Indeed, while it has been reported that mitochondrial content and/or function decline with advancing age [[45-48](#_ENREF_45)], studies that have matched subjects for physical activity status generally do not report any effect of aging *per se* [[16-18](#_ENREF_16), [49](#_ENREF_49), [50](#_ENREF_50)]. Thus, the present data indicate that muscle IMTG accumulation and declines in whole body insulin sensitivity occur with advancing age independently of changes in muscle oxidative capacity.

In line with the notion that physical activity status is a key contributor to the age-related decline in metabolic health [[16](#_ENREF_16), [17](#_ENREF_17)], it has previously been shown that prolonged (i.e. >2 weeks) muscle disuse atrophy is also accompanied by increased IMTG deposition, declines in muscle oxidative capacity and impaired whole body insulin sensitivity [[8-15](#_ENREF_8)]. Here, we aimed to see if such impairments in metabolic health also occur following short-term (i.e. five days) disuse. Despite significant muscle atrophy, we did not see any impact of immobilization on IMTG content (Figure 1), or markers of mitochondrial content/function (Figure 2) in either young or elderly men. However, mRNA levels of peroxisome proliferator-activated receptor c co-activator 1 alpha isoform (PGC1α; the gene thought to be a master regulator of mitochondrial biogenesis) [[51](#_ENREF_51)], and citrate synthase decreased considerably following immobilization in both young and elderly men (Figure 3). In addition, as an indicator of how disuse may modulate resting muscle fuel selection, we also determined muscle pyruvate dehydrogenase complex activity (PDCa) in the biopsy samples. Interestingly, we saw an increase in PDCa following immobilization in the elderly subjects only (Figure 2), suggesting an accelerated use of carbohydrate and an impaired ability to oxidize lipids. Collectively, these data demonstrate that changes in metabolic flux and the transcriptional regulation of mitochondrial genes precede changes in IMTG accumulation and muscle oxidative capacity during disuse, and therefore may be an important mechanism bringing about insulin resistance following a more prolonged period of disuse, particularly in older individuals.

Concomitant with a decline in metabolic health, aging is also associated with the gradual loss of muscle mass (sarcopenia) which dramatically reduces quality of life in our later years [[1](#_ENREF_1), [3](#_ENREF_3)]. Although the causes of sarcopenia are multifactorial [[7](#_ENREF_7), [52](#_ENREF_52)], it has previously been proposed that a key factor is the accumulated effects of successive periods of (short-term) muscle disuse atrophy throughout the lifespan [[4](#_ENREF_4), [6](#_ENREF_6), [7](#_ENREF_7)]. With this in mind, an interesting observation of the present study was the novel finding that muscle disuse atrophy during five days of limb immobilization was greater (both from an absolute and relative perspective) in young compared to older men (Table 1). This is in agreement with previous studies which have employed more prolonged limb immobilization protocols [[53](#_ENREF_53), [54](#_ENREF_54)]. When taking all twenty four subjects together, we observed a weak but significant correlation between initial quadriceps muscle mass and subsequent quantity of muscle loss during immobilization (Figure 5). This suggests that, rather than aging *per se*, muscle mass and/or training/activity status are key factors dictating the amount of muscle lost during a period of disuse, as has previously been suggested [[55](#_ENREF_55)]. In agreement with previous research [[53](#_ENREF_53)], disuse induced detriments in muscle strength did not differ between young and older men. This underlines the clinical impact of a small amount of muscle loss after just a few days of disuse in elderly individuals who may already be compromised from a functional capacity standpoint. Contrastingly, it has previously been reported that the elderly are actually *more* susceptible to muscle atrophy during whole body bed-rest [[25](#_ENREF_25)]. The reason(s) for the discrepancy between this suggestion and the current (and previous) limb immobilization studies are not clear, but may be related to the systemic adaptations that occur during bed-rest [[56](#_ENREF_56)], which are largely avoided during more localized models of disuse.

The present study has demonstrated that muscle loss during a brief period of disuse precedes measurable alterations in muscle lipid storage and mitochondrial content/function. However, the rapid transcriptional changes in mitochondrial genes and alterations in muscle PDCa suggest that, if disuse had have been continued, profound declines in muscle metabolic health would soon ensue. Indeed, whether increased PDCa with disuse in older men reflects an inability to use fat during periods of disuse requires further investigation, particularly as IMTG content increases with age. Our data underline the importance of clinically applying interventions immediately after the onset of disuse. Moreover, such strategies should not only target maintenance of muscle mass, but also muscle metabolic capacity, especially in older individuals. Future studies should assess the efficacy of strategies which have proved successful at attenuating muscle loss during a period of disuse, such as nutritional modulations [[4](#_ENREF_4), [20](#_ENREF_20)] or surrogates for physical activity [[57](#_ENREF_57)], to maintain metabolic capacity during short and more prolonged periods of disuse.

To conclude, the loss of muscle mass and transcriptional changes associated with mitochondrial metabolism induced by five days of limb immobilization do not translate into increased intra muscular lipid deposition or declines in muscle oxidative capacity in healthy young or elderly men.

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**FIGURE LEGENDS**

**Figure 1** Intramyocellular lipid content assessed by biochemical triglyceride extraction analyses (expressed as micromoles per gram of dry muscle tissue (µmol.g-1) before (PRE) and after (POST) 5 days of one-legged knee immobilization in healthy young (*n*=12) and elderly (*n*=10) men. Data are expressed as means±SEM. Data were analyzed with a two-way ANOVA (immobilization x age). \* = *P*<0.05 significant main effect of age.

**Figure 2** Skeletal muscle maximal enzyme activities of citrate synthase (**A**), β-hydroxyacyl coenzyme A dehydrogenase (β-HAD; **B**) and cytochrome C oxidase (**C**), and the skeletal muscle activation status of the pyruvate dehydrogenase complex (PDC; **D**) before (PRE) and after (POST) 5 days of one-legged knee immobilization in healthy young (*n*=12) and elderly (*n*=10) men. All enzyme activities are expressed as micromoles of product (citrate, 3-ketoacyl CoA, reduced cytochrome C and acetyl-CoA for A, B, C and D, respectively) generated per gram of wet muscle tissue per minute during the assay (µmol.g-1.min-1). Data are expressed as means±SEM. Data were analyzed with a two-way ANOVA (immobilization x age). In case of a significant main effect, Bonferroni post-hoc tests were applied to locate the individual differences. A, B and C: no significant effects. D: Significant time (*P*<0.01) and interaction (*P*<0.05) effects. \*\* = *P*<0.01 significantly different from corresponding PRE value.

**Figure 3** Relative skeletal muscle mRNA expression of peroxisome proliferator-activated receptor c co-activator 1 alpha isoform (PGC1α; **A**) and citrate synthase (**B**) before (PRE) and after (POST) 5 days of one-legged knee immobilization in healthy young (*n*=12) and elderly (*n*=12) men. Data are expressed as means±SEM. Data were analyzed with a two-way ANOVA (immobilization x age). In case of a significant main effect, Bonferroni post-hoc tests were applied to locate the individual differences. A: Significant effect of immobilization (*P*<0.001). B: Significant effect of immobilization (*P*<0.001) and age (*P*<0.01). \*\* and \*\*\* = *P*<0.01 and *P*<0.001 significantly different from corresponding PRE value, respectively.

**Figure 4** Relative skeletal muscle mRNA expression of Muscle Atrophy F-Box/atrogin-1 (MAFbx; **A**) and Muscle-Specific RING-finger protein 1 (MuRF1; **B**) before (PRE) and after (POST) 5 days of one-legged knee immobilization in healthy young (*n*=12) and elderly (*n*=12) men. Data are expressed as means±SEM. Data were analyzed with a two-way ANOVA (immobilization x age). In case of a significant main effect, Bonferroni post-hoc tests were applied to locate the individual differences. A: Significant effects of immobilization (*P*<0.001), age (*P*<0.001) and immobilization x age interaction (*P*<0.05). B: Significant effect of age (*P*<0.001). \*\*\* = *P*<0.001 significantly different from corresponding PRE value, respectively.

**Table 1**: Subjects’ characteristics

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Characteristic** | **Young Elderly** | | | | **Statistics** | | |
|  | **Pre** | **Post** | **Pre** | **Post** | **Age** | **Immobilization** | **Interaction** |
| **Age (y)** | 23±1 | --- | 69±1 | --- | *P*<0.001 | --- | --- |
| **Body mass (kg)** | 74.4±3.9 | --- | 82.9±3.5 | --- | NS | --- | --- |
| **BMI (kg/m2)** | 22.0±1.0 | --- | 27.3±0.7 | --- | *P*<0.01 | --- | --- |
| **HbA1c (%)** | 5.1±0.1 | --- | 5.4±0.1 | --- | *P*<0.10 | --- | --- |
| **Lean body mass (kg)** | 60.0±2.7 | 58.1±2.3 | 61.3±2.4 | 60.6±2.3 | NS | NS | NS |
| **Body fat (%)** | 15.6±1.4 | 15.1±1.4 | 21.8±1.2 | 22.1±1.2 | *P*<0.01 | NS | NS |
| **Fasting plasma glucose (mmol.L-1)** | 5.0±0.1 | 4.9±0.1 | 5.7±0.2 | 5.6±0.1 | *P*<0.001 | NS | NS |
| **Fasting plasma insulin (mU.L-1)** | 8.8±0.8 | 9.0.±0.8 | 11.7±1.6 | 12.6±1.1 | *P*<0.05 | NS | NS |
| **HOMA-IR** | 2.0±0.2 | 2.0±0.2 | 3.0±0.5 | 3.2±0.3 | *P*<0.01 | NS | NS |
| **Immobilized quadriceps CSA (mm2)** | 7504±395 | 7238±374 | 6864±358 | 6770±361 | NS | *P*<0.001 | *P*<0.01 |
| **Immobilized leg lean mass (kg)** | 10.1±0.5 | 9.7±0.4 | 10.4±0.3 | 10.2±0.3 | NS | *P*<0.05 | NS |
| **Immobilized leg fat mass (kg)** | 2.3±0.3 | 2.1±0.3 | 3.3±0.2 | 3.2±0.2 | *P*<0.05 | NS | NS |
| **Immobilized leg 1-RM (kg)** | 78±5 | 71±5 | 58±3 | 53±3 | *P*<0.001 | *P*<0.001 | NS |

Values are means±SEM. Abbreviations: BMI, Body Mass Index; ; CSA, cross sectional area; 1-RM, one-repetition maximum; NS, non-significant; Pre/Post, before and after a 5 day period of one-legged knee immobilization.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

**Figure 1**



**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

