Divergent skeletal muscle respiratory capacities in rats artificially selected for high and low running ability: a role for Nor1?

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Stephenson EJ, Stepto NK, Koch LG, Britton SL, Hawley JA. Divergent skeletal muscle respiratory capacities in rats artificially selected for high and low running ability: a role for Nor1? J Appl Physiol 113: 1403–1412, 2012. First published August 30, 2012; doi:10.1152/japplphysiol.00788.2012.—Inactivity-related diseases are becoming a huge burden on Western society. While there is a major environmental contribution to metabolic health, the intrinsic properties that predispose or protect against particular health traits are harder to define. We used rat models of inbred high running capacity (HCR) and low running capacity (LCR) to determine inherent differences in mitochondrial volume and function, hypothesizing that HCR rats would have greater skeletal muscle respiratory capacity due to an increase in mitochondrial number. Additionally, we sought to determine if there was a link between the expression of the orphan nuclear receptor neuron-derived orphan receptor (Nor)1, a regulator of oxidative metabolism, and inherent skeletal muscle respiratory capacity. LCR rats were 28% heavier (P < 0.0001), and fasting serum insulin concentrations were 62% greater than in HCR rats (P = 0.02). In contrast, HCR rats had better glucose tolerance and reduced adiposity. In the primarily oxidative soleus muscle, maximal respiratory capacity was 21% greater in HCR rats (P = 0.001), for which the relative contribution of fat oxidation was 20% higher than in LCR rats (P = 0.02). This was associated with increased citrate synthase (CS; 33%, P = 0.009) and β-hydroxyacyl-CoA (β-HAD; 33%, P = 0.0003) activities. In the primarily glycolytic extensor digitorum longus muscle, CS activity was 29% greater (P = 0.01) and β-HAD activity was 41% (P = 0.0004) greater in HCR rats compared with LCR rats. Mitochondrial DNA copy numbers were also elevated in the extensor digitorum longus muscles of HCR rats (35%, P = 0.049) and in soleus muscles (44%, P = 0.16). Additionally, HCR rats had increased protein expression of individual mitochondrial respiratory complexes, CS, and uncoupling protein 3 in both muscle types (all P < 0.05). In both muscles, Nor1 protein was greater in HCR rats compared with LCR rats (P < 0.05). We propose that the differential expression of Nor1 may contribute to the differences in metabolic regulation between LCR and HCR phenotypes.

intrinsic running capacity; mitochondria; skeletal muscle; metabolism; neuron-derived orphan receptor

THE ABILITY to deliver and utilize O2 is an essential component of metabolic regulation, with an individual’s maximal aerobic power [maximal O2 consumption (V\textsuperscript{\textcircled{O}2\textsubscript{max}})] a good correlate of whole body health status (13). Aerobic power is determined by the interaction of intrinsic (i.e., genetic) and environmental (i.e., lifestyle) factors, with the heritability of endurance capacity estimated to be 40% or higher (10). However, V\textsuperscript{\textcircled{O}2\textsubscript{max}} and its phenotypic expression (i.e., endurance running ability) can be rapidly modified by embarking on a vigorous exercise training program or, conversely, by adopting an inactive lifestyle. While such phenotypic plasticity can be harnessed to study the time course of progression (or reversal) of a number of metabolic disease traits, the contribution of potential intrinsic mechanisms to such diseases becomes difficult to define because of the confounding impact of numerous environmental influences (13).

Through two-way artificial selection, we have generated an animal model of low or high aerobic exercise capacity [low (LCR) or high running capacity (HCR), respectively] from a population of genetically heterogeneous rats. Divergent artificial selection for a complex trait (e.g., superior aerobic phenotype) produces a useful genetic model to study gene-exercise interactions because contrasting allelic variation is concentrated at the extremes from one generation to the next. In this model, 11 generations of two-way selection produced rats that differ substantially in aerobic exercise capacity (374% difference between LCR and HCR rats, P < 0.0001) while simultaneously presenting with markedly different metabolic and cardiovascular disease risk factors (48).

Because skeletal muscle mitochondrial respiration is a limiting factor for V\textsuperscript{\textcircled{O}2\textsubscript{max}}, and because skeletal muscle oxidative capacity correlates with whole body insulin sensitivity (4), it has been proposed that differences in mitochondrial function in LCR-HCR animals are the major determinants of their divergent running capacities and metabolic phenotypes. In support of this contention, HCR rats are better at using O\textsubscript{2}, an adaptive variation likely to be present at the level of the skeletal muscle (10). Results from a series of studies from our laboratory (16, 17, 35) and others (24, 25, 41, 45, 48) have provided direct evidence that skeletal muscle from HCR rats has superior substrate handling ability (i.e., “metabolic flexibility”) and mitochondrial enzyme activities compared with LCR rats. However, there is some contention as to whether this is due to increases in the mitochondrial reticulum (35) or an enhanced capacity of some or all of the oxidative enzymes (24, 41, 45). Additionally, we have shown that the differences in metabolic flexibility are linked to β-adrenergic signaling through the nuclear receptor (NR)4A orphan NR neuron-derived clone (Nur)77 (16, 17). The NR4A family of orphan NRs [Nur77, Nur1, and neuron-derived orphan receptor (Nor1)] has recently emerged as a key player in the regulation of a number of important metabolic processes (4, 16, 31, 33). All three have been linked to the regulation of skeletal muscle substrate
handling and whole body glucose homeostasis (4, 16, 31, 33), and Nor1 has been shown to be essential for oxidative metabolism (32) and may also be important in promoting several oxidative adaptations in the muscle (21).

Since previous studies have been inconclusive as to whether skeletal muscle respiratory capacity is due to an increased number of mitochondria (35) or increased mitochondrial activity (24, 41), we aimed to determine the ex vivo activity and protein expression of the key electron transfer system (ETS) respiratory complexes in muscle from LCR and HCR rats. We hypothesized that skeletal muscle respiratory capacity would be greater in HCR rats compared with LCR rats and that this difference would be due to a greater mitochondrial density in the muscle. Furthermore, we hypothesized that the expression of Nor1 would be greater in the muscle of HCR rats compared with LCR rats.

**METHODS**

**Experimental animals.** Rat models for LCR and HCR were derived from genetically heterogeneous N:NIH stock rats by artificial selection for treadmill running capacity, as previously described (12). Female offspring of either LCR rats (N = 12, generation 27) or HCR rats (N = 12, generation 27) were housed under a controlled 12:12-h light-dark cycle at a constant temperature of 22°C. Animals were provided ad libitum access to water and a standard chow diet. Breeding and phenotyping of parent rats was conducted at the University of Michigan (Ann Arbor, MI). Rats arrived at RMIT University at ~8 wk of age and were allowed to acclimate to the RMIT Animal Facility for 2 wk before the commencement of any experimental procedures. This study was undertaken with approval from both the University of Michigan and RMIT University Animal Ethics Committees.

**Fasting blood and insulin measurements.** After a 5-h fast, ~15-μl aliquot of blood was collected via a tail cut, and the glucose concentration was measured on a hand-held glucometer (Roche Diagnostics, Mannheim, Germany). A separate aliquot of blood (~15–20 μl) was allowed to clot over ice and centrifuged to obtain serum. Serum was kept frozen at −20°C for later analysis of fasting serum insulin concentrations using a commercially available ELISA (no. 80-INSRT-E01, ALPCO Immunodiagnostics).

**Intraperitoneal glucose tolerance testing.** After the fasting blood collection, rats received a single glucose bolus (2 g/kg body wt) via intraperitoneal injection, and blood glucose concentrations were monitored at 30-min intervals throughout the subsequent 120 min. Additionally, aliquots of blood were collected at 30, 60, and 120 min postinjection for the later determination of serum insulin concentrations in response to the glucose challenge.

**Physical activity monitoring.** Before being monitored, rats were familiarized with activity monitoring chambers comprising infrared beams in the x-, y-, and z-axes (MED Associates, St. Albans, VT) during three 10-min sessions in the week before testing. Spontaneous activity was monitored during both diurnal (1200 hours) and nocturnal (0200 hours) sessions. Horizontal, vertical, and ambulatory locomotor activities were determined in 20-s intervals over 2 × 10-min sessions.

**Tissue collection and analyses.** Tissue collection procedures were conducted after a 5-h fast in 11-wk-old rats, which were weighed and anesthetized with pentobarbital sodium (60 mg/kg body wt), and hindlimb muscles were immediately excised. To limit any possible differences that might occur as a result of differing cage activity, the soleus and extensor digitorum longus (EDL) muscles were chosen for study. The soleus (~87% type I fibers and ~12% type IIA fibers) is predominantly a postural muscle and therefore has a high daily activity level, whereas many of the fibers in the EDL muscle (~2% type I fibers, ~42% type IIA fibers, and ~56% type IIB fibers) are recruited only during rapid, high-intensity tasks (1). Similar experiments were performed on soleus and EDL portions from the same leg. The left soleus muscle was placed in ice-cold muscle preservation media (BIOPS; 50 mM KCl, 20 mM taurine, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, and 20 mM imidazole (pH 7.1 adjusted with 5 N KOH at 0°C); Ca2+-EGTA buffer (10 mM; 2.77 mM CaK2EGTA + 7.23 mM K2EGTA, 0.1 μM free calcium)) for respirometry experiments (34, 36, 42), whereas the contralateral soleus muscle was carefully dissected into longitudinal strips from tendon to tendon (15–20 mg/strip) and placed in pregassed Krebs-Henseleit buffer (KHB) for measurements of basal and insulin-stimulated glucose uptake. The remaining hindlimb muscles were freeze clamped in liquid N2 and stored at −80°C for later analyses. Perivascular fat pads were removed and weighed as indicators of adiposity.

**Glucose uptake.** All reagents used were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise specified. Soleus muscle strips from the right leg were allowed to recover for 30 min in preoxygenated KHB (118.5 mM NaCl, 25 mM NaHCO3, 4.74 mM KCl, 1.19 mM MgSO4·7H2O, 1.18 mM KH2PO4, and 2.5 mM CaCl2 (pH 7.4)) containing 32 mM mannitol, 2 mM pyruvate, 8 μM succinate, and 0.1% fatty-acid free BSA. Vials were maintained at 30°C in a shaking water bath throughout the experiment. Basal and insulin-stimulated glucose uptake were determined according to the method of Young et al. (49) with modifications (8, 17, 44). After the recovery incubation, muscle strips were incubated for an additional 30 min in continuously gassed modified KHB with or without insulin (60 nM, Humulin, Eli Lilly, Indianapolis, IN). Muscle strips were then washed for 10 min in KHB modified with 40 mM mannitol, 2 mM pyruvate, 0.1% fatty-acid free BSA, and 60 mM insulin, if it was present in the previous incubation. After glucose washout, strips were transferred to vials containing oxygenated KHB containing 0.1% fatty-acid free BSA, 2 mM pyruvate, 1 mM 2-[1-14C]deoxyglucose (1 μCi/ml, Perkin-Elmer), 39 mM 3-[3-14C]-mannitol (0.1 μCi/ml, Perkin-Elmer), and 60 mM insulin, if it was present in the previous incubations. After 20 min, the reaction was stopped by washing the muscle strips in KHB twice, blotting the tissue on filter paper, and then freeze clamping it with tongs precooled in liquid N2. Muscle strips were weighed, digested in 10% tricarboxylic acid (TCA), and transferred to vials containing 5 ml scintillation fluid (Ultima Gold XR, Perkin-Elmer). Duplicate aliquots were counted in a liquid scintillation counter (Packard Tri-Carb, Perkin-Elmer) set for simultaneous 3H and 14C counting. Rates of basal and insulin-stimulated skeletal muscle 2-[1-14C]deoxyglucose transport were calculated as previously described (15).

**High-resolution respirometry.** Approximately 10 mg of the soleus (n = 10 soleus muscles/group) were mechanically separated over ice using fine forceps (34) and then chemically permeabilized with saponin (50 μg/ml in BIOPS) for 30 min. This was followed by a 10-min wash period in mitochondrial respiratory media (MIRO5; 110 mM sucrose, 60 mM K-lactobionate (ACROS Organics), 0.5 mM EGTA, 3 mM MgCl2, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES (adjusted to pH 7.1 with KOH at 37°C), and 1 g/l fatty acid-free BSA (36)). Duplicate tissue samples (2–3.5 mg) were transferred to the chambers of an O2/K-Oxygraph high-resolution respirometer (Oroboros, Innsbruck, Austria) containing 2 ml MIRO6 (MIRO5 plus 280 IU/ml catalase) and calibrated to air saturation. Individual chambers were oxygenated to ~485 nmol/ml with pure O2 (BOC Australia). Chambers were maintained at a temperature of 37°C, and O2 saturation was kept between 450 and 300 nmol/ml with regular titrations of H2O2. The remaining, nonpermeabilized muscle was frozen for later analyses.

**Substrate-uncoupler-inhibitor titration protocol.** Malate (2 mM) was added, and the mass-specific O2 flux (in pmol·s−1·mg wet wt−1) was stabilized for 5–10 min. Subsequently, tissue samples were subjected to a substrate-uncoupler-inhibition titration (SUIT) protocol, which sequentially evaluates an electron-transferring flavoprotein (ETF; fat oxidation) in leak state through the addition of octanoylcar-
nitate (0.2 mM, TOCRIS Bioscience; state 2 respiration), oxidative phosphorylation (OXPHOS) with electron flux through the ETF by titration of ADP (1 and 2.5 mM; state 3 respiration), OXPHOS with electron flux through the ETF and complex I (CI) with the addition of glutamate (10 mM), OXPHOS with electron flux through the ETF, CI, and complex II (CII) with the addition of succinate (10 mM), maximal coupled OXPHOS with electron flux through the ETF, CI, and CII with the addition of ADP (5 mM), the integrity of the outer mitochondrial membrane with the addition of cytochrome c (10 µM), the maximal capacity of the ETS by uncoupling with a stepped titration of FCCP (0.5 and 1 µM), uncoupled respiration with electron flux through the ETF and CII with the addition of the CI inhibitor rotenone (0.5 µM), and residual O2 consumption with the addition of antimycin A (2.5 µM). Additionally, complex IV (CIV) activity was determined after the addition of the artificial electron donor N,N,N,N′,N′-pentamethyl-p-phenylenediamine dihydrochloride (0.5 mM) in the presence of ascorbate (2 mM). The reaction was terminated by the addition of NaN3 (50 mM), after which the chambers were reoxygenated for the determination of auto-oxidation. Mass-specific O2 flux was determined using DATLAB (Orboros) from steady-state O2 flux normalized to tissue wet weight and adjusted for instrumental background, residual O2 consumption, and auto-oxidation. In addition, flux control ratios (results normalized to the maximal capacity of the ETS) were calculated to determine mitochondrial function independent of mitochondrial density.

Mitochondrial enzyme activities. Muscle homogenates (n = 10 homogenates/group) were prepared over ice from freeze-clamped soleus and EDL muscles (10–20 mg in buffer [175 mM KCl and 2 mM EDTA (pH 7.4), 1:50 or 1:100 dilution]. Homogenates were then subjected to three freeze-thaw cycles. Citrate synthase (CS) and complex III (CIII; 48 kDa, no. MS331, MitoSciences), CIV subunit II (15 kDa, no. MS407, MitoSciences), CS (37 kDa, no. ab96600, Abcam), and uncoupling protein 3 (UCP3; 35 kDa, no. PA1-055, Affinity BioReagents). α-Tubulin (50 kDa, Sigma) was used as a protein loading control. Protein expression was detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry. Statistical analyses. All values are expressed as means ± SEM.

RESULTS

HCR and LCR rats have divergent metabolic health parameters. The metabolic health characteristics of each rat phenotype are shown in Table 1. Although there were no differences in the ages of the animals, LCR rats were 28% heavier than HCR rats (P < 0.0001) and had 48% more perivascular fat (P = 0.02). There were no differences in interscapular brown adipose masses. Fasting blood glucose concentrations were not different, but fasting serum insulin concentrations were 62% higher in LCR rats compared with HCR rats (P = 0.02). HCR rats were more glucose tolerant than LCR rats, as determined by the area under the glucose curve during the IP-GTT (LCR rats: 1,267 ± 47.4 and HCR rats: 1,100 ± 27.8, P = 0.01; Fig. 1, A and B). Similarly, the insulin response to the IP-GTT was 135% greater in LCR rats compared with HCR rats, as determined by the area under the insulin curve during the IP-GTT (LCR rats: 196.2 ± 15.8 and HCR rats: 83.6 ± 11.3, P = 0.001; Fig. 1, C and D).

<table>
<thead>
<tr>
<th>Age, days</th>
<th>LCR Rats</th>
<th>HCR Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>80.9 ± 0.6</td>
<td>81.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>214.1 ± 3.7</td>
<td>167.7 ± 4.2*</td>
</tr>
<tr>
<td>Perivascular fat pad mass, g</td>
<td>1.2 ± 0.1</td>
<td>0.8 ± 0.1*</td>
</tr>
<tr>
<td>Interscapular brown adipose mass, mg</td>
<td>183.0 ± 13.7</td>
<td>185.1 ± 10.2</td>
</tr>
<tr>
<td>Fasting blood glucose concentration, mM</td>
<td>6.5 ± 0.1</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Fasting serum insulin concentration, ng/ml</td>
<td>0.62 ± 0.07</td>
<td>0.39 ± 0.01*</td>
</tr>
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</table>

Values are means ± SE. N = 8–12 rats/group. LCR rats, rats with low running capacity; HCR rats, rats with high running capacity. *P < 0.05.

Table 1. Body composition and metabolic parameters

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The glucose uptake response to insulin is enhanced in HCR rats. Rates of glucose uptake in the soleus are shown in Fig. 2. There were no differences in the rate of basal glucose uptake between LCR and HCR rats (3.21 ± 0.35 and 3.57 ± 0.16 μM·g⁻¹·h⁻¹, respectively). Insulin stimulation increased glucose uptake in both groups (LCR rats: 5.16 ± 0.21 μM·g⁻¹·min⁻¹, P < 0.0001, and HCR rats: 6.00 ± 0.23 μM·g⁻¹·min⁻¹, P < 0.0001), and this increase was of a greater magnitude in HCR rats (14%, P = 0.02).

HCR rats are more active than LCR rats. Physical activity data for each group are shown in Table 2. During the diurnal period, HCR rats completed a greater number of horizontal beam breaks (P = 0.0004), spent more time ambulatory (P = 0.02) and less time stationary (P = 0.02), and traveled a greater total distance (P = 0.04) compared with LCR rats. During the nocturnal phase, HCR rats completed fewer horizontal beam breaks, had fewer stereotypic movements, and spent more time stationary than they did during the diurnal phase. For LCR rats, movement patterns were not different during diurnal or nocturnal testing periods. Compared with LCR rats during the nocturnal period, HCR rats spent more time ambulatory (P = 0.007) and completed a greater number of horizontal beam breaks (P = 0.07). Distance traveled or time spent stationary was not different between phenotypes during the nocturnal monitoring period.

Mitochondrial respiratory capacity is greater in HCR rats compared with LCR rats. To evaluate the capacity of the ETS and thus determine maximal O₂ flux through the mitochondria, we measured O₂ flux during a SUIT protocol with substrates for the ETF, CI, CII, and CIV (Fig. 3). State 2 respiration with electron leak through the ETF was not different between groups (LCR rats: 5.55 ± 0.42 pmol·s⁻¹·mg wet wt⁻¹ and HCR rats: 6.61 ± 0.83 pmol·s⁻¹·mg wet wt⁻¹); however, state 3 respiration (OXPHOS) with electron flux through the ETF was greater in HCR rats than in LCR rats at 1 mM ADP (17.6 ± 1.2 and 27.9 ± 1.7 pmol·s⁻¹·mg wet wt⁻¹, respec-

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**Fig. 1.** Glucose tolerance test. A: blood glucose responses over 120 min. B: areas under the blood glucose curve (Glucose AUC). C: serum insulin responses over 120 min. D: areas under the serum insulin curve (Insulin AUC). HCR rats, rats with high running capacity; LCR rats, rats with low running capacity. Values are means ± SE; N = 8–10. *P < 0.05.

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**Fig. 2.** Basal and insulin-stimulated glucose uptake in the soleus muscles of LCR and HCR rats. Values are means ± SE; N = 11. *P < 0.05, different from the basal value; †P < 0.05 different from the LCR value under the same conditions.
Table 2. **Physical activity levels of animals**

<table>
<thead>
<tr>
<th></th>
<th>Day</th>
<th>Night</th>
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<tbody>
<tr>
<td></td>
<td>LCR rats</td>
<td>HCR rats</td>
</tr>
<tr>
<td></td>
<td>LCR rats</td>
<td>HCR rats</td>
</tr>
<tr>
<td>No movement, %</td>
<td>67.1 ± 0.7‡</td>
<td>61.9 ± 1.9+†</td>
</tr>
<tr>
<td>Stereotomopulive, %</td>
<td>22.8 ± 0.6</td>
<td>22.3 ± 0.5†</td>
</tr>
<tr>
<td>Ambulatory movement, %</td>
<td>7.5 ± 0.3‡</td>
<td>10.0 ± 0.9†</td>
</tr>
<tr>
<td>Horizontal beam breaks, counts</td>
<td>54.8 ± 3.4‡</td>
<td>133.6 ± 16.7+†</td>
</tr>
<tr>
<td>Total distance traveled, cm</td>
<td>1280.0 ± 79.3‡</td>
<td>1721.0 ± 187.9+†</td>
</tr>
<tr>
<td></td>
<td>70.9 ± 1.6</td>
<td>71.4 ± 2.7‡</td>
</tr>
<tr>
<td></td>
<td>20.4 ± 1.1‡</td>
<td>17.8 ± 1.2+†</td>
</tr>
<tr>
<td></td>
<td>7.4 ± 0.4</td>
<td>10.6 ± 0.6†</td>
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<tr>
<td></td>
<td>49.8 ± 6.5‡</td>
<td>76.0 ± 12.4‡</td>
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<tr>
<td></td>
<td>1185.0 ± 79.4‡</td>
<td>1336.0 ± 132.1‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; N = 8–12 rats/group. *P < 0.05, different from LCR rats during the day; †P < 0.05, different from LCR rats during the night; ‡P < 0.05, different from HCR rats during the day.

Table 3. **Mitochondrial flux control ratios**

<table>
<thead>
<tr>
<th></th>
<th>LCR Rats</th>
<th>HCR Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETF and leak (state 2)</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>ETF and OXPHOS (state 3)</td>
<td>0.23 ± 0.01</td>
<td>0.29 ± 0.02*</td>
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<tr>
<td>ETF, CI, and OXPHOS (state 3)</td>
<td>0.32 ± 0.01</td>
<td>0.39 ± 0.2*</td>
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<tr>
<td>ETF, CI, CI, and OXPHOS (state 3, submaximal)</td>
<td>0.77 ± 0.02</td>
<td>0.76 ± 0.03</td>
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<tr>
<td>ETF, CI, CII, and OXPHOS (state 3, maximal)</td>
<td>0.96 ± 0.02</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>ETF, CI (uncoupled, state 4)</td>
<td>0.57 ± 0.01</td>
<td>0.51 ± 0.01*</td>
</tr>
</tbody>
</table>

Values are means ± SE; N = 8–12 rats/group. ETF, electron-transferring flavoprotein; OXPHOS, oxidative phosphorylation; CI, complex I; CII, complex II. *P < 0.05.
Mitochondrial protein expression is greater in HCR rats. In the soleus muscle, expression of all individual ETS complex proteins was greater in HCR rats compared with LCR rats \((P < 0.05; \text{Fig. 5})\) except for CIV subunit I. Additionally, expression of CS was 14% greater in HCR rats compared with LCR rats \((P = 0.03; \text{Fig. 5})\). In the EDL muscle, expression of all individual ETS complexes was greater in HCR rats compared with LCR rats \((P < 0.05; \text{Fig. 6})\) except for CIV subunit I, which was not different. CS protein expression was 38% greater in HCR rats \((P < 0.0001; \text{Fig. 6})\).

**DISCUSSION**

The motivation for developing the LCR/HCR model system originated from noting the strong statistical association between low endurance exercise capacity and increased morbidity and mortality in humans \((4)\). From this, we formulated the general hypothesis that aerobic energy metabolism is a central mechanistic determinant of the divide between disease and health, which we termed the “aerobic hypothesis.” As an unbiased test of this hypothesis, we applied divergent artificial selection for intrinsic LCR and HCR. As predicted by this hypothesis, disease risks and reduced longevity segregated strongly with low aerobic capacity \((14)\). Here, we provide specification for features that can explain the intrinsic aerobic exercise capacity differences between the LCR and HCR and, ostensibly, disease risks.

Alterations to mitochondrial function typically occur in parallel with disease progression, and although environmental influences undoubtedly play an important role in the development of metabolic diseases \((6)\), there is a growing body of evidence to suggest that a substantial genetic component underlies many complex metabolic disorders \((22, 29)\). Here, we present data showing that skeletal muscle respiratory capacity is greater in oxidative muscle from HCR rats compared with LCR rats \((\text{Fig. 3})\) and that this enhanced respiratory capacity is likely due to greater relative mitochondrial enzyme activities [particularly those with a role in fat oxidation (Figs. 3 and 4)] coupled with increased mitochondrial number \((\text{Figs. 4–6})\). This increase in muscle oxidative capacity is associated with an increase in the protein expression of the NR Nor1 \((\text{Figs. 5 and 6})\), which has previously been linked to the adaptive response of skeletal muscle in response to exercise training \((21, 50)\) and the expression of several proteins involved in glucose metabolism \((4, 16, 31, 33)\). These novel findings complement our previous reports in which we have shown that there are a greater number of mitochondria present in the glycolytic muscle from HCR rats \((35)\) and that muscle substrate handling is superior in these animals \((15–17)\).

Low aerobic capacity, independent of physical activity levels, is a key predictor of early mortality \((23, 47)\). Along with a reduced lifespan \((14)\), LCR rats express a number of characteristics common to metabolic disease phenotypes, such as increased body weight and adiposity \((\text{Table 1})\) \((10, 24, 25, 39)\), hyperinsulinemia \((\text{Table 1})\) \((25, 40, 48)\), and impaired glucose tolerance \((\text{Fig. 1})\) \((24, 25, 35)\). In contrast, HCR rats live 6–8 mo longer \((14)\) and present with superior metabolic health, as characterized by resistance to weight gain \((24–26)\) and an increased capacity for the uptake and oxidation of both glucose \((\text{Fig. 2})\) \((17, 35)\) and long-chain fatty acids \((17, 24, 25, 35)\). The differential expression of these characteristics appears to be

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**Fig. 4.** Enzyme activities and relative mitochondrial (mt)DNA copy numbers in both the soleus and EDL muscles of LCR and HCR rats. A: citrate synthase \((CS)\) activity \((N = 10)\). B: \(\beta\)-hydroxyacyl-CoA \((\beta\text{-HAD})\) activity \((N = 10)\). C: mtDNA-to-nuclear \((n)\)DNA ratio \([\text{mtDNA:}n\text{DNA}]; \text{in arbitrary units (AU)}, N = 7]\). Values are means \(\pm\ SE. *P < 0.05.\)

**Nor1 expression is greater in HCR rats.** In both the soleus and EDL muscles, protein expression of the orphan NR Nor1 was greater in HCR rats \((\text{soleus: } 21\%, P = 0.009, \text{and EDL: } 23\%, P = 0.016; \text{Figs. 5 and 6})\). Expression of Nur77 \((\text{soleus: } 40\%, P = 0.02, \text{and EDL: } 25\%, P = 0.01)\) and NR4A target proteins fatty acid translocase/CD36 \((\text{soleus: } 4\%, P = 0.04, \text{and EDL: } 9\%, P = 0.02)\) and UCP3 \((\text{soleus: } 32\%, P = 0.01, \text{and EDL: } 20\%, P = 0.0003)\) was also increased in both muscle types from HCR rats \((P < 0.05; \text{Figs. 5 and 6})\).
linked to muscle oxidative capacity (25), although previous studies have been inconclusive with regard to the precise mechanisms involved. As noted, HCR rats have superior O2 handling capacity, an adaptive variation that resides at the level of the muscle (10). We (35, 48) have previously reported a greater number of mitochondria in the muscle of HCR rats, although results from previous studies have not supported this observation (24, 45) and have concluded that the difference in oxidative capacity is not due to mitochondrial size or number (24, 41, 45) but to a difference in mitochondrial enzyme activity.

To address this issue, we performed respirometry experiments, monitoring O2 consumption through the mitochondria of permeabilized soleus muscles from LCR and HCR rats in response to specific substrate combinations. In contrast to individual enzyme activity assays, this is a dynamic measurement that reflects the integrated activities of β-oxidation, the TCA, cycle, and the ETS. During ADP-stimulated (state 3) respiration, we observed greater O2 consumption with electron flux through the ETF, CI, and CII. Additionally, maximal activity of the ETS was greater in muscles from HCR rats. Previously, we have shown that the oxidation of the long-chain fatty acid palmitate is greater in vitro soleus muscle incubations from HCR rats (17) and during hindlimb perfusion experiments (35). Here, we confirmed that the capacity for total lipid oxidation is enhanced in the muscle of HCR rats and that this is due to the increased activity of mitochondrial enzymes involved in the oxidation of fatty acids. Specifically, respiratory flux control ratios indicated that independent of mitochondrial volume, the relative contribution of the ETF to maximal flux was greater in HCR rats when the muscle was exposed to the medium-chain fatty acid octanoylcarnitine (Table 3). Furthermore, β-HAD activity (the third enzyme of the β-oxidation pathway) was greater in muscles from HCR rats (Fig. 4B). In contrast, when data for the remaining substrate combinations were normalized to maximal uncoupled respiratory capacity, combined O2 flux was not different, which suggests that the coupling of OXPHOS and CI and CII activity are similar for LCR and HCR rats. This indicates that along with an increased capacity for fat oxidation per mitochondrion, an increase in the mitochondrial reticulum is also likely to contribute to the increased respiratory capacity of muscles from HCR rats. Since respirometry and enzyme activity measurements are indirect determinants of mitochondrial content that must be supported by additional measurements, we also measured mtDNA copy numbers (Fig. 4C) and the expression of a number of mitochondrial proteins (Figs. 5 and 6) in both soleus and EDL muscles. We found that the mtDNA copy number was greater in both muscles under investigation and, moreover, that the expression of a number of mitochondrial proteins was increased in both muscle types, supporting our hypothesis of a greater mitochondrial volume in the muscle of HCR rats. Furthermore, the TCA cycle protein CS, which has long been regarded as a surrogate marker of mitochondrial volume, was expressed to a greater degree (Figs. 5 and 6) along with elevated maximal activities (Fig. 4A) in both the soleus and EDL muscles of HCR rats. This difference in skeletal muscle respiratory capacity is no doubt a major contributing factor to

Fig. 5. Protein content of the soleus muscle from LCR and HCR rats as determined by Western blot analysis. PGC-1, peroxisome proliferator-activated receptor-γ coactivator-1; Nur77, neuron-derived clone 77; Nor1, neuron-derived orphan receptor 1; FNDC5, fibronectin type III domain-containing 5; GLUT4, glucose transporter 4; FAT/CD36, fatty acid translocase/CD36; UCP3, uncoupling protein 3; CI, complex I; CII, complex II; CIII, complex III; CIV, CIV subunit I; CIV, CIV subunit II; CIV, CIV subunit IV; CV, complex V. Values are means ± SE; N = 8–12. *P < 0.05.
the overall phenotype of these rats. The greater capacity for lipid oxidation observed in HCR rats is likely to result in the sparing of glycogen during exercise. Indeed, even in the untrained state, HCR rats exhibit muscle substrate storage profiles similar to that of endurance-trained athletes. Increased fuel availability as a result of greater intramuscular storage of glycogen and triglycerides (16, 17, 35), along with an increased capacity for lipid oxidation and overall enhanced rates of OXPHOS, would allow HCR rats to run longer distances than LCR rats.

Another finding of the present study is the elevated protein levels of FNDC5, which was confined to the glycolytic muscle of HCR rats. This protein has recently been identified as a PGC-1α-dependent membrane protein that is cleaved to become the putative myokine irisin (3). Although we were unable to measure circulating irisin concentrations, the reduced adiposity and superior glucose tolerance exhibited by HCR rats may, at least in part, be related to the increased expression of FNDC5 in the glycolytic muscle. Exercise training has been shown to increase the concentration of irisin in both mice and humans (3), so it is quite plausible that HCR rats have higher concentrations of circulating irisin than LCR rats. Furthermore, the “browning” effect seen in white adipose tissue after irisin administration (3) may not be restricted to this tissue, and we cannot discount the possibility that irisin elicits a similar effect on glycolytic skeletal muscle. Characterization of the adipose tissue from LCR and HCR rats will determine whether differences in mitochondrial function are restricted to the muscle or if they are also present in other highly plastic tissues.

Previously, we (16) have shown that the orphan NR Nur77 and its downstream targets are expressed to a lesser extent in LCR rats and that this is linked to impaired β-adrenergic signaling in the skeletal muscle. Another NR4A NR, Nor1, shares a high sequence homology with Nur77 and has been shown to be induced under many of the same conditions (31). Pearen et al. (32) demonstrated that Nor1 is essential for oxidative metabolism in cultured myotubes and that muscle-specific Nor1 transgenic mice have a more oxidative skeletal muscle phenotype, a greater running capacity, and better glucose tolerance compared with their wild-type littermates (30). Here, we provide evidence showing that HCR rats have greater Nor1 protein levels in both the soleus and EDL muscles (Figs. 5 and 6) and suggest that Nor1 may serve as a possible mechanism for increasing the cell’s oxidative machinery. In support of a possible Nor1-mediated response, we found no differences in PGC-1α mRNA expression between phenotypes. Furthermore, little (in the EDL; Fig. 6) or no difference (in the soleus; Fig. 5) in PGC-1 protein levels was observed, suggesting that an alternative program is responsible for the greater respiratory capacity seen in the muscle of HCR rats. At present, little is known about the molecular mechanisms through which Nor1 is likely to drive changes in oxidative metabolism. Thus, more indepth analyses of the function of Nor1 and clarification of its primary targets will be essential in determining its precise role in the regulation of skeletal muscle metabolism.

Physical training increases skeletal muscle insulin sensitivity and oxidative capacity and induces a rapid increase in Nor1 transcription (21, 46, 50). Although both LCR and HCR animals were kept under identical housing and feeding regimens that excluded formal exercise training, we cannot discount the possibility that habitual activity levels may play a role in the development of their distinct phenotypes. Therefore,
we monitored the activity of the rats during both diurnal and nocturnal periods and found that HCR rats were considerably more active than LCR rats (Table 2). This observation is in agreement with others (26, 27, 40) who report differences in the habitual activity levels of LCR and HCR rats from earlier generations. Furthermore, Novak et al. (28) identified different activity levels in another strain of obesity-resistant rats, whereas others (7, 19) identified a type of “inactivity physiology” in humans. Since there is a link between intrinsic exercise capacity and daily habitual activity in rodents, and a link between inactivity and poor metabolic health in humans (7, 11, 18, 27), the extent to which spontaneous cage activity contributes to the distinct phenotypes of these rodent models is an important question that needs to be addressed. Although controlling for habitual activity is difficult, we propose that it is partially responsible for the divergent metabolic phenotype displayed by these two rat strains, as we (9, 17) have previously shown that exercise training of LCR rats reverses many of negative metabolic health traits observed in these animals.

In summary, we used an animal model in which artificial selection for HCR and LCR (in the total absence of exercise training) simultaneously controls for unknown environmental influences and allows the two phenotypes to act as controls for one another. In addition to their divergent running capacities, these rats displayed differences in a number of metabolic health traits and, importantly, markedly different muscle oxidative capacities that underlie the major differences between phenotypes. Given that the NR Nor1 has been shown to play a key role in metabolic regulation and is necessary for oxidative metabolism, we propose that the differential expression of Nor1 observed in HCR and LCR rats may contribute to their distinct phenotypes and overall metabolic regulation. “Gain/loss of function” experiments will be required to confirm this hypothesis.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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