The Impact of Lower Limb Immobilization and Rehabilitation on Angiogenic Proteins and Capillarization in Skeletal Muscle

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ABSTRACT

GLIEMANN, L., N. RYTTER, T. S. JØRGENSEN, P. PIIL, H. CARTER, M. NYBERG, M. GRASSI, M. DAUMER, and Y. HELLSTEN. The Impact of Lower Limb Immobilization and Rehabilitation on Angiogenic Proteins and Capillarization in Skeletal Muscle. *Med. Sci. Sports Exerc.*, Vol. 53, No. 9, pp. 1797–1806, 2021. **Purpose:** Skeletal muscle vascularization is important for tissue regeneration after injury and immobilization. We examined whether complete immobilization influences capillarization and oxygen delivery to the muscle and assessed the efficacy of rehabilitation by aerobic exercise training. **Methods:** Young healthy males had one leg immobilized for 14 d and subsequently completed 4 wk of intense aerobic exercise training. Biopsies were obtained from musculus vastus lateralis, and arteriovenous blood sampling for assessment of oxygen extraction and leg blood flow during exercise was done before and after immobilization and training. Muscle capillarization, muscle and platelet content of vascular endothelial growth factor (VEGF), and muscle thrombospondin-1 were determined. **Results:** Immobilization did not have a significant impact on capillary per fiber ratio or capillary density. The content of VEGF protein in muscle samples was reduced by 36% (P = 0.024), and VEGF to thrombospondin-1 ratio was 94% lower (P = 0.046). The subsequent 4-wk training period increased the muscle VEGF content and normalized the muscle VEGF to thrombospondin-1 ratio but did not influence capillarization. Platelet VEGF content followed the trend of muscle VEGF. At the functional level, oxygen extraction, blood flow, and oxygen delivery at rest and during submaximal exercise were not affected by immobilization or training. **Conclusions:** The results demonstrate that just 2 wk of leg immobilization leads to a strongly reduced angiogenic potential as evidenced by reduced muscle and platelet VEGF content and a reduced muscle VEGF to thrombospondin-1 ratio. Moreover, a subsequent period of intensive aerobic exercise training fails to increase capillarization in the previously immobilized leg, possibly because of the angiostatic condition caused by immobilization.
Key Words: ANGIOGENESIS, INACTIVITY, EXERCISE TRAINING, SKELETAL MUSCLE

Complete immobilization of a limb is a clinical procedure used for the remedial treatment of serious injury and bone fracture. Depending on the type of injury, the duration of immobilization varies, but it often lasts 3 wk or more. Although a number of studies have examined the effect of immobilization on muscle mass and metabolism (1,2), the consequence of a period of complete immobilization on capillarization and oxygen supply to skeletal muscle and the efficacy of subsequent rehabilitation training on these parameters has been sparsely examined.

Previous studies have shown that aerobic exercise training for 4 wk improves capillarization in skeletal muscle and that growth then stagnates, despite continued training (3). Moreover, exercise training improves oxygen extraction and lowers blood flow during submaximal exercise (4), an effect that has been reproduced by a pharmacological increase in capillarization in humans (5). It is, however, plausible that a period of immobilization will disturb the angiogenic potential and thereby impair the response to subsequent exercise training and the efficacy of a rehabilitation program.

A key factor for capillary growth in skeletal muscle is vascular endothelial growth factor (VEGF), which acts on VEGF receptor 2 on endothelial cells, influencing endothelial activation, proliferation, and migration (6–8). VEGF stored in skeletal muscle myocytes has been shown to be of particular importance for exercise-induced angiogenesis (9). In opposition

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of the proangiogenic effect of VEGF is the antiangiogenic factor thrombospondin-1 (TSP-1) (10). TSP-1 can interfere with VEGF by inhibiting migration and proliferation and promoting apoptosis of endothelial cells (11). Both VEGF and TSP-1 can be influenced by exercise training (12–14) but also by aging (15,16) and lifestyle-related disease (17). It is, however, unclear whether inactivity *per se* in humans causes a reduction in the levels of VEGF and TSP-1 (18) and thus an antiangiogenic state condition in skeletal muscle.

In addition to its location within myocytes, VEGF can also be carried in the blood stream bound to platelet α-granules. Platelet-rich plasma has been used to promote angiogenesis in wound healing (19), and depletion of platelets in rodents has been shown to eliminate overload-induced sprouting angiogenesis in skeletal muscle, further supporting a role of platelets in angiogenesis (19,20). However, to what extent the amount of VEGF carried by platelets is altered by changes in muscle activity is unclear. In the current study, we applied a newly developed method for isolating plasma-free platelets and assessed the amount of VEGF before and after immobilization and exercise training.

The hypotheses of this study were that i) a period of immobilization would reduce the VEGF to TSP-1 ratio and capillarization with a consequent reduction in muscle oxygen extraction during submaximal exercise; ii) a subsequent period of intense aerobic exercise training would reverse the effects of immobilization on capillarization, VEGF and TSP-1 levels, and oxygen extraction; iii) platelet content of VEGF would be decreased during immobilization and increased with training as a consequence of the change in physical activity. To test these hypotheses, young healthy and habitually active men completed a 2-wk full-leg immobilization of one leg, followed by 4 wk of cycle training. Analysis included skeletal muscle capillarization, angiogenic proteins, and at the functional level, determination of skeletal muscle oxygen extraction and blood flow during exercise.

MATERIALS AND METHODS

Subjects

Habitually active, healthy male subjects (n = 12) age 20–24 yr with body mass index <28 kg·m^{-2} and nonsmokers were recruited to the study. Before enrollment, all participants underwent a medical screening examination. The participants then performed an incremental exercise test on a cycle ergometer for the determination of pulmonary peak oxygen uptake ($\dot{V}O_{2\ peak}$). The subject characteristics are presented in Table 1 and have been published previously (21).

The study was approved by the Ethics Committee of the Capital Region of Denmark (H-17001344) and conducted in accordance with the latest guidelines of the Declaration of Helsinki. Written informed consent was obtained from all subjects before enrollment in the study (ClinicalTrials.gov Identifier: NCT03054376).

Study Design

The subjects underwent 2 wk of unilateral full-leg immobilization and then completed 4 wk of bilateral intense aerobic cycle ergometer exercise training (Fig. 1). Each subject underwent two experimental days before the intervention (baseline), on the day of cast removal (immobilized), and after 4 wk of aerobic exercise training, within 48 to 72 h after the last training session (4 wk trained). Muscle biopsies were obtained from the immobilized thigh muscle on all three occasions.

TABLE 1. Anthropometrics.

Variable	Baseline	Immobilized	4 wk Trained
Age, yr	22.1 ± 0.4		
Height, cm	184.8 ± 1.6		
$\dot{V}O_{2\ max}$, mL O_2·min^{-1}	4063 ± 193		
Body weight, kg	77.3 ± 2.0	76.8 ± 1.9	77.5 ± 1.9
Body fat mass, kg	14.4 ± 1.1	14.2 ± 1.0	14.2 ± 1.0
Body lean mass, kg	59.5 ± 1.7	59.3 ± 1.6	60.0 ± 1.6[a]
Leg lean mass, kg			
Immobilized leg	10.4 ± 0.3	10.0 ± 0.2[b]	10.4 ± 0.3[a]
Control leg	10.4 ± 0.3	10.4 ± 0.3	10.4 ± 0.3
Leg fat mass, kg			
Immobilized leg	2.6 ± 0.2	2.5 ± 0.2	2.6 ± 0.2
Control leg	2.6 ± 0.2	2.6 ± 0.2	2.6 ± 0.2

Data are presented as mean ± SD (n = 12).
[a]Significantly different from immobilized.
[b]Significantly different from baseline.

Intervention

Immobilization. Unilateral leg immobilization was achieved by a full-leg cast (Woodcast; Onbone Oy, Oulu, Finland) from just below the groin and to the toes, fixed in 20°–30° flexion to disable walking. The leg to be immobilized was determined by left–right randomization, without control for the dominant leg. Nine of 12 subjects were right dominant. Controlling for dominant versus nondominant leg did not change any of the outcome variables. Subjects were instructed to keep the leg muscles as inactive as possible and to use crutches during all movement. Furthermore, the subjects were requested to wear a pedometer (Garmin VivoSmart, Olath, KS) and a triaxial accelerometer (actibelt; Trium Analysis Online, Munchen, Germany) and not to exceed 3000 steps per day. The cast was removed on day 15 in the morning, and intact knee-joint movement was confirmed before experiments.

Exercise training. Within 4 d of cast removal, subjects initiated a supervised bilateral aerobic exercise training protocol consisting of 50-min sessions conducted three times per week for 4 wk. The exercise training protocol consisted of supervised high-intensity interval training on a cycle ergometer, and intensity of the training sessions was strictly controlled and gradually increased throughout the training period using heart rate monitors (TEAM2; Polar, Kempele, Finland). The protocol aimed at achieving heart rates of between 70% and 100% of maximal heart rate for at least 80% of the training sessions.

Experimental Days

Subjects were instructed to refrain from caffeine for 12 h and exercise training for 24 h before each experimental day.

On the first experimental day, subjects arrived at the laboratory in the morning after a light standardized breakfast. After local anesthesia (Lidocaine, 20 mg·mL^{-1}; Astra Zeneca, Cambridge, United Kingdom), catheters (20 gauge; Arrow

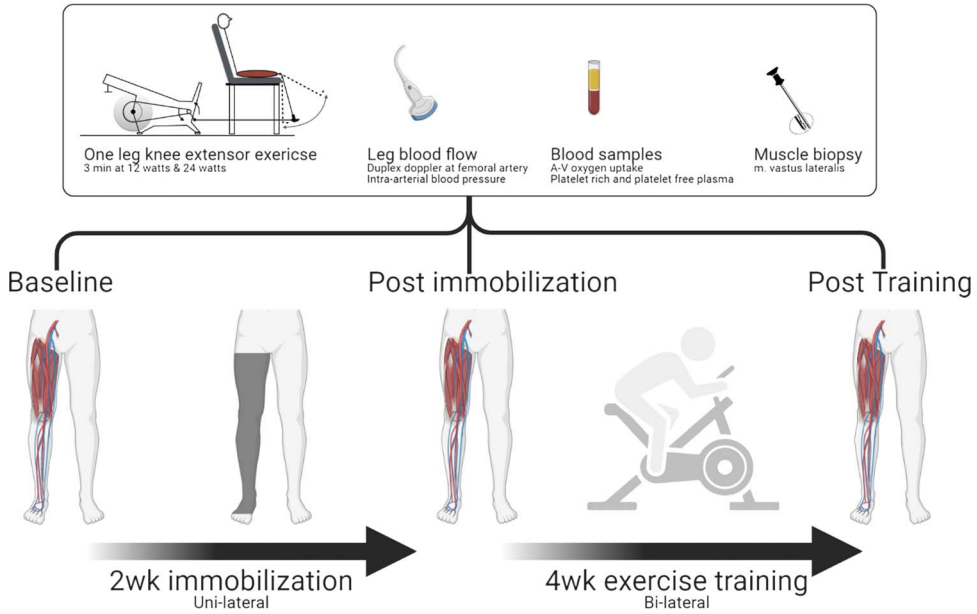

FIGURE 1—Illustrative presentation of the study timeline and important measures. At rest and during one-leg knee extensor exercise, leg hemodynamics and oxygen uptake were determined by duplex ultrasound Doppler and intra-arterial catheters. Arterial and venous blood samples were collected, and a skeletal muscle biopsy was obtained from musculus vastus lateralis. Measurements were done at baseline, after 2 wk of unilateral full-leg immobilization, and after 4 wk of bilateral cycle ergometer training.

International, Reading, PA) were inserted in the femoral artery and vein of the experimental leg. After at least 30 min of rest, the participant was seated in a supine position in a knee extensor ergometer. After 10 min of supine rest, the lower leg (experimental) was passively moved back and forth at a rate of 60 times per minute for 5 min. Subsequently, the participant conducted active knee extensions at 12 and 24 W, each for a duration of 5 min. Blood flow was measured by ultrasound Doppler, and blood samples were drawn from the femoral artery and vein for the determination of oxygen content at rest and within 30 s of the end of each intervention. Experimental leg mass was calculated from a whole-body dual-energy x-ray absorptiometry scanning (Prodigy; GE Healthcare, Chicago, IL).

On the second experimental day, subjects arrived at the laboratory in the morning in a fasted state. After 30 min of rest in a supine position and under local anesthesia (lidocaine, 20 mg·mL^{-1}; Astra Zeneca), a muscle biopsy was obtained from musculus vastus lateralis using the percutaneous needle biopsy technique (22). The biopsies were immediately frozen in liquid nitrogen or embedded in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, the Netherlands) and frozen in liquid nitrogen-cooled isopentane. Frozen samples were stored at −80°C until analysis.

Peak Oxygen Uptake

Before the intervention, all subjects conducted a graded exercise test on a cycle ergometer (Monark Ergomedic 839E; Monark, Vansbro, Sweden) in which pulmonary maximal oxygen uptake ($\dot{V}O_{2\ peak}$) was determined (Oxycon Pro; Viasys Healthcare, Hoechberg, Germany). After a standardized warm-up of 8 min at 100 W, the workload was increased by 25 W·min^{-1} until exhaustion. For recognition of $\dot{V}O_{2\ peak}$, three of the following five criteria had to be met: 1) individual perception of exhaustion, 2) $\dot{V}O_2$ curve plateau, 3) respiratory exchange rate >1.10, 4) heart rate approaching age-predicted maximum, and 5) inability to maintain pedaling frequency greater than 60 rpm. Verbal encouragement was given throughout the test, and $\dot{V}O_{2max}$ was calculated as the average of the three highest consecutive 15-s values. Results from this test have previously been described (21).

Measurements and Calculations

Femoral arterial blood flow was measured using ultrasound Doppler (Vivid E9; GE Healthcare, Pittsburgh, PA) equipped with a linear probe (L9) operating at an imaging frequency of 4/8 MHz and a Doppler frequency of 4.2 MHz. The site of measurement in the common femoral artery was distal to the inguinal ligament but above the bifurcation into the superficial and profound femoral branch to avoid turbulence from the bifurcation. All recordings were obtained at the lowest possible insonation angle and always less than 60°. Sample volume was maximized by choosing the widest section of the vessel, and recordings were made without interference of the vessel wall. A low-velocity filter rejected noise caused by turbulence at vascular wall and tissue movement. Doppler traces and B-mode images were recorded continuously and averaged greater than 30 s. Arterial diameter was determined for each Doppler measurement during systole from B-mode images obtained with the probe positioned perpendicular to the vessel.

Immunohistochemical Determination of Capillarization, Fiber Size, and Type

The embedded muscle samples were cut using a cryostat, and transverse sections of 8 μm in thickness were mounted

on glass slides. To verify the cross-sectional orientation of the individual muscle fiber, multiple samples were cut and examined under light microscopy until a cross section of desirable size, orientation, and uniform polygonal appearance was visible. For immunohistochemical staining, the cross sections were fixed for 2 min in phosphate-buffered saline (pH 7.2; Gibco 70013-016; Life Technologies Denmark, Nærum, Denmark) containing 2% formaldehyde followed by a washing sequence in a 1:10 wash buffer (Dako S3006, Glostrup, Denmark) and blocking for 10 min in phosphate-buffered saline containing 1% BSA.

Capillaries were visualized using biotinylated *Ulex europaeus* agglutinin I lectin (1:100; VECTB-1065; VWR, Bie and Berntsen, Herlev, Denmark) with secondary antibody (1:200; Streptavidin, Alexa Fluor® 568 conjugate, Invitrogen S11226; Life Technologies Denmark). Myofiber borders were visualized using an antibody against laminin (1:500; Dako Z0097) with secondary antibody (1:1000, AF 405 goat antirabbit). MHC-I was stained with MB421 (1:1000; Sigma-Aldrich, Merck, Darmstadt, Germany) with secondary antibody (1:1000, Alexa Fluor® 568; Invitrogen, Life Technologies Denmark), and MHC-IIa was stained with sc-71 (1:200; SC-71 was deposited to the DSHB by Schiaffino, S. (DSHB Hybridoma Product SC-71; University of Iowa, Iowa City, IA)), with secondary antibody (1:1000, Alexa Fluor® 488; Invitrogen, Life Technologies Denmark). Specificity of the staining was assessed by staining without the primary antibody. Visualization was performed on a computer screen using a fluorescence microscope (Carl Zeiss, Jena, Germany), and all morphometric analyses were performed using a digital analysis program (ImageJ, NIH ImageJ). Two or more separate sections of a cross section were used for analysis, and only sections without artifacts or tendency to longitudinal cuts were analyzed. Muscle fiber type, mean muscle fiber area, number of muscle fibers, and capillaries within each section were counted, and capillary supply was subsequently expressed as capillaries per fiber (C:F ratio) and capillary density ($cap \cdot mm^{-2}$). All analyses were carried out by the same blinded investigator and results were verified by full analysis by a second blinded investigator.

Platelets and Platelet-Free Plasma

Venous blood was drawn in citrate-anticoagulated tubes and kept at room temperature until centrifuged (10 min at 200*g* and 20°C) to obtain platelet-rich plasma. The platelet-rich plasma was layered on top of a gradient density medium (Iodixanol solution; OptiPrep, Sigma-Aldrich, St. Louis, MO) and centrifuged (15 min at 450*g* and 20°C) after which a fraction of isolated platelets and platelet-poor plasma was collected. To obtain plasma completely free of platelets, the platelet-poor plasma was centrifuged (15 min at 450*g* and 20°C) once more on the density gradient medium, and a fraction of the plasma was collected as platelet-free plasma. In all collected samples, platelets were counted using a hematology analyzer (XP-300; Sysmex, Kobe, Japan).

Sample Preparation and Protein Determination for VEGF and TSP-1 Analysis

From the muscle biopsy, a part (~30 mg) of the muscle sample was immediately frozen in liquid nitrogen and stored at −80°C. After freeze-drying, the samples were dissected to remove connective tissue, visible fat, and blood. The muscle tissue was then homogenized in a buffer (10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (Hepes), 1% nonyl phenoxylpoly-ethoxylethanol (NP-40), 20 mMβ-lycerophosphate, 2 mM sodium orthovanadate (Na_3VO_4), 10 mM NaF, 2 mM phenylmethylsulphonyl-fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 10 $\mu g \cdot mL^{-1}$ aprotinin, 10 $\mu g \cdot mL^{-1}$ leupeptin, and 3 mM benzamidine). Samples were rotated end over for 60 min at 4°C and then centrifuged for 30 min at 17,000*g* at 4°C. The lysate was collected, and protein concentration was determined by a BSA protein assay (Pierce Biotechnology, Inc., Rockford, IL). After counting, isolated platelets were lysed in a fresh of batch lysis buffer (MSD Tris Lysis Buffer; Meso Scale Diagnostics, Rockville, MD) on ice before being centrifuged (10 min at 10,000*g* and 4°C), and the supernatant was kept as platelet lysate. All samples were stored at −80°C until time of analysis.

Quantification of VEGF Protein by Electrochemiluminescence Assay Kit

$VEGF_{165}$ protein levels in muscle lysate, platelets, and platelet-free plasma were determined by electrochemiluminescence multiplex assay for $VEGF_{165}$ (Meso Scale Diagnostics) according to the manufacturer's guidelines. The skeletal muscle lysates were diluted 20-fold in Meso Scale diluent 43 (Meso Scale Diagnostics) to a concentration of approximately 0.5 $\mu g \cdot \mu L^{-1}$. The platelet lysates were diluted to a concentration of $\sim 1 \times 10^8$ platelets per microliter. The analysis was conducted on a sample volume of 50 μL. The muscle and platelet-free plasma samples were normalized to the total protein content in each sample, and the platelet samples were normalized to total platelet count.

Quantification of TSP-1 Expression in Skeletal Muscle Lysates by Western Blot

A volume of 5 μL lysate (2 mg protein$\cdot \mu L^{-1}$) was loaded on 5%–14% TGX-gels (Bio-Rad, Hercules, CA) and transferred semidry to PVDF membranes (Millipore AMC, Billerica, MA). The membranes were incubated overnight at 4°C with a primary antibody to TSP-1 (1:600, 2% nonfat milk; Abcam AB85762, Cambridge, United Kingdom). After washing 3 × 15 min, the membrane was incubated with secondary horseradish peroxidase–conjugated antibody (1:3000; Dako) for 1 h. The membrane staining was visualized by incubation with a chemiluminescent horseradish peroxidase substrate (Luminata Forte; Merck Millipore, Darmstadt, Germany), and the images were digitalized on a ChemiDoc MP system (Bio-Rad). All proteins were expressed in arbitrary units normalized to the average

of all samples loaded on the same gel. TSP-1 was not determined in platelets because of too low protein content.

Statistical Analysis

Number of subjects was based on *a priori* power calculation for capillary to fiber ratio in order to detect a 10% change in capillary to fiber ratio, with an α level of 0.05 and β level of 0.8 (23). Statistical analyses were performed with R (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria) using the interface RStudio (version 1.2.5033; RStudio Team, Boston, MA) with the extension packages *lme4* (24) and *multcomp* (25).

A linear mixed-model approach was used to detect differences with the intervention. The fixed factor was "time" (baseline, immobilization, exercise training). Subjects were specified as a repeated factor and identifier of random variation. Model checking was based on Shapiro–Wilk's tests, and residual and Q-Q plots. In case of heteroscedasticity, log transformation was applied before analysis. The Tukey honestly significant difference *post hoc* procedure was used to detect all pairwise differences, performed with multicomparison, and single-step adjusted *P* values are reported (25). Data are reported as mean ± SD, and the significance level was set at $P < 0.05$.

RESULTS

Subject compliance. Subject compliance with the intervention was high (94%), and there were no adverse events. On average, duration of immobilization was 13.7 ± 0.4 d, and 11.3 ± 0.3 training sessions out of 12 were completed. Training intensity corresponded to 79% of training time greater than 70% of maximum heart rate and to 18% greater than 90% of maximum heart rate. Based on waist-worn accelerometer and wrist-worn step counter, the subjects did 3505 ± 1358 and 2703 ± 897 steps per day, respectively.

Anthropometrics. Total body weight, fat mass, and lean mass were not changed after the immobilization period (Table 1). After the exercise training period, lean mass was increased (0.7 ± 0.3 kg, $P = 0.044$), whereas total body weight and fat mass were unchanged. In the immobilized leg, lean mass was decreased (by 0.4 ± 0.1 kg, $P = 0.003$) after immobilization and increased (by 0.4 ± 0.1 kg, $P < 0.001$) after the training period. Leg fat mass of the immobilized leg was unchanged throughout the study. Lean mass and fat mass of the control leg remained unchanged throughout the study. Compliance and anthropometrics have previously been reported (21). $\dot{V}O_{2\ peak}$ was not determined after immobilization or the training intervention.

Skeletal muscle histology. At baseline, the mean capillary per fiber ratio, capillary density, and mean muscle fiber area were 2.1 ± 0.3 capillaries per fiber, 342 ± 47 capillaries per square millimeter, and 6211 ± 752 μm^2, respectively (Figs. 2A, B). There were no changes in capillaries per fiber or capillary density with immobilization or training. Mean muscle fiber area was reduced after immobilization ($P = 0.019$, Fig. 2C) but not different to baseline after the training period ($P = 0.274$). The distributions of muscle fiber type at baseline were 56.1% ± 13.4% and 43.9% ± 13.4%, type I and type II, respectively. After immobilization, muscle fiber distributions were 48.3% ± 10.7% and 51.7% ± 10.7%, and after the training period, the values were 44.3% ± 11.4% and 55.7% ± 11.4%, type I and type II, respectively (main effect of time: $P = 0.029$ vs baseline: $P = 0.010$). Representative images of histology from one subject are presented in Figure 2D.

Skeletal muscle VEGF and TSP-1 protein. Skeletal muscle protein levels of VEGF were reduced by 35% ($P = 0.024$) after the immobilization period, and the levels were normalized after 4 wk of training ($P = 0.06$ vs baseline, Fig. 3A). There was no main effect of time ($P = 0.146$), but

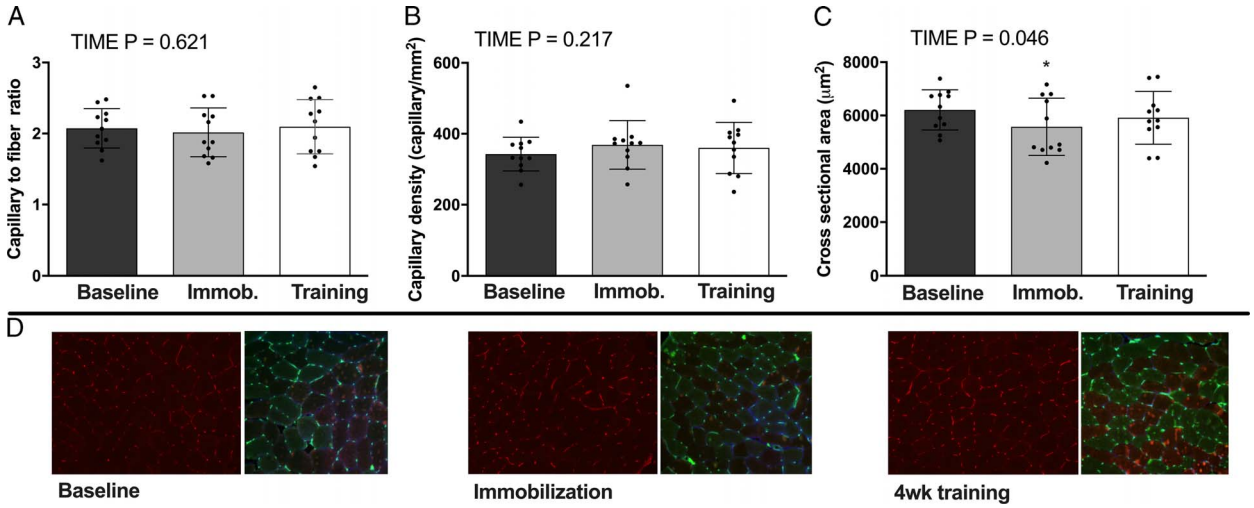

FIGURE 2—Skeletal muscle capillarization and fiber area. Capillary to fiber ratio (A), capillary density (B), and muscle fiber cross-sectional area (C) at baseline, after 2 wk of immobilization, and after 4 wk of exercise training. D, Representative histochemical staining of skeletal muscle fibers and capillaries from one subject. Myofiber borders were visualized by using an antibody against laminin (blue), and capillaries were visualized using biotinylated Ulex (red). Muscle fiber type was determined by staining for MHC-I (green) and MHC-II (black). Data are presented as mean with SD and individual data points. Main time effect is presented. *Different from baseline.

the protein content of TSP-1 was ~50% higher after the immobilization period and the levels were normalized after 4 wk of training (Fig. 3B). The ratio between VEGF and TSP-1 was reduced by 94% ($P = 0.046$) and normalized ($P = 0.84$ vs baseline) after 4 wk of training (Fig. 3C). Representative blots and stain-free TGX gel (protein stain of gels used for loading control) are presented from one subject in Figure 3D.

Platelet and platelet-free plasma protein levels of VEGF. Platelet number in full blood was not different between baseline and after immobilization or after training, but in platelet-rich plasma, the number of platelets was reduced by 13% ($P = 0.001$) after immobilization and increased by 11% after training ($P < 0.001$, Table 2). VEGF in isolated platelets was reduced by 13% ($P = 0.040$) after immobilization and increased by 17% after training ($P < 0.001$). VEGF in full blood was not different between baseline and after immobilization but was increased by 20% ($P = 0.021$) after training. VEGF in platelet-free plasma was not different between baseline and after immobilization or after training but was reduced in platelet-rich plasma by 45% ($P = 0.040$) after immobilization and increased by 29% after training ($P = 0.014$).

Leg hemodynamics and oxygen extraction. Oxygen extraction, blood flow, oxygen delivery, and oxygen uptake at rest and during submaximal exercise were not significantly different between baseline and after immobilization or after training (Figs. 4A–D). Leg arteriovenous lactate difference and leg lactate release were not different between baseline and after immobilization or after training (data not shown).

DISCUSSION

The main findings of the study were that a 2-wk period of full-leg immobilization had no effect on skeletal muscle capillarization but markedly reduced skeletal muscle VEGF content and the VEGF to TSP-1 ratio, suggesting an angiostatic condition. The subsequent 4-wk training period increased the muscle VEGF levels and restored the VEGF to TSP-1 ratio but did not result in increased capillarization. Oxygen extraction, blood flow, and oxygen uptake at rest and during submaximal exercise remained unaltered with the interventions.

The effect of immobilization on skeletal muscle capillarization. In contrast to our hypothesis, there was no reduction in capillarization with 2 wk of immobilization. Observations in previous studies examining the effect of 2 wk of immobilization in humans are disparate; Vigelsø et al. (26) reported a 10% reduction in capillary to fiber ratio

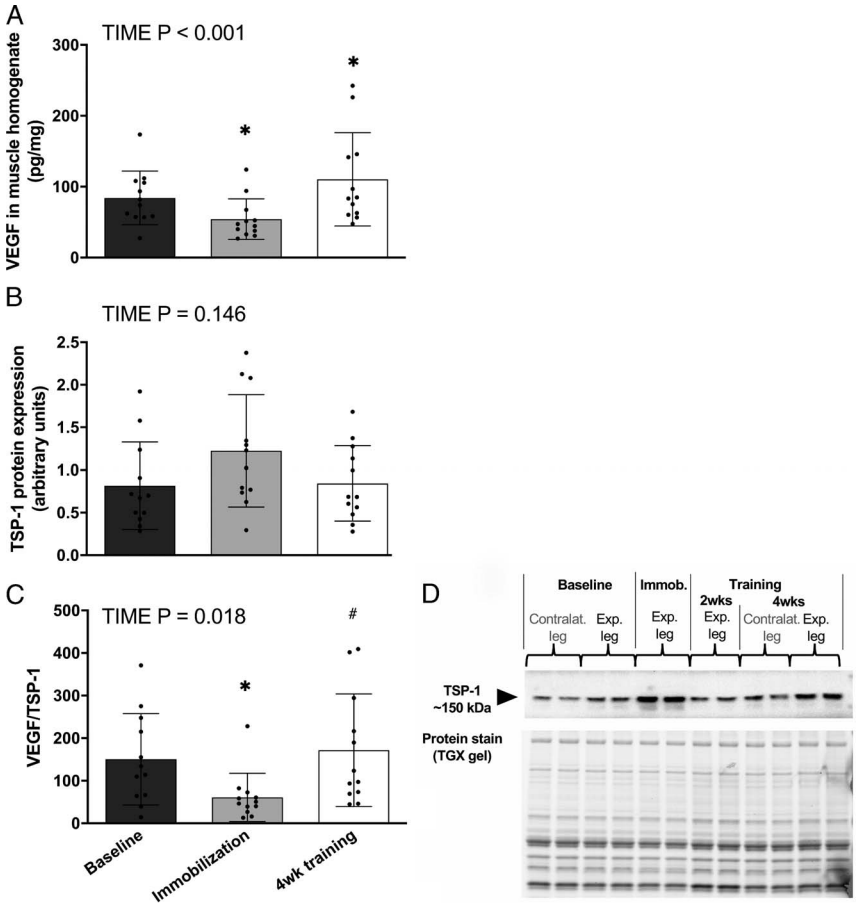

FIGURE 3—Angioregulatory proteins in skeletal muscle homogenates. VEGF (A), TSP-1 (B), and VEGF to TSP-1 ratio (C) in skeletal muscle homogenates at baseline, after 2 wk of immobilization, and after 4 wk of exercise training. D, Representative blots and stain-free TGX gel from one subject. Data are presented as mean with SD and individual data points. VEGF protein content in muscle homogenates was determined by Meso Scale, and TSP-1 expression was quantified by Western blot. Main time effect is presented. *Different from baseline. #Different from immobilization.

TABLE 2. Platelet number and VEGF content.

	Baseline	Immobilized	4 wk Trained
Platelet count			
Full blood, 10^9 plt·L^{-1}	195.0 ± 25.4	185.6 ± 26.1	188.7 ± 30.0
Platelet-rich plasma, 10^9 plt·L^{-1}	373.6 ± 59.5	323.2 ± 56.1[a]	360.1 ± 61.3[b]
Platelet-free plasma, 10^9 plt·L^{-1}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
VEGF content			
Platelets, pg·10^9 plt^{-1}	693.6 ± 320.4	658.7 ± 256.7	768.3 ± 398.7[a]
Platelets, pg·mL^{-1}	72.8 ± 32.6	63.1 ± 25.5[a]	73.9 ± 33.0[b]
Platelets in full blood, pg·mL^{-1}	125.7 ± 51.2	118.0 ± 39.4	141.7 ± 64.4[b]
Platelet-free plasma, pg·mL^{-1}	9.41 ± 4.06	9.13 ± 3.51	8.77 ± 3.53
Platelet-rich plasma, pg·mL^{-1}	250.3 ± 107.8	214.6 ± 80.3[a]	277.7 ± 138.4[a,b]
Platelet to plasma VEGF ratio	9.23 ± 6.10	8.28 ± 4.77[a]	10.1 ± 6.56[b]

Platelet number (plt) and VEGF in full blood, plasma, and platelets. Data are presented as mean ± SD (n = 10).
[a]Significantly different from baseline.
[b]Significantly different from immobilized.

compared with baseline in young and older men, whereas Al-Shammari and coworkers (27) reported unaltered capillary to fiber ratio after 2 wk of immobilization in older men. A similar discrepancy in findings exists from bed rest studies, with some showing no effect on capillarization (28,29) and others a reduction (30). The reason for the discrepancy between studies on severe inactivity is somewhat unclear but may be related to aspects such as the original physical activity level of subjects, or the degree of inactivity during the immobilization period and/or methodological aspects concerning the analysis. In the present study, the subjects were habitually active as indicated by the relatively high $\dot{V}O_2$ peak. The degree of inactivity of the immobilized leg was high, as the full-length cast prevented not only movement but also weight support on the leg. In addition, the overall activity of the subjects was very limited with an average step count per day of less than 3000. Regarding methodological aspects, staining quality was good; the size of the muscle samples, as determined by the number of fibers counted on, was for most samples over 200; and there was no trend for a change in capillary per fiber ratio. Therefore, we are convinced that 2 wk of immobilization is too short for capillary necrosis to occur in this population of young healthy, habitually active subjects. Evidence of resilience of capillary loss in healthy trained skeletal muscle is supported by results from detraining studies, in which capillarization has been shown to remain high after periods of up to 3 months (31,32). Nevertheless, the marked reduction in VEGF levels and the reduced VEGF to TSP-1 ratio observed after immobilization in the present study would suggest an angiostatic status after 2 wk of immobilization. Thus, it would be expected that, with a longer time of immobilization, which is often the case after bone fracture, capillarization may be reduced. Previous studies have shown an increase in TSP-1 protein levels in rodents with hindlimb unloading (18) and detraining (33); however, this is the first time a marked reduction in VEGF combined with a reduction in the VEGF to TSP-1 ratio has been demonstrated in human skeletal muscle with immobilization. The substantial reduction in VEGF amount in human muscle tissue is particularly interesting and differs from findings in rodents (18,33). Nevertheless, it is in line with reduced skeletal muscle levels of VEGF in older individuals (15) and individuals with hypertension (16,17). Such a loss in VEGF would seem to be critical as VEGF stored in myocytes is central in skeletal muscle angiogenesis (9). The importance of the absolute amounts of VEGF in skeletal muscle for exercise-induced angiogenesis is further evidenced by the finding that the release of VEGF, which is an important step in the initiation of angiogenesis in response to exercise (13,34), is related to the amount of VEGF stored in the muscle (7,17). In the present study, we demonstrate that inactivity may be a major factor in the reduced levels of VEGF observed in

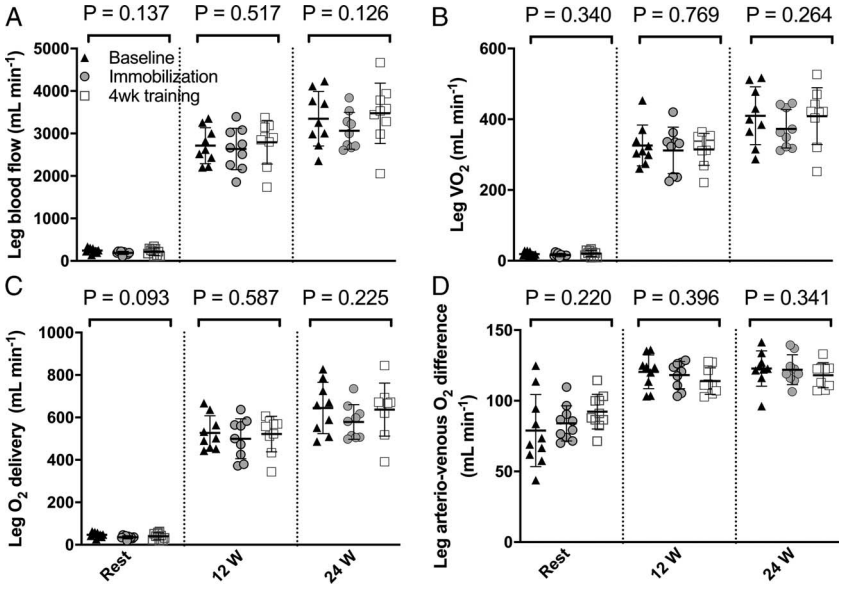

FIGURE 4—Blood flow and oxygen uptake. Leg blood flow (A), leg oxygen uptake ($\dot{V}O_2$; B), leg O_2 delivery (C), and leg arteriovenous O_2 difference (D) at baseline, after 2 wk of immobilization, and after 4 wk of exercise training. Data are presented as mean with SD and individual data points. Main time effect is presented.

aging and hypertension, as only 2 wk of immobilization in healthy young men reduced VEGF amounts markedly. The concurrent trend for an increase in TSP-1, which is known to oppose angiogenesis by inhibiting proliferation of endothelial cells and interfering with VEGF activity (35,36), would further reduce the angiogenic propensity in the muscle tissue. Although muscle protein levels of TSP-1 have not been found to be high in age or hypertensive individuals, very high levels of TSP-1 have been observed in the skeletal muscle interstitium of individuals with peripheral arterial disease (37).

The 4 wk of intense aerobic cycle training after the immobilization period did not influence capillarization in the immobilized leg but did normalize the individual levels of VEGF to TSP-1 and their ratio at 4 wk. Combined with results from previous studies, showing that 4 wk of exercise training is sufficient to induce capillarization in young men (38), these data suggest that immobilization induces a degree of resilience to capillary growth, as also indicated by the reduced VEGF to TSP-1 ratio. Our findings suggest that intense aerobic training is an effective treatment modality to reverse the negative consequences of immobilization at the angiogenic protein level. However, to achieve an increase in muscle capillarization in an immobilized muscle, a somewhat longer time of training may be required compared with a muscle that has not experienced immobilization.

Circulating VEGF bound to platelets. The present study applied a newly developed method for isolation of plasma-free platelets with the purpose of examining whether altered muscle activity influences the amount of VEGF carried by platelets. By this method, it is possible to determine the fraction of VEGF carried in blood bound to platelets and the fraction freely dissolved in plasma. The platelet to plasma VEGF ratio was ~9 at baseline, indicating that by far the largest amount of VEGF in the blood is carried by platelets and only ~10% is freely dissolved in plasma. The immobilization and the training period did not alter plasma VEGF levels, whereas VEGF levels in platelets followed the trends of the skeletal muscle VEGF levels with a reduction after immobilization and an increase after the 4 wk of exercise training. This finding suggest that platelets may alter their content of VEGF in relation to physical inactivity and activity, but whether such alterations have a direct relation to skeletal muscle angiogenesis is unclear. Nevertheless, our data provide an indication of platelets that can provide a reservoir of VEGF, supporting the previous proposition that platelet-bound VEGF contributes to angiogenesis (19,20). To our knowledge, this is the first study to demonstrate that leg immobilization and exercise training can influence platelet-bound VEGF levels, and overall, the findings suggest that determination of VEGF in plasma-free platelets may be a more sensitive indicator of platelet bound circulating VEGF.

Skeletal muscle oxygen extraction and blood flow. At the functional level, there was no influence of the immobilization period or the 4 wk of training on oxygen delivery, oxygen extraction, or oxygen uptake of the thigh muscle at rest or during submaximal exercise. The finding of unaltered oxygen extraction and blood flow agrees with the lack of change in capillarization with the interventions, as a change in oxygen extraction and thereby blood flow, would have been expected as a consequence of altered capillarization (5) or altered mitochondrial capacity. Mitochondrial respiration was not measured in the current study, and the lack of change in oxygen extraction would indicate that mitochondrial capacity was not markedly altered, although based on previous findings, some reduction may have occurred with immobilization (39,40). Combined the available data suggest that 2 wk of one-leg immobilization does not induce changes to oxygen delivery, extraction, or uptake.

Immobilization induces a shift in skeletal muscle fiber type distribution. The present study found a shift in skeletal muscle fiber type distribution from 56%/44% of type I/type II before the intervention to 48%/52% of type I/type II after immobilization. A similar change in fiber type distribution has previously been reported (41,42); however, an interesting novel observation in the present study was that the change in fiber type distribution with immobilization was not reversed by the 4-wk exercise training with a distribution of 44%/56% of type I/type II fibers at the end of training. We have no clear mechanistic explanation for this finding, but a slow reversal of immobilization induced fiber type change has previously been reported in injured athletes (43) where resistance exercise with the nonimmobilized leg was found to oppose the muscle fiber shift (41). Thus, it is well possible that the training stimuli, and in particular the duration of the training period, were too short to achieve a measurable shift.

Limitations. The leg that was not immobilized could not be used as a control in the study, as the activity of this leg was altered by the intervention. Therefore, only preintervention values were used for comparison with postimmobilization and training. It should also be considered that the activity level of the control leg may interfere with the direct local impact of the immobilization, as studies have previously shown that unilateral strength training causes adaptations in the contralateral immobilized limb (44,45). To minimize this possible crossover effect, physical activity was limited during the immobilization period. Activity of daily living was not controlled during the exercise intervention period, and it is possible that some subjects were more active than others, for example, by commuting on bike. However, the impact of this is likely minimal compared with the extreme change from 2 wk of immobilization to supervised intense spinning training three times per week. Only men were included in the present study. The reason for this was that the 2-wk immobilization period made it impossible to control for change in hormonal status of women because of the menstrual cycle.

CONCLUSIONS AND PERSPECTIVE

The results show that 2 wk of immobilization of one leg does not influence skeletal muscle capillarization or oxygen

extraction during exercise in young habitually active men. However, immobilization leads to an angiostatic condition as indicated by a loss of VEGF and a marked increase in the VEGF to TSP-1 ratio and by the lack of effect of intense aerobic training on skeletal muscle capillarization. In addition, the level of platelet-carried VEGF followed the levels of VEGF in the skeletal muscle and supports a role of platelet carried VEGF in the regulation of angiogenesis. We propose interventions such as passive movement (46) of the injured limb or exercise training with the noninjured limbs to maintain angiogenic potential during periods of immobilization.

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