Low intrinsic running capacity is associated with reduced skeletal muscle substrate oxidation and lower mitochondrial content in white skeletal muscle

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Rivas DA, Lessard SJ, Saito M, Friedhuber AM, Koch LG, Britton SL, Yaspelkis BB 3rd, Hawley JA. Low intrinsic running capacity is associated with reduced skeletal muscle substrate oxidation and lower mitochondrial content in white skeletal muscle. Am J Physiol Regul Integr Comp Physiol 300: R835–R843, 2011. First published January 26, 2011; doi:10.1152/ajpregu.00659.2010.— Chronic metabolic diseases develop from the complex interaction of environmental and genetic factors, although the extent to which each contributes to these disorders is unknown. Here, we test the hypothesis that artificial selection for low intrinsic aerobic running capacity is associated with reduced skeletal muscle metabolism and impaired metabolic health. Rat models for low- (LCR) and high- (HCR) intrinsic running capacity were derived from genetically heterogeneous N:NIH stock for 20 generations. Artificial selection produced a 530% difference in running capacity between LCR/HCR, which was associated with significant functional differences in glucose and lipid handling by skeletal muscle, as assessed by hindlimb perfusion. LCR had reduced rates of skeletal muscle glucose uptake (~30%; P = 0.04), glucose oxidation (~50%; P = 0.04), and lipid oxidation (~40%; P = 0.02). Artificial selection for low aerobic capacity was also linked with reduced molecular signaling, decreased muscle glycogen, and triglyceride storage, and a lower mitochondrial content in skeletal muscle, with the most profound changes to these parameters evident in white rather than red muscle. We show that a low intrinsic aerobic running capacity confers reduced insulin sensitivity in skeletal muscle and is associated with impaired markers of metabolic health compared with high intrinsic running capacity. Furthermore, selection for high running capacity, in the absence of exercise training, endows increased skeletal muscle insulin sensitivity and oxidative capacity in specifically white muscle rather than red muscle. These data provide evidence that differences in white muscle may have a role in the divergent aerobic capacity observed in this generation of LCR/HCR.

insulin signaling; mitochondria; oxidative capacity

DURING THE PAST 50 YEARS, the prevalence of a cluster of interrelated chronic metabolic disease states, including coronary heart disease, insulin resistance, type 2 diabetes mellitus (T2DM), and obesity has reached epidemic proportions (2). The etiological basis of these disorders is polygenic and highly dependent on the environment (i.e., existing genes interact with environmental factors to result in phenotypic expression of these diseases). One environmental factor to have changed dramatically in this time and strongly associated with a plethora of chronic metabolic disorders is the decline in physical activity (8). Indeed, the increased prevalence of cardiovascular disease, insulin resistance, type 2 diabetes, and obesity and their strong association with inactivity has produced an “exercise-deficient phenotype,” in which individuals with a particular combination of disease-susceptible genes (i.e., risk factors) interact with undefined environmental conditions (e.g., level of physical activity) and cross a threshold of biological significance that results in overt clinical conditions. Evidence in support of this premise comes from studies in which multiple genes involved in aerobic metabolism are downregulated in several metabolic states and may be linked to the pathogenesis of these disorders (31, 38).

Skeletal muscle plays a vital role in locomotion and makes an important contribution to whole-body energy metabolism, disposing of up to ~80% of a postprandial glucose load (7, 56). Whole-body metabolic health is associated with the ability of skeletal muscle to transition between the uptake and oxidation of carbohydrate- and lipid-based fuels in response to their availability and the prevailing hormonal milieu (20). In several lifestyle-related diseases, such as obesity and type 2 diabetes, there is a loss of skeletal muscle plasticity such that rates of substrate oxidation do not increase effectively in response to fuel availability (10, 20, 22). Impaired muscle oxidative capacity coupled with a decreased ability of muscle to oxidize lipids are strong predictors of insulin resistance (3, 42). In support of this premise, several groups have reported that skeletal muscle mitochondrial content, mitochondrial function, and/or oxidative capacity are reduced in individuals that are insulin resistant or have T2DM (15, 18, 21, 42). Taken collectively, the results from these studies suggest that the lower oxidative capacity observed in individuals with obesity, T2DM, or both, may play a significant functional role in the development of insulin resistance. However, it is not known to what extent low oxidative capacity in muscle and the concomitant insulin resistance are a result of genetically predetermined or environmental factors.

To identify the genetic contribution to oxidative capacity, we have developed a novel rat model through two-way artificial selection for either low (LCR) or high (HCR) aerobic treadmill running capacity (24). After 11 generations of selective breeding, there was a 374% difference in running capacity between phenotypes that were associated with an increase in risk factors for cardiovascular disease, such as hyperinsulin-
emia, hyperlipidemia, and increased adiposity in LCR compared with HCR (36, 52). HCR rats were also resistant to the development of high-fat, diet-induced obesity, and insulin resistance (35, 36).

The aim of the present investigation was to test the hypothesis that low intrinsic aerobic running capacity is associated with impaired skeletal muscle oxidative capacity and blunted insulin sensitivity. This was accomplished by mimicking a “fasted” and “fed” environment in the skeletal muscle of the LCR and HCR and determining rates of insulin-stimulated glucose uptake and oxidation and basal lipid uptake and oxidation using hindlimb perfusion techniques. We have attempted to elucidate potential mechanisms that impart the impaired metabolic health associated with a low intrinsic running capacity by measuring substrate storage, insulin signal transduction, and mitochondrial activity and density in both oxidative and glycolytic muscle, from these divergent phenotypes.

METHODS

Experimental animals. Forty-eight female LCR/HCR rats from generation 20 (15 to 16 wk old) and generation 22 (20 wk old) (G20 and G22) were used. Rat models for high and low aerobic capacity were derived from genetically heterogeneous N:NIH stock rats by artificial selection for low and high aerobic running capacity (52). Animals were phenotyped for intrinsic running capacity at 11 wk of age using an incremental running test with the treadmill constantly at an uphill incline of 15° (52). Rats were housed two per cage in a temperature-controlled animal room (21°C) maintained on a 12:12-h light-dark cycle. Animals were provided with standard chow diets and water ad libitum. All animal experimentation procedures were carried out with the approval of animal ethics committees from California State University, Northridge; the University of Michigan; and RMIT University (AEC 0805).

Blood measures. Fasting blood values were taken after a 5-h fast, and glucose concentrations were determined with the Medisense2 Blood Glucose Testing system (MediSense Australia; Melbourne, Australia). Fasting serum nonesterified fatty acid measures were obtained using an enzymatic colorimetric method (NEFA C; Wako Pure Chemicals, Osaka, Japan).

Glucose tolerance test. Animals from G20 were fasted for 5 h before receiving an intraperitoneal injection of d-glucose (1 g/kg body wt). Blood glucose concentrations were measured at 0, 15, 30, 45, 60, 90, and 120 min following the glucose dose. The area under the blood glucose curve (AUC; m/mnin) was calculated for each animal.

Hindlimb perfusions. Animals from G20 were fasted for 5–7 h before undergoing hindlimb perfusion for the measurement of insulin-stimulated \( \left[ \text{U-}^{14}\text{C} \right] \)-glucose uptake/oxidation (\( n = 8 \)/group) or \( \left[ 1^{14}\text{C} \right] \)-palmitate uptake/oxidation (\( n = 8 \)/group). Rats were anesthetized and surgically prepared for hindlimb perfusion, as previously described (26, 54). Just prior to cannulation, portions of the red gastrocnemius (RG) and white (WG) gastrocnemius were excised from the nonperfused left leg, freeze-clamped in liquid N₂, and stored at −80°C until later analysis. In the nonperfused RG, palmitate uptake, perfusate samples were anaerobically taken from the arteriovenous difference, the perfusate flow rate, and the weight of the muscle perfused.

Arterial and venous samples for the analysis of \( ^{14}\text{CO}_2 \) were taken immediately at the end of a 5-min equilibration period and at the end of the perfusion (30 min). The liberation and collection of \( ^{14}\text{CO}_2 \) from perfusate samples were performed by injecting 2 ml of anaerobically collected perfusate into a sealed flask containing an equal volume of 1 M acetic acid. The released \( ^{14}\text{CO}_2 \) was trapped by an insert containing a strip of filter paper saturated with 500 \( \mu \)l benzethonium hydroxide and quantified using liquid scintillation counting. The rate of glucose oxidation was determined from the arteriovenous difference in \( ^{14}\text{CO}_2 \) and flow rate. Lactate was determined as previously described (54). Lactate accumulation was calculated from the arteriovenous difference, perfusate flow rate, and the weight of the muscle perfused.

\( ^{14}\text{C} \) palmitate uptake/oxidation rates. Skeletal muscle fatty acid metabolism was assessed as previously described (26). In brief, immediately after cannulation, the rats were killed via an intracardiac injection of pentobarbital sodium, while the hindlimbs were washed out with 20 ml of heparinized (10 U/ml) KHB. The catheters were then placed in line with a nonrecirculating perfusion system, and the hindlimb was allowed to stabilize during a 5-min washout period. The perfusate flow rate was set at 5 ml/min during the 5-min stabilization period and subsequent perfusion. Perfusates were performed in the presence of insulin (1 mU/ml) for all experimental groups. Following the stabilization period, the perfusate was changed to one containing 8 mM glucose [0.25 \( \mu \)Ci/ml \( d-[1^{14}\text{C}] \) glucose; PerkinElmer Life Sciences, Boston, MA]. At the completion of the 30-min perfusion, portions of the RG and WG were removed, blotted on gauze, dampened with cold KHB, clamp frozen in liquid N₂, and stored at −80°C until later analysis. For determination of glucose uptake, perfusate samples were taken from the arterial perfusate, and well-mixed venous effluent, deproteinized in 10% TCA, was centrifuged and quantified using liquid scintillation counting. Muscle glucose uptake was calculated from the arteriovenous difference, the perfusate flow rate, and the weight of the muscle perfused.

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detected after extraction with 2M HCl and neutralization with 0.67 M NaOH. Portions (3–4 mg) of freeze dried, powdered RG (8–10/group), and WG (8–10/group) were homogenized in 100 mM potassium phosphate buffer (pH 7.3, 1:400 dilution), and CS was assayed spectrophotometrically at 25°C by the reduction of DTNB.

Western blot analysis. Portions of muscle were cut from the RG and WG, weighed frozen and homogenized in an ice-cold homogenization buffer (1:8 wt/vol) containing 50 mM Tris·HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (vol/vol), 1% Triton-X, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 µg/ml trypsin inhibitor, and 2 µg/ml aprotinin. Following centrifugation (21,000 g, 4°C) for 15 min, the supernatant was collected and assayed for protein content. RG muscle lysates (60 µg) were solubilized in Laemmli buffer, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were then blocked (5% nonfat dry milk), and incubated overnight at 4°C with primary antibodies specific for either phospho-Akt Ser473, phospho-Akt Thr308, Akt1, Akt2, phospho-Akt substrate (1:1,000; Cell Signaling, Beverly, MA), phospho-insulin receptor substrate 1 (IRS1) Thr308, CPTI (1:200 –500; Santa Cruz Biotechnology, Santa Cruz, CA), or AS160 (TBCD14), which was produced as previously described (25), using a region of human AS160 from amino acids 621–766 fused with glutathione S-transferase (1:1,000, a gift from Prof. David James, Garvan Institute, Sydney, Australia). The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry.

Transmission electron microscopy for determination of mitochondrial content. Soleus (SOL) [4/group] and extensor digitorum longus (EDL) [4/group] skeletal muscle from a separate cohort of animals (G22) were excised, cut into small longitudinal strips (1 × 1 × 2 mm) fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 2% osmium tetroxide solution. After dehydrating in graded acetone, tissue was embedded in araldite/epon resin. Thick sections (0.5 µm) were cut using Ultratcut S ultramicrotome (Leica, North Ryde, New South Wales, Australia) and then stained with 1% uranyl acetate and lead citrate (Reynold’s stain). Randomly sampled transverse sections of muscle fibers were obtained followed by micrographs acquired with an electron microscope at 60 V (Siemens Elmispkop 102 electron microscope) on a final magnification of X10,000. To obtain a valid representation of the whole muscle, two micrographs (1 from the subsarcolemmal region and 1 from the adjacent interfibrillar region) in three separate muscle fibers for each muscle (SOL, EDL) in each group were acquired for a total of six micrographs per muscle per animal. Mitochondrial content was determined using the point-counting stereological analysis methodology with Image J software (Image J 1.41, National Institutes of Health, Bethesda, MD). Each micrograph was counted and then recounted in a double-blind fashion.

Statistical analysis. Differences between LCR and HCR were identified using a two-tailed t-test with GraphPad Prism version 4.04 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).
Markers of metabolic health are divergent in HCR and LCR. Intrinsic treadmill running capacity was 530% greater in HCR compared with LCR (generation 20, \( P = 0.0001 \), Table 1). HCR had lower body mass and fasting serum glucose levels (\( P < 0.0002 \) vs. LCR; Table 1) but had no difference in the levels of circulating free-fatty acids compared with LCR. HCR were more glucose tolerant than LCR, as measured from the AUC after an intraperitoneal glucose tolerance test following a 5-h fast (\( P = 0.01 \); Table 1).

HCR group has superior substrate handling in skeletal muscle. To determine whether intrinsic differences in aerobic capacity were associated with alterations in glucose and lipid handling, “fasted” and “fed” states were mimicked during hindlimb perfusions. HCR had superior insulin-stimulated glucose metabolism, as demonstrated by a 30% higher rate of glucose uptake (\( P = 0.04 \) vs. LCR; Fig. 1A) and a 50% higher rate of glucose oxidation (\( P = 0.04 \) vs. LCR; Fig. 1B). HCR also had superior lipid metabolism, with a higher rate of palmitate oxidation (\( P = 0.02 \) vs. LCR; Fig. 1D), and a trend for a higher palmitate uptake (\( P = 0.10 \) vs. LCR; Fig. 1C). Lactate accumulation during the 30-min perfusion was similar for both phenotypes (4.464 ± 1.2 \( \mu \)mol·g\(^{-1}\)·h\(^{-1} \) vs. 4.052 ± 0.7 \( \mu \)mol·g\(^{-1}\)·h\(^{-1} \)).

Enhancement of insulin signaling in WG muscle of HCR. To determine insulin-stimulated cell signaling responses in white and red muscle, the phosphorylation of proximal and distal components of the insulin signaling cascade was assessed. There were no differences between phenotypes in the phosphorylation of IRS1 on tyrosine (Y)632 in the RG. However, there was an increase in IRS1 phosphorylation in the WG of HCR (\( P = 0.002 \) vs. LCR; Fig. 3A). The phosphorylation of Akt on threonine (T)308 and serine (S)473 was also greater in the white muscle from HCR compared with LCR with a 24% and 26% increase in the WG (\( P = 0.016, P = 0.03 \) vs. LCR; Fig. 3, B and C), but not RG. The phosphorylation of AS160, a downstream substrate of Akt, was also greater in WG (21% increase, \( P = 0.03 \) vs. LCR; Fig. 3D) and tended to be higher in the RG (\( P = 0.12 \) vs. LCR; Fig. 3D). There was a 46% increase in the expression of the Akt1 isoform in the RG of HCR (\( P = 0.02 \) vs. LCR; Fig. 3E) and a 95% increase in the WG (\( P = 0.03 \) vs. LCR; Fig. 3E). In accordance with these observations, the expression of Akt2 was also increased in the RG (35\%, \( P = 0.026 \) vs. LCR; Fig. 3E) and in the WG (47\%, \( P = 0.008 \) vs. LCR; Fig. 3E) of HCR. There was a decrease in the expression of AS160 in the RG (\( P = 0.04 \) vs. LCR; Fig. 3E) but no change in the WG of the HCR. There were no differences in RG and WG when phosphorylated Akt/AS160 was normalized to total Akt/AS160 for either group.

Enhanced mitochondrial oxidative capacity in white but not red muscle of HCR. To determine whether there were differences in markers of mitochondrial capacity and density between phenotypes, CS activity, the expression of mitochondrial proteins and mitochondrial content, as quantified by TEM, was determined. There were no differences in the maximal activity of CS in RG of HCR and LCR, but CS activity was increased in WG of HCR (51\%, \( P = 0.0004 \) vs. LCR; Fig. 4A). There were no differences in the expression of CPTI in the RG for either phenotype (Fig. 4B). However, the expression CPTI tended to be higher (\( P = 0.098 \) vs. LCR; Fig. 4B). Representative TEM images of Sol and EDL of LCR/HCR are shown in Fig. 5A. There was no significant difference in mitochondrial content in the SOL (a red, oxidative muscle) between groups (Fig. 5B). However, there was a ~70% higher mitochondrial content in the EDL (a white, glycolytic muscle) of the HCR (\( P = 0.0002 \) vs. LCR; Fig. 5B).

DISCUSSION

Divergent selection for low and high intrinsic aerobic capacity has generated a powerful model system for dissection of the role of aerobic endurance capacity and its correlated traits. Importantly, simultaneous breeding of the LCR and HCR at each generation also allows for each line to serve as a control
for unknown environmental changes. The LCR/HCR model of divergent intrinsic aerobic capacity was developed to specifically study some of the potential mechanisms associated with the metabolic syndrome and cardiovascular disease in a system in which there is a degree of control over both environmental and genetic variables. Hence, the results from the present investigation provide novel insight into the fundamental molecular events underlying increased risk for metabolic disease.

The inability of skeletal muscle to adjust fuel oxidation to fuel availability is strongly associated with the development and progression of several chronic disease states, such as obesity, type 2 diabetes, and cardiovascular disease (10, 20).
In the present investigation, we have analyzed both red (soleus and red gastrocnemius) and white (extensor digitorum longus and white gastrocnemius) muscle to determine whether the metabolic phenotype of LCR/HCR is fiber-type specific. Differences in the physiological properties and function of red and white skeletal muscle have been well characterized (14, 29, 46, 50). Red muscle has a higher oxidative capacity, partly because of higher concentrations of mitochondria, and is more insulin sensitive than white muscle (17, 37). In response to endurance exercise training, several kinases have been found to be differentially activated in red and white skeletal muscle (28, 34), and insulin signaling has been found to be impaired in both fiber types in the skeletal muscle of diabetic animals (45).

A novel finding from the present study was that the most significant changes in mitochondrial content occurred in white muscle rather than in red muscle between the divergent phenotypes (Fig. 5B). We found a significantly lower mitochondrial content in the EDL muscle from LCR (P < 0.001 vs. HCR; Fig. 5B), but no significant difference in the soleus (Fig. 5B). These findings paralleled the decrease in CS activity (Fig. 4A) observed in the WG of LCR. Interestingly, other groups, using genetically manipulated mouse models (transgenic or knockout for PGC1α, PPARγ, or calsarcin-2), have also observed a fiber-type shift with white glycolytic muscle taking on the functional properties of red fibers (e.g., increased myoglobin, succinate dehydrogenase activity, fatigue resistance, etc.).
Such fiber-type changes were positively correlated with an increased endurance capacity (9, 27, 51). Hood et al. (16) have previously noted that the coordination of metabolic plasticity (i.e., mitochondrial content) can, in large part, be attributed to change in the properties of white glycolytic as opposed to red oxidative muscle. Interestingly, it was recently observed in generation 18 of LCR/HCR that there is a tendency for a more oxidative phenotype in the EDL of HCR (23), which is consistent with our present finding of an increased mitochondrial content in this muscle. The similarity in mitochondrial content in the soleus muscle between phenotypes observed in the present study may, in part, be due to its constant loading observed in caged animals (53).

Reduced mitochondrial content in the skeletal muscle of LCR was associated with increased body weight, decreased fatty acid oxidation, and reduced insulin sensitivity in these animals. These results are in agreement with the fact that in obesity and insulin resistance, there is impairment to mitochondrial oxidative capacity in skeletal muscle (30, 44, 48). Furthermore, insulin-resistant subjects with a family history of diabetes have low mitochondrial oxidative capacity that is correlated with decreased mitochondrial density in skeletal muscle as measured by electron microscopy (1, 32). We have previously reported a reduction in PPARγ coactivator 1α (PGC1α) protein abundance and in the concentration of the oxidative enzyme, cytochrome-c oxidase subunit 1, and in skeletal muscle from LCR (36). Reductions in the expression or activation of mitochondrial enzymes or proteins, like PGC1α, CPTI, or CS, are related to a decline in the oxidative capacity of skeletal muscle (4, 33, 43).

Our investigation demonstrates that selection for the trait of low intrinsic aerobic capacity alters the ability of skeletal muscle to oxidize substrate in response to their availability. The LCR/HCR models used in the present investigation provide novel evidence of a genetic contribution to muscle oxidative capacity. This is in agreement with previous studies that have reported data suggesting that reduced oxidative capacity may represent an early stage in the progression of metabolic disease states (i.e., insulin resistance) in some humans, and that low oxidative capacity may be an inherited defect in such people (32, 48, 49). Indeed, rates of lipid oxidation at rest have been observed to be persistently low even in formerly obese people who subsequently lose weight (41, 47).

**Perspectives and Significance**

In conclusion, selection for low intrinsic aerobic running capacity was associated with reduced insulin signaling, decreased skeletal muscle oxidative capacity, and lowered mito-
chondrial content. The impaired fuel-handling capacity in animals bred for inferior running capacity can, in part, be explained by a fiber-type specific decrease in insulin-stimulated phosphorylation of the IRS1, Akt, and the Akt substrate of 160 kDa (AS160), along with a substantial decrease in mitochondrial content. Using skeletal muscle-specific measures of substrate metabolism, we provide the first evidence that low intrinsic running capacity is associated with impaired oxidative capacity and substrate handling and decreased insulin signaling and mitochondrial content. Furthermore, selection for high running capacity, in the absence of exercise training, endows increased skeletal muscle insulin sensitivity and oxidative capacity, the latter of which is mainly confined to white rather than red muscle. These data provide some evidence that differences in the white muscle may have a role in the divergence in aerobic capacity observed in this generation of LCR/HCR.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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