Age-related changes in conducted vasodilation: effects of exercise training and role in functional hyperemia

Shawn E. Bearden,¹ Erik Linn,¹ Blair S. Ashley,² and Robin C. Loft-Wilson²

¹Idaho State University, Department of Biological Sciences, Pocatello Idaho; and ²College of William and Mary, Department of Kinesiology, Williamsburg, Virginia

Submitted 22 November 2006; accepted in final form 24 July 2007


To address hypothesis 1, young (4–5 mo), adult (12–14 mo), and old (19–21 mo) C57BL6 male mice were sedentary or given access to running wheels for 8 wk. Voluntary running distances were significantly different (in km/day): young = 5.8 ± 0.1, adult = 3.9 ± 0.1, old = 2.2 ± 0.1 (P < 0.05). In gluteus maximus muscles, conducted vasodilation was greater in adults than in young or old mice (P < 0.05) and greater in young sedentary than in old sedentary mice but was not affected by exercise training. Citrate synthase activity was greater with exercise training at all ages (P < 0.05). mRNA for endothelial nitric oxide synthase did not differ among ages, but endothelial nitric oxide synthase protein expression was greater in adult and old mice with exercise training (P < 0.05). Connexin 37, connexin 40, and connexin 43 mRNA were not affected by exercise training and did not differ by age. To address hypothesis 2, perfusion of the gluteus maximus muscles during light to severe workloads was assessed by Doppler microprobe at 3–26 mo of age. Maximum perfusion decreased linearly across the lifespan. Perfusion at the highest workload, absolute and relative to maximum, decreased across the lifespan, with a steeper decline beyond ~20 mo of age. In this model, exercise training does not alter conducted vasodilation and 2) muscle perfusion is maintained up to near maximum workloads despite age-related changes in conducted vasodilation.

endothelial; smooth muscle; gap junction; microcirculation; aging

METHODS

All procedures were approved by the Animal Care and Use Committee of Idaho State University and performed in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on male C57BL6 mice (23–35 g, n = 99; from Harlan, Indianapolis, IN, or Jackson Laboratory, Bar Harbor, ME). Mice were housed at 24°C on a 12:12-h light-dark cycle with ad libitum access to food and water. Chemicals for these experiments were purchased from Sigma-Aldrich or Fisher Scientific.

Experiment 1: to Test the Hypothesis That Exercise Training Enhances the Capacity for Conducted Vasodilation

Young (4–5 mo), adult (12–14 mo), and old (19–21 mo) mice were studied. Median lifespan is 24 mo for this strain. Three days after arrival, animals were housed individually for 8 wk in cages equipped with computer-monitored running wheels (Mini-Mitter, Bend, OR). Sedentary controls were kept in groups of three to five.

Intravital microscopy. The gluteus maximus muscle was prepared as previously described (4). Six trained and sedentary animals from each age group were studied (n = 36 total), one vessel per mouse. Rectal temperature was maintained at 37 ± 1°C using a heating pad with thermistor feedback. Po2 of the superfusion fluid was 10–11 Torr in the superfusion line and 28–29 Torr over the preparation (Strathkelvin model 781). With the use of transillumination, arterioles were observed through a ×20 objective (numerical aperture = 0.4) coupled to a video camera; final magnification on the CCTV monitor was ×1,663. Internal vessel diameter was measured with a video caliper (model 308A; Colorado Video, Boulder, CO) with spatial resolution of 1 µm. Data were acquired at 10 Hz using PowerLab and software (SSP, Chart 5; ADInstruments, Colorado Springs, CO). Second-order branches of the arteriolar network were studied because they govern...
the distribution of blood flow within the muscle. Because of the consistent arteriolar architecture among animals (4, 5), the same vessel branch in each preparation was studied, i.e., one vessel per animal. To evaluate conducted vasodilation, ACh was delivered as 1-μA pulses for 1,000 ms by microiontophoresis (model 260; World Precision Instruments, Sarasota, FL), 170–220 nA retaining current, and an Ag/AgCl reference wire as previously described (4). At the end of each experiment, maximum diameter was recorded (10−4 M sodium nitroprusside).

### Tissue collection and biochemistry.
For citrate synthase activity and endothelial nitric oxide synthase (eNOS) and connexin expression, six to nine animals per group were studied (n = 43 total). Gluteus maximus muscles were either 1) snap frozen in liquid nitrogen and stored at −80°C or 2) submerged in RNAlater and stored at −20°C until analysis. Citrate synthase activity was measured as previously described (6). For Western blotting, tissue proteins were separated by SDS-PAGE, blotted to polyvinylidene difluoride membranes, and probed with rabbit anti-mouse eNOS antibody (Transduction Laboratories, BD Pharmingen; 1:1,000) and rabbit anti-mouse connexin 37 (Cx37), connexin 40 (Cx40), and connexin 43 (Cx43) (purified and anti-sera; Alpha-Diagnostics and Chemicon; 1:100–1:2,000). Alkaline phosphatase anti-rabbit secondary antibody (Pierce, Rockford, IL; 1:20,000) was followed by chemiluminescent detection and band densitometry (FluorS; Bio-Rad). Control experiments included omission of primary antibody or preincubation with a 10-fold excess of peptide. Immunoprecipitation for connexin proteins in skeletal muscle was carried out with a commercially available kit according to manufacturer’s directions (Sieze Classic; Pierce) and the antibodies listed for Western blotting. RNA was isolated using the RNasy fibrous tissue MiniKit (Qiagen, Valencia, CA), and real-time PCR was performed at the Nucleic Acids Research Facilities at Virginia Commonwealth University using TaqMan one-step RT-PCR Master Mix reagent kit (Applied Biosystems). Samples were tested in triplicate with the following cycling conditions: 48°C for 30 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min; results were quantified with a relative standard curve generated with pooled RNA from control mouse hindlimb muscle. Probes and primers are listed in Table 1. No significant genomic DNA contamination was detected. mRNA expression for each gene was normalized to smooth muscle α-actin mRNA expression to control for potential differences in vascularity among treatment groups.

### Experiment 2: to Test the Hypothesis That Age-Related Changes in Conducted Vasodilation Are Reflected in Concomitant Changes in Functional Hyperemia

Mice (n = 17, 3–26 mo) were anesthetized and kept warm as in experiment 1. The animal was positioned on its right side, and the left knee and lower back were held in place, using a device built in our laboratory for this purpose, to minimize movement for isometric contraction. Skin overlying the gluteus maximus muscle was removed, two platinum-iridium wires were positioned alongside rostral and caudal edges of the muscle, and the preparation was covered with clear plastic wrap. Superfusion solution (see experiment 1) bathed the muscle during the time of exposure (∼1–2 min).

**Capillary perfusion.** A fine-needle Doppler probe (tip diameter = 480 μm; 1-mm-deep voxel) was positioned on the surface of the muscle using a micromanipulator, and data were acquired through Powerlab (8SP) at 100 Hz (Chart 5; ADInstruments). The probe was placed over a capillary bed (region of lowest resting readings) to determine perfusion through nutrient exchange vessels. One region supplied by the second-order arteriole vessel studied in experiment 1 was studied in each muscle though two regions were studied in four of the animals (one region supplied by a different second-order arteriole; 3.5, 11, 15.5, and 22 mo) to ensure consistent responses throughout the muscle. Data from the two regions differed by <10% and were averaged before statistical analysis. Muscle contraction was stimulated using a Grass Stimulator [0.2-ms square-wave pulses at 40 and 80 Hz for bouts of 1 min at 0.5, 1, 2, and 4 V with 3 trains/s and 50% duty cycle (i.e., each second contained 3 contractions interspersed with 3 relaxations of equal duration)]. These parameters mimic physiological contraction of this muscle and span low intensity through near-maximum workloads. Perfusion measurements were acquired during 1- to 5-s postcontractions; the perfusion reading remained stable during this time because recovery had not yet begun. At the end of the experiments, maximal perfusion was determined by sodium nitroprusside superfusion.

### Statistics
Data were compared by one-way ANOVA with Tukey’s post hoc analysis, across age and training conditions for experiment 1 and for the effect of contraction intensity on muscle perfusion in experiment 2. Linear regression was used to determine whether age was a significant predictor of muscle perfusion at any contraction intensity in experiment 2. Differences were deemed significant at P ≤ 0.05. Data are presented as means ± SE.

### RESULTS

#### Experiment 1

**Voluntary run training.** Mice voluntarily engaged in wheel running for the majority of the dark period but for very little of the light period of the day (Fig. 1). Daily accumulated distance decreased with age. The pattern of running was not significantly different among ages. The pattern of running (pooled data) was 5.0 ± 0.37 min of running with 5.4 ± 0.73 min of inactivity (zero wheel turns) for 4–7 h beginning within 10–15 min of lights off. Muscle mass was not significantly different among ages (68 ± 5 mg for all muscles). When muscles were homogenized for biochemistry, we acquired similar protein yields among ages.

**Response to run training.** Citrate synthase activity was significantly higher for run training vs. sedentary conditions. Values for the three age groups were (in units activity/g wet wt) as follows: young sedentary = 6.4 ± 0.3, young trained = 7.7 ± 0.9, adult sedentary = 6.2 ± 0.8, adult trained = 7.6 ± 0.6, old sedentary = 6.7 ± 0.7, and old trained = 8.2 ± 0.9.

### Table 1. Real-time PCR primer and Taqman probe sequences

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Forward Primer</th>
<th>Taqman Probe</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx37</td>
<td>NM_008120</td>
<td>CCGGCTCTCATCGGACATGAGCAAGACT</td>
<td>TCACTCTCTCCCTCTGTTGTCACGCTG</td>
</tr>
<tr>
<td>Cx40</td>
<td>NM_008121</td>
<td>TGGGCTCTCATCGGACATGAGCAAGACT</td>
<td>CATCGAGGTTACAGAGAAGA</td>
</tr>
<tr>
<td>eNOS</td>
<td>NM_008713</td>
<td>AATGGCCACCGCAGGACATGAGCAAGACT</td>
<td>TCACTCTCTCCCTCTGTTGTCACGCTG</td>
</tr>
<tr>
<td>SAA</td>
<td>NM_007392</td>
<td>CCGGCTCTCATCGGACATGAGCAAGACT</td>
<td>TCACTCTCTCCCTCTGTTGTCACGCTG</td>
</tr>
</tbody>
</table>

Cx, connexin; eNOS, endothelial nitric oxide synthase; SAA, smooth muscle α-actin.
These values are consistent with fast-twitch glycolytic muscle. eNOS mRNA (Fig. 2) was not changed with run training or age. Expression of eNOS protein increased significantly with run training in the adult and old groups. These responses show that run training recruited the gluteus maximus muscle and was sufficient to stress pathways for oxidative metabolism and that the muscle and vasculature adapted to these stresses.

**Connexin expression.** Preliminary immunolabeling showed positive staining for Cx37 and Cx40 but not for Cx43 (not shown). Connexin mRNA (Fig. 2) was not changed with run training or age. We were unable to quantify connexin protein expression.

**Conducted vasodilation.** Microiontophoresis of ACh resulted in near-maximal vasodilation at the local site at ~1 s after application and appreciable vasodilation at the conducted site (1 mm upstream), also at ~1 s. This rapid transmission is consistent with the electrical nature of conducted vasodilation (12, 13). As shown in Fig. 3, conducted responses in the young sedentary group were greater than in the old sedentary group; conducted responses in both adult groups (sedentary and trained) were greater than in any young or old group. Eight weeks of voluntary run training did not significantly affect the capacity to conduct vasodilation.

**Experiment 2**

**Capillary perfusion.** Individual data points are presented in Fig. 4. Maximum perfusion declined ~0.2% per month of age ($P < 0.05$). From 3–26 mo, perfusion at the highest workload declined 0.6% per month ($P < 0.05$, absolute) and 0.4% as a percentage of maximum ($P < 0.05$, relative). Beyond 20 mo of age, this rate steepened to 1.2% absolute and 1.0% relative.
In previous work in conscious rats, blood flow to the gluteus maximus was measured by the microsphere technique (1, 2, 17). These authors reported that blood flow to the gluteus maximus muscle increased in a roughly linear fashion from baseline through sprinting speeds (~105 m/min) with an approximately fivefold range (~30–150 ml·min⁻¹·100 g⁻¹) (1, 2, 17). Our data are remarkably consistent using the gluteus maximus of the anesthetized mouse. We observed an approximately fivefold range of muscle perfusion values from a baseline of (in arbitrary relative units) 45 to a contraction induced peak of 238 and a pharmacological maximum of 251. For seven of the eight contraction intensities, age did not change capillary perfusion until animals neared median lifespan. We hypothesized that changes in conducted vasodilation, increasing during the first half of the lifespan and decreasing over the second, would result in concomitant changes in muscle perfusion during contraction. This hypothesis was not supported. Therefore, if conducted vasodilation is necessary for functional hyperemia in this muscle, then even the lowest capacity for conduction, measured in mice up to 21 mo of age, is sufficient. A steeper decline in perfusion to workloads above 80% of maximum perfusion as median lifespan is approached suggests that declining capacity for conducted vasodilation may only impair muscle perfusion at very heavy and severe workloads.

We studied second-order arterioles because these vessels control the regional distribution of blood within the gluteus maximus muscle and may lie at the point of integration between sympathetic vasoconstriction (to maintain systemic blood pressure) and the local vasodilation (to permit perfusion of active muscle downstream) (25). Despite exercising for hours each night with substantial accumulated running distances, 8 wk of exercise training did not alter the capacity to conduct vasodilation in the gluteus maximus, which is a fast-twitch glycolytic muscle (Ref. 16, present citrate synthase data, Ref. 19). Others have shown that the vasomotor control of fast-twitch oxidative muscles, but not of slow- or fast-twitch glycolytic, is most affected by exercise training (20). Therefore, arterioles in more oxidative muscles may have a different response to exercise training.

The effect of exercise training on the expression of vascular connexins has not been previously examined. Oxidative stress and increased shear stress, two stressors that promote vascular remodeling during exercise training (15), have been shown to increase both mRNA and protein for Cx43 in cultured vascular smooth muscle cells and endothelial cells, respectively (3, 9, 10). In the present study, chronic exercise training did not alter the mRNA expression of Cx43 or of Cx37 or Cx40, which may indicate differences in responses between cultured cells and intact blood vessels in vivo or that the blood vessels had adapted to these stressors. Exercise training increased eNOS protein expression without a change in eNOS mRNA. Our attempts to quantify connexin protein expression were unsuccessful despite the use of a variety of isolation and purification methods. Therefore, we do not know whether exercise training changes connexin protein expression. The luminal surface area of the primary arteriolar network in this muscle is ~6 mm² (4). This network comprises ~60% of the muscle. The muscle also contains two smaller anastomozing arteriolar networks. We may estimate that the arteriolar network in the entire muscle has a luminal surface area of ~10 mm². From the present data, Ref. 19).
immunocytocchemical observations and previous reports (18), we expect that the bulk if not all of the connexin expression is confined to the endothelial monolayer of the arteriolar branches. In endothelial cell culture, a 35-mm dish has an area of ∼962 mm² and will yield ∼165 μg of protein. Our muscles may contain ∼1.7 μg of total endothelial protein. The limit of detection of our immunoprecipitation with silver staining is ∼5 ng. Therefore, the individual expression of Cx37 and Cx40 was <0.3% of the total cell protein.

Limitations

A function of conducted vasodilation may be to coordinate a drive for increased perfusion that originates from within capillary beds with dilation of vessels immediately upstream (8), fourth- and third-order arterioles. It is possible that conduction in these more distal branches was affected by exercise training. The present data demonstrate that exercise training does not affect conduction along the vessels responsible for regional control of blood flow.

Although ACh is considered the “gold standard” stimulus for evaluating conduction (21, 22), there is little support for its endogenous role in blood flow control. It is used because it reproducibly and consistently initiates vasodilation and conduction of vasodilation through endogenous vasomotor pathways. The details of these pathways are still being explored (e.g., Refs. 11, 24), although cell-to-cell communication through gap junctions is required (14). Therefore, the present study specifically addresses the role of exercise training in affecting the capacity for cell-to-cell conduction of vasodilation through gap junctions. This does not exclude the possibility that adaptations of other communication pathways may compensate to ensure that functional hyperemia remains intact, as we observed.

Conclusions

This study is the first to address the mechanistic requirement of conducted vasodilation during physiological changes in the capacity for conduction. Eight weeks of heavy exercise training does not affect conducted vasodilation in the gluteus maximus muscle of the mouse. Moreover, capillary perfusion during muscle contractions is maintained for all but the heaviest workloads. As animals age beyond 20 mo, diminishing capacity for conduction may impact perfusion near maximum intensity workloads. Muscle perfusion during light and moderate activity is preserved despite changes in conducted vasodilation from young to adult and adult to old. Collectively, these data demonstrate that the mechanisms governing skeletal muscle blood flow in support of light to heavy workloads are sufficient or adapt sufficiently to maintain perfusion despite changes in microvascular cell-cell coupling.

ACKNOWLEDGMENTS

We thank Caleb Hixson and Amanda Petersen for analysis of the mouse running data. We also thank Dr. Fred Risinger (Idaho State University) for the gift of some of the older mice used in these experiments.

GRANTS

This work was supported by National Institutes of Health Grant P20 RR-016454, American Heart Association Grant 0435339T, Idaho State University Grants 00602120 and FRC08167501, and The Borgenicht Program For Aging Studies and Exercise Science.

REFERENCES


Downloaded from http://ajpregu.physiology.org/ by 10.220.32.247 on March 31, 2017