Regression of capillary network in atrophied soleus muscle induced by hindlimb unweighting

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Fujino, Hidemi, Hisaharu Kohzuki, Isao Takeda, Takahiko Kiyooka, Takehiro Miyasaka, Satoshi Mohri, Juichiro Shimizu, and Fumihiko Kajiya. Regression of capillary network in atrophied soleus muscle induced by hindlimb unweighting. J Appl Physiol 98: 1407–1413, 2005. First published December 10, 2004; doi:10.1152/japplphysiol.00961.2004.—Little is known about the mechanisms responsible for the adaptation and changes in the capillary network of hindlimb unweighting (HU)-induced atrophied skeletal muscle, especially the coupling between functional and structural alterations of intercapillary anastomoses and tortuosity of capillaries. We hypothesized that muscle atrophy by HU leads to the apoptotic regression of the capillaries and intercapillary anastomoses with their functional alteration in hemodynamics. To clarify the three-dimensional architecture of the capillary network, contrast medium-injected rat soleus muscles were visualized clearly using a confocal laser scanning microscope, and sections were stained by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) and with anti-von Willebrand factor. In vivo, the red blood cell velocity of soleus muscle capillaries were determined with a pencil-lens intravital microscope brought into direct contact with the soleus surface. After HU, the total muscle mass, myofibril protein mass, and slow-type myosin heavy chain content were significantly lower. The number of capillaries paralleling muscle fiber and red blood cells velocity were higher in atrophied soleus. However, the mean capillary volume and capillary luminal diameter were significantly smaller after HU than in the age-matched control group. In addition, we found that the number of anastomoses and the tortuosity were significantly lower and TUNEL-positive endothelial cells were observed in atrophied soleus muscles, especially the anastomoses and/or tortuous capillaries. These results indicate that muscle atrophy by HU generates structural alterations in the capillary network, and apoptosis appears to occur in the endothelial cell of the muscle capillaries.

intercapillary anastomosis; tortuosity; capillary volume; capillary lumen; erythrocyte velocity; disuse atrophy; endothelial terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling

SKELETAL MUSCLE CAPILLARIES run tortuously along muscle fibers in the relaxed resting state (2, 4, 18, 20, 33). These capillaries are connected with anastomoses, which run orthogonally to muscle fiber direction like parallel rungs of ladder (12, 33). Capillary-to-fiber (C/F) ratio is frequently used to evaluate O2 supply capacity in skeletal muscle (27). In fact, the C/F ratio is higher in rat soleus muscle, which mainly contains slow oxidative fibers (type I), than in extensor digitorum longus, which is predominantly comprised of fast glycolytic fibers (type IIb) (1, 8, 13). C/F ratio was increased by the augmentation of muscle activity, e.g., exercise or electrical stimulation (9, 14, 20, 25), and was decreased by disuse (14, 31, 33). Histological sections from earlier studies of capillary remodeling in skeletal muscle with disuse atrophy demonstrate a decrease in capillary luminal diameter (16, 36) and in the C/F ratio (13, 31, 32) despite an increase in capillary density (13, 31, 32).

Although the regression of anastomoses in atrophied skeletal muscle has not been well studied, it was demonstrated that reduction of the number of anastomosis decreases the net flow greatly in a coronary capillary network by a simulation study (17). Tortuosity is characteristic of muscular capillaries, but its change after disuse has received little attention. Moreover, data on in vivo capillary hemodynamics before and after disuse are lacking. We hypothesized that muscle atrophy by hindlimb unweighting (HU) causes regressive morphological changes in the capillary and anastomosis with apoptosis, resulting in the alteration of hemodynamics in the capillary networks. The purposes of this study were to 1) examine the changes in the architecture of capillaries and intercapillary anastomosis three dimensionally (3D) with confocal laser scanning microscopy (CLSM), 2) evaluate possible capillary endothelial cell apoptosis after disuse and its predominant location if any, and 3) measure in vivo red blood cell velocities (V RBC) in capillaries and anastomoses after disuse using a pencil-lens probe intravital microscope.

METHODS

Animals and muscle atrophy procedure. All experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and permitted by the Animal Experimental Committee of the Okayama University and the Suzuka University of Medical Science. Twenty-two male rats (Wistar; 225–315 g; Japan SLC, Hamamatsu) aged 8–12 wk were used in the experiments. All rats were housed in a temperature-controlled room at 22 ± 2°C with a light-dark cycle of 12 h and maintained on rat chow and water ad libitum. The rats were randomly divided into either the HU (n = 11) or control (n = 11) groups; five rats randomly selected from each group were used for capillary structure and biochemical analyses, and the remaining six rats underwent V RBC visualization. The rats subjected to HU were suspended by their tails according to the Morey technique (22, 23) for 2 wk. The suspension height was adjusted by 10.220.33.2 on June 10, 2017 http://jap.physiology.org/ Downloaded from http://jap.physiology.org/ by 10.220.33.2 on June 10, 2017

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and to prevent any contact between the hindlimbs and the cage surfaces. The rats of the control and HU group were housed in individual cages.

3D visualization and capillary structural analysis. The 3D capillary architecture was visualized by laser fluorescent mode of CLSM (Fluoview, Olympus, Tokyo, Japan) with an argon laser (488 nm), with a slight modification to the method by our laboratory’s earlier study (29).

After 2 wk of HU treatment, the rats of the HU and the matched control groups were weighed and anesthetized with pentobarbital sodium (50 mg/kg ip). The abdominal cavity was opened rapidly, and a perfusion apparatus catheter was inserted into the abdominal aorta for perfusion and fixation of the soleus muscle. The muscle was first perfused for 3 min with 0.1 M phosphate buffer (pH 7.2) containing 10,000 IU/l of heparin and adenosine (1 mg/min) to wash out the intravascular blood and to induce maximal vasodilation with perfusion pressure of 110–120 mmHg, then the contrast medium (42°C) was administered into the muscle circulation. Our contrast medium consisted of 1) 20% vol/vol of India ink (Saga Koku Nosen, Kaimeri, Saitama, India) for CLSM and optical microscope observations in transmission imaging mode, 2) 1% vol/vol of fluorescent material (PUSR80, Mitsubishi Pencil, Tokyo, Japan) for CLSM observation in fluorescent mode, 3) 8% w/v of gelatin (Nakalai Tesque, Kyoto, Japan), and 4) distilled water. The whole body was quickly immersed into a cold saline-circulating bath (0°C) and kept there for 10 min to solidify the contrast medium under perfusion pressure without chemical fixation. This procedure enabled us to fill the entire microvasculature completely under a physiologically relevant perfusion pressure as described in our laboratory’s earlier paper (29).

The soleus muscle was removed and weighed, and slices from the midbelly were frozen in isopentane precooled in liquid nitrogen. This sample block was sliced into 200-μm sections using a cryostat (5040 Microtome, Bright Instrument, London, UK).

Microscopic images were obtained at a magnification of ×20 and scanned for 100 μm in depth divided by 1 μm for 1-slice thickness. Microscopic observations were performed in longitudinal section. The CLSM images were automatically rendered and displayed as 3D images.

Capillary morphometry in soleus muscle. Digital images were converted into stack files for morphometric analysis (NIH Image 1.63, NIH, Bethesda, MD). To measure capillary diameters, we acquired Z stacks of images that entirely encompassed the vessels. We then projected each stack onto a two-dimensional image and measured the internal diameter of the vessel by counting the pixels.

To evaluate the effect of HU on the capillary architectures, we compared three parameters between HU and control conditions: 1) how many capillaries exist in muscle volume unit (no. of capillaries/106 μm3); 2) how much capillary volume (CV) occupies in muscle volume unit (CV μm3/106 μm3); 3) how much volume occupies in relative numbers of muscle fibers (CV/mass/fiber diameter: CV/106 μm3). CV/mass represents the sum of the gray values in the stack image and CV/mass/fiber diameter allows evaluations of the change of CV relation to that of muscle fiber diameter.

The capillary tortuosity was represented by tortuosity index (3, 14) as follows: tortuosity index = actual vessel length (in μm)/straight-line distance (in μm).

Evaluation of \( V_{\text{BAC}} \) in capillary and pencil-lens probe intravital microscopy. Details of our system were described in our laboratory’s earlier studies (15, 25, 34, 35). In brief, the experimental system consists of a pencil-lens probe intravital microscope with a charge-coupled device camera, micromanipulator, light source, monitor, digital videocassette recorder, and a computer for image analysis (Nihon Kohden, Tokyo, Japan) (see Fig. 1 in Ref. 35). This probe had a magnification of ×520, depth of field of <60 μm, and spatial resolution of 0.86 μm, which permitted identification of individual erythrocytes (25, 34, 35). The scale of the captured video image was 360 × 270 μm on the display. Analysis of \( V_{\text{BAC}} \) was performed using the NIH Image program combined with MATLAB software (The MathWorks, Natick, MA), as shown by Ogasawara et al. (25) and Yamamoto et al. (34) (see Fig. 3 in Ref. 34). In addition, the number of capillaries and anastomoses with flowing erythrocytes were measured from 60 frame images in the captured stacks.

The in vivo red blood cell velocimetry was performed as follows. First, the animals were placed on a heated surgical table. After tracheotomy, heparin (1,000 IU/kg ip) was administered under artificial ventilation, and then a cannula was inserted into the carotid artery for monitoring of the systemic blood pressure. After decapsulation of the calf, red blood cell flow within soleus capillaries was recorded with a pencil-lens probe brought into contact with the muscle surface, which had been superfused with Tyrode’s solution to prevent evaporation and to maintain physiological temperature. Images were recorded on digital videocassette tapes in the steady state at rest.

Biochemical analysis. Frozen soleus muscles were used for myofibril protein content analysis; the standard curve was provided by BSA using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) and SDS-PAGE of myosin heavy chain (MHC) protein. Skeletal muscle myofibrils and MHCs were prepared by the method of Tsika et al. (30). Myofibril yields were reported as milligrams of protein per gram of muscle. Also, myofibril yield was expressed on a per muscle basis (mg/g soleus muscle wt). Purified myofibril protein was run on SDS gels under nondissociating conditions to separate myosin into its isofoms. Quantification of the myosin isofom was based on peak area densitometric analysis of the individual myosin bands derived from SDS gel electrophoresis.

Analysis of apoptosis on cross-sectional specimens. Serial 10-μm-thick cryosections from the soleus muscle were cut and collected on polyllysine precoated slides. In situ nick-end labeling (TUNEL) of fragmented DNA was performed using terminal deoxynucleotidyl transferase and fluorescein-conjugated nucleotides with the in situ apoptosis detection kit ApopTag (Aptoptosis in situ detection kit, Wako Pure Chemical, Osaka, Japan), as described in the manufacturer’s instructions, and were then counterstained with FITC-conjugated secondary antibody. In the second reaction, these sections stained with TUNEL reagents were incubated with mouse anti-βnucleolin factor antibody (Sigma, St. Louis, MO) to identify endothelial cells and were then counterstained with rhodamine-conjugated anti-mouse secondary antibody, washed, mounted, and examined by CLSM with an argon laser (488 nm) and a HeNe laser (543 nm). The former detects FITC on TUNEL-positive nuclei, and the latter detects rhodamine on endothelial cells.

### Table 1. Changes in body mass, muscle mass, and myofibril protein by hindlimb unweighting

<table>
<thead>
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<th>Body Mass, g</th>
<th>Muscle Mass, mg</th>
<th>Myofibril Protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>284±14</td>
<td>117±7</td>
<td>120±6</td>
</tr>
<tr>
<td>HU</td>
<td>268±10</td>
<td>89±12*</td>
<td>70±5*</td>
</tr>
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Values are means ± SE. HU, 2-wk hindlimb-unweighting group. *HU significantly different from control (P < 0.05).

### Table 2. Myosin isofom distribution in soleus muscle

<table>
<thead>
<tr>
<th>Group</th>
<th>MHC2A, %</th>
<th>MHC2d/X, %</th>
<th>MHC1, %</th>
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<tbody>
<tr>
<td>Control</td>
<td>11.1±1.3</td>
<td>0</td>
<td>88.9±1.3</td>
</tr>
<tr>
<td>HU</td>
<td>10.9±0.7</td>
<td>7.4±0.7*</td>
<td>81.7±1.0*</td>
</tr>
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</table>

Values are means ± SE. MHC, myosin heavy chain *HU significantly different from control (P < 0.05).
Statistical analyses. Experimental data is expressed as means ± SE of each group. A Mann-Whitney U-test was used when appropriate. Values of \( P < 0.05 \) were considered statistically significant.

RESULTS

Muscle atrophy. The HU group soleus muscle weight was significantly smaller than that of the control group (Table 1). The relative muscle wet weight per unit body weight (mg/g) also decreased in HU. The myofibril protein content per unit muscle mass also decreased significantly in the HU group relative to the control group. The composition of MHC (Table 2) in the control group was 89% MHC I and 11% MHC IIa, whereas in the HU atrophied soleus the proportions were 82% MHC I, 11% MHC IIa, and 7% MHC IIId/x, indicating the relative decrease in slow-type MHC I and the expression of fast-type MHC IIId/x, which was not observed in the control group.

Comparison of characteristics of 3D capillary structure between normal and unweighted soleus muscles. The 3D images of microvessels were visualized using CLSM under control conditions and after HU in the soleus muscle (Fig. 1). CLSM images were reconstructed, the slices were scanned for 100 μm by 1 μm into a 3D representation, and a rotatable projection of the 3D specimen was created by software running on a personal computer (see movies 1 and 2; supplemental data can be found at http://jap.physiology.org/cgi/content/full/00961.2004/DC1).

Typical CLSM images of capillaries running along muscle fibers and capillary-interconnecting anastomoses under control conditions and after HU are shown in Fig. 1. The tortuosity of capillaries running parallel to muscle fibers (simply called “capillaries” hereafter) was clearly observed in both control and HU. The mean luminal diameter of capillaries was 5.8 ± 0.3 μm (range 1.8–9.5 μm) in the control group and 2.9 ± 0.2 μm in the HU group.

![Fig. 1. Confocal laser scanning microscopic images of capillaries and anastomoses in the soleus muscles under control conditions (A) and after 2 wk of hindlimb unweighting (HU; B). Capillaries run tortuously along the muscle fibers (indicated by arrows) in control and after HU, but the degree of tortuosity is more remarkable in the control group. Intercapillary anastomoses (indicated by triangles) were observed more clearly in the control group. In HU, the capillaries were shrunken with fewer anastomoses and less tortuosity.](image-url)

![Fig. 2. Influence of HU on luminal diameter (A) and number of vessels per unit mass (B) of capillaries and anastomoses in the soleus muscle. Values are means ± SE. Open bars, under control conditions; closed bars, after HU. *Significant difference between control and HU (\( P < 0.05 \)). †Significant difference between capillaries and anastomoses (\( P < 0.05 \)). After HU, the luminal diameter of capillaries and anastomoses was significantly smaller than that of the control group.](image-url)
A 107 μm (1.7–5.7 μm) after HU (Figs. 2A and 3; P < 0.01). The mean luminal diameter of interconnecting anastomoses (simply called “anastomoses” hereafter) was 3.1 ± 0.3 μm in the control group, which was significantly greater than that after HU (1.9 ± 0.1 μm; P < 0.01). The luminal diameter of anastomoses was significantly smaller than that of capillaries both in control and after HU (Fig. 2A). The number of capillaries and anastomoses per unit mass (10⁶ μm³) in control group was 3.0 ± 0.1 and 7.0 ± 1.0, respectively, whereas those after HU were 3.7 ± 0.3 and 4.0 ± 0.9 (P < 0.05 vs. control), respectively.

The calculated volume projected from the region of interest of 10⁴ μm² in 100-μm Z stacks of 3D CLSM images was 5,335 ± 314/10⁶ μm³ in the control group and 2,164 ± 350/10⁶ μm³ after HU (P < 0.01), i.e., 60% decrease compared with control group. The CV/fiber diameter in the control group was 107 ± 6/10⁶ μm³ and 62 ± 10/10⁶ μm³ after HU (P < 0.05).

The tortuosity index of control group (2.0 ± 0.2) was greater than HU (1.3 ± 0.1; P < 0.01) (Fig. 4). This indicated the capillaries were shrunken with less tortuosity after HU.

Capillary VRBC in soleus muscle. Figure 5A shows the capillaries and anastomoses visualized by our intravital microscope. Mean arterial blood pressures were 125 ± 5 and 123 ± 4 mmHg at the beginning of the experiments in control and HU groups, respectively. The measured VRBC in capillaries and anastomoses in the control group were 352 ± 42 and 229 ± 27 μm/s, respectively, and those in HU were 471 ± 38 and 289 ± 34 μm/s, respectively (Fig. 6B; see movies 3 and 4; supplemental data can be found at http://jap.physiology.org/cgi/content/full/00961.2004/DC1). In both control and HU groups, the VRBC in anastomoses was significantly lower than that in capillaries (both P < 0.05). The capillaries and anastomoses diameters with flowing erythrocytes were 3.9 ± 0.2 and 3.7 ± 0.2 μm per the stack frame in the control group and 3.3 ± 0.2 and 3.4 ± 0.2 μm after HU, respectively. Both capillaries and anastomoses diameters with flowing erythrocytes were not significantly different between control and HU.

Apoptosis of capillary endothelial cells. DNA nick-end labeling in the soleus revealed, when muscle atrophy occurs, the presence of apoptotic nuclei in the endothelial cells. Endothelial cells were distinguished from interstitial and myocyte cells on the basis of von Willebrand factor staining (Fig. 6, A, B, and D). There was a significantly greater number of endothelial TUNEL-positive cells in capillaries and anastomoses after HU. The numbers of apoptotic endothelial cells were 7.3% in the atrophied muscle vs. 0.53% in the controls (P < 0.01; Fig. 6C). It should be noted that the TUNEL-positive cells were seen predominantly in anastomoses (Fig. 6D).
DISCUSSION

In this study, we visualized 3D ex vivo and in vivo architecture of capillary network and hemodynamics in rat soleus muscle with and without 2-wk HU. Oxygen supply for muscle is maintained by vascular network, and therefore regression of capillaries and anastomoses is important to adjust the blood flow for decreased oxygen demand, e.g., after HU. Although the present study mainly focused on structural and hemodynamic changes of capillary network and did not provide the basic molecular mechanism of microvascular regression after HU, it is important to analyze this adaptation phenomenologically to understand physiological significance. We also demonstrated that the characteristics of apoptotic degradation of capillary architecture, i.e., predominance of vascular regression in interconnecting anastomoses after HU, causing a decrease in net capillary flow.

3D capillary architecture in skeletal muscles. To characterize the capillary structure change after HU more integratively, we projected capillary area, number of capillaries, and tortuosity using CLSM in soleus muscle. Earlier papers reported that the mean capillary diameter under control conditions measured by two-dimensional histochemical study was 5.3 μm (16) and 6.9 μm (36), which were in good agreement with our 3D observation, i.e., 5.8 μm. After HU, however, the 3D capillary diameter decreased significantly to 3.1 μm, which is consistent with earlier reports (16, 36). The reported critical diameter for rat red blood cell passage through capillaries was 2.5 μm (11). If this value is valid in our study, we estimate that only 1% of the capillaries may display plasma-only flow, but 30% of anastomoses show plasma-only flow in the control group. After HU, 33% of capillaries and 86% of anastomoses may exhibit plasma-only flow, suggesting a remarkable increase in plasma flow channels over red blood flow channels, especially in anastomoses. These findings suggest a remarkable decrease in oxygen supply to the muscle fibers after HU.

Changes in capillary 3D structure due to atrophy. Previous studies have reported that muscle atrophy is associated with a decrease in the C/F ratio (13, 31, 32) or in the capillary...
diameter (16, 36). Desplanches et al. (7) reported that hindlimb suspension of rats for 5 wk resulted in a decrease of 37% in the C/F ratio but an increase of 48% in capillary density. HU causes atrophy of skeletal muscle fibers, reducing the muscle mass (7, 28). If the degree of muscle atrophy is greater than that of capillary regression, the capillary density should increase despite the decrease in the C/F ratio. This was the case in our study. Although earlier reports (13, 31, 32) indicated the decrease of the C/F ratio after HU, they did not focus on the regression of anastomoses. Our 3D observation clearly indicates that the regression of microvessels in HU is greater in the anastomoses than in capillaries, resulting in a significant decrease in blood flow in the capillary network.

Brey et al. (3) stated that two-dimensional analysis does not allow detailed quantitation of complex vascular parameters such as tortuosity. In our present 3D study, we could evaluate the effects of HU on capillary tortuosity with the tortuosity index. We found that HU decreased CV in muscle volume unit, which compositely reflects the number, diameter, and tortuosity of capillaries. In our experiments, the decrease of tortuosity resulted in a decrease of CV in muscle volume unit despite an increase in the number of capillaries. Therefore, structural change of capillaries to straight configuration is an important morphological change caused by HU because it may decrease the ability of oxygen delivery. Our result of decreased tortuosity was compatible with an earlier report with transmission electron microscopy (26) that showed that the wave-like structure of capillaries changed into a straight configuration in the immobilized soleus. The vascular endothelial cells in atrophied muscles were seen to be TUNEL positive with nuclear fragmentation, suggesting that vascular endothelial cell apoptosis is an important factor in the changes in the remodeling of capillary network after HU. The apoptotic process was involved more in anastomoses, but the capillary straightening after HU might be also related to an apoptotic process.

McDonald et al. (21) examined the changes in the rat soleus blood flow after acute and chronic HU using microspheres and showed that blood flow was reduced. We suggest that HU caused a reduction in blood flow, which may have changed the number, diameter, and tortuosity of capillaries, through an apoptotic process.

Analysis of capillary \( v_{RBC} \). The rat skeletal muscle \( v_{RBC} \) has been reported to range widely from 200 to 700 \( \mu m/s \) (4). In this study, the mean capillary \( v_{RBC} \) measured with an intravital microscope was 363 \( \mu m/s \) and the mean anastomosis \( v_{RBC} \) (210 \( \mu m/s \) ) was significantly lower. Dawson et al. (4) reported that the \( v_{RBC} \) was slower in slow-twitch fiber muscles than in fast-twitch fiber muscles. Because the soleus muscle contains the highest percentage of slow type I muscle fibers (89%) of the hindlimb muscles (1), it may exhibit slightly lower velocities among the reported range. The increase of \( v_{RBC} \) in atrophied muscles of our HU group was consistent with the report of Tyml et al. (32). They reported that tetrodotoxin-induced muscle atrophy was associated with an increase in \( v_{RBC} \) in the extensor digitorum longus muscle. Because of plasma skimming, i.e., the number of capillaries that include only plasma increased after HU, the hematocrit should increase in the capillaries with red blood cells. This may increase the \( v_{RBC} \) because the capillaries with higher hematocrit exhibits higher velocities (5). Also, atrophied muscle may decrease mechanical compressive force on the capillaries, causing the increase in \( v_{RBC} \) as well as other factors, e.g., reduction of the number of anastomoses.

In conclusion, we found that HU caused reductions in capillary diameter and tortuosity, particularly in anastomoses, in association with vascular endothelial cell apoptosis. Hemodynamically, plasma skimming increased and \( v_{RBC} \) increased after HU. These results reveal HU-induced adaptive remodeling in capillary networks.

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