Vascularity of myocardium and gastrocnemius muscle in rats selectively bred for endurance running capacity

Patricia E. Beighley,† Mair Zamir, Robert J. Wentz, Lauren G. Koch, Steven L. Britton, and Erik L. Ritman

1Department of Physiology and Biomedical Engineering, Mayo Clinic, College of Medicine, Rochester, Minnesota; 2Departments of Applied Mathematics and of Medical Biophysics, University of Western Ontario, London, Ontario, Canada; and 3Department of Anesthesiology, University of Michigan, Ann Arbor, Michigan

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Beighley PE, Zamir M, Wentz RJ, Koch LG, Britton SL, Ritman EL. Vascularity of myocardium and gastrocnemius muscle in rats selectively bred for endurance running capacity. Physiol Genomics 45: 119–125, 2013. First published December 11, 2012; doi:10.1152/physiolgenomics.00110.2012.—We tested the hypothesis that changes in the arteriolar branching architecture contributed to increased running capacity of rats subjected to two-way artificial selection for intrinsic aerobic endurance treadmill running capacity resulting in strains of low-capacity and high-capacity endurance rats. Hearts and gastrocnemius muscles were harvested from each strain, and the microvasculature’s branching geometry measured from micro-CT images. The vascular branching geometry of the hearts and skeletal muscle from the high capacity strain was indistinguishable from low-capacity rats. Our hypothesis was not supported. Neither remodeling nor an increase in arteriolar microvasculature branching appears to play a role in the enhanced performance of the high capacity rats. We are led to speculate that endothelial tolerance for shear stress and/or increased coupling of myocardial muscle fiber metabolic-to-contractile function is increased in the high-capacity runner strain to the effect of allowing either higher flow rate per unit volume of muscle or more efficient use of oxygen and nutrients in the high-capacity endurance rats.

arterial branching; metabolic efficiency; micro-CT; perfusion territory

ADAPTATION OF THE MAMMAL TO GREATER EXPENDITURE OF ENERGY, either through genetic alterations, chronic workload, or just growth due to maturation, can conceivably be accomplished by a range of mechanisms. Such mechanism would include increased oxygen delivery by increased blood flow, increased oxygen carrying capacity of the blood, reduced impediment to oxygen transfer across membranes in the lung, or from the blood to the intracellular contractile mechanism. If increased blood flow is a mechanism, then arteries would have to increase in diameter so as to reduce resistance, or, if not, the endothelium would have to tolerate higher shear stress with the increased velocity of blood flow. That blood vessels adapt to maintain endothelial shear stress within certain limits is consistent with the widespread finding that the cube of the radius of an artery is equal to the sum of the cubes of the radii of the two branches of that artery (16).

Koch and Britton used two-way artificial selection for intrinsic aerobic endurance treadmill running capacity in rats to make strains of low-capacity runner (LCR) and high-capacity runner (HCR) rats (14). In brief, two-way artificial selection of an heterogeneous rat population from the N:NIH stock produced rat strains differing in inherent aerobic capacity. We assessed endurance running capacity at 11 wk of age using run time and distance to exhaustion on a treadmill (15 degree incline; initial velocity 10 m/min and increased 1 m/min every 2 min) as parameters. The top 20% of each sex was randomly bred to produce the HCR strain, whereas the bottom 20% was mated to initiate the LCR strain. Each subsequent generation was stratified and bred in a similar fashion with precaution being taken to minimize inbreeding (1% per generation). The general goal of this selection process was to use this model system to evaluate the association of low aerobic capacity with increased disease risks at all levels of biological organization. After only six generations of selection the LCR and HCR rats differed in running capacity by 171% and the LCR rats were 16–20% heavier than the HCR rats. Initial physiological studies by Henderson et al. (5) and Howlett et al. (6) in generation 7 rats demonstrated that the HCR rats had a V̇O2max that was 11.8% higher than in LCR rats. This difference was explained by higher transfer of oxygen at the tissue level (increased extraction ratio and tissue diffusion capacity) with no evidence for participation by the heart or lung. Subsequent study of generation 15 rats by Gonzalez et al. (3) and Kirkton et al. (12) revealed that V̇O2max was now 50% higher in HCR rats compared with LCR rats and that this continued divergence was mostly produced by increases in cardiac output and pulmonary function. Gonzalez et al. (3) reported that peripheral tissue oxygen diffusive conductance increased in the HCR rats from generations 7 to 15, but not in the LCR rats. Although the HCR had a higher tissue oxygen extraction relative to the LCR at generation 7, this difference was not found at generation 15. This relative decline presumably occurred because of the offsetting effect of greater HCR blood flow on tissue oxygen extraction.

That change in mitochondrial function in LCR rats may also play a role in increased cardiac and skeletal muscle function is supported by the data of Wisloff et al. (21). Reduced molecular signaling, decreased muscle glycogen and triglyceride storage, as well as lower mitochondrial content in primarily white skeletal muscle may also be contributors (18).

Hussain et al. (9) found in generation 3 rats that hearts from HCR had a 48% greater cardiac output relative to the LCR as assessed by a Langendorff-Neely working heart preparation. Coronary flow was 40% higher in the HCR vs. LCR in these isolated heart studies. Hoydal et al. (7) showed that NOS-1-derived nitric oxide production accounts for some of the difference in cardiac contractility between LCR and HCR rats.
Here we examined the possibility that the arteriolar branching structure of LCR and HCR rats changes significantly to provide increased blood flow to the myocardium and skeletal muscle. A lack of change of branching architecture would provide increased blood flow to the myocardium and skeletal muscle. A lack of change of branching architecture would provide increased blood flow to the myocardium and skeletal muscle. A lack of change of branching architecture would provide increased blood flow to the myocardium and skeletal muscle.

Materials and Methods

The protocol for this animal study was approved by Mayo Clinic’s Institutional Animal Care and Use Committee.

Eight LCR and 8 HCR female rats for both 9th and 14th generations (G9 and G14) of selection were obtained from the NCRR Facility at the University of Toledo, Toledo, OH (Resource currently at the University of Michigan, Ann Arbor, MI).

Preparation of the specimens. The micro-CT imaging was performed on the hearts and gastrocnemius muscles removed from euthanized rats at 32 wk for the G9 and 24 wk for the G14 rats.

The rats were intravenously injected with 5,000 units of heparin in saline solution and after 5 min were euthanized with a lethal dose of intravenous pentobarbital. Next, the abdominal aorta and adjacent inferior vena cava were exposed and cannulated. The aorta was then infused with heparinized saline until clear fluid emerged from the inferior vena cava. The aorta was then injected with Microfil (Flow Tech, Carver, MA) (15) at 100 mmHg pressure until it emerged from the vena cava, at which point the aorta and inferior vena cava were ligated and the rat placed in a refrigerator overnight to allow for polymerization of the injected microfil. The next morning the heart and both gastrocnemius muscles were excised and immersed in a 10% formalin solution for 24 h. The specimens were then removed from the solution and patted dry and then immersed in low melting point paraffin wax.

Micro-CT scans. Each of the 32 heart and 32 gastrocnemius specimens was positioned on a computer-controlled rotating stage in our custom built micro-CT scanner (11). In any one rotational position they were exposed to X-ray until an image of sufficient signal was acquired, following which the charge-coupled device (CCD) imaging array was read out, and the data transferred to a computer memory. This process was repeated for each of the 360 angular steps around 360°. The X-ray tube consisted of a molybdenum anode and a zirconium foil filter that produced a quasi-monochromatic X-ray beam and then subjected to a modified Feldkamp cone-beam tomographic reconstruction program that generated the three-dimensional (3D) image of up to 10^7 20 μm on-a-side cubic voxels, the number of voxels depending on the size of the organ. In some of the gastrocnemius muscles, the proximal and/or distal ends could not be fully encompassed by this image. As a consequence, the volume of the gastrocnemius muscle that was imaged generally did not represent the true volume of the entire muscle.

In addition to these bench-top micro-CT scans, two hearts and two gastrocnemius muscles from each group were scanned at the Brookhaven National Laboratories’ Synchrotron micro-CT facility at 4 μm voxel resolution (2). No capillaries could be resolved because this would require a voxel resolution of 1–2 μm resolution.

Image analysis. The three-dimensional (3D) images were reconstructed by point spread function deconvolution (22) to correct for the imaging system’s modulation transfer function limitations. The 3D images were displayed and preprocessed with the Analyze (19) image analysis program. The opacified microvasculature was then segmented by virtue of its CT brightness values being much higher than those of the surrounding muscle. The segmented vascular trees were then analyzed by a program developed recently for that purpose (13). The core of that program is morphological erosions and dilations (4) of increasing size, which allow us to measure the volume of vessel segments of a given diameter. Erosion involves removing all voxels on the surface of a segmented blood vessel lumen, and dilation reverses that process by adding a layer of voxels to that surface. A copy of the segmented 3D-CT image was created, converted to grayscale (1 for the opacified vessel lumens and 0 for the surroundings), and then eroded once and dilated once. This left the larger vessels essentially unchanged but removed any vessels less than two voxels in diameter. A second pass over the image using two erodes followed by two dilates removed all vessels less than four voxels in diameter. Repeated iterations of this process remove increasingly larger vessels, and the process was repeated until all vessels had been removed from the image. After every erode/dilate cycle, the new image was subtracted from the previous erode/dilate image, leaving only the newly eroded vessels. This allowed us to calculate the volume (i.e., total number of voxels with value = 1) of all the vessel segments of that diameter. Finally, any data on vessels less than two voxels in diameter (i.e., <40 μm for the 20 μm voxel images and <8 μm for the 4 μm voxel images) were discarded.

While this process is fairly simple, the images used in this study were quite large, and standard erode/dilate programs took multiple hours to complete. In response to this, a new program was developed that used Manhattan distances (1) to quickly calculate the result of repeated erode or dilate operation. Using repeated erode/dilate calls on a data-set that requires n erode-dilate cycles would take n(n + 1) function calls to complete the analysis. With Manhattan distances, this can be done in only 2n function calls. Since the gastrocnemius datasets frequently required up to 25 erode-dilate cycles, the Manhattan distance method was an estimated 13 times faster than standard erode/dilate techniques for this data.

While erode/dilate analysis provides fast access to information about vessel diameters, it does not track the connectivity of the vessel branches that it is measuring. To account for this, a second program was used to provide vessel tree analysis (13). This program utilizes a fast-marching algorithm (20) to identify branch segments and tree endpoints. It then combined the branch segments into a vascular tree and converted any branches that had been labeled as trifurcations by splitting them into two successive bifurcations. After this, it generated a table of all branch diameters and hierarchical positions. These data were used to analyze the relationship between the radii of the “mother” vessel and its “daughter” branches.

The volume of myocardium perfused by a selected artery (whose lumen cross-sectional area was measured from the micro-CT image) was estimated by dilating the arterial tree downstream from each lumen cross section at sequential branch points along the selected artery (17). After each dilation step the total number of voxels in the dilated vessel was counted. When the spaces between the branches were filled by the dilation process then the rate of increase of the dilated vessel’s volume decreased. When this reduction in the rate of increase was detected, the dilated volume was taken to be the volume of the myocardium perfused by the arterial tree that was dilated. The volume of muscle within that piece of myocardium was then computed by subtracting the arterial lumen volume from the myocardial volume. As illustrated schematically (see Fig. 2, right), this process was repeated at each downstream branch point until the terminal branch was reached. An identical approach was used for the gastrocnemius muscle arterial trees.

The volume of the LV myocardium (including septum) was estimated by counting the voxels making up the segmented myocardium after the RV free wall was “removed” (10). Of all the G9 animals scanned, six of the HCR and six of the LCR rats had micro-CT images suitable for image analysis of both the heart and gastrocnemius muscle. The corresponding numbers for G14 rats was seven and five rats. An image was considered unsuitable if there were multiple
discontinuities in the contrast agent along any one vessel (due to a saline or air bubble within the Microfil polymer or a break in the polymer) or in some cases the lack of complete filling to the periphery (usually due to premature setting of the Microfil polymer and/or inadequate infusion pressure and/or due to a “blow-out” in which the polymer escapes through a rupture in the wall of the arterial tree).

RESULTS

Table 1 summarizes the vital statistics of each of the animals whose hearts and gastrocnemius muscles were studied. The HCR rats consistently had lower body weights and myocardial volumes relative to the LCR rats. Although the G14 and G9 rats were euthanized at 32 and 24 wk, respectively, this weight difference is not due to this age difference. Unpublished data of similar rat cohorts showed that the rats’ body weights plateaued after 24 wk. The increment of body weight between 24 and 32 wk was 3% in the LCR rats and 5% in the HCR rats. We could find only two rats in each HCR group with body weights above 200 g, whereas in the LCR rats, we had many with body weights above 200 g. The increment of body weight between 24 and 32 wk was 3% in the LCR rats and 5% in the HCR rats. We considered the heart weight-to-body mass relationship, at the same body weight, is similar for the HCR and LCR rats. Myocardial volumes divided by body mass yielded ratios that were not different between the LCR and HCR.

Figure 1 shows typical images obtained by our micro-CT of the vessels in the heart wall and within the gastrocnemius muscle. We analyzed both the entire vasculature and an isolated arterial “tree” (highlighted in color) within the heart and the imaged segment of the gastrocnemius muscle. The “isolated” vessel analysis allowed normalization of the vascular segments’ dimensions with respect to the lumen cross-sectional area of the root vessel of that isolated vascular tree.

In Fig. 2, the two left panels show how a binary representation of an isolated arterial tree is expanded (“dilated”) by adding voxels to the surface of the segmented tree until the space between the branches are filled. The added volume represents the volume of muscle perfused by that artery. This volume could also be normalized by relating it to either the lumen volume of the vascular tree and/or to the cross-sectional area of the lumen of the root vessel perfusing that tree. The right panel is a schematic of the progressive application of the dilation method applied at increasingly peripheral arteriolar perfusion territories, shown here for myocardium perfused by arterioles ≤40 μm, 41–80 μm, and 81–120 μm in lumen diameter. When the HCR vessel lumen volume distribution is plotted against the same parameter in the LCR rats, we get regressions as shown in Fig. 4. These regressions show that there is no significant difference between the HCR and LCR coronary and gastrocnemius arterial tree vessel volume distributions.

![Fig. 1. Computer-generated volume renderings of 1 view of the contrast-filled lumens of representative of 1 heart’s and of 1 gastrocnemius muscle’s vasculature. The flattened vessels in the heart image are the veins, and the round vessels are the arteries. The highlighted tree is an artery selected for analysis of its hierarchical branching geometry and inter-branch segment interconnectedness. Top right: highlights the selected coronary by itself. These arterial trees are color-coded for lumen diameter in which cyan (18–36 μm), light blue (37–72 μm), dark blue (73–108 μm), indigo (109–144 μm), purple (145–180 μm), magenta (181–216 μm), red (217–252 μm), orange (253–288 μm).](http://physiolgenomics.physiology.org/)

Table 1. Mean values for groups

<table>
<thead>
<tr>
<th>Generation</th>
<th>Body Weight, g</th>
<th>Myocardial Vol. (mm³)</th>
<th>Myocardial Vol., mm³</th>
<th>Running Best Duration, min</th>
<th>Running Best Distance, m</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>G9 LCR/G9 HCR</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>8</td>
</tr>
<tr>
<td>G14 LCR/G14 HCR</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>8</td>
</tr>
<tr>
<td>G9 HCR/G14 HCR</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.005</td>
<td>8</td>
</tr>
<tr>
<td>G9 LCR/G14 LCR</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>8</td>
</tr>
<tr>
<td>9 LCR</td>
<td>212.8 ± 13.6</td>
<td>2.265 ± 0.492</td>
<td>483.1 ± 54.9</td>
<td>14.7 ± 1.3</td>
<td>194.0 ± 22.1</td>
<td>8</td>
</tr>
<tr>
<td>9 HCR</td>
<td>171.7 ± 11.2</td>
<td>2.300 ± 0.541</td>
<td>394.0 ± 91.7</td>
<td>55.4 ± 4.9</td>
<td>1297.6 ± 184.0</td>
<td>8</td>
</tr>
<tr>
<td>14 LCR</td>
<td>217.9 ± 23.0</td>
<td>1.865 ± 0.499</td>
<td>405.3 ± 114.2</td>
<td>18.8 ± 2.0</td>
<td>268.6 ± 31.4</td>
<td>8</td>
</tr>
<tr>
<td>14 HCR</td>
<td>152.9 ± 9.4</td>
<td>2.008 ± 0.363</td>
<td>307.1 ± 60.3</td>
<td>88.9 ± 10.8</td>
<td>2846.8 ± 638.8</td>
<td>8</td>
</tr>
</tbody>
</table>

LCR, low capacity runner; HCR, high capacity runner; G, generation. P > 0.05 not significant (NS).
Figure 5 shows the relationship between the volume of muscle perfused by vessel interbranch segment of lumen cross-sectional areas at each branch point from the proximal root segment down to the distal terminal vessel segment in the HCR and LCR G14 rats. Although the HCR rats’ arteries seem to perfuse a larger volume of muscle than do the LCR rats’ arteries of the same cross-sectional area, there is sufficient variability in the relationships that statistically we cannot confidently distinguish the two populations. Note, however, that the myocardial and gastrocnemius arteries do perfuse different muscle volumes per arterial cross-sectional area. The lumen diameter at which there is a change in regression slope corresponds to the point at which the cross-sectional area, there is sufficient variability in the relationships that statistically we cannot confidently distinguish the two populations. Note, however, that the myocardial and gastrocnemius arteries do perfuse different muscle volumes per arterial cross-sectional area. The lumen diameter at which there is a change in regression slope corresponds to the point at which the
intramyocardial (and intragastrocnemius muscle) arterioles transition to the epicardial (and surface of gastrocnemius muscle).

**DISCUSSION AND CONCLUSION**

The most important finding of this study is the result that high endurance running and aerobic capacity in the HCR are achieved without alterations in the architecture of the microvasculature. This result has not been previously reported and is somewhat counterintuitive. It is important because past studies have been based largely on capillary density as a measure of the rate of blood flow. While our results did not include the capillaries, they suggest clearly that higher rates of blood flow were achieved in the HCR with no change in microvascular architecture.

Another important finding of our study is the remarkable difference seen in Fig. 5 between the “perfusion capacities” of epicardial and intramicrovessels serving the myocardial or the gastrocnemius muscles. This intrinsic difference has not been reported previously and is pertinent to our understanding of the functional aspects of vascular architecture, particularly in the heart.

The results show further that the volume of muscle perfused by a vessel of a given luminal cross-sectional area in the HCR rats was not statistically different from that relationship in LCR rats, hence the flow per unit volume of muscle must be greater in the HCR relative to the LCR rats. This leads us to speculate that the endothelial tolerance for shear stress is increased in the HCR strain, or conversely that it is reduced in the LCR rats.

This operation is consistent with the assessment of endothelial...
function in the two strains of rats by Wisloff et al. (21), who assayed nitric oxide-mediated (acetylcholine) vascular relaxation in isolated ring segments of carotid arteries. In this assay, higher vessel relaxation is interpreted as better endothelial function. For maximal absolute relaxation, the HCR rats demonstrated a 48% increase compared with the LCR rats. Furthermore, the concentration of acetylcholine that provoked a half-maximal response [medial effective concentration (EC50)] was 8.8-fold greater in LCR than HCR rats. These findings (that the HCR have increased vascular dilation in response to nitric oxide mediated factors) are consistent with the speculated reduction of endothelial shear stress at higher blood flow.

This study highlights the great utility of the “erode-dilate” method, which we have used to assess the perfusion territory of a microvessel. The digital nature of this technique makes it particularly useful because it can deal with digital images of the vasculature rather than requiring more invasive procedures. This method allowed us to observed that the volume of muscle perfused by a vessel of a given luminal cross-sectional area in the HCR rats was not statistically different from that relationship in LCR rats, hence the flow per unit volume of muscle must be greater in the HCR relative to the LCR rats. Another factor that may contribute to the extra endurance of the HCR is an increased capacity of the myocardial and gastrocnemius muscles to extract oxygen and nutrients from the blood (18).

Previous work (generation 7 rats) by Howlett et al. (6) found that total capillary and fiber numbers in the medial gastrocnemius were similar in HCR and LCR, but, because fiber area was 37% lower in HCR, the number of capillaries per unit area (or mass) of muscle was higher in HCR by 32%. Consistent with this, they also reported a positive correlation (r = 0.92) between capillary density and muscle O2 conductance. It follows therefore that the extra endurance of the rats is partly due to increased capacity of the myocardial and gastrocnemius muscles to extract oxygen and nutrients from the blood (18). Transport of oxygen from the air to mitochondria can be modeled as a series-coupled cascade composed of convective and diffusive steps via the lungs, heart, circulation, and muscles (7). In theory, divergent artificial selection for a complex trait works because contrasting allelic variation is concentrated at the extremes from one generation to the next, and the strength of selection is dependent upon the magnitude of the additive genetic variation (14). At least through G14 of selection, we have no evidence that genetic variation for remodeling of the vasculature participates as a feature explanatory of differential for oxygen transport between the LCR and HCR rats. However, as we did not image the capillaries we cannot address any possible differences in capillary network geometry.

Limitations

Our quantitation of microvascular branching architecture has several sources of inaccuracy. First, the Microfil contrast polymer injection into the arterial tree most likely maximally dilated the arteries, but we can’t be sure. Second, the spatial resolution of the micro-CT images is limited by the voxel size (cannot expect to resolve structures smaller than 2 voxels) and because of the limited modulation transfer function (MTF) resulting from the optical characteristics of the X-ray imaging process (i.e., X-ray source focal spot diameter, the X-ray-to-light conversion process within the crystal plate, and the inherent characteristics of the optical lens projecting the light image emitted from the crystal plate onto the CCD imaging array). For this reason, in this study in which we used 20 µm-sized voxels, we cannot expect to have meaningful data for microvessels <40 µm in diameter. For the 4 µm voxel data we cannot expect to resolve vessel lumens below 8 µm diameter. The variability in our measurements of arterial dimensions in the gastrocnemius muscle is higher than in the coronary arteries. This is possibly in part due to the fact that the “root” arteries in the gastrocnemius muscles are of smaller diameter than in the coronary arterial tree.

The image analysis method we used (13) to extract the vascular tree does compensate for the MTF characteristics of the scanner but is not exact. Another issue contributing to variability in our data is the biological variability, i.e., the branching geometry follows the “cube” rule on average but there is considerable variation about this value (23). Hence, it is not surprising that our results also show considerable variation. Nonetheless, despite this variation, our results are still consistent with the previous findings obtained with biomolecular methodologies (8).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.E.B. and E.L.R. performed experiments; P.E.B. and R.J.W. analyzed data; P.E.B., R.J.W., and E.L.R. prepared figures; M.Z. and E.L.R. interpreted results of experiments; M.Z., L.G.K., S.L.B., and E.L.R. edited and revised manuscript; L.G.K., S.L.B., and E.L.R. conceived and designed of research; L.G.K., S.L.B., and E.L.R. approved final version of manuscript; E.L.R. drafted manuscript.

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