Microvascular dynamics regulate post-ischemic muscle damage and regeneration in experimental hindlimb ischemia

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Abstract

Tissue microvasculature is known to remodel in response to ischemia. Yet, how this remodeling contributes to the process of post-ischemic recovery is still not completely understood. We studied how microvascular changes relate to post-ischemic muscle repair. Muscle-level microvascular alterations of blood flow and hemoglobin oxygenation, and post-ischemic myofiber and capillary responses were analyzed in aged, healthy C57Bl/6J mice (n = 48) and aged, hyperlipidemic LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice (n = 69) after induction of acute hindlimb ischemia using contrast ultrasound, photoacoustic imaging, and histological analyses, respectively. Microvascular responses leading to successful post-ischemic muscle repair in C57Bl/6J mice included an early capillary dilation phase preceding the return of arterial driving pressure followed by an increase in capillary density that further supported satellite cell-induced muscle regeneration. Failure of initial capillary enlargement due to a life-long moderate hypercholesterolemia in LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice led to inability to recover arterial driving pressure and resulted in an increase in distal necrosis, chronic tissue damage and a delay in overall recovery after ischemia. To conclude, these data reveal the important role of transient capillary enlargement in initiating a cascade of capillary events that are crucial for a successful post-ischemic muscle recovery. Essentially, the observed dynamic nature of the post-ischemic capillaries should be considered when designing novel treatments targeting the microvasculature in ischemic diseases.

Introduction

The fundamental task of an organ-serving vasculature after an arterial occlusion is to re-establish tissue perfusion as soon as possible to minimize ischemic tissue damage. The central role of the collateral arterial network in restoring blood flow to ischemic skeletal muscle tissue has been well characterized [1, 2]. Instead, the role of microvascular angiogenesis in this process has been considered almost negligible [1]. In general, upon an ischemic insult a complex network of responses is activated at the tissue level initially by hypoxia and later by ischemia-induced tissue damage. These responses promote angiogenesis and acute inflammation in the affected muscle [3, 4] leading to increased capillary density. Also, the crosstalk between capillary endothelial and myogenic satellite cells during muscle regeneration further induces angiogenesis [5, 6]. The presence of cardiovascular risk factors, such as increasing age and hypercholesterolemia, has also been associated with impaired arteriogenic/angiogenic regenerative capacity under ischemic conditions [7–9]. The tissue microvasculature is thus known to undergo remodeling in response to ischemia and likely does so for a purpose. How this remodeling is exactly related to the tissue recovery process is still not completely understood and may limit the translation of experimental pro-angiogenic therapies to the clinic.

In this study, the contribution of the microvasculature in post-ischemic blood flow and tissue recovery after acute hindlimb ischemia in aged, healthy C57Bl/6J mice and aged, hyperlipidemic LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice were investigated by using a high-resolution contrast enhanced ultrasound, state-of-the-art photoacoustic imaging, and histological analyses. We demonstrate that ischemic muscle...
capillaries undergo dynamic changes that are associated beyond muscle regeneration also with changes in blood flow parameters, microvascular hemoglobin oxygenation as well as with severity and duration of the ischemic damage. These data add to our understanding of the importance of microvasculature controlling the fate of skeletal muscle tissue after ischemia.

Materials and Methods

Experimental animals

Healthy C57Bl/6J mice (n = 48, The Jackson Laboratory, USA) and hyperlipidemic LDLR−/−ApoB100/100 mice (n = 69) with a lifetime of a 3-fold increase in blood cholesterol levels and a humanized lipoprotein profile, predisposing the animals to develop atherosclerosis even on a regular chow diet [10] were used. Notably, all mice were of old age (8–21 months) as advanced age is one of the most important risk factors for poor recovery and to better represent the human population suffering from ischemic diseases. Mice were subjected to unilateral acute ischemia in the right hindlimb via permanent ligation of both common femoral artery and vein proximal to the origin of the profound femoral artery branch. The femoral nerve was left undamaged. For the surgical procedure and imaging, the mice were anesthetized with isoflurane inhalation (induction: 4.5% isoflurane, 450 ml min⁻¹ air, maintenance: 2.5% isoflurane, 250 ml min⁻¹ air; Baxter International). Post-operatively, the mice were treated with analgesia (Rimadyl, 10mg/kg, Pfizer). All experimental procedures for animal studies described here were approved by The National Animal Experiment Board of Finland (license number: ESAVI/5343/04.10.07/2014) and carried out in accordance with the guidelines of The Finnish Act on Animal Experimentation. The animals were kept in standard housing conditions and fed on a standard chow diet in the National Animal Laboratory Center of the University of Eastern Finland, Kuopio, Finland.

Contrast enhanced ultrasound imaging of skeletal muscle perfusion

Resting blood flow in calf muscles was monitored and quantitatively measured pre-operatively and 0, 1, 4, 7, 11 and 29 days after the surgery with Acuson Sequoia 512 ultrasound system and a 15L8 transducer (Siemens) using contrast enhanced Power Doppler (CED; frequency = 14MHz, power = -5 dB, Doppler gain = 50) and Cadence contrast pulse sequencing (CEU; frequency = 14 MHz, power = -8 dB, mechanical index = 0.25, CEU gain = 0 and depth = 20 mm) software [11]. Whereas CED imaging allows measurement of the flux of red blood cells primarily through high pressure arteries and large veins, CEU imaging enables measurement of real-time tissue perfusion at a microvascular level with superior spatial resolution showing blood flow in vessels with a diameter of 10 to 20 µm in addition to bigger vessels [11, 12]. After isoflurane anesthesia both hindlimbs, operated and contralateral, were placed in a prone position over the holder, fixed with tape and immersed in Aquasonic ultrasound transmission gel (Parker Laboratories Inc.). The transducer was placed on top of the calf muscle bundle in a transverse position. Perfusion video clips (20 seconds in length, 15 clip frames / 1 second) were recorded upon the administration of an intravenous bolus injection of 50 µl of SonoVue contrast agent (a sulfur
hexafluoride gaseous core in a phospholipid shell, approx. $2 \times 10^8$ bubbles/ml, mean size 2.5 µm, Bracco) via the jugular vein. CED and CEU peak signal intensities (dB; relative to both blood flow and volume) of the video clips were quantified with Datapro software (Noesis). In addition, contrast arrival time, i.e., the time (in seconds) from the administration of the bolus injection to the arrival of the contrast agent into the imaging plane, was calculated from CEU video clips as a surrogate of changes in arterial driving pressure. In cases in which the contrast agent did not arrive at the imaging plane within the 20-seconds-measurement window, the maximal time was recorded for the arrival time.

**Photoacoustic imaging of microvascular hemoglobin oxygenation**

Microvascular hemoglobin oxygenation in calf muscles was monitored and quantitatively measured pre-operatively and 0, 1, 4, 7, 11 and 29 days after the ischemia operation with Vevo 2100 LAZR photoacoustic imaging (PAI) system equipped with a linear-array transducer (LZ250, center frequency = 21 MHz, 256 elements; VisualSonics Inc.) [13]. The measurements were made in oxy-hemoglobin quantification mode with the following parameters: frequency = 21 MHz, PA Gain = 50 dB, 2D Gain = 18 dB, depth = 20 mm, width = 23.04 mm and dual laser wavelength = 750/850 nm. The method has already been used in several applications for non-invasive measurement of microvascular blood oxygenation levels in animals and humans [14–18]. Upon isoflurane anesthesia both, the operated and contralateral hindlimb were positioned in similar fashion to contrast enhanced ultrasound imaging and immersed in Aquasonic Clear ultrasound transmission gel (Parker Laboratories Inc.). Upon placement of the transducer across both calves, transverse plane photoacoustic clips superimposed on B-mode ultrasound clips were acquired. Average microvascular oxygen saturation of hemoglobin at a muscle level ($mHbO_2\%$) was measured from at least 30 clip frames using Vevo LAB software v1.7.2 (VisualSonics Inc.).

**Histological approaches**

After euthanization with carbon dioxide, mice were perfusion-fixed through the left ventricle with 1% paraformaldehyde. The posterior calf muscle bundle, including two-headed gastrocnemius, plantaris, and soleus muscles, was immersion-fixed in 4% paraformaldehyde in 7.5% sucrose (pH 7.4) for 4 h and rinsed in 15% sucrose (pH 7.4) for at least 12 h. After fixation, the samples were embedded in paraffin and prepared to 4 µm thick transversal sections.

To analyze ischemia-induced myofiber changes, calf muscle bundle sections were stained with hematoxylin-eosin (HE). Images of the histological sections were taken with Olympus AX-70 light microscope (Olympus Optical) at 1.25x magnification. The entire cross-sectional muscle area was examined using analySIS imaging software (Soft Imaging System GmbH) and the following six areas, representing different myofiber morphologies (Fig. 1) were measured: 1) normal area; 2) rounded myofibers; 3) necrosis; 4) early regenerative changes; 5) advanced regeneration; 6) late regeneration. Each area was calculated as a percentage of the whole cross-sectional muscle area. The damaged
muscle area was calculated as the sum of areas 2–3. The regenerative muscle area was calculated as the sum of areas 4–6.

For immunohistochemical assessment of post-ischemic muscle capillary reactivity, calf muscle bundle sections were immunostained with antibodies against CD31 [platelet endothelial cell adhesion molecule (PECAM-1), dilution 1:25, rat anti-mouse monoclonal CD31, MEC 13.3, BD BioSciences Pharmingen] and α-SMA (dilution 1:50, anti-alpha smooth muscle actin-Cy3 mouse monoclonal, clone 1A4, Sigma-Aldrich). For all stainings biotinylated rabbit anti-rat secondary antibody (dilution 1:200, Vector Laboratories) was used. Avidin-biotin-horseradish peroxidase system (Vector Laboratories) with 3,3’-Diaminobenzidine (DAB) as a chromogen (Zymed) was used to visualize immunoreactivity for light imaging. Tyramide signal amplification (TSA, PerkinElmer) was used for an enhancement of the signal. Calf muscle slides were mounted with Permount (TermoFisher Scientific) for brightfield imaging or Vectashield antifade mounting media with DAPI (Vector Laboratories) for fluorescent imaging. Fluorescent imaging was performed with Zeiss LSM700 confocal microscope (Oberkochen, Germany) where 405/488/555 nm diode lasers together with the appropriate emission filters were used (20×/0.5 PlanApo objectives, 1024×1024 frame sizes).

**Capillary measurements**

To analyze post-ischemic muscle capillary responses, images of the CD31 stained histological sections were taken with Nikon H550L light microscope (Tokyo, Japan) and NIS-Elements advanced research imaging software at 20x magnification. To characterize the type of alterations occurring in the capillary responses over time, only areas with myofiber histopathological changes (if applicable) were selected for the evaluation of CD31-based capillary reactivity as described before [19]. Capillary area (endomysial CD31<sup>+</sup> mean vessel luminal area in µm<sup>2</sup>), capillary density (endomysial CD31<sup>+</sup> vessels/muscle area mm<sup>2</sup>), and capillary size distribution [fraction (%) of endomysial CD31<sup>+</sup> vessels bigger than an average normal capillary, here defined as 33 µm<sup>2</sup> based on counting of 12326 capillaries from intact muscle sections of C57Bl/6J mice] were measured from two to three fields for each calf muscle using NIS-Elements software. Calf muscle bundle cross-sections derived from intact C57Bl/6J mice (n = 6) and LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> mice (n = 8) were used to define the baseline for the corresponding mouse strains. All measurements were performed in a blind manner.

**Statistical analysis**

All statistical analyses were performed using SPSS software (version 27.0 for Windows, SPSS Inc.) and p < 0.05 was considered as statistically significant. Data were expressed as mean ± standard error of the mean (SEM) with individual data points displayed on top of the plots. Significant changes within each strain were assessed using Kruskal-Wallis test followed by Mann-Whitney U test. The difference in prevalence of necrosis between the two mouse strains was assessed using Chi-squared test.

**Results**
The “favorable” and “delayed” patterns of ischemic damage and regeneration

Following acute ischemia operation, presentation of macroscopic signs of ischemia was most commonly seen in C57Bl/6J mice at day 1 but in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice at day 4. The most prevalent macroscopic symptoms were swelling and/or stiffness of the operated hindlimbs and were observed in 18% and 39% of C57Bl/6J and LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice, respectively. More severe symptoms, such as toe/toenail necrosis (2% in C57Bl/6J and 28% in LDLR\(^{-/-}\)/ApoB\(^{100/100}\), Chi-squared, \(p < 0.001\)) or toe autoamputation (5% in both strains) were less common or rare, respectively. Histologically, myofiber responses to acute ischemia in C57Bl/6J mice were presented in two phases – an initial phase of ischemic damage followed by a phase of regeneration. HE-analysis of the skeletal muscles (Fig. 1) revealed that on day 1, on average 48% of the calf muscle in C57Bl/6J was acutely injured by the operation (\(p < 0.05\); Fig. 2A). On day 4, on average 11% of the total muscle area was displaying necrosis (Fig. 2A). The regenerative changes (Fig. 1) were first presented on day 4, persisted at least until day 11 (Fig. 2B) and led to the complete recovery of the myofiber morphology by day 29 in C57Bl/6J mice (Fig. 1). In LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice, the ischemic muscle damage already presented two phases. Acute damage on day 1 was detected on average in 17% of the calf muscle area, whereas a second peak of damage occurred on day 11 affecting on average 9% of the calf muscle (\(p < 0.05\); Fig. 2C). Hallmarks of early regeneration (Fig. 1) in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice were observed as early as on day 4 but persisted until day 11 leaving the calf muscles still under repair on day 29 (\(p < 0.05\); Fig. 2D). Unlike C57Bl/6J, the LDLR\(^{-/-}\)/ApoB\(^{100/100}\) muscles on day 29 also showed atrophic, non-regenerating myofibers as indication of possible chronic damage resulting from the acute ischemia under hyperlipidemic conditions (Fig. 2E). The ischemic damage and regeneration in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice appeared therefore delayed, whereas C57Bl/6J mice displayed a more favorable, swift recovery regardless of old age.

**Skeletal muscle blood flow recovers under decreased arterial driving pressure**

CED and high-resolution CEU ultrasound were used to differentiate between arterial/venous and microvascular blood flow, respectively (Fig. 3A). Whereas both methods uniformly displayed significant reduction in blood flow after ischemia operation, the level of post-operative flow as well as the detection of flow recovery differed between the methods. In C57Bl/6J mice, CED showed no significant recovery of large arterial/venous flow in the operated hindlimbs throughout the follow-up (Fig. 3B). Instead, high-resolution CEU imaging in C57Bl/6J mice showed some contrast signal appearance immediately after the operation and a recovery of microvascular flow to pre-operative values as early as 4 days after the operation (Fig. 3C). Importantly, CEU contrast arrival time in C57Bl/6J mice recovered back to baseline levels only 7 days post operation, reflecting the recovery of arterial driving pressure (Fig. 3D). Also, in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice CED flow remained significantly reduced throughout the 29-day follow-up (Fig. 3E) and CEU contrast signal was restored to baseline levels 4 days after the operation (Fig. 3F). However, CEU contrast arrival time in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice remained significantly reduced.
throughout the whole 29-day follow-up (Fig. 3G). The recovery of skeletal muscle blood flow on the level of the microvasculature, therefore, seems to take place under decreased arterial driving pressure and in the absence of large arterial flow in both studied mouse strains. Arterial driving pressure was recovered in C57Bl/6J but not in LDLR<sup>−/−</sup>/ApoB<sup>100/100</sup> mice, based on CEU contrast arrival time.

**Oxygen delivery and its tissue demand affect post-ischemic microvascular hemoglobin oxygenation**

To assess the functionality of the tissue microcirculation after ischemic injury, PAI was used to measure alterations in microvascular hemoglobin oxygen saturation (mHbO<sub>2</sub>%) in the affected calf muscles (Fig. 4A). Immediately after the operation, mHbO<sub>2</sub>% in the operated hindlimbs significantly decreased from pre-operative values as expected, reflecting the suddenly reduced arterial inflow of oxygenated blood and increased oxygen extraction from hemoglobin in the acutely ischemic muscles. However, only hours after the operation mHbO<sub>2</sub>% in individual C57Bl/6J mice reached baseline values (Fig. 4A), leading to the normalization of mean mHbO<sub>2</sub>% already by day 1 (Fig. 4B). Interestingly, the normalized mHbO<sub>2</sub>% in C57Bl/6J mice was maintained only until day 4, after which a second significant decrease in mHbO<sub>2</sub>% was detected on days 7 and 11. mHbO<sub>2</sub>% recovery in LDLR<sup>−/−</sup>/ApoB<sup>100/100</sup> mice took place only on day 7 and thereafter again significantly decreased 11 days post-surgery (Fig. 4C). In both strains, the second reduction in mHbO<sub>2</sub>% seemed to take place in the presence of fully restored microvascular blood flow, suggesting increased oxygen demand of the tissue rather than reduced arterial delivery of oxygenated blood. However, the recovery of mHbO<sub>2</sub>% under still significantly reduced muscle blood flow in C57Bl/6J mice on day 1 could suggest impairment of oxygen extraction in the severely hypoxic muscles.

*Initial capillary enlargement launches a cascade of post-ischemic microvascular changes that leads to increased capillary density in C57Bl/6J but not in LDLR<sup>−/−</sup>/ApoB<sup>100/100</sup> mice*

CD31-immunostainings were used to analyze the responses of the muscle capillary bed to acute ischemia (Fig. 5A). In C57Bl/6J mice, capillaries in areas of muscle damage on days 1–4 post-operation were significantly enlarged when compared to the intact muscle (<p < 0.05; Fig. 5B). Especially on day 1, C57Bl/6J capillaries displayed relatively thin capillary walls as compared to capillaries in the healthy skeletal muscle (Fig. 5A, black arrow). Capillary size distribution, representing the fraction of capillaries undergoing significant enlargement, demonstrated that as much as 24% of endomysial blood vessels in ischemic muscles of C57Bl/6J mice had an increased luminal area on day 4 (<p < 0.05; Fig. 5C). The enlarged endomysial blood vessels in C57Bl/6J on day 1 also displayed no α-SMA-smooth muscle cell coverage observed by immunofluorescence (Fig. 5D, ischemic). In contrast, vessels of a similar caliber in the perimysial space of the intact muscles (small arterioles or venules) were α-SMA-positive (Fig. 5D, intact). During the period of capillary enlargement, capillary density in C57Bl/6J mice was initially decreased (<p < 0.05; Fig. 5E) but quickly normalized and further significantly increased along normalization of capillary size on days 7 to 11 (<p < 0.05; Fig. 5E). Possibly mediating this transition, CD31-positive endothelial pillars, resembling vascular structures in intussusceptive angiogenesis, were
detected crossing lumens of enlarged capillaries on days 1 and 4 (Fig. 5A, **yellow arrows**) and possible splitted vessels located in a very close proximity to each other on days 4 and 7 (Fig. 5A, **white arrows**). Capillary dilation, therefore, appears as the first response of the tissue capillary bed to ischemia leading to further normalization of the capillary size and an increase in capillary density that seems to mediate tissue recovery in C57Bl/6J mice. Capillaries in LDLR−/−/ApoB100/100 mice neither in areas of muscle damage nor in areas of regeneration at any studied time point showed significant changes in the mean capillary area (Fig. 5F), capillary size distribution (Fig. 5G) or capillary density (Fig. 5H).

**Discussion**

This study offers an explanation of how capillary remodeling may contribute to post-ischemic muscle damage and repair after experimental arterial occlusion. A sequence of dynamic microvascular responses was associated with successful post-ischemic muscle repair in aged but otherwise healthy C57Bl/6J mice. Instead, the lack of microvascular responses in aged, hyperlipidemic LDLR−/−/ApoB100/100 mice was associated with a delay in overall post-ischemic recovery. Beyond muscle regeneration, the differential capillary responses in the two mouse strains were also linked to changes in blood flow parameters, microvascular hemoglobin oxygenation as well as to extent of the ischemic damage.

Capillary enlargement identified here as the primary post-ischemic capillary response (on days 1 to 4) in C57Bl/6J mice affected only about 20% of capillaries in the capillary bed. The quick, selective enlargement of capillaries could suggest the involvement of physical forces, such as collateral flow distribution in initiating this process. In support of flow-mediated capillary vasodilation, the walls of enlarged capillaries in C57Bl/6J mice were relatively thin on day 1. Through decreasing peripheral resistance, flow-mediated post-ischemic capillary enlargement could also facilitate collateral maturation. Enlarged capillaries allow high flow through the microvascular bed [20], which in turn can be expected to help the maturation of upstream nascent collaterals into high-pressure - enduring functional conduits through, e.g., shear stress mediated mechanisms [2, 21]. Supporting this conclusion, arterial arrival time of CEU contrast agent was recovered in C57Bl/6J but not in LDLR−/−/ApoB100/100 mice. Based on CEU imaging, the gradually decreasing but still significantly slower arrival of contrast agent on day 4 suggests that blood flow to the ischemic calf muscles in both strains was initially reinstated via relatively narrow collateral arteries with steadily increasing arterial driving pressure. The normalization of contrast arrival time on day 7 in C57Bl/6J mice could imply further growth and maturation of collaterals to the point of normalizing arterial driving pressure. Notably, upon the restoration of arterial driving pressure the effect of significant capillary enlargement also disappeared as capillary size distribution returned to normal in C57Bl/6J mice. Instead, the lack of capillary enlargement may have impaired proper collateral remodeling in LDLR−/−/ApoB100/100 mice, which resulted in inability to retrieve perfusion pressure, as indicated by the significantly delayed contrast arrival time still on day 29. Nonetheless, the lack of capillary enlargement in LDLR−/−/ApoB100/100 mice did not seem to affect the initial opening of collaterals as the return of tissue blood flow took place on day 4 similar to C57Bl/6J mice. Initial post-ischemic capillary enlargement, therefore, seems an important initiator of post-ischemic microvascular remodeling that could facilitate
collateral maturation and the normalization of arterial driving pressure rather than the initial recovery of tissue blood flow.

Capillary enlargement was also associated with paradoxically increased microvascular hemoglobin oxygenation in the acutely ischemic C57Bl/6J muscles. Computational modelling has predicted that the rate of oxygen diffusion from blood capillaries to myofiber mitochondria might be accelerated through a network of cell membrane phospholipids [22]. The small diameter of capillaries, therefore, could serve to maximize the contact area between cell membrane phospholipids of erythrocytes and capillary endothelium for optimal diffusion of oxygen to adjacent myofibers. Capillary enlargement detected in C57Bl/6J mice on day 1 reduced the contact area between red blood cells and capillary endothelium. According to Pias, this might paradoxically compromise oxygen delivery through enlarged capillaries and further exacerbate acute muscle damage. In support of this theory, in this study normalization of hemoglobin oxygenation was detected in the acutely ischemic muscles without fully recovered microvascular blood flow in C57Bl/6J mice. In contrast, the absence of initial capillary enlargement in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice did not seem to hamper oxygen extraction postoperatively as mHbO\(_2\) \% remained significantly decreased on days 1 to 4. However, a trend towards an increase in the fraction of capillaries undergoing some degree of enlargement in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice was observed on days 7 to 11. This coincided with the normalization of mHbO\(_2\) \% in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice on day 7 and with the time of the second significant peak of muscle damage observed in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice on day 11. Hence, capillary enlargement seems to be able to affect oxygen diffusion from erythrocytes to tissues and potentially could also cause direct tissue damage.

Interestingly in this study, the degree and timing of post-ischemic damage seen in the two mouse strains did not relate to the amount of blood flow, but rather to the extent of capillary enlargement and its impact on arterial driving pressure return. Despite equivalent reduction of post-operative blood flow in both strains, macroscopic signs of ischemic damage, such as swelling of the leg, were most common in C57Bl/6J on day 1 whereas most prevalent in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) only on day 4. Tissue edema is commonly associated with angiogenesis and capillary enlargement [20] and could explain the differential macroscopic findings also here. The presence of transient capillary enlargement in C57Bl/6J may have also protected from distal necrosis. Irrespective of similar recovery of post-operative microvascular blood flow on day 4, 28% of LDLR\(^{-/-}\)/ApoB\(^{100/100}\) whereas only 2% of C57Bl/6J mice showed distal necrosis. This may be explained by insufficient arterial driving pressure in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) not able to mediate enough blood to the most distal parts of the limb. The insufficient arterial driving pressure in LDLR\(^{-/-}\)/ApoB\(^{100/100}\) likely also contributed to the formation of chronic damage in the ischemic muscles. The role of capillary enlargement in post-ischemic recovery may thus be controversial. The initial phase of capillary vasodilation may increase acute tissue damage but may protect from more chronic damage through facilitating collateral maturation and the recovery of arterial driving pressure.
The phase of capillary enlargement rapidly ended in C57Bl/6J mice along the normalization of arterial driving pressure on day 7. After this, a period of increased capillary density (days 7 to 11) took over and displayed abundant myofiber regeneration. This clear switch of capillary response points towards the possible involvement of intussusceptive angiogenesis in expanding the capillary network. Increased blood flow has been previously found to initiate intussusceptive splitting of enlarged capillaries [23, 24]. As compared to sprouting angiogenesis, intussusception has been also reported to be both faster and more energy efficient [25], providing a possible benefit in ischemic tissues to expand the capillary network according to the needs of the regenerating muscle. Pointing towards intussusception, CD31-positive intraluminal pillars were found in enlarged capillaries on days 1 to 4. Later, on days 4 to 7, small “paired” capillaries were often spotted representing possible splitted daughter vessels. Supportive evidence of intussusceptive angiogenesis, being responsible for the regeneration of microvasculature in ischemic skeletal muscle, was also described in a recently published study by Arpino et al. [26]. The phase of increased capillary density in C57Bl/6J mice was also associated with another decrease in hemoglobin oxygenation during already recovered microvascular blood flow and arterial driving pressure. It is likely that this decrease in mHbO₂% was induced by the energy consuming process of satellite cell-induced muscle regeneration [27]. Adequate functional perfusion and optimal oxygen diffusion through small-sized capillaries is likely a preset for efficient regeneration. Activated satellite cells have been previously reported to proliferate near capillaries [5, 6] and to be actively involved in recruiting endothelial cells through VEGF signaling [6]. Similarly, in C57Bl/6J mice hallmarks of advanced regeneration, such as proliferation, maturation, and fusion of satellite cells, were exclusively seen in areas with increased capillary density, leading to a complete morphological recovery on day 29. Despite impaired capillary responses, LDLR⁻/⁻/ApoB¹⁰⁰/¹⁰⁰ muscles did also display regeneration. However, the regenerative process in LDLR⁻/⁻/ApoB¹⁰⁰/¹⁰⁰ was delayed and revealed signs of even chronic damage such as myofiber atrophy on day 29.

Conclusions

Based on these results, the activation of a “favorable” microvascular response is required for the successful post-ischemic tissue recovery. That is (1) an early capillary dilation phase that facilitates the return of arterial driving pressure prior to (2) an intussusceptive increase in capillary density supporting efficient oxygen diffusion and muscle regeneration. Failure of this microvascular response, again, leads to an inability to recover arterial driving pressure resulting in an increase in distal necrosis, induction of chronic damage and slowing down myofiber regeneration. In conclusion, microvasculature reveals an important role in regulating post-ischemic muscle recovery. Understanding the dynamic nature of the microvascular changes under ischemia should be considered when designing novel therapies targeting the ischemic microvasculature.

Declarations

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Author Contributions


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Competing interests

The authors declare no conflict of interest.

Ethics approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by The National Animal Experiment Board of Finland (license number: ESAVI/5343/04.10.07/2014).

References


Figures

**ACUTE POST-ISCHEMIC MYOFIBER CHANGES**

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**Figure 1**

Myofiber morphologies during post-ischemic damage and regeneration. Representative Hematoxylin-Eosin (HE) stained images displaying intact calf muscle and different stages of morphological changes
to myofibers following acute femoral arterial ligation. In intact muscles eosinophilic myofibers displayed an angular shape with peripherally located condensed, dark nuclei in addition to relatively uniform fiber diameter and organized muscle fascicles. Ischemic myofiber damage was presented either as rounding, i.e., a loss of polygonal shape and a pale eosinophilic sarcoplasmic coloring of myofibers with still preserved muscle fascicle organization, and/or necrosis, i.e., very pale non-nucleated rounded myofibers and a loss of muscle fascicle organization. Arrows indicate hypertrophic, non-condensed nuclei of activated satellite cells in the periphery of scattered rounded myofibers. Basophilic satellite cell rings surrounding necrotic myofibers were considered as signs of early regeneration. The appearance of small rounded myofibers with multiple hypertrophic, non-condensed centralized nuclei together with necrotic myofiber remnants and/or fusing together to form larger units marked advanced regeneration. Angularly shaped eosinophilic myofibers organized in muscle fascicles with centrally oriented, condensed, dark nuclei and significantly varying fiber diameter were considered late regeneration, and preceded completely recovered myofiber morphology. Scale bar, 50 µm.
Aged, healthy C57Bl/6J and aged, hyperlipidemic LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice displayed differential patterns of post-ischemic damage and regeneration. HE-based quantitation of A) myofiber damage and B) regeneration in aged, healthy C57Bl/6J mice. HE-based quantitation of C) myofiber damage and D) regeneration in aged, hyperlipidemic LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice. E) Post-ischemic recovery led to complete normalization of myofiber morphology in C57Bl/6J but not in LDLR\(^{-/-}\)ApoB\(^{100/100}\), where on top of still
late regenerative changes (D) also myofiber atrophy (asterisks) was detected on day 29 as shown in the HE-staining. *p < 0.05, **p < 0.01, ***p < 0.001 vs. intact muscle. Scale bar, 50 µm.

Figure 3

Post-ischemic restoration of microvascular blood flow took place under decreased arterial driving pressure in both strains, but arterial driving pressure further recovered only in C57Bl/6J mice. A) Contrast-enhanced Power Doppler (CED) and Contrast-enhanced ultrasound (CEU) were used to detect macro- and microvascular blood flow recovery, respectively. Dashed, white circles display the analysis area of a single leg cross-section in each image. Quantitation of changes in B) macrovascular blood flow using CED, C) microvascular blood flow using CEU and D) contrast arrival time reflecting arterial driving pressure in aged, healthy C57Bl/6J showing a recovery of tissue level microvascular blood flow on day 4 and a recovery of arterial driving pressure of day 7 but still significantly reduced CED signal on day 29. Quantitation of changes in E) macrovascular blood flow using CED, F) microvascular blood flow using CEU and G) contrast arrival time reflecting arterial driving pressure in aged, hyperlipidemic LDLR^{−/−} ApoB^{100/100} showing a recovery of tissue level microvascular blood flow on day 4 but still significantly altered CED signal and contrast arrival time on day 29. Dashed, red line in (B-G) indicates the baseline level. *p < 0.05, **p < 0.01, ***p < 0.001 vs. pre op values. The timing of phases of histological damage and regeneration (from Figure 2.) are displayed under the graphs (B-D and E-G) of the corresponding mouse strains.
Figure 4

Normalization of microvascular hemoglobin oxygenation happened unpredictably fast in C57Bl/6J mice but decreased again after normalization in both mouse strains. A) Photoacoustic imaging was used to non-invasively study changes in microvascular hemoglobin oxygenation (mHbO₂%). Pink circles on the images represent the analysis area of a single leg cross-section in each image. Scale bar, 2 mm. B) Quantitation of mHbO₂% in aged, healthy C57Bl/6J displays an initial normalization on day 1 followed by a significant decrease on days 7-11. C) Normalization of mHbO₂% in aged, hyperlipidemic LDLR⁻/⁻ ApoB¹⁰⁰/¹⁰⁰ takes place on day 7 after which mHbO₂% decreases again on day 11. Dashed, red line in (B-C) indicates the baseline level. *p < 0.05, **p < 0.01 vs. pre op values. The timing of phases of histological damage and regeneration (from Figure 2.), and recovery of microvascular CEU blood flow and contrast
arrival time (Tca) (from Figure 3.) are displayed under the graphs (B and C) of the corresponding mouse strains.

Figure 5

Initial capillary enlargement was followed by an increase in capillary density in C57Bl/6J mice, whereas LDLR⁻/⁻/ApoB¹₀₀/₁₀₀ mice displayed no change. A) Representative CD31 (brown) immunohistochemical stainings displaying morphological appearance of skeletal muscle capillaries in aged, healthy C57Bl/6J and aged, hyperlipidemic LDLR⁻/⁻/ApoB¹₀₀/₁₀₀ at different timepoints. Appearance of thin walled, enlarged capillaries (black arrow) was detected on day 1 in C57Bl/6J. These vessels often displayed CD31⁺ intraluminal pillar-like structures (yellow arrows). On days 4-7, small, paired capillaries were found next to each other (white arrows) in C57Bl/6J indicating possible daughter vessels formed through intussusceptive angiogenesis. Quantitation of B) capillary area and C) capillary size distribution as well as D) immunostaining with CD31 (green), α-sma (red) and DAPI (blue) demonstrate that the capillaries are enlarged and lack α-sma-positive smooth muscle cells in C57Bl/6J on days 1-4, which was followed by E) an increase in capillary density on days 7-11. Quantitation of F) capillary area, G) capillary size
distribution and capillary density showed no significant differences in LDLR⁻/⁻ApoB^{100/100}. Dashed, red line in (B-C, E-H) indicates the baseline level. *p < 0.05, **p < 0.01 vs. intact. The timing of phases of histological damage and regeneration (from Figure 2.), recovery of microvascular CEU blood flow and contrast arrival time (Tca) (from Figure 3.), and hemoglobin oxygenation recovery (mHbO₂% recovered) and hemoglobin oxygenation second decrease (mHbO₂% 2^{nd} drop) (from Figure 4.) are displayed below the graphs (B-C, E-H) of corresponding mouse strains. Scale bars, 50 µm.