Increased skeletal muscle capillarization independently enhances insulin sensitivity in older adults after exercise training and detraining

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Abstract

Intramuscular signaling and glucose transport mechanisms contribute to improvements in insulin sensitivity after aerobic exercise training. This study tested the hypothesis that increases in skeletal muscle capillary density (CD) also contribute to exercise-induced improvements in whole-body insulin sensitivity (M/I) independent of other mechanisms. The study design included 6-month aerobic exercise training followed by a 2-week detraining period to eliminate short-term effects of exercise on intramuscular signaling and glucose transport. Before and after exercise training and detraining, 12 previously sedentary older (65±3 years) men and women underwent research tests including hyperinsulinemic-euglycemic clamps and vastus lateralis biopsies. Exercise training increased VO_{2\text{max}} (2.2±0.2 vs. 2.5±0.2L/min), CD (313±13 vs. 349±18cap/mm^2), and M/I (0.041±0.005 vs. 0.051±0.007µmol/kgFFM/min), (P<0.05 for all). Exercise training also increased insulin activation of glycogen synthase by 60%, glucose transporter-4 expression by 16%, and 5’ AMP-activated protein kinase-α1 expression by 21%, but these reverted to baseline levels after detraining. Conversely, CD and M/I remained 15% and 18% higher after detraining, respectively (P<0.05) and the changes in M/I (detraining - baseline) correlated directly with changes in CD in regression analysis (partial r=0.70, P=0.02). These results suggest that an increase in CD is one mechanism contributing to sustained improvements in glucose metabolism after aerobic exercise training.
Introduction

Aging is associated with physical inactivity and lifestyle behaviors that contribute to vascular dysfunction and microvascular rarefaction. Skeletal muscle capillary density (CD) is lower in older compared to younger adults (1,2) and is associated with glucose intolerance and lower insulin-stimulated glucose uptake (3-5). This reduction in CD decreases the available surface area for diffusion of glucose into the interstitium (6,7) and may also limit insulin action (8). Therefore, strategies to increase skeletal muscle CD may ameliorate age-related declines in insulin sensitivity and glucose tolerance by enhancing muscle perfusion to promote glucose uptake and metabolism.

Interventions including aerobic exercise (AEX) training and weight loss reduce insulin and glucose responses to glucose tolerance tests (9,10), increase insulin-stimulated glucose uptake (11-13), and reduce progression to type 2 diabetes in insulin resistant people (14,15). AEX training also increases skeletal muscle CD (16,17) and we recently showed that AEX training and weight loss-induced increases in skeletal muscle CD are directly associated with improvements in glucose tolerance and insulin-stimulated glucose uptake in older adults with impaired glucose tolerance (18). However, it is often difficult to distinguish the contribution of CD to improvements in insulin sensitivity independent of intramuscular signaling mechanisms and significant weight loss.

Increases in skeletal muscle glucose transporter-4 (GLUT4) expression (11,19-23), 5’ AMP-activated protein kinase (AMPK) expression (24,25), and insulin activation of glycogen synthase (GS) (13,26-28) all contribute to increase insulin sensitivity after AEX training. While AEX training enhances all of these mechanisms, the effects are not all long-lasting. For example, 5-10 days of detraining causes a reversal of exercise-induced increases in skeletal muscle GS activity (29) and GLUT4 expression (19-22). Conversely, exercise training-induced increases in skeletal muscle CD are largely maintained for up to 12 weeks of detraining in endurance-trained subjects (30,31); Thus, we anticipated that 2 weeks of detraining after 6-month AEX training would eliminate AEX-induced improvements in intramuscular signaling and
glucose transport protein expression, allowing us to test the hypothesis that longer-lasting increases in CD independently contribute to improvements in insulin sensitivity in older adults. We assessed the responses of skeletal muscle CD and insulin sensitivity to AEX training and detraining, and we measured GS activity, citrate synthase activity, GLUT4 and AMPKα1 expression, and pAkt\textsuperscript{Ser473} as benchmark indices of the intramyocellular responses to training and detraining in older adults.
Subjects and Methods:

Subjects

Men and postmenopausal women 50-80 years of age were recruited from the Baltimore, MD area to participate in studies examining metabolic responses to AEX training. All subjects were previously sedentary (self-reported exercise less than 20 minutes on 2 or fewer days per week), non-smokers, and reported no previous diagnosis of diabetes or cardiovascular disease. Subjects were excluded if they had liver or renal disease, chronic pulmonary disease, cancer, or physical impairment that would limit exercise participation. The women in the study had not menstruated for at least 1 year and were not prescribed hormone replacement therapy. Subjects taking medications for hypertension or dyslipidemia were included if medically stable and if medications were not known to affect glucose metabolism. This study was approved by the Institutional Review Board at the University of Maryland School of Medicine and all subjects provided written informed consent.

Study Design

Before and after 6 months of AEX training, subjects underwent research testing consisting of body composition measurement, OGTTs, maximal exercise tests, and hyperinsulinemic-euglycemic clamps with basal and insulin-stimulated vastus lateralis biopsies (Figure 1). After AEX training, subjects underwent each metabolic test 24-36 hours after one of their usual exercise sessions. Subjects then stopped all exercise training for 2 weeks and repeated research testing at the detraining time point. Subjects were counseled to maintain body weight during all phases of the study.

AEX training and detraining interventions: Subjects exercise on motorized treadmills at the Baltimore Veterans Affairs Medical Center Geriatric Research, Education and Clinical Center exercise facility 3 times per week, 45 minutes per session, for 6 months. Exercise intensity was prescribed as a target heart rate range using the Karvonen formula (32) and heart rate was monitored during exercise using heart rate monitors (Polar Electro Inc., Lake Success, NY). AEX training began at a volume of 3 sessions/week of 20 minutes at 50% of heart rate reserve, and gradually increased to 45 minutes at ~75%
of heart rate reserve, a level maintained for >4 months.

**Research Testing**

**Hyperinsulinemic-euglycemic clamp:** Insulin-stimulated glucose uptake per unit plasma insulin (M/I) was measured as an index of insulin sensitivity after a 12-hour overnight fast using a 3-hour hyperinsulinemic–euglycemic glucose clamp (33,34) with an insulin infusion rate of 555 pmol/m²/min. Data are reported as M/I during the third hour of the clamp [µmol of glucose infused per kilogram of fat-free mass per pmol of plasma insulin per minute (µmol/kgFFM/pmol insulin/min)]. Plasma glucose levels were analyzed at 5-minute intervals using the glucose oxidase method (Beckman Instruments, Fullerton, CA). Plasma insulin levels were determined by radioimmunoassay (Millipore, St. Charles, MO). The mean insulin and glucose levels during the clamp were 1255±49pmol/L and 5.1±0.1mmol/L, respectively, and did not differ across time points (P≥0.4). Subjects were provided with all meals for two days preceding the clamp to control nutrient intake.

**Vastus lateralis biopsies:** Bergström needles (Stille, Solna, Sweden) were used to obtain biopsies from the right vastus lateralis, approximately 12-14 cm above the patella as previously described (35). Muscle samples were obtained immediately prior to, and two hours after the onset of insulin infusion. One portion of each muscle sample was rapidly embedded in OCT-tragacanth gum mixture and frozen for histochemical analyses. The remaining muscle was immediately freeze-clamped using tongs frozen in liquid nitrogen and stored at -80°C; this portion of the sample was then lyophilized for 48 hours and then dissected free of connective tissue, fat, and vascular cells prior to protein and enzyme assays.

**Skeletal muscle CD:** Muscle was sectioned to a thickness of 14µm and capillaries were identified by immunohistochemistry using *Ulex europaeus* agglutinin I to detect endothelial cells and mouse anti-collagen IV antibody to detect muscle fiber perimeters as previously described (18). Stained muscle sections were imaged (Eclipse Ti, Nikon Instruments Inc., Melville, NY) and analyzed using NIS Elements software (Nikon Instruments Inc., Melville, NY). CD (capillaries per mm² of muscle cross-sectional area) was quantified using more than 50 fibers for each sample (mean = 70±2 fibers/sample);
sampling a larger number of fibers does not improve the estimation of capillarization in human muscle (36).

**GLUT4, AMPKα1, pAkt, and Akt expression:** Skeletal muscle was homogenized in lysis buffer (20mM Hepes, 100mM NaCl, 1.5mM MgCl2, 0.1% Triton X-100, and 20% glycerol) containing 1mM DTT and 1 tablet of cOmplete mini EDTA-free Protease Inhibitor Cocktail (Roche, Nutley, NJ). After homogenization, samples were kept on ice for 20min, followed by centrifugation for 10min at 20,000 x g. The supernatant was collected and stored at -80°C. GLUT4 and AMPKα1 expression were measured in basal samples; Akt and pAkt<sup>Ser473</sup> were measured in both basal and insulin-stimulated samples to assess insulin action. Total protein was determined using Pierce BCA<sup>®</sup> Protein Assay Kits (Thermo Scientific, Waltham, MA) and either 20µg (Akt and pAkt<sup>Ser473</sup>) or 30µg (GLUT4 and AMPKα1) of total protein was loaded on sodium dodecyl sulfate polyacrylamide gels. After gel electrophoresis, proteins were transferred to PVDF membranes (Millipore, Billerica, MA) and blocked with 5% non-fat dry milk for 1hr at room temperature. Membranes were washed briefly with Tris-buffered saline (TBS) and incubated with primary antibodies detecting GLUT4 (anti-GLUT4, 5 µg/mL, R&D Systems, Minneapolis, MN), AMPKα1, total Akt, or pAkt<sup>Ser473</sup> (anti-AMPKα1, anti-Akt, and anti-pAkt<sup>Ser473</sup>, 1:1000, Cell Signaling Technology, Danvers, MA). β-actin (anti-β-actin, 1:1000, Cell Signaling Technology, Danvers, MA) was used for total protein normalization. After primary antibody incubation, membranes were washed with TBS and incubated with horseradish peroxidase (HRP)-linked anti-rabbit IgG or anti-mouse IgG (1:1000, Cell Signaling Technology, Danvers, MA) secondary antibodies for 1hr at room temperature. For GLUT4 and AMPKα1, target bands were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) and detected using the GeneGnome imaging system (Syngene, Frederick, MD). Band densities were quantified using densitometry (Image J Software, Rockville, MD). For pAkt, signal was detected by adding an enhanced chemiluminescence (ECL) HRP substrate (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific, Waltham, MA) and for total Akt, signal was detected by adding Clarity™ ECL (Bio-Rad, Hercules, CA). Protein bands were
visualized using Bio-Rad Image Lab System (Bio-Rad, Hercules, CA). All protein expression data are expressed as arbitrary units (AU).

**GS activation, glycogen content, and citrate synthase (CS) activity:** GS independent, total, and fractional activities were determined in muscle samples as previously described (28). The independent and total activities of GS (non-phosphorylated) were determined in the presence of physiological (0.1mmol/L) and saturating (10mmol/L) concentrations of glucose-6-phosphate, respectively. GS fractional activity is the ratio of independent activity to total activity expressed as a percent. The difference in GS fractional activity between the insulin-stimulated and basal conditions is a measure of insulin activation of GS. Glycogen content was measured in the same supernatant as GS. Supernatant was boiled for 5min then centrifuged at 13,000 x g for 5min. Ten µL of this supernatant was used for fluorometric determination of glycogen using the Glycogen Assay Kit (Sigma, St. Louis, MO). For CS activity, 1mg of muscle was homogenized in 150µL ice-cold buffer containing 250mM sucrose, 10mM Tris–HCl, 1mM Methylene-diaminetetraacetic acid, pH 7.4, and cOmplete mini EDTA-free Protease Inhibitor Cocktail (Roche, Nutley, NJ). CS activity was measured by continuous spectrophotometric rate determination as previously described (37). All measures were corrected for total protein content using Coomassie Plus (Thermo Scientific, Waltham, MA) and assayed in triplicate.

**Maximal oxygen consumption (VO\textsubscript{2max}):** VO\textsubscript{2max} was measured by indirect calorimetry during a graded treadmill exercise test to maximal effort on a motorized treadmill. VO\textsubscript{2max} was defined as the highest oxygen consumption value obtained for a 30-second increment. Attainment of VO\textsubscript{2max} was verified by standard physiological criteria (respiratory exchange ratio >1.10 or a plateau in VO\textsubscript{2} with an increase in workload).

**Body Composition:** Height and weight were measured to calculate body mass index. Fat mass and fat-free mass were measured by DXA (Prodigy, LUNAR Radiation Corp., Madison, WI). Intra-abdominal (IAF) and subcutaneous abdominal (SAF) fat areas were determined by a computed tomography (CT) scan at L\textsubscript{4}-L\textsubscript{5} region (Siemens Somatom Sensation 64, Fairfield, CT) and Medical Image Processing, Analysis
and Visualization software v.7.0.0 (NIH, Bethesda, MD). CT data were not available for one subject due to a technical problem.

**Oral glucose tolerance tests (OGTT) and lipid profiles:** Subjects underwent 2-hour OGTTs after a 12-hour overnight fast. A catheter was placed in an antecubital vein and blood samples were drawn for glucose, insulin and lipoprotein-lipids. After ingestion of a 75-gram glucose solution, blood samples were drawn every 30min for 2hrs for measurement of glucose and insulin levels. Blood samples were centrifuged and plasma triglycerides and lipoprotein-lipid levels were analyzed using an automated colorimetric assay as previously described (38). Plasma samples were stored at –80°C for analysis of glucose levels (2300 STAT Plus, YSI, Yellow Springs, OH) and insulin by radioimmunoassay (Millipore, St. Charles, MO).

**Statistical Analyses**

The primary study outcomes were M/I and skeletal muscle CD. Secondary outcomes included GLUT4, AMPKα1, Akt, and pAktSer473 protein expression, skeletal muscle glycogen content, GS fractional activity, CS activity, VO2max, fasting plasma glucose (FPG), plasma glucose response to an OGTT, and body composition. Data are presented as means ± SEM. Statistical analyses were performed using SPSS v12.0 (IBM, Armonk, NY). Repeated measures analysis of variance was used to test for differences in outcome variables before and after exercise training and detraining. Regression analyses were used to test for associations between M/I and other variables. A type I error rate of α = 0.05 was selected and two-tailed probabilities are reported for all analyses.
Results

Subject characteristics (Table 1)

Subjects were all middle-aged to older men and women with no recent history of exercise. At baseline, the body mass index (BMI) of subjects ranged from normal weight to class I obesity (24-32kg/m²). Participants all had normal or well-controlled blood pressure (112±3mmHg systolic; 70±2mmHg diastolic) and lipoprotein-lipid levels. Classified by baseline OGTTs, 6 subjects had normal glucose tolerance and 6 had impaired glucose tolerance (3 men and 3 women).

Metabolic effects of exercise training and detraining

AEX training increased VO₂max (L/min) by 15% (P=0.006), the majority of which (10%) was maintained after 2-week detraining (Table 1, P=0.03). A goal of the study was for subjects to maintain body weight, and although statistically significant, the subjects lost only 2±1kg of body weight after exercise training (Table 1, P=0.04). Subjects did lose a small amount of body fat; however, there were no changes in IAF or SAF after exercise training (Table 1). Six-month AEX training increased M/I (µmol/kgFFM/pmol insulin/min) by 25%, and an 18% increase was maintained after 2-week detraining (Figure 2, ANOVA P=0.03). In the entire group of subjects, a 12% decrease in 120-minute postprandial glucose was detected after detraining, largely due to subjects with impaired glucose tolerance who reduced 120-minute postprandial glucose by 13-17% (Table 1, P=0.02). Half of the subjects with impaired glucose tolerance reverted to NGT after the interventions. Subjects had 17% lower triglyceride levels and 6% higher HDL-C levels after 6-month exercise training (Table 1, P<0.05) and the changes were largely maintained after detraining.

Effects of exercise training and detraining on mechanisms affecting skeletal muscle glucose metabolism

AEX training increased GLUT4 protein expression by 16% and AMPKα1 protein expression by 21%, and both reverted to levels baseline levels after 2-week detraining (Figure 3A, ANOVA P=0.02 for both). Total Akt protein expression was higher than baseline after exercise training and detraining (Figure 3B, P<0.05); however, although pAktSer473 increased in response to insulin at each time point (Figure 3C,
P<0.01), neither basal nor insulin-stimulated pAkt<sup>Ser473</sup> changed after exercise training or detraining (Figure 3C). The same pattern was observed when pAkt<sup>Ser473</sup> was expressed relative to total Akt protein (pAkt<sup>Ser473</sup>/Akt, Figure 3D). Total GS activity did not change across time points, nor with insulin stimulation (Table 2), but GS independent activity and fractional activity increased during insulin stimulation at each time point (Table 2, P<0.01). The 60% increase in insulin activation of GS after exercise training approached significance, and GS activation reverted to baseline levels after detraining (14.3±2.3 vs. 22.4±5.0 vs. 12.8±1.7%, respectively, P=0.08). Changes in CS activity followed the same pattern with a ~30% increase after exercise training that returned to baseline levels after detraining, but this was not statistically significant (Table 2). Skeletal muscle glycogen content was numerically higher than baseline after both exercise training and detraining (204±51 vs. 274±62 vs. 292±44µg/mg protein, respectively, P=0.2), indicating that subjects were not glycogen-depleted after exercise training or detraining.

Exercise training had a durable effect to increase CD, as the 15% increase was maintained after detraining (Figure 4, ANOVA P=0.009). The proportion of type I or II muscle fibers did not change from baseline after exercise training and detraining (67±4 vs. 70±5 vs. 69±4% type I fibers, respectively, P=0.80) nor did skeletal muscle fiber area (4732±421 vs. 4961±460 vs. 4784±494µm<sup>2</sup>, respectively, P=0.89), indicating that increases in CD were not due to changes in muscle fiber size or type. Thus, of the measured factors that would contribute to increases in M/I, CD was the only variable to increase and remain significantly higher after exercise training and detraining. We assessed the correlation between these variables in regression model accounting for sex and small changes in percent body fat finding that the change in M/I (detraining - baseline) correlated directly with the change in CD (Figure 5, partial r=0.70, P=0.02; model R=0.82, P=0.02).
Discussion

This study demonstrates that in previously sedentary older adults at risk for developing type 2 diabetes, the AEX training-induced increase in skeletal muscle CD is one mechanism contributing to improvements in insulin sensitivity. Our previous study (18) shows that changes in insulin sensitivity after AEX training with significant weight loss (8%) are related to improvements in CD; however, that study did not allow us to distinguish the independent effects of exercise training and weight loss, or effects of CD independent of other mechanisms. By minimizing the effects of weight loss and reversing acute exercise-induced changes in intramuscular mechanisms with detraining, the present study could better assess the effect of increased CD to improve M/I. As skeletal muscle is responsible for the majority of insulin-stimulated glucose uptake, identifying microvascular mechanisms that mediate skeletal muscle uptake of glucose has significant implications for the prevention and treatment of insulin resistance and type 2 diabetes.

The design of this study allowed allows us to distinguish the chronic and acute effects of AEX training on metabolic outcomes. For example, 6 months of AEX training increased VO$_{2\text{max}}$ by 15% in previously sedentary older subjects and VO$_{2\text{max}}$ decreased slightly after detraining, but two-thirds of the increase remained. While the change in CS activity (a marker of mitochondrial enzyme activity) was not statistically significant, the magnitude of the change is likely physiologically significant as it is similar to those reported in previous studies using AEX training (16) and detraining (39), and the pattern of changes in CS activity was consistent with the changes in VO$_{2\text{max}}$. This suggests that part of the reduction in VO$_{2\text{max}}$ after detraining may be attributed to the reversal of short-term changes in mitochondrial enzyme activity. The sustained improvement in VO$_{2\text{max}}$ after detraining is likely attributable to the effects of chronic exercise training on the cardiovascular system, including increases in CD to enhance oxygen delivery.

After 6-month AEX training, M/I increased by 25%. This corresponded with an increase in skeletal muscle CD, increases in GLUT4 and AMPKα1 expression, and an increase in insulin activation
of GS. The direction and magnitude of these changes are similar to previous studies of CD (16-18), GLUT4 (11,23) and AMPKα1 (24,25), and insulin activation of GS (13,27,28). These findings indicate that a number of intracellular and extracellular mechanisms likely contribute to improvements in M/I immediately following AEX training; however, insulin action did not appear to contribute to this increase (Figure 6). Although total Akt protein expression increased after exercise training, basal or insulin-stimulated pAkt\textsuperscript{Ser473} did not change after exercise training or detraining. This finding is consistent with previous studies of AEX training with or without weight loss in non-diabetic young and older subjects (27,40,41). Furthermore, Wasserman et al. [for review see (42)] show that in response to insulin infusion similar to that used in this study, resistance to muscle glucose uptake shifts away from insulin signaling and glucose transport toward glucose delivery, so it is not surprising that insulin action may not have contributed to the increase in M/I seen in this study. There is evidence that a larger transcapillary barrier is required to limit insulin delivery and action compared to that required to limit glucose delivery and uptake (42,43). In the present study, the lower CD at baseline may not have been low enough to limit insulin delivery and action; therefore, increases in CD would not be expected to further enhance insulin action, but may reduce the barrier to glucose delivery to increase M/I. One experimental model does show that occlusion of capillaries reduces insulin action (44); however, it is likely that the occlusion of capillaries created a significant barrier to insulin delivery in that study.

After detraining, M/I decreased ~7%, but still remained 18% higher than baseline levels. The maintenance of higher skeletal muscle glycogen content after detraining shows that the increase in M/I was not an artifact of glycogen depletion which could have enhanced insulin-stimulated glucose uptake (45). The partial decrease in M/I with detraining paralleled the decrease in GS activation, GLUT4 and AMPKα1 expression back to baseline levels. These findings are consistent with previous reports showing that GS activation and GLUT4 expression decrease in humans within days of ceasing exercise (19,22,29,46). In contrast, the increase in CD was maintained after detraining and represents one mechanism underlying the sustained increase in M/I. This is consistent with the finding that CD is
maintained even after 12 weeks of detraining in endurance-trained subjects (30). While the present study does not account for all possible mechanisms affecting exercise-induced increases in M/I, of the measured factors, CD was the only measured variable to remain higher after detraining (Figure 6).

Our results show that short-lasting adaptations to exercise, including but not limited to increases in GLUT4, AMPKα1, and GS activation, account for approximately one-fourth of the exercise-training induced improvement in M/I (i.e., the 7% improvement in M/I that dissipated with detraining vs. the 18% improvement that was maintained), and that other long-lasting mechanisms are responsible for the remaining proportion of the improvement in M/I. The results of the regression analysis suggest that as much as one-half of the 18% increase in M/I that remains after detraining could be attributable to increases in CD ($r^2=0.49$), while the remaining half is likely due to other chronic adaptations not addressed in the present study. This is consistent with reports that 45-65% of the resistance to insulin-stimulated glucose uptake is due to limitations in delivery of glucose to muscle (42). The contribution of CD to M/I is supported by experimental data from animal models in which either occlusion of capillaries (44) or deletion of vascular endothelial growth factor causing low CD (47) reduce insulin-stimulated glucose uptake. Conversely, the AEX-induced increase in CD would increase the diffusible surface area to enhance glucose flux from blood to muscle. This is supported by a recent study assessing the effects of experimental increase in capillarization on insulin sensitivity in a rodent model (48). Concordant with our findings, this group reported that increased capillarization (17-20%), in the absence of other metabolic adaptations (e.g., no effects on AMPK, GLUT4, pAkt, or GS activation), increased insulin sensitivity by 24%. The present findings, along with data from animal models, provide evidence that AEX-induced increases in skeletal muscle CD contribute to improvements insulin sensitivity in older adults.

By measuring several intramuscular mechanisms known to enhance insulin sensitivity, the design of this study allowed us to identify a contribution of changes in CD to M/I. However, we acknowledge that other mechanisms such as decreases in inflammatory cytokine expression or intramyocellular lipid, as well as improved glucose phosphorylation or mitochondrial adaptations in muscle may also contribute to
the sustained increase in insulin sensitivity. Also, the study design controlled for substantial changes in body weight, but we cannot exclude the possibility that the modest (2kg) weight loss and decrease in body fat contributed to increases in M/I. Improvements in blood flow (49) and insulin-mediated recruitment of capillaries [for review see (50)] may also contribute to exercise-induced improvements in M/I. While the present study is unable to distinguish the independent effects of CD per se from improvements in blood flow and recruitment of capillaries, it is clear that having higher CD in skeletal muscle provides for greater diffusion of glucose.

While the sample size is a potential limitation of this study, a strength of the study is measurement of M/I using the hyperinsulinemic-euglycemic clamp, with muscle sampling immediately before and during the clamp for measurement of CD and intramuscular mechanisms that affect insulin-stimulated glucose uptake, all within the same experiment. Another strength is the selection of older men and women in good health, but with lower CD than their younger counterparts (1,2) and risk factors for cardiovascular disease and diabetes, including a sedentary lifestyle. AEX training in older adults can increase CD to levels similar to younger subjects (2,16); therefore, this represents one mechanism by which age-associated declines in insulin sensitivity could be ameliorated. The study included subjects with a range of glucose tolerance, but subjects with type 2 diabetes were excluded to avoid confounding effects of medications and comorbid diseases on study outcomes. Future studies will need to confirm these findings in subjects with type 2 diabetes and other diseases associated with impaired glucose metabolism.

In conclusion, this integrative, translational investigation demonstrates that AEX training increases skeletal muscle CD in older adults, and that the increase in CD contributes to the sustained improvement in glucose metabolism independent of several intramuscular glucose transport mechanisms. If sustained, this increase in CD has the potential to mitigate and possibly prevent declines in glucose metabolism in susceptible older adults and potentially reduce progression to impaired glucose tolerance and type 2 diabetes.
Author Contributions

S.J.P., A.P.G., and A.S.R. conceived and designed the research. All authors were involved in data collection, performance of the experiments and analysis of the data. S.J.P. wrote the manuscript; all authors edited and revised the manuscript. S.J.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors have no conflict of interest to declare.

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References


Table 1. Subject Characteristics and Responses to Aerobic Exercise Training and Detraining

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<th>6-month Exercise</th>
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<td>Age (yrs)</td>
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<td>7.40 ± 0.53</td>
<td>7.13 ± 0.61</td>
<td>6.50 ± 0.54 *</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.55 ± 0.28</td>
<td>4.47 ± 0.31</td>
<td>4.44 ± 0.25</td>
</tr>
<tr>
<td>LDL-Cholesterol (mmol/L)</td>
<td>2.78 ± 0.28</td>
<td>2.74 ± 0.30</td>
<td>2.67 ± 0.26</td>
</tr>
<tr>
<td>HDL-Cholesterol (mmol/L)</td>
<td>1.16 ± 0.09</td>
<td>1.23 ± 0.09 *</td>
<td>1.23 ± 0.12</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.35 ± 0.14</td>
<td>1.12 ± 0.13 *</td>
<td>1.18 ± 0.13 *</td>
</tr>
</tbody>
</table>

\(^*\)Significant difference from Baseline, P < 0.05; \(^\dagger\)Significant difference from Baseline, P < 0.01.BMI, body mass index; IAF, intra-abdominal fat; SAF, subcutaneous abdominal fat; VO\(_{2}\)max, maximal oxygen consumption; G\(_{120}\), 120-minute postprandial glucose; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
### Table 2. Glycogen Synthase and Citrate Synthase Activities

<table>
<thead>
<tr>
<th>Glycogen Synthase</th>
<th>Baseline</th>
<th>6-month Exercise</th>
<th>Detraining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Insulin Stimulated</td>
<td>Basal</td>
</tr>
<tr>
<td>Fractional Activity (%)</td>
<td>7.6 ± 1.0</td>
<td>21.9 ± 3.0†</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>Independent Activity (nmol/min/mg protein)</td>
<td>0.46 ± 0.14</td>
<td>1.52 ± 0.26†</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td>Total Activity (nmol/min/mg protein)</td>
<td>6.4 ± 1.6</td>
<td>7.1 ± 0.9</td>
<td>8.5 ± 1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Citrate Synthase</th>
<th>Baseline</th>
<th>6-month Exercise</th>
<th>Detraining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (µmol/min/mg protein)</td>
<td>0.16 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.15 ± 0.03</td>
</tr>
</tbody>
</table>

†Significant effect of insulin (difference from basal) P < 0.01
Figure Legends

Figure 1. Study Design

Figure 2. Changes in insulin sensitivity (M/I) after exercise training and detraining. M/I was measured after a 12-hour overnight fast using a 3-hour hyperinsulinemic–euglycemic glucose clamp (n=12). Data are reported as means ± SEM during the third hour of the clamp [μmol of glucose infused per kilogram of fat-free mass per pmol of plasma insulin per minute (μmol/kgFFM/pmol insulin/min)]. *Significant difference from Baseline, P < 0.05

Figure 3. Changes in skeletal muscle GLUT4 and AMPKα1 (A), Akt (B), and pAkt\textsuperscript{Ser473} (C) expression, and pAkt\textsuperscript{Ser473}/Akt ratio (D) after exercise training and detraining. Protein levels for GLUT4, AMPKα1, total Akt, and pAkt\textsuperscript{Ser473} were determined in vastus lateralis by Western blot analyses. Representative Western blots are shown directly above the corresponding bar in each graph. Protein expression levels were determined by densitometry of the chemiluminescence signal and are expressed as arbitrary units (AU). Data are presented as means ± SEM (n=12). *Significant difference from Baseline, P < 0.05. †Significant difference from Basal condition (without insulin stimulation) at same time point, P < 0.01.

Figure 4. Changes in skeletal muscle capillary density (CD) after exercise training and detraining. CD was measured in vastus lateralis samples by immunohistochemistry and expressed as capillaries per mm\textsuperscript{2} of muscle cross-sectional area. Data are reported as means ± SEM (n=12). *Significant difference from Baseline, P < 0.05

Figure 5. Scatterplot depicting the partial correlation between the differences in insulin sensitivity (M/I) and skeletal muscle capillary density (CD) between the baseline and detraining timepoints. AU: arbitrary units. Data are transformed residuals from the regression analysis accounting for sex and percent body fat (model R = 0.82, P = 0.02, n=12).

Figure 6. Summary of the measured contributors to increases in insulin sensitivity (M/I) after aerobic exercise training and detraining. GLUT4: glucose transporter-4, AMPK: 5’ AMP-activated protein kinase, GS: glycogen synthase, p-Akt: phosphorylated Akt\textsuperscript{Ser473}.
Exercise 3x/week 45min @ 75% VO$_{2\text{max}}$  Detrain

<table>
<thead>
<tr>
<th>Week 0</th>
<th>24</th>
<th>26</th>
</tr>
</thead>
</table>

Research Testing

- Hyperinsulinemic-Euglycemic Clamp: X  X  X
- Muscle Biopsy (Basal & Insulin-Stimulated): X  X  X
- Oral Glucose Tolerance Test: X  X  X
- Maximal oxygen consumption (VO$_{2\text{max}}$): X  X  X
- Body Composition (DXA & CT): X  X
**A)**

- GLUT4
- β-Actin

**Protein expression (AU)**

- Baseline
- 6-mo Exercise
- Detraining

**B)**

- AMPKα1

**Akt (AU)**

- Basal
- Insulin

**C)**

- pAkt<sup>Ser473</sup>
- β-Actin

**D)**

- pAkt<sup>Ser473</sup>/Akt (AU)

In all panels, asterisks (*) indicate statistical significance, typically compared to baseline conditions, while plus signs (+) denote other significant differences.
Partial $r = 0.70$, $P = 0.02$