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Maximal oxidative capacity during exercise is associated with skeletal muscle fuel selection and dynamic changes in mitochondrial protein acetylation

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Summary

Maximal exercise-associated oxidative capacity is strongly correlated with health and longevity in humans. Rats selectively bred for high running capacity (HCR) have improved metabolic health and are longer-lived than their low capacity counterparts (LCR). Using metabolomic and proteomic profiling, we show that HCR efficiently oxidize fatty acids (FA) and branched-chain amino acid (BCAA), sparing glycogen and reducing accumulation of short- and medium-chain acylcarnitines. HCR mitochondria have reduced acetylation of mitochondrial proteins within oxidative pathways at rest, and there is rapid protein deacetylation with exercise, which is greater in HCR than LCR. Fluxomic analysis of valine degradation with exercise demonstrates a functional role of differential protein acetylation in HCR and LCR. Our data suggest efficient FA and BCAA utilization contribute to high intrinsic exercise capacity and the health and longevity benefits associated with enhanced fitness.

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Conceived and designed the experiments: KO, CE, NQ, J. Coon, CB, LK, SB. Performed the experiments: KO, CE, CM, J. Carson, CCS, DP, NQ. Analyzed the data: KO, CE, CM, CCS, CB. Wrote the paper: KO, CE, CB.

Introduction

Exercise capacity and cardiovascular fitness are highly predictive of metabolic health, including lower fat mass, higher insulin sensitivity, lower blood pressure and, importantly, age adjusted mortality (Blair et al., 1996; Church et al., 2004; Dvorak et al., 2000; Kodama et al., 2009). The mechanisms underlying these associations are not fully understood. One important link between exercise capacity and overall metabolic health is the fuel selection for energy production. Higher exercise capacity is associated with increased fatty acid (FA) oxidation during exercise (Hall et al., 2010; Morris et al., 2013; Nordby et al., 2006; Venables et al., 2005), while poor metabolic health is associated with high basal use of carbohydrates and impaired fuel switching during the fast-fed transition (Kelley and Mandarino, 2000). The glucose-fatty acid cycle described by Randle et al. (1963) states that fat availability will drive fat oxidation and reciprocally lead to decreased glucose oxidation; however, this theory cannot explain instances when fat availability is high but carbohydrates are preferentially oxidized, as is the case during high-intensity exercise and insulin resistance (Kelley and Mandarino, 2000; Mittendorfer and Klein, 2001; Sidossis et al., 1997).

Recent advances in metabolomics and proteomics allow the quantification of tens to thousands of metabolites or peptides in a single biological sample. Integrating these techniques can provide insight into the changes in nutrient utilization under different physiological conditions. In these studies, we employed a combination of metabolomics and proteomics to investigate fuel selection in rats selectively bred for high and low intrinsic running capacity (HCR and LCR). The HCR-LCR rat model was derived from a heterogeneous founder population (N:NIH) with breeder selection based solely on intrinsic (untrained) treadmill running capacity (Koch and Britton, 2001). In this model, as in humans, exercise capacity is a heritable trait (Fagard et al., 1991; Ren et al., 2013), and like humans who differ in running capacity, HCR and LCR diverge in susceptibility to metabolic and related disease traits (Koch et al., 2011; Naples et al., 2010; Noland et al., 2007; Novak et al., 2010; Wisloff et al., 2005). Compared to LCR, HCR animals diverge more strongly in running capacity from the founder stocks and show a 2.4-fold increased running capacity over the highest capacity observed in inbred lines (Ren et al., 2013). HCR weigh significantly less than LCR throughout their lifespan, despite similar food consumption, and there is evidence of increased capacity of substrate oxidation (Rivas et al., 2011). A recent study (Gavini et al., 2014) showed that HCR and LCR have similar resting energy expenditure, but HCR have small elevations in exercise energy expenditure and greater exercise-induced heat production from their skeletal muscle. The phenotype of HCR is coincident with a host of health benefits (Wisloff et al., 2005), including a 28–40% increased lifespan (Koch et al., 2011).

In this study, we found that the respiratory quotient (RQ) is lower at rest in HCR compared to LCR, indicative of enhanced FA oxidation, and FA oxidation is even more markedly enhanced in HCR during exercise. Metabolomic and fluxomic profiling demonstrate that during exercise, HCR use FA and branched chain amino acids (BCAA) more efficiently than LCR. Assessment of the muscle mitochondrial proteome of HCR and LCR, as well as post-translational modifications (phosphorylation and acetylation), show specific differences

between HCR and LCR within oxidative pathways of FA and BCAA metabolism and provide evidence that rapid changes in protein acetylation during exercise could play a role in augmenting the fuel selection differences. These differences in fuel selection and proteome modification mirror those found in caloric restriction (Hallows et al., 2011) and implicate fuel selection and mitochondrial oxidative efficiency as mechanisms linking enhanced exercise capacity with improved metabolic status and longevity.

Results

HCR efficiently use fat during exercise

Using a protocol identical to that used for selecting breeders, HCR have 1.4-fold higher VO_2max and run 4.3-fold longer distance than LCR (Table S1). To estimate fuel use, we determined VO_2 and VCO_2 (normalized to lean mass) at rest and during exercise in HCR and LCR. Carbohydrate utilization rose rapidly in both HCR and LCR with onset of exercise (Figure 1A). In LCR, carbohydrate utilization continued to increase until exhaustion, with a concomitant decrease in fat oxidation. In contrast, HCR maintained comparatively high levels of fat oxidation throughout exercise. As HCR approached exhaustion, carbohydrate oxidation increased and fat oxidation decreased (Figures 1A–B). Similarly, at maintained submaximal exercise (75% VO_2max), the rate of fat oxidation was significantly higher in HCR than LCR (Figure S1), providing further evidence that HCR, compared to LCR, have enhanced capacity to oxidize FA.

We evaluated metabolites in plasma and gastrocnemius muscle from these same animals following a separate exercise bout (Table S2A). Subsets of rats (n=5–6) were sampled at rest (0 min), at 10 min (mean time to exhaustion for LCR), and at 45 min (mean time to exhaustion for HCR) (Figure 1C). At rest, LCR and HCR show no difference in levels of primary fuel sources blood glucose (Figure 1D), muscle glycogen (Figure 1E), or plasma non-esterified fatty acid (NEFA)(Figure 1F). Blood glucose increased in both LCR and HCR at 10 min of exercise but was reduced in HCR at 45 min (Figure 1D). Muscle glycogen decreased with exhaustion in LCR (10 min) and HCR (45 min), but glycogen levels were not significantly changed in HCR from 0 to 10 min of exercise (Figure 1E). These data suggest that muscle glycogen contributes significantly to the higher whole-body carbohydrate oxidation in LCR during the first 10 min of exercise (Figure 1A), while HCR have delayed mobilization of glycogen.

Although HCR have significantly greater fat oxidation throughout exercise, plasma NEFA declined similarly in HCR and LCR at 10 minutes of exercise (Figure 1F). However, plasma lipids (primarily triglycerides) declined more in HCR than LCR at 10 min of exercise (Figure 2A). Alterations in fat metabolism can also be observed through changes in acylcarnitines, which are derived from fatty acyl-chains exchanging CoA with L-carnitine in the mitochondria. At rest, muscle medium- and long-chain acylcarnitine species were lower in HCR than LCR (Figure 2B), while the same acylcarnitine species were higher in plasma of HCR than LCR (Figure 2C). With exercise, LCR muscle long-chain acylcarnitines changed minimally, and we observed only small rises in plasma long-chain acylcarnitines at 10 min, consistent with reduced FA use. In contrast, HCR muscle long-chain acylcarnitines increased at 10 min of exercise, indicative of increased FA import. In addition, minimal

changes in muscle medium-chain acylcarnitines (C6–C12) were found, suggesting efficient oxidation of the FA. Only near exhaustion do HCR muscle and plasma medium- and long-chain acylcarnitines increase above resting levels (Figure 2B–C and S2). The increases in plasma acylcarnitines in HCR and LCR near exhaustion likely reflect the limit of mitochondrial FA oxidation, and the delayed rise in plasma acylcarnitines in HCR reflects their greater capacity to oxidize FA during exercise.

Although FA and carbohydrates account for 85–95% of the fuel used during exercise, amino acids also contribute to overall energy production (Horton et al., 1998; Wagenmakers et al., 1991). At rest, HCR have higher muscle lysine and lower plasma leucine levels than LCR (Figures 2D–E). With exercise, there are greater changes in amino acids in HCR than LCR. Muscle BCAA (leucine, isoleucine, valine) and ornithine were lower in HCR than LCR at 10 min; these changes were paralleled by a fall in plasma BCAA and ornithine in HCR but not LCR (Figure 2D–E), suggesting increased utilization in HCR during exercise. Of all amino acids, BCAA contribute the most toward energy production, and BCAA metabolism increases with exercise (Wagenmakers et al., 1991). The first step in BCAA degradation is loss of nitrogen through transamination to yield branched-chain keto-acids (BCKA). We found that plasma BCKA, α-ketoisocaproic acid (KIC), α-ketomethlyvaleric acid (KMV), and α-ketoisovaleric acid (KIV), were significantly increased in LCR at 10 min but not in HCR (Figure 2F). Further metabolism of BCKA results in the formation of CoA derivatives that can be transferred to carnitine, primarily as C3-, C4- and C5-carnitine. These short-chain acylcarnitines were also elevated in LCR muscle and plasma near exhaustion (10 min) but only C4-carnitine increased in HCR at 10 min, likely reflecting increased n-butyryl-carnitine derived from fat oxidation. Muscle and plasma C3-, C4-, C5-carnitine similarly increased near exhaustion (45 min) in HCR (Figure 2B–C). As with FA oxidation, these results are consistent with greater utilization of BCAA in HCR during exercise.

Metabolite changes coincident with exhaustion

Carbohydrate use increases and FA use decreases with approach to exhaustion in both HCR and LCR. To identify metabolite changes that are coincident with fuel selection and exhaustion, we determined correlation coefficients (Pearson *r*) of metabolites with estimated FA use, carbohydrate use, and percent exhaustion (time at collection/previous time to exhaustion). We found 19 metabolites to be correlated with both fat and carbohydrate oxidation and coincident with exhaustion; most of these increased with longer exercise duration (Table S2B). Six of these metabolites were confirmed to change significantly with exhaustion in a second independent metabolomic data set (Table S2B) in which HCR and LCR were run to quarter, half, and full exhaustion (Table S2C). Muscle malate consistently increased with approach to exhaustion, as did blood lactate and acetylcarnitine (Figure 3A–C). In parallel, muscle glycogen levels declined with approach to exhaustion (Figure 1E). Muscle acetylcarnitine levels were positively correlated with carbohydrate oxidation and inversely correlated with fat oxidation (Figure 3D–E). A correlation between acetylcarnitine levels and fuel selection (RQ) has been reported previously (Kiens, 2006), but unlike previous reports, we found no association between depletion of muscle L-carnitine and the decline in fat oxidation (Figure 3F), suggesting that in this model, carnitine availability is not limiting fat oxidation. Rather, accumulation of acetylcarnitine indicates overproduction

of acetyl units relative to downstream utilization through the citric acid cycle at exhaustion in both HCR and LCR. The similar pattern of change in metabolite levels relative to exhaustion in HCR and LCR suggests that the mechanism of exhaustion is similar in both strains, but is simply delayed in HCR.

Specific upregulation and differential acetylation of mitochondrial proteins in HCR and LCR

Changes in fuel use, though dependent on fuel availability, are largely controlled by enzyme availability and activity. Previous studies have demonstrated increased mRNA and protein expression in pathways related to oxidative metabolism in HCR vs. LCR (Burniston et al., 2011; Kivela et al., 2010). To investigate potential changes in the proteome and post-translation modifications in skeletal muscle mitochondria with exercise, we performed quantitative proteomic analysis. Mitochondria were isolated from HCR and LCR extensor digitorum longus muscle at rest and after 10 min of exercise, and isobaric tags were used for multiplexed quantification (Hebert et al., 2013). We identified 428 mitochondrial proteins (Table S3A), of which 73 were phosphorylated and 85 were acetylated (Figure 4A–B; Table S3A–C). In prior studies, HCR were reported to have no change (Naples et al., 2010) or a fiber type-specific upregulation of mitochondrial mass (Rivas et al., 2011) when compared to LCR rats; thus we normalized our proteomic data to mitochondrial protein. In the mitochondrial proteome, 174 proteins were significantly different ($p<0.05$, permutation t-test) between HCR and LCR. Pathway analysis is a common tool to understand global changes in phenotypes in cells and tissues. Due to our focus on metabolism, we chose to use KEGG Pathway Ontology for analysis, which has well annotated metabolic pathways. KEGG pathway analysis by Enrichr (Chen et al., 2013) showed that the differentially expressed proteins were enriched in oxidative phosphorylation, FA metabolism, BCAA degradation, and tRNA biosynthesis pathways (Figure 4C). Of the 174 proteins, only 22 were significantly higher in HCR and included multiple enzymes involved in FA oxidation (Figure 4C, Table S3A). When we examined changes with exercise, we found few alterations in the proteome or phosphoproteome in either strain (Figure 4D–E). However, we found evidence for decreased acetylation in both HCR and LCR mitochondria following exercise (Figure 4F).

Overall, mitochondrial acetylation was lower in HCR at rest (Figure 5A), and the difference was amplified following exercise (Figure 5B). KEGG pathway analysis revealed that acetylation sites were enriched within the oxidative phosphorylation, citric acid cycle, BCAA degradation, propionate metabolism and FA metabolism pathways. By estimating the relative level of acetylation of all peptides within each of these pathways, we found that there was significantly lower mitochondrial acetylation in each pathway in HCR compared to LCR, both at rest and following exercise (Figure 5C). In addition, HCR had significant deacetylation in 4 of the 5 acetyl-rich pathways with 10 min of exercise, while LCR had no significant pathway changes in acetylation. These pathways are targets of the mitochondrial lysine deacetylase SIRT3 (Rardin et al., 2013). In particular, MDH2 K239 is a major Sirt3 target (Hebert et al., 2013) and is significantly differentially acetylated between HCR and LCR at 10 min of exercise ($p=0.02$) and becomes less acetylated in HCR with exercise ($p=0.057$; see Table S3C), suggesting a possible role of SIRT3 in mediating the differences

in acetylation. However, we found no difference in the SIRT3 levels between HCR and LCR in mitochondrial extracts by proteomic analysis (Table S3A) or Western blotting (Figure S3). If SIRT3 is mediating these differences, then it is likely due to differential activation of SIRT3.

To assess the role of differential acetylation of MDH2, we measured MDH activity, previously shown to be modulated by acetylation state (Hebert et al., 2013; Zhao et al., 2010), in isolated mitochondria from HCR and LCR. While MDH2 protein level were similar in HCR and LCR (Table S3A), there was a significant inverse relationship between average acetylation state of MDH2 and MDH activity, but less significant inverse relationship between individual acetylation sites and MDH activity (Figure S4). These data suggest a complex interplay between sites, and potentially other post-translational modifications, in regulation of individual enzyme activity.

Deacetylation of proteins in the BCAA pathway is associated with increased BCAA catabolism during exercise

To further assess the functional consequence of differential acetylation in HCR and LCR, we estimated flux through the BCAA degradation pathway by intraperitoneal injection of U-$^{13}C^{15}N$ valine. We determined the isotopic enrichment of downstream metabolites in serum and gastrocnemius muscle at rest and immediately after 10 min of exercise (Table S4A–B, Figure 6). We confirmed that valine injection does not significantly alter running capacity or estimated fuel preference (Figure S5) and that serum and muscle isotopic enrichment of valine in was not significantly different between HCR and LCR (Table S4A, Figure 6A). U-$^{13}C^{15}N$ valine (mass shift of 6Da; M+6 isotope) is reversibly transaminated to M+5 KIV (Figure 6B) and can be reaminated to M+5 valine by the incorporation of ^{14}N. HCR-Run had a greater M+5:M+6 ratio of valine in serum and muscle (Table S4A), indicating greater valine transamination-reamination in HCR. Muscle glutamate nitrogen enrichment (M+1) was also significantly greater in HCR-Run, supporting the conclusion that HCR have greater valine transamination in skeletal muscle (Table S4B).

The irreversible branched-chain keto-acid dehydrogenase (BCKDH) complex is the rate-limiting step of BCAA degradation. With exercise, there is greater ^{13}C-labeled C4-carnitine and β-hydroxyisobutryate (HIB) in muscle in both HCR and LCR (Figure 6C–D), indicating increased metabolism of valine. HCR showed greater flux through the BCKDH complex and downstream enzymes, as demonstrated by greater accumulation of ^{13}C-labeled C4- and C3-carnitines and succinate in muscle of HCR-Run vs. LCR-Run (Figure 6C, E and F). However, accumulation of ^{13}C-labeled succinate was quantitatively quite small (Table S4A), suggesting that valine contributes minimally to citric acid cycle anaplerosis.

The BCKDH complex can be activated by dephosphorylation, but the role of dephosphorylation in exercise-associated increase in BCAA utilization is unclear (Howarth et al., 2007). Our proteomics analysis showed a non-significant decrease in Ser293 phosphorylation of BCKDHA subunit with exercise in HCR (Figure 6, Table S3B). Notably, dihydrolipoamide dehydrogenase (DLD), a subunit of the BCKDH complex, contained 10 acetylation sites that were all less acetylated in HCR compared to LCR with exercise (Figure 6, Table S3C). As DLD is a subunit of the pyruvate dehydrogenase and α-ketoglutarate

dehydrogenase complex, future studies are warranted to dissect the potential modulation of these complexes secondary to changes in DLD acetylation.

Discussion

The selection for enhanced exercise capacity in HCR recapitulates many of the health benefits observed in humans (Blair et al., 1996; Church et al., 2004; Dvorak et al., 2000; Kodama et al., 2009) and provides a model to understand the underlying mechanisms linking oxidative capacity and metabolic health. Based on our results, we propose that animals with enhanced oxidative capacity have increased FA and BCAA utilization and ATP generation through upregulation and deacetylation of proteins within these oxidative pathways (Figure 7). In conditions of increasing energy demand such as exercise, HCR are able to continue production of ATP through oxidative phosphorylation, at a time when LCR have reached their maximal oxidative capacity. When the ability to oxidize substrate becomes limiting, glycogen is mobilized and lactate is generated through anaerobic glycolysis. The buildup of acetylcarnitine and other substrates in muscle at exhaustion in both HCR and LCR points to a profound imbalance between the influx of substrates and ability to oxidize these substrates.

Like others (Hall et al., 2010; Morris et al., 2013; Nordby et al., 2006; Venables et al., 2005), we show that higher exercise capacity is associated with increased FA oxidation during exercise. Akin to adaptations observed with exercise training (Battaglia et al., 2012; Holloszy et al., 1998; Kiens et al., 1993), non-trained HCR have upregulation of enzymes within the FA oxidation pathway (Table S3A), increased FA oxidation, slower lactate production and delayed glycogen utilization (Figure 1). Accumulated evidence suggest that deacetylation of enzymes within FA and BCAA pathways increases their activity (Hallows et al., 2011; Hirschey et al., 2010; Rardin et al., 2013; Still et al., 2013). Our finding of mitochondrial protein deacetylation within specific catabolic pathways with exercise provides an additional potential mechanism by which muscle can increase substrate oxidation in response to increased energy demand. Similar to observations in mice (Hirschey et al., 2010), we find that reduced acetylation of enzymes in FA oxidation pathways (Figure 5D) is associated with increased whole-animal FA oxidation (Figure 1B) and increased skeletal muscle efficiency (delayed medium-chain acylcarnitine accumulation) in HCR vs. LCR. Differential acetylation of BCAA pathway enzymes also parallels differential valine flux (Figure 6). The finding that mitochondrial MDH activity was inversely correlated with average MDH2 acetylation (Figure S4), but not as well correlated with individual acetyl-sites, supports a complex rheostat-like role for acetylation (and likely other modifications) of lysine residues (Choudhary et al., 2014).

Change in lysine acetylation of mitochondrial proteins is a balance between NAD^+-dependent SIRT3 deacetylase activity (Lombard et al., 2007) and non-enzymatic addition of acetyl groups to lysine (Wagner and Payne, 2013). In contrast to the lower acetylation observed with exercise training and calorie restriction (Palacios et al., 2009), the lower level of acetylation observed in HCR is not explained by SIRT3 content (Table 3A and Figure S3). Rather, the dynamic deacetylation could be due to SIRT3 activation by increases in mitochondrial NAD^+ that occurs with exercise (White and Schenk, 2012). In addition, the

differences in acetyl-unit availability may also contribute to the observed differences in acetylation between HCR and LCR. Acetylcarnitine accumulation with increasing exercise intensity is a consistent phenomenon (Constantin-Teodosiu et al., 1991; Gollnick et al., 1974; Hiatt et al., 1989; Sahlin, 1990; van Loon et al., 2001), and like others (Kiens, 2006), we show that acetylcarnitine levels correlate positively with carbohydrate oxidation and negatively with FA oxidation (Figure 3D–E). Thus, HCR mitochondria appear to be exposed to less acetyl groups at rest and during activity, contributing to the lower mitochondrial protein acetylation when compared to LCR.

Importantly, metabolic differences between HCR and LCR during exercise inform us about altered metabolic pathways that underlie risk for metabolic disease. Decreased fat oxidation during exercise (Hall et al., 2010) and impaired switching between glucose and FA oxidation are both linked to insulin resistance and diabetes (Kelley and Mandarino, 2000). Recent studies highlight a link between altered BCAA metabolism to the development of insulin resistance and diabetes, reviewed in (Lynch and Adams, 2014). Notably, resting plasma BCAA levels are also inversely correlated with exercise capacity (Morris et al., 2013), and disruption of BCAT2, the enzyme responsible for the initial transamination step, in skeletal muscle leads to decreased exercise capacity (She et al., 2010). Based on our data, changes in the capacity to oxidize FA and BCAA could be due to differential expression and post-translational modification of enzymes in FA and BCAA metabolism and oxidative phosphorylation (Figure 7). Intrinsic genetic differences could be amplified by mitochondrial acetylation in the setting of inactivity and overnutrition (Hirschey et al., 2011). Thus, phenotypes associated with metabolic disease, such as increased circulating BCAA and metabolic inflexibility, may reflect differences in the capacity to utilize BCAA and FA.

Beyond implications to metabolic health, these data suggest a possible mechanistic link between exercise capacity and longevity. Like HCR, a number of longevity models have elevated FA and BCAA oxidation, including Ames dwarf and growth hormone receptor knock-out mice (Westbrook et al., 2009) and caloric restriction in drosophila (Katewa et al., 2012), mice (Bruss et al., 2010) and humans (Huffman et al., 2012). In addition to enhanced FA and BCAA oxidation, HCR have reduced weight gain, lower percent body fat, and small elevations in non-resting energy expenditure (Gavini et al., 2014), and yet HCR have greater food consumption (Thyfault et al., 2009), implying energy wasting. Thus, the enhanced efficiently of FA and BCAA utilization is not accompanied by enhanced efficiency of energy production and suggests that HCR have mitochondrial uncoupling. This uncoupling may be in part due to expression of uncoupling protein 3 (UCP3) (Table S4A) (Gavini et al., 2014), in addition to the lower ATP yield per mole of oxygen consumed during of FA oxidation (compared to carbohydrate) and the intrinsic ability of lipids to uncouple mitochondria (Skulachev, 1998). Indeed, it has been shown that exercise training results in increased substrate utilization humans, accompanied by mitochondrial uncoupling (Befroy et al., 2008). Uncoupling in individuals with increased VO_2max appears to be associated specifically with FA oxidation (Jacobs and Lundby, 2013). Despite the uncoupling there is reduced oxidative damage in HCR vs. LCR (Tweedie et al., 2011), similar to caloric restriction (Lanza et al., 2012). Coincidently, long-lived organisms with mitochondrial mutations have elevated FA utilization and display upregulation of energy producing

pathways (Martin et al., 2011; Munkacsy and Rea, 2014). Like these models of longevity, HCR and LCR show pathway specific differences in mitochondrial protein abundance (Figure 4B–C). It has been suggested that metabolites mediate the response of the organism to mitochondrial dysfunction (Butler et al., 2013). Ongoing studies directed towards understanding the genetic basis of maximal exercise capacity (Ren et al., 2013) and the commonalities of metabolites in long-lived animals, may provide important insights into the integration of signals in modulating the whole-animal response to exercise and aging.

Methods

Animal exercise protocol

Animals were housed at the University of Michigan and the University Committee on Use and Care of Animals (Ann Arbor, Michigan) approved the study. HCR and LCR rats were used for indirect calorimetry and metabolomics analysis (male, generation 26 at 4.5 months of age), proteomic analysis (male, generation 31 at 3 months of age), and the isotope tracer study (female, generation 31 at 3 months of age). Exercise capacity was determined using an increasing intensity exercise test (Koch and Britton, 2001) on a motorized treadmill (Columbus Instruments, Columbus, OH), and this protocol was used for all subsequent tests.

For metabolomic profiling, rats were run for 0 min, 10 min, 45 min (HCR only). For the proteomics analysis, rats were run for 0 min and 10 min (n=5). For the isotope tracer study, HCR and LCR were intraperitoneally injected with U-$^{13}C^{15}N$ valine (100 mg/kg) and placed on a stationary treadmill for 10 min. Between 10 and 20 min post injection, the Run groups underwent the treadmill running protocol and the Rest groups remained on the stationary treadmill (n=4–6).

Immediately after removal from the treadmill, animals were euthanized by decapitation. Trunk blood was used for immediate glucose and lactate quantitation (Accu-Chek Aviva meter and Nova Biomedical Lactate Plus meter) and to prepare plasma and/or serum. Tissues were harvested and frozen in liquid nitrogen. For subsequent assays, frozen tissues were pulverized with a mortar and pestle that had been pre-chilled with liquid nitrogen.

Indirect Calorimetry

Volume of oxygen consumed (VO_2) and carbon dioxide produced (VCO_2) were recorded using a Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments), with a 5-second sampling period every 2 minutes. VO_2 and VCO_2 were scaled to lean mass determined using an NMR-based analyzer (Minispec LF90II, Bruker Optics, Billerica, MA). Whole body carbohydrate and fat oxidation were estimated from non-protein RQ (Peronnet and Massicotte, 1991). We adjusted the carbohydrate oxidation as reported previously (Jeukendrup and Wallis, 2005), see supplemental methods.

Muscle glycogen

Gastrocnemius muscle was digested for 15 min at 37°C in 30% KOH (m/v). Ethanol and Na_2SO_4 (64% and 0.32% final) were added to samples to precipitate glycogen overnight at −20°C. Sample pellets were washed twice with 10% KOH and 66% ethanol. Pellets were

dissolved with 4 N H_2SO_4 at 100°C for 2 hr and then neutralized with 4 N NaOH. Glucose concentrations was determined using Sigma's glucose assay kit (HK; St. Louis, MO) and was normalized to starting tissue mass.

Plasma lipid analysis

Total plasma non-esterified FA was measured with a kit from Wako (Richmond, VA). For lipid species analysis, lipids were extracted from plasma (Bligh and Dyer, 1959). Methyl esters were purified by thin layer chromatography and analyzed by gas chromatography (Sattler et al., 1991).

Muscle and plasma Metabolites

For detailed methods, see supplemental methods. Briefly, metabolites from frozen tissue and serum/plasma were extracted with a solvent mixture consisting of 8:1:1 HPLC grade methanol:chloroform:water. Polar metabolites were analyzed by hydrophilic interaction chromatography – electrospray time of flight mass spectrometry (HILIC-ESI-TOF)(Lorenz et al., 2011). Acylcarnitines were analyzed by reversed phase liquid chromatography – tandem quadrupole mass spectrometry (RPLC-ESI-QQQ). Amino acids and other polar metabolites were derivatized and analyzed by gas chromatography – electron ionization mass spectrometry (GC-EI-MS) (Badawy et al., 2007; Fiehn et al., 2000). Keto acids were analyzed by RPLC-ESI-TOF in negative ion mode (Evans et al., 2013). Targeted metabolite quantitation was performed by peak area using Agilent Masshunter Quantitative Analysis software, and natural isotope abundance correction was performed using MATLAB (2012a, The MathWorks, Natick, MA).

Mitochondrial isolation and proteomic analysis

For detailed methods, see supplemental methods. Intact mitochondria were isolated from homogenized extensor digitorum longus muscle. Proteins were reduced, alkylated, and digested with LysC followed by trypsin. The resulting peptides were labeled with TMT isobaric labels, mixed in equal amounts by mass, and fractionated by strong cation exchange chromatography. Phospho peptides were enriched with immobilized metal affinity chromatography (IMAC) with magnetic beads. Acetylated peptides were enriched with pan-acetyl lysine antibody-agarose conjugate. Enriched and non-enriched fractions were analyzed by nano-RPLC coupled to an Orbitrap Fusion (Thermo). Spectra were searched using the open mass spectrometry search algorithm and results were filtered to 1% FDR at the unique peptide level using the COMPASS software suite. TMT quantification and protein grouping were performed according to previously reported rules (Phanstiel et al., 2011). Proteins were identified as mitochondrial by mapping rat genes to a previously compiled MitoCarta compendium of mitochondrial mouse proteins (Pagliarini et al., 2008).

Statistics and Figures

Metabolite data are presented as mean ± standard error of the mean (SEM). Proteomic data was expressed as log2 fold change between rest and run and HCR and LCR. Statistical analysis and figures were made with the R statistical and graphing environment (R Core

Team, 2013). P-values were determined by permutation t-test using R *perm* package (Fay, 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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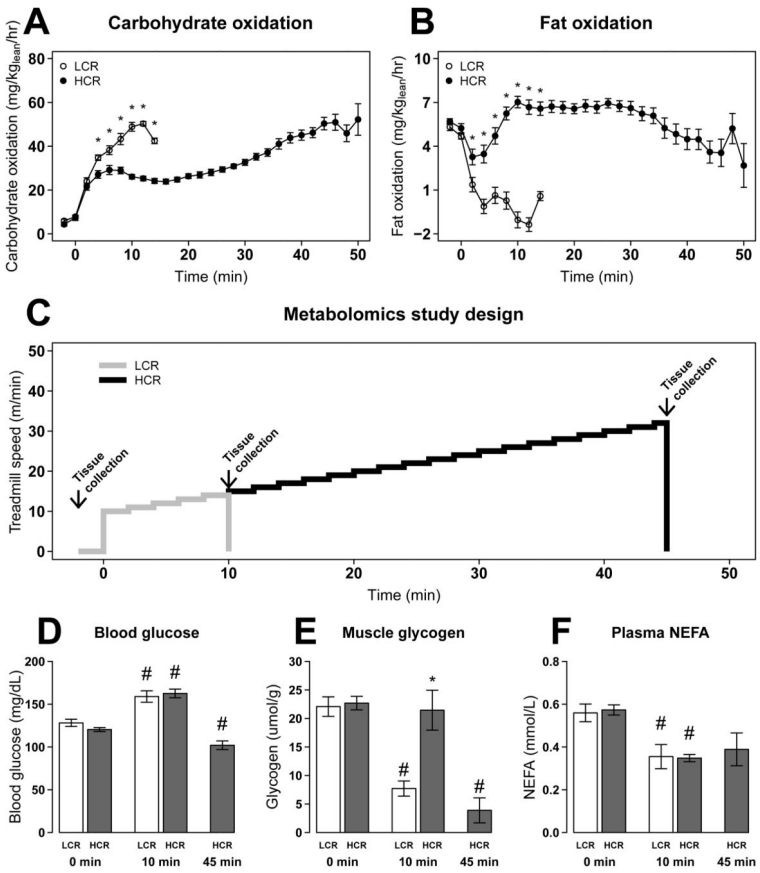


Figure 1. HCR have lower glycogen utilization and higher fat oxidation during exercise

Carbohydrate (A) and fat oxidation (B) were estimated from VO_2 and VCO_2 during an exhaustive exercise test for LCR (○) (n = 16) and HCR (●) (n = 23). Animals were separated into groups (n=4–6) and run for 0, 10, and 45 min (HCR only)(C). Blood glucose concentration (D), muscle glycogen (E), plasma non-esterified fatty acid (NEFA) (F) are presented as mean ± SEM for each group. * $p<0.05$ between HCR and LCR at a specific time point; # $p<0.05$ difference from rest (0 min).

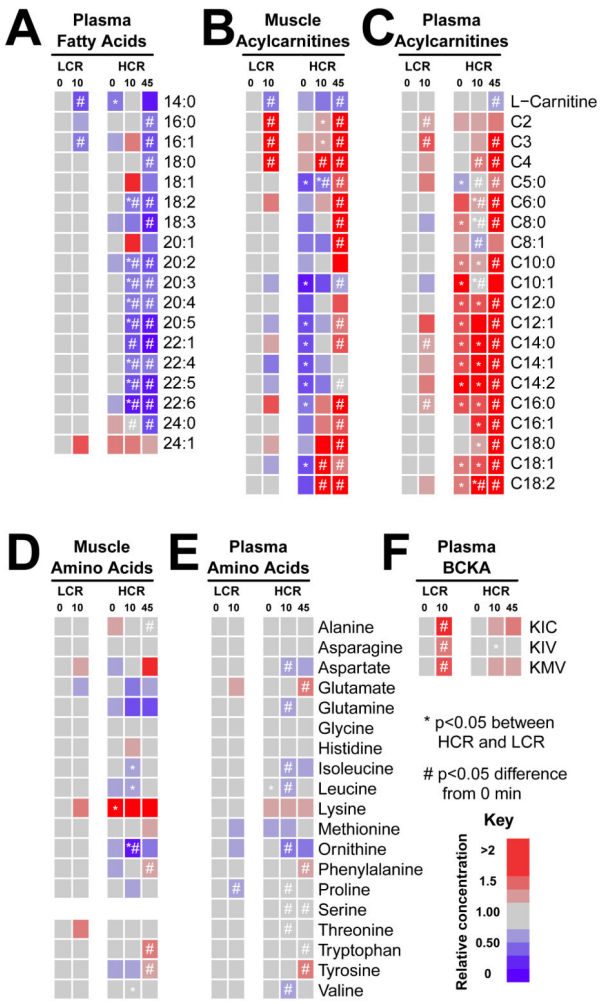


Figure 2. Muscle and plasma long-chain acyl-carnitines increase more with exercise in HCR

Metabolite values at 0, 10 and 45 min of exercise (LCR and HCR) are expressed relative to LCR rest (0 min) for each group (n = 4–6). Plasma fatty acids (primarily triglycerides) (A) and muscle and plasma acylcarnitines (B and C) are listed by carbon chain-length. Muscle and plasma amino acids (D and E) are less dynamic with exercise, but branched-chain keto acids (F) α-ketoisocaproic acid (KIC), α-ketoisovaleric acid (KIV), and α-ketomethylvaleric acid (KMV) show similar trends as short-chain acylcarnitines. * $p<0.05$ between HCR and LCR at a specific time point; # $p<0.05$ difference from baseline.

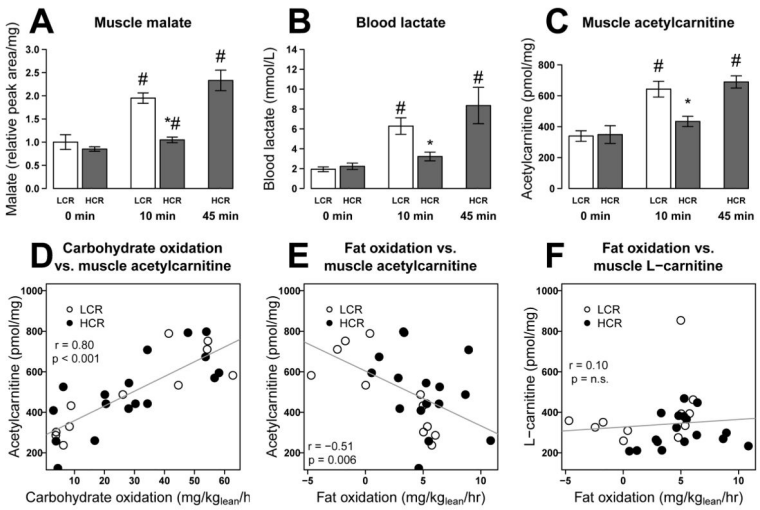
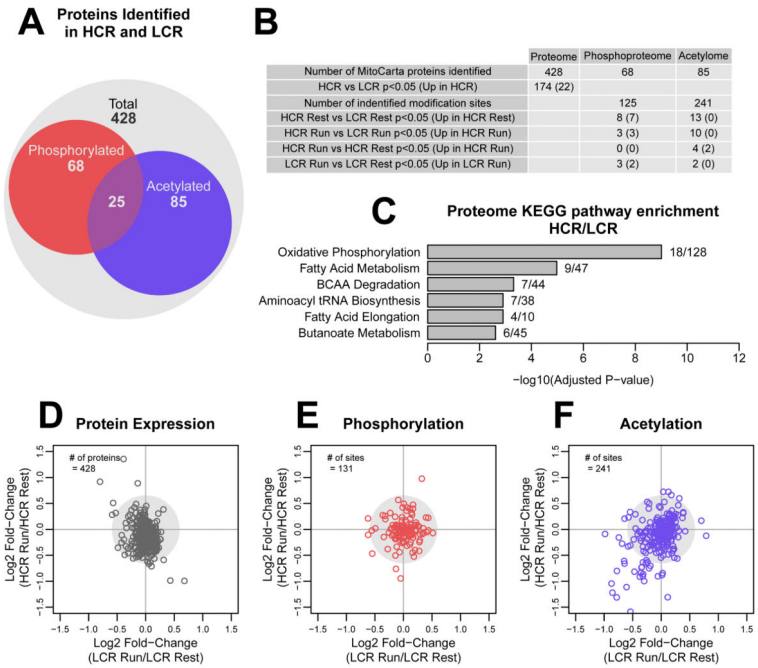


Figure 3. Citric acid cycle intermediates, blood lactate and short-chain acylcarnitines increase with exhaustion

Muscle malate (A), blood lactate (B) and muscle acetylcarnitine (C) values are shown at 0, 10 and 45 min of exercise as mean ± SEM for each group (n = 4–6). Muscle acetylcarnitine (C2) is also strongly correlated with previous estimates of carbohydrate oxidation (D) and fat oxidation (E) at 0, 10, and 45 min of exercise. Muscle L-carnitine (F) was not correlated with fat oxidation. * $p<0.05$ between HCR and LCR at a specific time point; # $p<0.05$ difference from baseline.



	Proteome	Phosphoproteome	Acetylome
Number of MitoCarta proteins identified	428	68	85
HCR vs LCR p<0.05 (Up in HCR)	174 (22)		
Number of indentified modification sites		125	241
HCR Rest vs LCR Rest p<0.05 (Up in HCR Rest)		8 (7)	13 (0)
HCR Run vs LCR Run p<0.05 (Up in HCR Run)		3 (3)	10 (0)
HCR Run vs HCR Rest p<0.05 (Up in HCR Run)		0 (0)	4 (2)
LCR Run vs LCR Rest p<0.05 (Up in LCR Run)		3 (2)	2 (0)

Figure 4. Mitochondrial protein acetylation is dynamic with exercise

We identified 428 mitochondrial proteins, of which 73 were phosphorylated and 85 were acetylated (A–B). For pathway enrichment, log2 fold-change values of differentially expressed proteins between HCR and LCR were input into Enrichr; the numbers of identified proteins out of total number of proteins in the ontology are listed to the right of the bars (C). Log2 fold-change (Run/Rest) values were used to plot changes with exercise in the proteome (D), phosphoproteome (E), and acetylome (F).

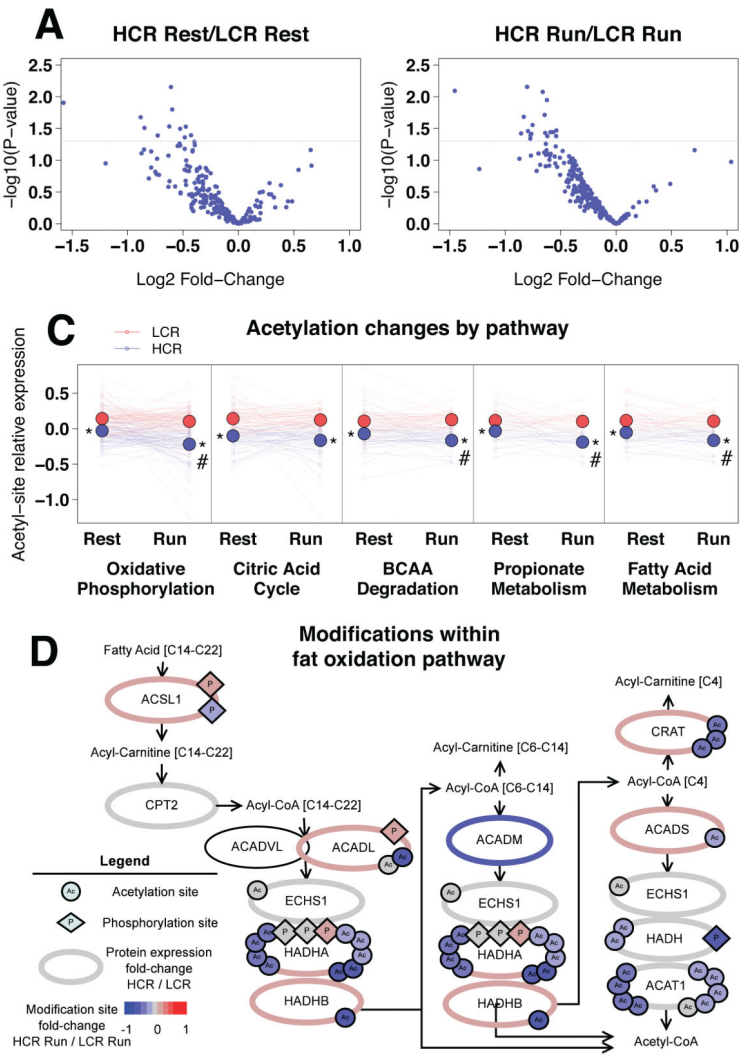


Figure 5. HCR have less mitochondrial protein acetylation than LCR

Log2 fold-change (HCR/LCR) values for the 141 acetyl-lysine sites were plotted against p-values at rest (A) and at 10 min run (B, n=5). Acetylation sites were enriched within oxidative pathways; for each enriched pathway, HCR proteins are less acetylated than LCR proteins (C). Average protein acetylation (small circles) is dynamic with exercise, and average pathway acetylation (large circles) is lower in HCR than LCR at 10 min of exercise. The fat oxidation pathway (D) is less acetylated in HCR with exercise. Proteins (ellipses) are labeled by gene symbol with identified acetylation (circle) and phosphorylation (diamond) sites. The color scale designates log2 fold-change (HCR/LCR comparison for protein; or HCR Run/LCR Run comparison for acetylation and phosphorylation).

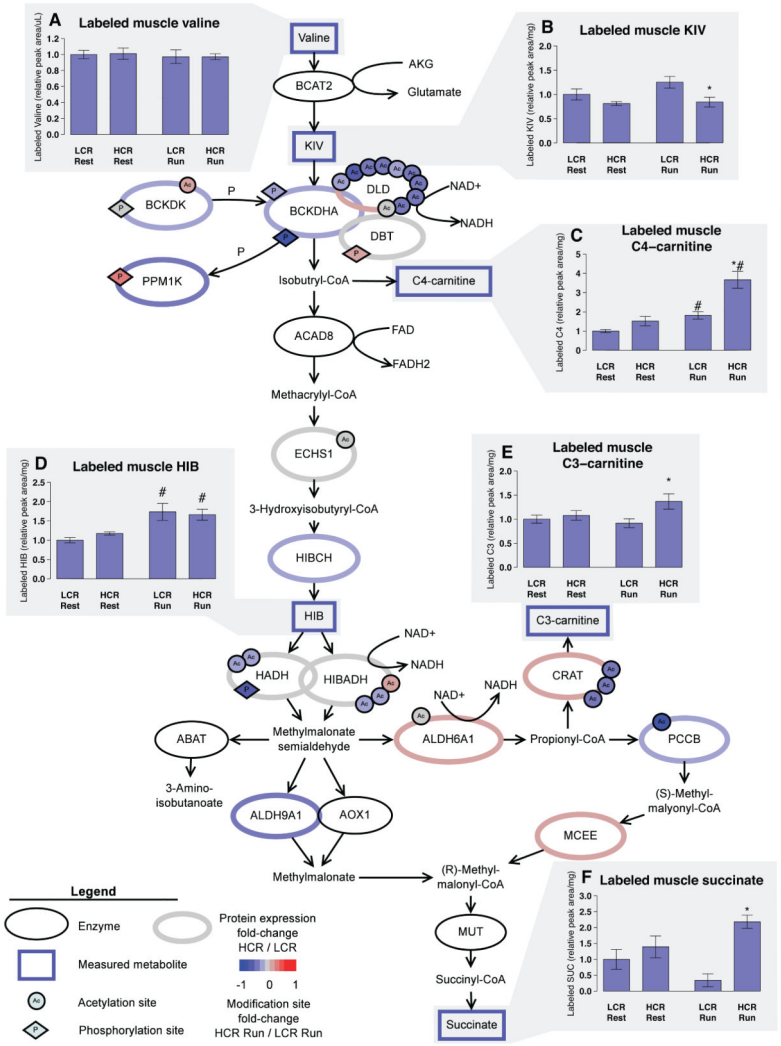


Figure 6. HCR have greater valine metabolism with exercise than LCR

Proteins involved in BCAA metabolism (ellipses) are labeled by gene symbol with identified acetylation (circle) and phosphorylation (diamond) sites. The color scale designates log2 fold-change (HCR/LCR comparison for protein; or HCR Run/LCR Run comparison for acetylation and phosphorylation). Animals were injected with U-$^{13}C^{15}N$ valine and rested for 10 min before an additional 10 min rest or 10 min run. LCR and HCR have similar amounts of labeled valine in gastrocnemius (A), but show differences in amount of labeled metabolites downstream of valine: α-ketoisovaleric acid (KIV, B), isobutyryl-carnitine (C4, C), β-hydroxyisobutyrate (HIB, D), propionyl-carnitine (C3, E) and succinate (F). Values are mean ± SEM relative to LCR rest for each group (n = 4–6). * $p<0.05$ between HCR and LCR; # $p<0.05$ difference within strain between Rest and Run.

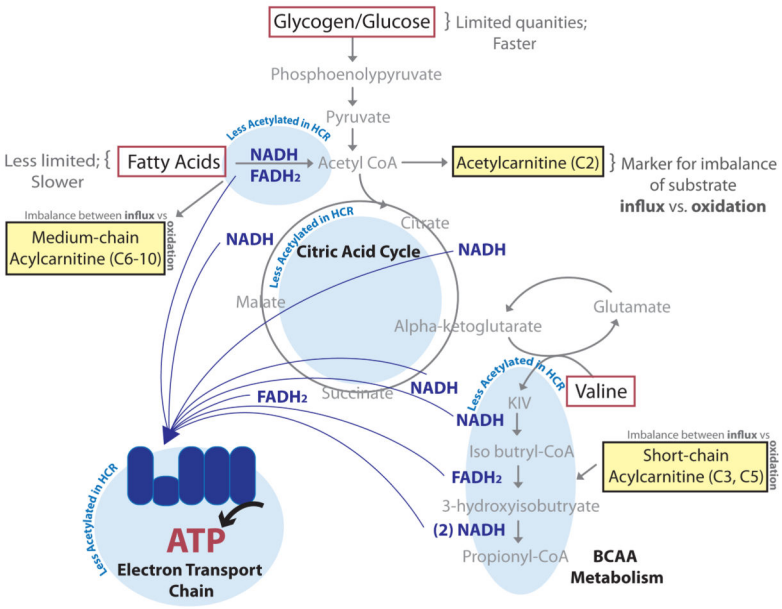


Figure 7. Increased fuel efficiency supports enhanced oxidative capacity

HCR have more efficient oxidation of fatty acid (FA) and branched-chain amino acid (BCAA); this is supported by lower protein acetylation within FA and BCAA metabolic pathways, as well as the citric acid cycle and the electron transport chain. Enhanced oxidative efficiency leads to slower accumulation of metabolic intermediates such as short- and medium- chain acylcarnitines, which indicate imbalances between substrate supply and downstream oxidation.