Mitochondrial Function in Metabolic Health

A thesis submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy

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Publications

Publications arising directly from this thesis

E.J. Stephenson and J.A. Hawley, Mitochondrial function in metabolic health: A genetic and environmental tug of war BBA General Subjects (invited review) In Review


Publications arising from work associated with this thesis


Other publications arising during the period of PhD candidature

Declaration

I, the candidate, declare that:

Except where due acknowledgement has been made, the work is that of the candidate alone and any editorial work, paid or unpaid, carried out by a third party is acknowledged.

The work has not been submitted previously, whole or in part, to qualify for any other academic award.

The content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program.

Ethics procedures and guidelines have been followed.

Erin J. Stephenson
July 2013
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<td>α-AR</td>
<td>α-Adrenergic Receptor</td>
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<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>AMP</td>
<td>Adenosine Monophosphate</td>
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<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>u</td>
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<td>UCP</td>
<td>Uncoupling Protein</td>
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<td>VO2max</td>
<td>Maximal O2 Consumption</td>
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<td>White Adipose Tissue</td>
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<td>Western Diet</td>
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Abstract

Inactivity-related diseases (such as obesity and insulin resistance) are a burden on Western society, with low cardiorespiratory fitness (maximal aerobic capacity, VO_{2max}) a strong independent predictor of metabolic disease and all-cause mortality. 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). The work undertaken for this thesis comprised a series of independent but related studies aimed at enhancing our understanding of the relationship between genetic factors and environmental stimuli in determining the capacity for aerobic energy production in skeletal muscle and white adipose tissue (WAT). Rodent models of divergent intrinsic running capacity (and, by association, metabolic health profile) and environmental interventions (i.e., diet and exercise) were employed in order to explore some of the mechanisms that determine the capacity for mitochondrial energy production in these two insulin-responsive tissues.

In the first investigation (Chapter 2), Long-Evans rats were given ad libitum access to either a Western Diet (WD; 40% energy (E) from fat, 17% protein, and 43% carbohydrate (30% sucrose); n=12) or a control diet (CON; 16% E from fat, 21% protein, and 63% carbohydrate (10% sucrose); n=12) for 12 wk. Rats fed the WD consumed 23% more E than CON (P=0.0001), which was associated with greater increases in body mass (23%; P=0.0002) and adiposity (17%; P=0.03). There were no differences in fasting blood glucose concentration or glucose tolerance between diets, although fasting insulin was increased by 30% (P=0.007). Fasting serum triglycerides were also elevated in WD (86%; P=0.001). The maximal respiratory capacity of m. soleus (soleus) was greater following the WD (37%; P=0.02), as were the maximal activities of several mitochondrial enzymes (citrate synthase, CS; β-hydroxyacyl-CoA dehydrogenase, β-HAD; carnitine palmitoyltransferase). Protein expression of CS, uncoupling protein (UCP)-3, and individual respiratory complexes was greater after WD (all P<0.05) despite no differences in the expression of peroxisome proliferator-

activated receptor-γ coactivator-1α (PGC-1α) mRNA or protein. The finding that the mitochondrial machinery was increased in skeletal muscle in response to the WD led to the conclusion that mitochondrial energy production pathways were up-regulated in order to cope with the sudden increased flux of energy substrates to metabolically active tissues. It was suggested that elevated skeletal muscle respiratory capacity would be, at least in the short term, protective against lipid-induced impairments in glycemic control.

Given that VO$_{2\text{max}}$ also has a genetic underpinning, the second investigation (Chapter 3) sought to identify whether intrinsic running capacity (and, by association, metabolic health) was associated with skeletal muscle mitochondrial content and/or oxidative capacity. Eleven-wk old genetically heterogeneous rats with inborn high- (HCR) and low- (LCR) running capacity were studied in the absence of exercise training. LCR rats ($n=12$) were 28% heavier ($P=0.0001$), and fasting serum insulin concentrations were 62% greater than in HCR rats ($n=12$; $P=0.02$). In contrast, HCR rats had better glucose tolerance ($P=0.01$) and reduced adiposity ($P=0.02$). In soleus, maximal respiratory capacity was 21% greater in HCR rats ($P=0.001$), for which the relative contribution of fat oxidation was 20% higher than LCR rats ($P=0.02$). This was associated with increased CS (33%; $P=0.009$) and β-HAD activities (33%; $P=0.0003$). In m. extensor digitorum longus (EDL), CS activity was 29% greater ($P=0.01$) and β-HAD activity was 41% greater ($P=0.0004$) in HCR compared to LCR rats. Mitochondrial DNA was also elevated in the EDL of HCR rats (35%; $P=0.049$) and soleus (44%; $P=0.16$). Additionally, HCR rats had increased protein abundance of individual mitochondrial respiratory complexes, CS, and UCP-3 in both muscle types (all $P<0.05$). The finding that both mitochondrial machinery and capacity were elevated in HCR compared to LCR rats is consistent with the observation that endurance trained individuals have a greater reliance on lipid-based fuels as an energy source, and explains, in part, how intrinsically determined skeletal muscle metabolism contributes to the phenotype of running capacity and its correlated traits.

WAT plays a central role in regulating whole-body lipid metabolism, with the metabolic activity of WAT being a crucial factor for substrate metabolism in other peripheral tissues. Thus, the primary aim of the third investigation (Chapter 4) was to
characterize the expression and activity of mitochondrial proteins important to energy production pathways in WAT from the LCR and HCR rat phenotypes. Additionally, since exercise training has recently been shown to alter important aspects of WAT energy metabolism (such as improved insulin action and elevated expression of PGC-1α and UCP-1), the effect of a short-term treadmill running protocol (same cumulative distance (~10 km) over 6 wk) on WAT from LCR and HCR rats was also investigated. LCR and HCR rats (n=10 per group, 22 wk old) were studied with or without exercise training. In untrained rats, the abundance of individual mitochondrial respiratory complexes, CS, and PGC-1α was similar for both phenotypes, although, CS activity showed a tendency to be greater in HCR (50%; $P=0.09$). Exercise training increased CS activity in both phenotypes but did not alter mitochondrial protein content. Training increased the expression and phosphorylation of proteins with roles in β-adrenergic signaling, including the β3-adrenergic receptor (increased 16% in LCR; $P<0.05$), neuron-derived orphan receptor-1 (decreased 24% in LCR and 21% in HCR; both $P<0.05$), phosphor-adipose triglyceride lipase (increased 25% in HCR; $P<0.05$), perlipin (increased 25% in HCR; $P<0.05$), comparative gene identification-58 (increased 15% in LCR; $P<0.05$), and the glucose transport protein GLUT4 (increased 16% in HCR; $P<0.0001$). A training effect was also observed for the phosphorylation status of the stress kinases p38 mitogen-activated protein kinase (decreased 12% in LCR and 20% in HCR; both $P<0.05$) and c-JuN terminal kinase 1/2 (increased 29% in LCR and 20% in HCR; both $P<0.05$). It was concluded that in the LCR-HCR rat model system, mitochondrial protein expression in WAT is not affected by intrinsic running capacity or short-term exercise training. However, training does induce alterations in the activity and expression of several proteins that are essential to the intracellular regulation of WAT metabolism.

In summary, this thesis has identified several novel mechanisms by which mitochondrial function/adaptations can influence the capacity for energy substrate metabolism in skeletal muscle and WAT.
Mitochondrial function in metabolic health: A genetic and environmental tug of war

Adapted, in part, from E.J. Stephenson & J.A. Hawley BBA General Subjects (invited review)

In Review

1.0 PHYSICAL INACTIVITY AND DISEASE: A METABOLIC CROSS ROAD.

The recent proliferation in the rate of diagnosis of many lifestyle-related diseases stems from the readiness of the vast majority of individuals from industrialized nations to adopt a sedentary lifestyle in the face of excess energy intake [1]. Accordingly, during the past 50 years there has been an explosion in the prevalence of a number of chronic metabolic disorders including obesity, type 2 diabetes and cardiovascular disease. 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). However, since no new major human gene mutations have occurred in the latter half of the 20th century to cause the greater frequency of chronic metabolic diseases, the increased incidence must principally be due to alterations in environmental conditions. One environmental factor to have changed dramatically in this time and strongly associated with a plethora of chronic metabolic disorders is a decline in daily physical activity [2]. In healthy individuals, acute but persistent sedentary behavior (inactivity for 2-7 hours/day) has been shown to impair whole-body insulin sensitivity and glucose tolerance [3] so, it is hardly surprising that an inactive lifestyle initiates a cascade of physiological events that are mechanistically linked to metabolic disease progression [4]. The results of several cross-sectional and epidemiological studies provide direct evidence that a lack of physical
activity is strongly associated with the prevalence of a number of risk-factors associated with obesity and other cardio-metabolic disease states [5-10].

The increased prevalence of obesity, insulin resistance and type 2 diabetes 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. Strong evidence in support of this premise comes from studies in which multiple genes involved in energy metabolism are coordinately down-regulated in individuals with obesity and associated conditions, and are linked to the pathogenesis of these disorders [11, 12]. As such, low cardio-metabolic fitness (i.e., whole-body aerobic capacity; VO\textsubscript{2max}) is now recognized as a strong independent risk-factor for not only several cardiovascular conditions, but also all-cause mortality [13, 14]. In line with this notion, it is now well accepted that regular physical exercise offers an effective therapeutic intervention to prevent, improve or, in some instances, reverse many of the hallmark features of cardio-metabolic disease [15, 16]. Thus, an understanding of the mechanisms that control how different biochemical pathways respond to physical activity (or inactivity) is critical for establishing the biological basis of obesity and its interrelated co-morbidities.

As noted earlier, obesity is central to the cardio-metabolic paradigm, with its progression directly linked to the development of insulin resistance, type 2 diabetes mellitus and cardiovascular disease [17]. Generally, the obesity sequelae promote disease states that are characterized by impaired regulation in the delivery, storage and utilization of energy substrates (primarily carbohydrate- and lipid-based fuels). The capacity of these substrate-related regulatory systems is highly dependent on several factors including the intrinsic metabolic program (as determined by genes) and environmental programming (Fig. 1.1) [18, 19]. Although complex interactions between intrinsic and environmental stimuli underpin an individual’s ability to respond to transient perturbations in energy availability and the ensuing hormonal milieu, the efficacy of this response is largely dependent upon environmental factors controlling energy intake and energy expenditure [20]. Thus, modulation of factors
linked to energy intake (i.e., the macronutrient composition of the diet) and/or energy expenditure (i.e., level of habitual physical activity), has the potential to enhance, or conversely, impair overall cardio-metabolic health [21].

This chapter presents a synopsis of studies that have examined the relationship between skeletal muscle and white adipose tissue (WAT) mitochondrial energy metabolism, whole-body aerobic capacity and cardio-metabolic disease risk, with a focus on some of the intrinsic (genetic/epigenetic) and environmental factors that are strongly associated with metabolic health status.

![Diagram](image)

**Figure 1.1** The metabolic program is determined through complex interactions between intrinsic factors (i.e., genes) and environmental stimuli. Independent of genetic mechanisms, modulation of factors linked to energy intake and energy expenditure has the potential to influence overall cardio-metabolic health.
1.1 Skeletal Muscle and Metabolic Health

Skeletal muscle comprises about 55% of individual body mass in sedentary humans and plays important roles in locomotion, heat production during periods of cold stress, and whole-body metabolism [22]. There is a remarkable capacity for skeletal muscle to adapt to a variety of external stimuli including habitual level of contractile activity, substrate availability, and the prevailing environmental conditions [23, 24]. This phenomenon of plasticity, common to all vertebrates [25], explains, in part, the marked differences observed in physical performance (such as feats of endurance or strength) between individuals, and also underpins the vastly divergent health profiles observed within a population. In this regard, skeletal muscle plays a major role in determining whole-body energy homeostasis because in healthy individuals it is the site of up to 80% of postprandial glucose disposal [26]. However, in inactivity-related conditions such as obesity, insulin resistance and type 2 diabetes, there is reduced insulin-stimulated glucose uptake into muscle, which has been associated with impairments in skeletal muscle mitochondrial content and/or function [27, 28]. The extent to which impaired skeletal muscle mitochondrial function play a causal or coincidental role in metabolic disease progression and whether these changes are secondary to lifestyle factors is a matter of current debate [29-32].

1.2 White Adipose Tissue and Metabolic Health

WAT was once considered an inert storage facility, however, it is now known to play a crucial role in metabolic regulation. Under fasting conditions, energy production in the skeletal muscle and other organs is almost entirely reliant on the oxidation of free fatty acids (FFA’s) [33] and the availability of FFA’s is largely dependent on the metabolic activity of WAT [34]. Although the metabolic activity of WAT is relatively low compared to other tissues, the major functions of WAT (i.e., lipolysis, FFA-re-esterification, de novo lipogenesis, glucose uptake and adipokine synthesis and secretion; Fig. 1.2) are all energy consuming processes that rely on substrates derived from the co-ordinated activities of the enzymes controlling β-oxidation, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation [34, 35]. Given that many of these important metabolic processes appear to be altered in the obesity sequelae, it
has been posited that disruptions in WAT mitochondrial metabolism may be associated with metabolic disease progression [35].

Figure 1.2 Lipid cycling in WAT is dependent on mitochondrial metabolism. α-AR, α-adrenergic receptor; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; ATP, adenosine triphosphate; β-AR, β-adrenergic receptor; CoA, coenzyme A; cAMP, cyclic AMP; CGI-58, comparative gene identification-58; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; FA, fatty acid; FFA, free fatty acid; G-3-P, glyceraldehyde-3-phosphate; G, inhibitory G protein-coupled receptor; Gs, stimulatory G protein-coupled receptor; HSL, hormone sensitive lipase; IRS-1, insulin receptor substrate-1; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; OA, oxaloacetate, PDE-3B, phosphodiesterase-3B; p-GA, phosphoglueraldehyde; PI3-K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKA, protein kinase A; PLIN1, perilipin; TAG, triacylglycerol; WAT, white adipose tissue.
2.0 Mitochondrial metabolism matters!

The relationship between \( \text{VO}_{2\text{max}} \) and metabolic health highlights the importance of the body's capacity to deliver and utilize \( \text{O}_2 \) in energy producing pathways [14, 36-38]. \( \text{VO}_{2\text{max}} \) is primarily limited by \( \text{O}_2 \) delivery systems [39-41], although the capacity for mitochondrial energy production in peripheral tissues, especially in skeletal muscle, is also of major importance [40]. Indeed, while the capacity for mitochondrial energy production is not likely to be rate-limiting for the attainment of \( \text{VO}_{2\text{max}} \) [39], it is a fundamental requirement for the tolerance of physical activity at a sustained intensity [42-44]. Therefore, skeletal muscle mitochondrial function indirectly influences whole-body aerobic capacity by determining the economy of exercise at different workloads [45] and, subsequently, the extent to which the cardiovascular system is challenged and whether or not adaptations occur in the \( \text{O}_2 \) delivery systems [41, 46, 47]. Moreover, this positive correlation between \( \text{VO}_{2\text{max}} \) and the aerobic capacity of skeletal muscle [48] strongly suggests that any factor with the potential to enhance skeletal muscle mitochondrial content and/or function will positively affect \( \text{VO}_{2\text{max}} \) (and possibly its correlated traits). Thus, an understanding of how physical activity induces favorable adaptations to the skeletal muscle mitochondrial network is essential to understanding the pathology of cardio-metabolic disease.

2.1 Mitochondrial function in health

To understand disease progression it is important to recognize what constitutes 'healthy' metabolism. In healthy individuals free from metabolic disease, skeletal muscle has the capacity to oxidize both carbohydrate- and lipid-based fuels and to transition between these substrates in response to hormones (predominantly insulin), substrate signals (such as elevated FFA flux) and the contractile status of the muscle (rest versus contraction) [49]. Similarly, the WAT must coordinate between lipolytic and lipogenic pathways in response to hormones (primarily insulin and catecholamines), substrate signals and the contractile status of skeletal muscle [33]. This 'metabolic flexibility' underpins the major roles that skeletal muscle and WAT have in whole-body energy homeostasis, and highlights the importance of well-coordinated mitochondrial function in maintaining metabolic control [50]. Thus,
functional defects in the energy producing systems of skeletal muscle and/or WAT mitochondria are likely to have profound systemic effects on overall metabolic health.

The main energy-producing processes in skeletal muscle and WAT are located in the mitochondria. Under fully aerobic conditions, pyruvate, (the end product of glycolysis) is converted to acetyl CoA in the mitochondrial matrix, where it enters the TCA cycle (or in the case of WAT, it may be directed toward lipogenesis, Fig. 1.2) and undergoes a series of reactions that reduce the co-enzymes nicotinamide adenine dinucleotide (NAD⁺) and flavin adenine dinucleotide (FAD; Fig. 1.3A) [51]. Similarly, the oxidation of free fatty acids occurs in the mitochondrial matrix and involves the reduction of NAD⁺ and FAD with the end product also being acetyl CoA (Fig. 1.3A) [52]. These reduced co-enzymes (from the TCA cycle and β-oxidation pathway) then pass electrons to the electron transfer system (ETS), a series of oxido-reductase enzymes located on the inner mitochondrial membrane where the transfer of electrons between enzyme complexes ultimately reduces oxygen to water in a process that is coupled to the production of adenosine triphosphate (ATP; Fig. 1.2B) [53]. Essentially, the skeletal muscle mitochondrial reticulum acts as a ‘sink’ for incoming energy substrates and uses them for the production of cellular energy, switching between the oxidation of lipid and carbohydrate substrates according to rates of supply and demand [54]. Conversely, the WAT mitochondrion coordinates switching between ATP production and lipid release/storage pathways (Fig. 1.2). Thus, rates of substrate uptake and the capacity for oxidation once inside the mitochondria are crucial points of metabolic regulation for not only skeletal muscle and WAT, but also whole-body metabolism.

2.1.1 MOVING MUSCLES MAKE MORE MITOCHONDRIA

The role and importance of skeletal muscle mitochondrial function in endurance training adaptation has long been recognized [55-59]. Classic work undertaken by John Holloszy almost 50 years ago demonstrated the potent role that exercise training plays in the biosynthesis of mitochondrial proteins [55]. For example, the capacity of the mitochondrial fraction from trained muscle to oxidize pyruvate doubled in rats subjected to a strenuous program of treadmill running. Mitochondria from the muscle
Figure 1.3 Mitochondrial energy production pathways. Reduced coenzymes from β-oxidation and the tricarboxylic acid cycle in the mitochondrial matrix (A) pass electrons to a series of oxido-reductase enzymes located on the inner membrane (B). The free energy released during electron transfer generates an electrochemical proton gradient between the matrix and the intermembrane space. Protons flow back into the matrix through CV, driving the synthesis of ATP [51-53].
of trained animals also exhibited a higher level of respiratory control and tightly
coupled oxidative phosphorylation compared to sedentary animals. Thus, the increase
in electron transport capacity was associated with a concomitant rise in the capacity to
produce ATP. This training-induced adaptation in mitochondrial function accounts for
the increase in aerobic work capacity that occurs with regularly performed, prolonged
exercise. Of importance when examining the role of exercise training in promoting
mitochondrial biogenesis is that a single bout of endurance exercise triggers a cascade
of molecular signaling events that ultimately lead to mitochondrial proliferation (Fig.
1.4 and 1.5 [60-68]) and other adaptations that favor the transport and oxidation of
carbohydrate and lipid substrates [62, 69, 70]. Notably, a rapid but transient elevation
in peroxisome proliferator activated receptor (PPAR)-γ coactivator-1α (PGC-1α) mRNA is observed following each acute bout of exercise [68], and this is accompanied
by a steady increase in PGC-1α protein, as well as an increase in the activity of several
oxidative enzymes over a period of training (Fig. 1.4 [68]). Thus, the cumulative
benefits of each (acute) bout of exercise result in chronic adaptations in skeletal muscle
(i.e., increased mitochondrial content) that favor improvements in fuel uptake and
utilization, exercise economy and, ultimately, whole-body aerobic capacity.

![Figure 1.4](image.png)

**Figure 1.4** Early time course of exercise training-induced mitochondrial biogenesis in
skeletal muscle. Representative figure showing that repeated transient PGC-1α mRNA bursts
precede increases in transcriptional- and mitochondrial- proteins (and their activities) over
the course of a two wk high-intensity interval training period. Image from Perry *et al.* (2010)
[68]. CS, citrate synthase. PGC-1α, peroxisome proliferator activated receptor-γ coactivator-
1α.
Figure 1.5  Contraction causes disturbances in the metabolic state of the muscle which leads to induction of PGC-1α and, subsequently, mitochondrial biogenesis. AMPK, adenosine monophosphate (AMP)-activated protein kinase; CaMK, Ca²⁺/calmodulin-dependent protein kinase; cAMP, cyclic AMP; CREB, cAMP response-element binding protein; NOR1, neuron-derived orphan receptor-1; NRF, nuclear respiratory factor; p38 MAPK, p38 mitogen-activated protein kinase; PGC-1α, PPARY coactivator-1α; PPARδ, peroxisome proliferator activated receptor-δ; TFAM, mitochondrial transcription factor A.
2.1.2 ADIPOCYTES IN (EXERCISE) TRAINING

Recent advances in our understanding of WAT metabolism have indicated that this dynamic tissue is capable of both responding and adapting to a number of external stimuli. Importantly, WAT has been shown to be exercise-responsive, with a number of metabolic changes occurring in response to various forms of endurance exercise training [71-78]. In 1991, Stallknecht et al. [71] reported an increase in the activities of TCA cycle (malate dehydrogenase) and oxidative phosphorylation (cytochrome C oxidase, CIV) enzymes in epididymal WAT in response to 10 wk of swimming training (6 hr/d, 5 d/wk). Similarly, Laye et al. [73] showed that 40 wk of voluntary wheel running can induce increases in citrate synthase (CS) activity and the protein content of CIV in omental WAT. Taken together, these findings suggest that chronic exercise training may be capable of inducing mitochondrial biogenesis in WAT. In support of this notion, Sutherland et al. [72] report a marked increase in PGC-1α mRNA in both the epididymal and retroperitoneal WAT depots immediately following an acute bout of endurance exercise (2 hr swimming), and an increase in CS activity and the protein abundance of CIV after four wk of swimming training (2 hr/d, 7 d/wk). Moreover, Xu et al. [79] observed an increase in mitochondrial number in epididymal WAT following 8 wk of treadmill running (40 min/d, 5 d/wk). Thus, despite the extreme nature of many of these training protocols [71-73], it is apparent that endurance training can induced mitochondrial biogenesis in WAT. However, unlike in skeletal muscle (Fig. 1.5), the mechanisms behind exercise-induced mitochondrial biogenesis in WAT are not well defined. Nonetheless, increased mitochondrial content and/or oxidative enzyme activity as a result of exercise training is an adaptation that would likely have favorable effects for the metabolic partitioning of FFA’s.

2.2 MITOCHONDRIAL (DYS)FUNCTION IN CARDIO-METABOLIC DISEASE

Skeletal muscle and WAT energetics have long been implicated in the pathogenesis of obesity and other lifestyle-related metabolic disorders in both humans [27, 80-83] and animals [73, 84-87]. However, the extent of mitochondrial involvement in metabolic disease progression, and whether changes observed in mitochondria are secondary to lifestyle factors (such as inactivity and/or diet) is a matter of much debate [30-32, 35].
One of the hallmark features of advancing cardio-metabolic disease is insulin resistance in peripheral tissues [26]. Insulin-resistant muscle demonstrates an impaired ability to transition between carbohydrate and lipid fuels and, as such, is often described as having ‘metabolic inflexibility’ [88]. Similarly, insulin-resistant WAT exhibits an inability to switch between lipolysis and re-esterification upon hormonal stimulation [89]. Thus, in both obesity and type 2 diabetes, the rates of both skeletal muscle lipid oxidation and WAT lipolysis do not suppress effectively in response to a meal (i.e., with insulin stimulation), whereas during the postprandial state, the rate of lipid oxidation is incapable of increasing sufficiently [89-91]. This leads to the accretion of triglyceride within both adipose and non-adipose tissues, a pathology strongly associated with both the insulin resistant state [92] and altered mitochondrial metabolism [35, 93]. As such, patients with type 2 diabetes [90], direct relatives of patients with type 2 diabetes [94, 95] and obese individuals [91, 96, 97] display an overt reduction in the capacity for lipid oxidation and an impaired ability to transition between fuel sources when subjected to changes in substrate availability [98, 99].

Given that the mitochondria are almost entirely responsible for the β-oxidation of FFA’s and the importance of TCA cycle intermediates in lipid storage pathways, it is perhaps not surprising that so-called mitochondrial ‘deficiencies’ have been observed in the skeletal muscle and WAT of obese rodents [73, 84, 86, 87, 100, 101] and humans [27, 81, 82, 102, 103]. These findings have led to the notion that abnormalities in skeletal muscle and/or WAT lipid metabolism may play a causative role in the pathogenesis of obesity and insulin resistance [27, 28, 84, 85, 104-106]. However, the issue of whether mitochondrial deficiency and/or defective mitochondrial metabolism contributes to insulin resistance has been questioned in light of two lines of evidence. First, Boushel et al. [107] reported that when O₂ flux was normalized for markers of mitochondrial content (such as mitochondrial DNA content or oxidative enzyme activity), oxidative phosphorylation and electron transport capacity in skeletal muscle extracts obtained from patients with type 2 diabetes were not different from those observed in muscle of age-matched healthy control subjects. In other words, they found no evidence for a decrease in the quality of the mitochondria in skeletal muscle from type 2 diabetic patients. A key feature of the data presented by Boushel et al. [107] was
that although mitochondrial respiration was indeed lower in the muscle of patients with type 2 diabetes than in a healthy control group, this was due to a reduction in muscle oxidative capacity (i.e. the number of mitochondria) rather than mitochondrial function per se. Importantly, this finding highlights the significance of nomenclature when discussing aspects related to muscle mitochondrial function: a true mitochondrial ‘dysfunction’ implies an inherent abnormality within the mitochondrial machinery rather than a decline in mitochondrial number or density (mass per unit volume) [108].

A second line of evidence that brings into question the issue of whether or not there is a mitochondrial ‘dysfunction’ in muscle of individuals with a range of chronic metabolic disease states are the recent reports demonstrating high-fat diet- (HFD) induced increases in mitochondrial protein abundance and enhanced oxidative enzyme activities [109-114]. Indeed, HFD’s cause elevated concentrations of circulating FFA’s which, acting as ligands to PPAR’s, can induce mitochondrial biogenesis through PGC-1α [112, 115-117] while concomitantly causing rapid gains in whole-body adiposity and peripheral insulin resistance [112, 118]. However, compared to a typical rodent HFD (60-80% energy (E) derived from fat), a typical human obesogenic diet (i.e., a Western diet; WD), is only moderately high in fat (~40% of total E from fat) yet still comprises a substantial proportion of E from carbohydrate (particularly sugars of high glycemic index) and protein [119, 120]. Although diets high in fat or high in sugar may result in similar outcomes (i.e., rapid gains in adiposity, insulin resistance, elevated circulating lipids) the mechanisms underlying the development of these pathologies are likely to be markedly different. Indeed, unlike with high-fat feeding, Brand et al. [119] did not observe defects in the insulin signaling cascade after using a cafeteria diet (70% E from carbohydrate) to induce insulin resistance in rats. Thus, although HFD’s are often used to study the underlying mechanisms of obesity and its associated conditions, the disparity between the macronutrient breakdown of different obesogenic diets is an important factor to consider when studying diet-induced metabolic disease progression.

Whether or not an obesogenic WD can induce mitochondrial changes in rodent skeletal muscle similar to that seen with a HFD is not clear. Regardless, the results of
recent studies indicate that increases in mitochondrial content and/or respiratory capacity are likely to precede insulin resistance in both rodents [121, 122] and humans [123, 124]. However, it has been proposed that the increase in mitochondrial content is a transient phenomenon [121-123] that disappears as the insulin resistance becomes more severe [121, 122]. This would suggest that a reduction in the mitochondrial content of skeletal muscle is not likely to be a causative factor in the pathogenesis of insulin resistance but, rather, that it is severely impaired insulin action which disrupts mitochondrial biogenesis. In line with this notion, the rate of insulin-stimulated mitochondrial protein synthesis is blunted in obese, insulin resistant individuals when compared to their lean counterparts [103]. Furthermore, in rodent models of HFD-induced obesity, attenuation of mitochondrial energy metabolism in both skeletal muscle and WAT appears to improve insulin action and glucose tolerance [125-133], whereas in WAT, insulin resistance develops prior to the down-regulation of mitochondrial protein expression [87]. Therefore, although diet undoubtedly plays a major role in the pathogenesis of obesity and insulin resistance, it is unlikely that ‘dysfunctional’ skeletal muscle or WAT mitochondrial energy metabolism (and in particular, a reduced rate of lipid oxidation) induced by a HFD is the direct cause of insulin resistance [32].

An alternative argument is that impaired lipid metabolism and reduced mitochondrial function is, in part, an adaptive response to an ‘inactivity cycle’, driven by diet-induced gains in adiposity coupled with other environmental influences which predispose to a reduced level of habitual physical activity. In support of this hypothesis, the respiratory capacity of skeletal muscle from the lower limb (m. vastus lateralis) is reduced in obese individuals and patients with type 2 diabetes, whereas the muscle from the upper limb (m. deltoideus) shows ‘normal’ mitochondrial function [134, 135]. Furthermore, despite differences in mitochondrial function in the leg musculature and a lower whole-body VO$_2$max, obese persons and patients with type 2 diabetes have a normal capacity for fat oxidation during exercise [99, 134], suggesting that the reduced mitochondrial content observed in the leg musculature of these metabolically challenged individuals is likely a result of reduced habitual locomotor activity, rather than an intrinsic deficit per se. Indeed, exercise training increases the oxidative capacity
of lower limb musculature in otherwise healthy, lean individuals, obese, insulin resistant individuals and patients with type 2 diabetes [136-140]. Moreover, despite improving insulin action, non-exercise induced weight loss does not appear to improve mitochondrial content/function in skeletal muscle [137, 138]. Thus, it seems entirely possible that the low skeletal muscle oxidative capacity observed in muscle from obese individuals reflects the lower levels of habitual physical activity (and therefore a reduced rate of ATP turnover in the locomotor musculature) typically displayed by obese, insulin resistant persons [141]. This line of reasoning is directly supported by findings which suggest that the oxidative capacity of lower-limb skeletal muscle is not impaired in non-obese, insulin resistant (but glucose sensitive) individuals compared with their non-obese, insulin-sensitive counterparts [124]. Furthermore, despite markedly elevated skeletal muscle lipid content in type 2 diabetic patients and different levels of whole-body glucose disposal, the best predictor of insulin sensitivity is skeletal muscle oxidative capacity rather than more 'traditional' markers of insulin resistance such as lipid status or body composition [140].

The level of habitual contractile activity may well be the missing link connecting skeletal muscle and WAT mitochondrial function with metabolic disease risk factors. Chronic inactivity (or disuse) results in a decrease in mitochondrial number [142], aberrant lipid handling [92] and an impaired health profile [2]. In contrast, chronic activity (exercise training) results in marked mitochondrial biogenesis in both skeletal muscle [142] and adipose tissue [71, 79], increased whole-body lipid oxidation and turnover [143] and an improved overall health status [2]. It therefore follows that interventions that result in either an increase or decrease in mitochondrial content should cause reciprocal changes in health/disease outcomes.

3.0 DO THE GENES FIT?

Despite the accumulating evidence suggesting that it is an 'inactivity cycle' which leads to a reduction in mitochondrial content during metabolic disease progression, a growing body of evidence is suggestive of a genetic component underlying the complexity of metabolic disease [11, 12, 144]. In the last decade a number of studies have demonstrated that cultured myotubes isolated from the skeletal muscle of obese
individuals retain the metabolic program of the donor tissue [97, 145-147], even though most of the adaptive factors have been removed (e.g., hormone and substrate fluctuations, neural input, contraction). Therefore, the remaining characteristics are primarily inherent, rather than due to environmental influences. This important finding suggests that several key features of metabolic inflexibility (such as impaired insulin action [145, 148-150] and a reduced capacity for lipid oxidation [97, 98, 145, 147]) may, in part, have a genetic or epigenetic origin [151]. In line with this notion, the heritability of endurance exercise capacity is estimated to be 40% or higher [152, 153] and has been linked to skeletal muscle mitochondrial gene expression [152]. However, familial environmental influence may also be important [154], with the heritability of exercise participation estimated to be as high as 70% [155]. Thus, while it is clear that a genetic component may underlie many of the features of cardio-metabolic disease, it is difficult to identify and attribute specific features to disease progression in living humans because of the profound environmental influence.

Given the polygenic nature of complex disease, another approach to study this problem has been to control for genetics in the hope of teasing out which facets of metabolic disease are primarily environmental and which are inherent. Studies in discordant monozygotic or same-sex dizygotic twins (where interpair differences are almost entirely environmental) have indicated that independent of genetic factors, acquired obesity and/or low aerobic capacity are associated with insulin resistance and a reduced expression of mitochondrial genes essential to lipid oxidation and oxidative phosphorylation in both muscle [156] and WAT [156, 157]. Furthermore, these reports have highlighted physical activity as a potent environmental modulator of body mass and other heritable traits linked to cardio-metabolic health [156-158]. However, the information obtained through these investigations is hampered by the same limitations as other human studies in that individuals are studied in a free-living environment making factors such as diet and lifestyle difficult to control. While these studies attempt to control genetics in order to investigate environmental factors, they provide little insight into the underlying genetic underpinning of cardio-metabolic health/disease.

The heterogeneous nature of human populations and the countless environmental variables make it difficult to study specific gene/environmental interactions in relation
to health outcomes in humans, an effort which is of utmost importance if the mechanistic role played by physical activity in determining cardio-metabolic health is to be understood [159]. Therefore, animal models that select for the phenotypic expression of polygenic traits provide valuable insight into the gene/environmental interactions that determine cardio-metabolic health. In 1996, Koch and Britton at the University of Michigan began a selective breeding program in a population of genetically heterogeneous rats, artificially selecting for high- and low- endurance running capacity in the absence of exercise training [160]. Selection of opposing phenotypic extremes has concentrated contrasting allelic variation for running capacity from one generation to the next, leading to two extreme divergent phenotypes. Remarkably, co-selecting for high- and low- running capacity also yielded divergent models of metabolic disease risk, with low capacity runners (LCR) displaying many characteristics common to metabolic disease phenotypes (such as increased body mass and adiposity, hyperinsulinaemia, impaired glucose tolerance and cardiovascular abnormalities) whereas high capacity runners (HCR) present with superior metabolic health, characterized by resistance to weight gain in the face of a HFD and an increased capacity for the uptake and utilization of both glucose and FFA’s [161-164]. Importantly, many of these features appear to be linked to the metabolic characteristics of the skeletal muscle [162, 165] and, in particular, the capacity for skeletal muscle oxidative energy production. However, there is some contention as to whether this is due to differences in the mitochondrial reticulum [165], an enhanced capacity of some (or all) of the oxidative enzymes [166-168], or a combination of both. Moreover, while the oxidative capacity of skeletal muscle is evidently a defining feature for these divergent phenotypes, little is known about the role played by WAT. Given the substantial overlap of both skeletal muscle and WAT metabolism in whole-body metabolic regulation, whether differences in WAT energy metabolism are associated with running capacity (and by association, metabolic health) is an important question that should be addressed in this rodent model.
**Table 1.1** Fasting parameters for LCR and HCR rats, generation 11

<table>
<thead>
<tr>
<th></th>
<th>LCR</th>
<th>HCR</th>
<th>% Difference LCR vs. HCR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mg/dL</td>
<td>110 ± 9</td>
<td>92 ± 5</td>
<td>20%</td>
<td>0.0007</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>684 ± 195</td>
<td>296 ± 172</td>
<td>131%</td>
<td>0.002</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>67 ± 24</td>
<td>25 ± 4</td>
<td>168%</td>
<td>0.013</td>
</tr>
<tr>
<td>Free fatty acids, meq/L</td>
<td>0.64 ± 0.22</td>
<td>0.33 ± 0.04</td>
<td>94%</td>
<td>0.031</td>
</tr>
<tr>
<td>Visceral adiposity/body mass, %</td>
<td>1.55 ± 0.39</td>
<td>0.95 ± 0.32</td>
<td>63%</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Blood was drawn from 11-12 wk old male LCR and HCR rats following a 12 hr fast. Values represent the mean ± standard deviation. n=8 per group. LCR, low capacity runner; HCR, high capacity runner. Table adapted from Wisløff et al. (2005) [161].

4.0 Scope of this work

The finding that mitochondrial adaptations occur in both skeletal muscle and WAT in parallel with changes in whole-body metabolic health highlights the importance of mitochondrial plasticity in responding to changing energy demands. However, there are still many gaps in our understanding of the gene/environment interactions that determine skeletal muscle and WAT mitochondrial metabolism. Accordingly, the aim of the work undertaken for this thesis was to assess the mitochondrial energy producing pathways in skeletal muscle and WAT, with the intention of investigating both the genetic and adaptive (i.e., diet and exercise training) mechanisms behind tissue respiratory capacity. Specifically, this thesis addresses the following questions:

1. Does an obesogenic WD alter the respiratory capacity of skeletal muscle and does it do so via the same signaling mechanisms as a HFD?
2. Is the difference in the respiratory capacity of LCR and HCR rat skeletal muscle due to increased mitochondrial number, increased oxidative enzyme activity or a combination of both?
3. Is intrinsic running capacity associated with the abundance and activity of oxidative enzymes in WAT?
4. Can exercise training alter WAT metabolism in rats with inborn high- and low- intrinsic running capacity?
Chapter 2

Skeletal muscle respiratory capacity is enhanced in rats consuming an obesogenic Western diet


2.1 Introduction

The macronutrient composition of the diet is an important factor in the pathogenesis of obesity [169, 170] and when combined with a sedentary lifestyle, a human obesogenic diet (characterised by the over-consumption of dietary fat and simple sugars [120, 170]), results in an energy imbalance and subsequent increase in the flux of energy substrates to metabolically active tissues [170]. When substrate delivery exceeds a tissue’s needs, substrates accumulate and are eventually stored [119, 171].

Skeletal muscle is a major site for both fatty acid (FA) and glucose disposal [33, 172]. In obesity, inappropriate lipid deposition in non-adipose tissues (such as skeletal muscle), and a concomitant reduction in the ability of cells to completely oxidize lipids has been frequently described [104, 173, 174]. Given that the mitochondria are entirely responsible for FA β-oxidation, it is not surprising that mitochondrial functional defects and deficiencies have been observed in the skeletal muscle of obese rodents [84, 100, 101] and humans [27, 102]. These findings have led to the notion that lipid-induced abnormalities in mitochondrial metabolism are a causative factor in the pathogenesis of obesity and its co-morbidities [27, 28, 84, 85, 104-106, 118].

The issue of whether mitochondrial ‘deficiency and/or dysfunction’ contributes to the obesity sequelae has been questioned in light of recent reports of HFD-induced increases in mitochondrial enzyme activities and protein expression [31, 110-112, 114]. Indeed, such diets cause an increase in circulating FFA [109, 112, 118, 175] which,
acting as ligands to the PPAR’s [115], are thought to increase mitochondrial biogenesis via the PGC-1α pathway (Fig. 1.5 [112, 115-117]). This suggests that certain aspects of mitochondrial function (i.e. β-oxidation and oxidative phosphorylation) may be up-regulated following a HFD.

High-fat fed rodents rapidly gain weight while concomitantly developing insulin resistance [176-178]. As such, this model is used frequently to investigate the underlying mechanisms of obesity and its associated conditions. Investigations are often undertaken based on the assumption that these pathologies develop similarly in rodents as they do in humans. However the typical human obesogenic diet (i.e. a WD), is moderately high in fat (~40-45% of total energy, E) yet still comprises a substantial proportion of E from carbohydrate (particularly simple sugars) and protein [120]. In contrast, a typical rodent HFD usually contains extreme levels of fat (up to 80% of total available E) and very low carbohydrate [119, 120]. Given the role diet composition plays in the mechanistic progression of obesity and insulin resistance, the disparity between the macronutrient breakdown of a rodent HFD and a typical human obesogenic diet is an important factor to consider when interpreting results from animal studies [119, 171]. While diets high in fat and diets high in sugar may result in the similar outcomes such as rapid weight gain [119, 171], increased circulating lipids [119, 171] and insulin resistance [119], the mechanisms underlying these changes are likely to be markedly different [119].

In the current investigation, rats were fed a commercially available diet that closely mimics a human WD (a diet both high in fat and in sucrose), and the mitochondrial respiratory capacity of the skeletal muscle was measured using high-resolution respirometry. It was hypothesized that 12-wk of a WD would increase the respiratory capacity of skeletal muscle mitochondria, via the up-regulation of PPARδ and PGC-1α.

2.2 Materials and methods

EXPERIMENTAL ANIMALS

Eight-wk old male Long-Evans rats (n=24) were obtained from Monash Animal Services (Monash, Australia) and housed under a controlled 12:12 hr light/dark cycle at
a constant temperature of 22 °C. Animals had *ad libitum* access to food and water and were allowed to acclimate to the RMIT Animal Facility for one wk prior to commencement of the study. All procedures were approved by the RMIT Animal Ethics Committee.

**Experimental diets**

Following the acclimation period, rats were randomly assigned to one of two 12 wk dietary interventions; a control diet (CON, \( n = 12 \); 16% of E from fat, 21% from protein and 63% from carbohydrate, 16.1 kJ/g; Specialty Feeds, Glen Forrest, WA, Australia) or a commercially available WD (\( n = 12 \); 40% E from fat, 17% from protein and 43% from carbohydrate; 19.4 kJ/g; Specialty Feeds, Glen Forrest, WA, Australia). The macronutrient breakdown of each diet is presented in Table 2.1. Body mass and food intake were monitored twice weekly.

**Glucose tolerance tests**

After 11 wk, all rats were subjected to a glucose tolerance test (GTT) to assess glucose clearance. Following an overnight fast (14 hr), ~15 µL of blood was collected via tail cut and glucose concentration was measured on a hand-held glucometer (Roche Diagnostics, Castle Hill, NSW, Australia). Rats then received a single glucose bolus (2 g/kg body mass) via intra-peritoneal injection, and blood glucose concentration was monitored at 30 min intervals over 120 min post-injection.

**Tissue collection and analyses**

After 12-wk, animals were decapitated and hind-limb muscles immediately excised. To limit any possible differences that might occur as a result of differing cage activity, the *m. soleus* (soleus) and *m. 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 fibers of the EDL muscle (~2% type I fibers, ~42% type IIA fibers, and ~56% type IIB fibers) are recruited only during rapid, high-intensity tasks [179]. The left soleus was placed in ice-cold muscle preservation media (BIOPS; K+-MES, 50 mM; taurine, 20 mM; dithiothreitol, 0.5 mM; MgCl₂, 6.56 mM; ATP, 5.77 mM; phosphocreatine, 15 mM; imidazole, 20 mM; pH 7.1 adjusted with 5 N KOH at 0 °C; Ca-EGTA buffer (10 mM;
2.77 mM CaK$_2$EGTA + 7.23 mM K$_2$EGTA; 0.1 µM free calcium) [180-182], while the contra-lateral soleus and EDL muscles were snap-frozen in liquid N$_2$ for later analyses. Epidydimal fat pads were removed and weighed as indicators of adiposity. All reagents used were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise specified.

**Blood serum analyses**

Trunk blood was allowed to clot over ice after which it was centrifuged to obtain serum. Serum was analyzed using commercially available kits for insulin (#80-INSRT-E01, ALPCO Immunoassays, Salem, NH) and lipids (free fatty acids, NEFA-C; triglycerides, Triglyceride E and Cholesterol, Cholesterol E, all from WAKO Pure Chemical Industries Ltd., Osaka, Japan).

**High-resolution respirometry**

High-resolution respirometry provides a direct measure of mitochondrial respiratory capacity *ex vivo*, from which information about specific components in a working system can be obtained [180]. Soleus (n=6 per dietary treatment) was mechanically separated over ice using fine forceps [180], then chemically permeabilized with saponin (50 µg.mL$^{-1}$ in BIOPS) for 30 min. This was followed by a 10 min wash period in mitochondrial respiratory media (MiR05; sucrose, 110 mM; K-lactobionate, 60 mM, ACROS Organics; EGTA, 0.5 mM; MgCl$_2$, 3 mM; taurine, 20 mM; KH$_2$PO$_4$, 10 mM; HEPES, 20 mM; adjusted to pH 7.1 with KOH at 37 °C; and 1 g. L$^{-1}$ fatty acid free BSA)[180]. Duplicate tissue samples (2-3.5 mg) were transferred to the chambers of an O$_2$K-Oxygraph high-resolution respirometer (Oroboros; Innsbruck Austria) containing 2 mL of MiR06 (MiR05 plus 280 IU.mL$^{-1}$ catalase) and calibrated to air saturation. Individual chambers were oxygenated to approximately 485 nmol.mL$^{-1}$ with pure O$_2$ (BOC; Australia).

**Substrate-uncoupler-inhibitor titration protocol**

Malate (2 mM) was added to the chamber and the mass specific O$_2$ flux ($J_{O_2}$; pmol.s$^{-1}$.mg$^{-1}$ wet weight) stabilized for 5-10 minutes. The tissue was then subjected to a substrate-uncoupler-inhibitor titration (SUIT) protocol that sequentially evaluates complex (C)I leak state (the addition of pyruvate, 5 mM and glutamate, 10 mM; state 2
respiration), oxidative phosphorylation with electron flux through CI, by titration (the addition of ADP, 0.25, 0.75, 2.5 and 5 mM; state 3 respiration), the integrity of the outer mitochondrial membrane (the addition of cytochrome C, 10 µM), maximal coupled respiration with electron flux through both CI+CII (the addition of succinate, 10 mM; state 3 respiration), maximal capacity of the electron transport system (ETS) by uncoupling with a stepped titration (the addition of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), 0.5 and 1 µM), uncoupled (u) oxidative phosphorylation with electron flux through CII (with the addition of the CI inhibitor rotenone, 0.5 µM) and, residual O₂ consumption (ROX; with the addition of NaN₃, 200 mM). Chambers were maintained at 37 °C, while O₂ saturation was maintained between 300 and 450 nmol.mL⁻¹, via regular titrations of H₂O₂. Mass-specific O₂ flux was determined using DATLAB (Oroboros, Innsbruck; Austria) from steady state J₀₂ normalized to tissue wet weight and adjusted for instrumental background and ROX. In addition, flux control ratios (FCR; results normalized to the maximal capacity of the ETS) were calculated to determine mitochondrial function independent of mitochondrial density [180].

MITOCHONDRIAL ENZYME ACTIVITIES

Muscle homogenates (n=10 per group) were prepared over ice from snap-frozen soleus or EDL (10-20 mg for each) in buffer (KCl, 175 mM; EDTA, 2 mM, pH 7.4; 1:50 or 1:100 dilution) then subjected to three freeze-thaw cycles. Citrate synthase (CS), β-hydroxyacyl-CoA dehydrogenase (β-HAD) and total carnitine palmitoyltransferase (CPT) activities were determined using the methods of Srere [183], Bergmeyer [184] and Bieber [185] respectively, with the following modifications. Briefly, aliquots of homogenate were added to the appropriate wells of a 96-well microplate with a working solution with final concentrations of either Tris-HCl, 72.5 mM; Acetyl CoA, 0.45 mM; DTNB, 0.1 mM (for CS), Tris-HCl, 50 mM; EDTA, 2 mM; NADH, 250 µM for (for β-HAD) or Tris-HCl, 116 mM; EDTA, 1 mM; L-carnitine, 1.1 mM; DTNB, 100 µM (for CPT). After monitoring the wells for background activity, reactions were initiated via the addition of oxaloacetic acid, 0.5 mM (for CS), acetoacetyl-CoA, 100 µM (for β-HAD) or palmitoyl-CoA, 1.9 mM (for CPT). Enzyme activities were determined by monitoring Δ absorbance at 412 nm (for CS and CPT) and 355 nm (for β-HAD) over 3
min at 25 °C. Extinction coefficients of 13.6 µmol/cm² (for CS and CPT) and 6.22 µmol/cm² (for β-HAD) were used to calculate rates of enzyme activity. Rates are expressed as µmol/min/g wet weight.

**INTRAMYOCYTOCELLULAR TRIGLYCERIDE DETERMINATION**

Intramyocellular triglyceride (IMTG) concentration was determined according to the method described by Spriet et al. [186]. Briefly, freeze-dried soleus and EDL (n=8 per group for each muscle) was powdered and cleaned of all visible blood and connective tissue. Total lipids in muscle fragments (5-10 mg dry muscle) were extracted in chloroform-methanol (13). The organic portion was then evaporated under a stream of N₂ and reconstituted in chloroform. Silicic acid was then added to the organic portion and the phospholipids removed by centrifugation. The resultant supernatant was evaporated and saponified in ethanolic KOH and glycerol content was analyzed fluorometrically [187].

**NUCLEIC ACID EXTRACTION AND QUANTIFICATION**

DNA and RNA were each extracted from approximately 10-15 mg of snap-frozen soleus and EDL. RNA was extracted using Trizol reagent (Invitrogen, Mulgrave, Australia), whereas DNA was extracted using a commercially available kit (Qiagen, Doncaster, Australia). The quality and quantity of both extracts was determined using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). For both DNA and RNA an aliquot was taken from each CON extract and pooled. Following quantification, the pooled sample was serially diluted in order to produce a standard curve for inclusion in each PCR run.

**REVERSE TRANSCRIPTION AND REAL-TIME PCR**

First-strand cDNA synthesis was performed for RNA extracts using Superscript VILO (Invitrogen, Mulgrave, Australia). Relative mRNA expression was determined using commercially available Taqman primer/probe sets (Applied Biosystems, Mulgrave, Australia) for PGC-1α (*Ppargc1a*; cat. no. Rno0580241_m1) and PPARδ (*Ppard*, cat. no. Rno0565707_m1). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; cat. no. Rno1775763_g1) was included as a housekeeping gene to normalize threshold cycle (*Cₜ*) values. Mitochondrial DNA (MtDNA) copy number was determined as previously
described [188, 189], using Taqman primer/probes sets for mitochondrially-encoded nicotinamide adenine dinucleotide (reduced; NADH) dehydrogenase-1 (MT-ND1; cat. no. Rn03296764_s1) and nuclear-encoded Gapdh. Quantification was performed in duplicate using a 72-well Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). PCR conditions were as follows: 2 min at 50 ºC, 10 min at 95 ºC, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s. Data was calculated using the ΔΔCt method [190].

IMMUNOBLOTTING

Approximately 50 mg of frozen soleus and EDL was homogenized in buffer (Tris HCl, 50 mM, pH7.5; EDTA, 1 mM; EGTA, 1 mM; DTT, 1 mM; NaF, 50 mM; Na Pyrophosphate, 5 mM; glycerol, 10%; Triton X-100, 1%; trypsin inhibitor, 10 µg/mL; aprotinin, 2 µg/mL; benzamidine, 1 mM; phenylmethylsulfonyl fluoride, 1 mM; 1:8 dilution), and centrifuged at 20,000 G for 30 min at 4 ºC. Protein concentration of the supernatant was determined using the bicinchoninic method (Pierce, IL). Muscle lysates containing either 5, 10 or 20 µg protein were prepared in 4 x Laemmli buffer, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked (5% non-fat dry milk) for 1 hr at room temperature, then incubated overnight at 4 ºC with primary antibodies specific for: PPARδ (~49 KDa, Thermo Scientific, #PA1-823A), PPARα (~52 KDa, Abcam, #ab24509), PGC-1 (~100 KDa, Chemicon, #ab3242), CPT1-M (~80 KDa, Santa Cruz Biotechnology, #sc20670), uncoupling protein 3 (UCP3; ~35 KDa, Affinity BioReagents, #PA1-055), mitochondrial respiratory complexes I, II, III, IV (subunit I) and V (~18, ~25, ~45, ~37, ~52 KDa, respectively, MitoSciences, #MA604), Complex IV subunit II (~25 KDa, MitoSciences, #MS405), Complex IV subunit IV (~15 KDa, MitoSciences, #MS407), CS (~52 KDa, Abcam, #ab96600) and as a protein loading control, α-tubulin (~50 KDa, Sigma, St. Louis, MO). Protein was detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry.
STATISTICAL ANALYSES

All values are expressed as the group mean ± standard error of the mean (SE). An unpaired t-test was used to compare groups for all analyses except the GTT and the stepped titration of ADP, where a two-way analysis of variance (ANOVA) was used. Significance is reported where P<0.05. All statistical analyses were performed using Graph Pad Prism software.

2.3 Results

WESTERN DIETS ARE OBESOGENIC

Rats fed the WD maintained a greater energy intake than CON throughout the study (Table 2.1; 335 ± 6 and 274 ± 4 kJ/d respectively; P<0.0001). From 6 wk, WD rats were heavier than CON (P=0.01; 379.1 ± 7.1 and 356.6 ± 3.9 g, respectively) and remained heavier throughout the intervention (Fig. 2.1A).

Table 2.1. Daily energy intake and nutritional parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Western diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily energy intake, kJ</td>
<td>273.52 ± 3.94</td>
<td>335.41 ± 5.92*</td>
</tr>
<tr>
<td>Protein (casein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E%</td>
<td>21.00</td>
<td>17.00</td>
</tr>
<tr>
<td>g/d</td>
<td>3.39 ± 0.05</td>
<td>3.37 ± 0.06</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E%</td>
<td>63.00</td>
<td>43.00</td>
</tr>
<tr>
<td>Sucrose, g/d</td>
<td>1.69 ± 0.02</td>
<td>5.89 ± 0.10*</td>
</tr>
<tr>
<td>Starch, g/d</td>
<td>9.10 ± 0.13</td>
<td>2.66 ± 0.05*</td>
</tr>
<tr>
<td>Lipid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E%</td>
<td>16.00</td>
<td>40.00</td>
</tr>
<tr>
<td>Total, g/d</td>
<td>1.19 ± 0.02</td>
<td>3.63 ± 0.06*</td>
</tr>
<tr>
<td>Saturated, g/d</td>
<td>0.08 ± 0.001</td>
<td>2.37 ± 0.04*</td>
</tr>
<tr>
<td>Unsaturated, g/d</td>
<td>1.10 ± 0.02</td>
<td>1.24 ± 0.02</td>
</tr>
</tbody>
</table>

Values represent mean ± SE, as determined over 12 wk. E, energy. n=12 animals per group. *P<0.05

As illustrated in Fig. 2.1B, epididymal fat pads were heavier in WD compared to CON (P=0.03; 8.1 ± 0.4 and 6.9 ± 0.3 g, respectively). IMTG’s were elevated 37% in WD compared to CON in the soleus (42.8 ± 5.1 and 31.2 ± 4.3 µmol/g dry wt respectively), although values did not attain statistical significance (P=0.10; Table 2.2). In the EDL, IMTG’s were not different between groups (P>0.05).
Figure 2.1. The WD ■ promotes (A) increased body mass and (B) epididymal fat pad mass when compared to the CON ○ diet over 12 wk. CON, control diet. WD, Western diet. Values represent mean ± SE. n=12 animals per group *P<0.05.
The western diet caused hyperinsulinaemia

No differences were observed in fasting blood glucose concentrations. However fasting serum insulin was greater in WD compared to CON ($P=0.007$; Table 2.2). During the GTT, there was no diet effect or interaction, although there was a main effect for time in response to the glucose challenge ($P<0.0001$). The areas under the blood glucose curves for WD and CON were not different (results not shown).

Table 2.2. Physiological parameters in blood and skeletal muscle

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Western Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood glucose, mM</td>
<td>5.70 ± 0.24</td>
<td>5.10 ± 0.18</td>
</tr>
<tr>
<td>Fasting serum insulin, ng/mL</td>
<td>1.70 ± 0.12</td>
<td>2.39 ± 0.20*</td>
</tr>
<tr>
<td>Fasting serum cholesterol, mM</td>
<td>2.99 ± 0.17</td>
<td>3.53 ± 0.19</td>
</tr>
<tr>
<td>Fasting serum triglycerides, mM</td>
<td>1.66 ± 0.13</td>
<td>3.09 ± 0.36*</td>
</tr>
<tr>
<td>Fasting serum free fatty acids, mM</td>
<td>0.61 ± 0.02</td>
<td>0.73 ± 0.07</td>
</tr>
</tbody>
</table>

Intramyocellular triglycerides, µM/g dry wt

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Western Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>31.17 ± 4.27</td>
<td>42.78 ± 5.14</td>
</tr>
<tr>
<td>EDL</td>
<td>5.93 ± 1.16</td>
<td>5.97 ± 1.26</td>
</tr>
</tbody>
</table>

Values represent mean ± SE, as determined after 12 wk. EDL, m. extensor digitorum longus. Soleus, m. soleus. n=8-12 animals per group. *$P<0.05$

The WD increased fasting serum triglycerides

Serum triglycerides were greater in WD compared to CON ($P=0.001$; Table 2.2). Total cholesterol and serum FFA’s were also greater in WD, however neither of these values attained statistical significance ($18\%, P=0.052$ and $20\%, P=0.12$, respectively; Table 2.2).

The capacity of the ETS was greater following consumption of a western diet

To evaluate the capacity of the ETS and thus determine maximal $O_2$ flux through the mitochondria, $O_2$ flux in the soleus was measured during a SUIT protocol with substrates for CI and CII (Fig. 2.2). State II respiration with electron leak through CI was greater in WD compared to CON ($P<0.001$; WD, 9.9 ± 0.6 pmol.s$^{-1}$.mg wet wt$^{-1}$ and CON, 5.8 ± 0.6 pmol.s$^{-1}$.mg wet wt$^{-1}$). A similar trend was observed for state III respiration with flux through CI, although this difference did not attain statistical significance (WD, 89.7 ± 8.9 pmol.s$^{-1}$.mg wet wt$^{-1}$ and CON, 70.6 ± 6.8 pmol.s$^{-1}$.mg wet wt$^{-1}$; $P=0.11$).
Figure 2.2. Under all SUIT conditions except CI OXPHOS, mass-specific O₂ consumption is greater in soleus from rats receiving the WD ■ compared to CON □. CI, complex I. CII, complex II. CIIu, uncoupled flux through CII. CON, control diet. ETS, electron transfer system. OXPHOS, oxidative phosphorylation. SUIT, substrate-uncoupler-inhibitor titration. WD, Western diet. Values represent mean ± SE. n=6 animals per group. *P<0.05

However, greater sensitivity to ADP was observed (P<0.05, Fig. 2.3). State III respiration with flux through both CI and CII together (WD, 137.2 ± 11.8 pmol.s⁻¹.mg wet wt⁻¹ and CON, 101.7 ± 9.8 pmol.s⁻¹.mg wet wt⁻¹), and non-coupled respiration with flux through CII (WD, 73.9 ± 5.3 pmol.s⁻¹.mg wet wt⁻¹ and CON, 56.5 ± 4.5 pmol.s⁻¹.mg wet wt⁻¹) was greater in WD (P=0.04 and P=0.03, respectively). Similarly, uncoupled/maximal flux through the ETS was greater in WD compared to CON (P=0.02; 152.0 ± 11.3 and 110.8 ± 9.8 pmol.s⁻¹.mg wet wt⁻¹ respectively). In order to assess O₂ flux independent of mitochondrial density, we calculated the FCR for each substrate combination (Table 2.3). There were no differences between dietary groups.
Figure 2.3 Soleus muscle from rats receiving the WD ■ displays greater sensitivity to a titration of ADP compared to CON ○. ADP, adenosine diphosphate. CON, control diet. Soleus, m. soleus. WD, Western diet. Values represent mean ± SE. n=6 animals per group. ***P<0.05 Two-way ANOVA. *P<0.05 T-test

Table 2.3. Flux control ratios

<table>
<thead>
<tr>
<th>Flux Control Ratios</th>
<th>Control</th>
<th>Western diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I, Leak</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Complex I, OXPHOS (state III)</td>
<td>0.62 ± 0.02</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td>Complex I and II, OXPHOS (state III)</td>
<td>0.92 ± 0.02</td>
<td>0.94 ± 0.02</td>
</tr>
<tr>
<td>Complex II, uncoupled</td>
<td>0.53 ± 0.03</td>
<td>0.53 ± 0.04</td>
</tr>
</tbody>
</table>

Flux control ratios are the respirometry data normalized to maximal O₂ consumption in uncoupled states. OXPHOS, oxidative phosphorylation. Values represent mean ± SE. n=6 animals per group.

CONSUMPTION OF A WESTERN DIET INCREASES THE ACTIVITY OF MITOCHONDRIAL ENZYMES

Both CS (WD, 23.5 ± 1.5 μM/min/g wet wt and CON, 19.4 ± 1.0 μM/min/g wet wt; P=0.03; Fig. 2.4A) and total CPT activity (WD, 0.8 ± 0.1 μM/min/g wet wt and CON, 0.4 ± 0.1 μM/min/g wet wt; P=0.01; Fig. 2.4C) were higher in the soleus after WD compared to CON. There were no differences between dietary groups for β-HAD activity in the soleus (WD, 4.9 ± 0.2 μM/min/g wet wt and CON, 4.1 ± 0.3 μM/min/g wet wt; P=0.11, Fig. 2.4B). In the EDL, CS (WD, 10.1 ± 0.5 μM/min/g and CON, 8.3 ± 0.5 μM/min/g; P=0.01; Fig. 2.4A), β-HAD (WD, 2.6 ± 0.1 μM/min/g and CON, 2.2 ± 0.2 μM/min/g; P=0.04; Fig. 2.4B) and CPT (WD, 0.13 ± 0.01 μM/min/g and CON 0.09 ± 0.01 μM/min/g; P=0.02; Fig. 2.4C) activities were all greater following the WD.
Figure 2.4 In the soleus, rats receiving the WD had greater (A) citrate synthase and (C) CPT activity compared to CON, whereas in the EDL, rats receiving the WD had greater (A) citrate synthase, (B) β-HAD and (C) CPT activities compared with CON. β-HAD, β-hydroxyacyl-CoA dehydrogenase. CON, control diet. CPT, carnitine palmitoyltransferase. EDL, m. extensor digitorum longus. Soleus, m. soleus. WD, Western diet. Values represent means ± SE. n = 10 animals per group. *P<0.05.
Figure 2.5 Relative mRNA expression was not different between WD ■ and CON □ for PPARδ or PGC-1α in either (A) soleus or (B) EDL. Also reported here are MtDNA copy number for both soleus and EDL. CON, control diet. EDL, *m. extensor digitorum longus*. MtDNA, mitochondrial DNA. PGC-1α, PPARγ co-activator-1α. PPARδ, peroxisome proliferator activated receptor-δ. Soleus, *m. soleus*. WD, Western diet. Values represent means ± SE. *n* = 7 animals per group.
Figure 2.6 (A) Representative immunoblots for the transcriptional regulators PPARδ, PPARα, and PGC-1; their targets CPT1 and UCP3; and mitochondrial proteins C.S. and respiratory complexes I-V in the soleus muscle from both WD and CON animals. (B) Relative protein expression as determined by densiometry for both WD and CON animals. AU, arbitrary units. CI, complex I. CII, complex II. CIII, complex III. CIV, complex IV subunit I. CIV, complex IV subunit II. CIV, complex IV subunit IV. CV, complex V. CON, control diet. CPT1, carnitine palmitoyl transferase. C.S., citrate synthase. PGC-1, PPARγ coactivator-1. PPAR, peroxisome proliferator activated receptor-α. Soleus, m. soleus. UCP3, uncoupling protein 3. WD, Western diet. Values are means ± SE; n = 8-10 animals per group. *P<0.05
Figure 2.7 (A) Representative immunoblots for the transcriptional regulators PPARδ, PPARα and PGC-1; their targets CPT1 and UCP3; and mitochondrial proteins C.S. and respiratory complexes I-V in the EDL muscle from both WD and CON animals. (B) Relative protein expression as determined by densiometry for both WD ■ and CON □ animals. AU, arbitrary units. CI, complex I. CII, complex II. CIII, complex III. CIV, complex IV subunit I. CIV', complex IV subunit II. CIV', complex IV subunit IV. CV, complex V. CON, control diet. CPT1, carnitine palmitoyl transferase. C.S., citrate synthase. EDL, m. extensor digitorum longus. PGC-1, PPARγ coactivator-1. PPAR, peroxisome proliferator activated receptor-α. UCP3, uncoupling protein 3. WD, Western diet. Values are means ± SE; n= 8-10 animals per group. *P<0.05
2.4 Discussion

The mitochondrion has long been implicated in the pathogenesis of obesity and metabolic dysfunction \([27, 80]\). However the extent of mitochondrial involvement and whether these changes are secondary to lifestyle factors (i.e., physical inactivity) are a matter of current debate. Previously it has been shown that a mitochondrial deficiency exists in obese humans \([27]\) and rodents \([84, 85]\). However the results of recent studies suggest there is dissociation between mitochondrial dysfunction and the progression of obesity \([109, 110, 112, 138]\). Indeed, accumulating evidence indicates that HFD’s induce increases in the expression \([109-112]\) and activity \([106, 110]\) of key mitochondrial enzymes, and that this process is likely to be driven by an increase in circulating FFA’s \([112]\). The data presented in this chapter supports this hypothesis and demonstrates for the first time that skeletal muscle respiratory capacity is enhanced after 12 wk on an obesogenic (high-fat, high-sucrose) WD (Fig. 2.2).

High-resolution respirometry allows the direct monitoring of \(O_2\) consumption by the ETS in permeabilized muscle fibers \([180]\), and reflects the integrated activity of the TCA cycle and ETS, providing a dynamic measurement of the oxidative capacity of tissue \(ex\ vivo\) \([180]\). Twelve wk of a WD was shown to induce an increase in the activity of the ETS in skeletal muscle with high oxidative capacity (Fig. 2.2). When the data is normalized to allow interpretation of function independent of mitochondrial volume (Table 2.3), this increase is attenuated, suggesting that mitochondrial biogenesis, rather than increased activity, is likely to be responsible for the increase in respiratory capacity. This finding is supported by the increased expression and activity of the mitochondrial enzyme CS (Fig. 2.4A), an enzyme long used as a marker enzyme for mitochondrial content. Additional mitochondrial enzyme activity and protein measurements in the primarily glycolytic EDL muscle further support this notion (Fig. 2.4-2.7).

Previously it has been shown in WD- \([171]\) and HFD-fed rodents \([109, 110, 112]\) that skeletal muscle has an increased capacity for fat oxidation after as little as four wk. In the current study, increased activity of total CPT (CPT1 and CPT2) was observed (Fig. 2.4C) and this is indicative of an increase in the delivery of long-chain FA’s into the mitochondria. While fat oxidation was not measured directly, HFD-induced
increases in the activity of CPT1 have previously been associated with greater rates of fat oxidation [110, 113]. Interestingly, no differences were observed in the activity of β-HAD, the third enzyme of the β-oxidation pathway, implying that the rate of FA delivery to the mitochondria may be discordant with the capacity for β-oxidation in muscle with high oxidative capacity. It is important to note however, that this data is of maximal enzyme activity *ex vivo* and is not indicative of *in vivo* demand.

Although it is only possible to speculate whether or not this miss-match is likely to contribute to cellular lipid deposition (IMTG’s were elevated 37% in the soleus, *P*=0.10, but remained similar in the EDL where β-HAD activity was elevated; Table 2.2), the notion that the β-oxidation and TCA cycle activities are disproportionate and contribute to lipid deposition in diet-induced obesity is not a new concept. Previously Hoehn *et al.* [191] demonstrated that simply driving metabolism toward increased fat oxidation is not sufficient to increase energy expenditure, while Koves *et al.* [104] suggest that incomplete FA oxidation and accumulation of lipid species occurs when the import of FA into the mitochondria exceeds metabolic demand. Moreover, human studies have shown that despite increases in IMTG storage, rates of FA oxidation may only be impaired in cases of extreme obesity where mobility is severely reduced [97, 138]. This implies that impaired FA oxidation *per se* is unlikely to be responsible for the accumulation of lipid species during obesity but rather the reduction in physical activity. These findings suggest that chronic feeding of a WD increases the cellular machinery necessary to cope with increases in energy expenditure yet, without the appropriate energy demand substrates will accumulate in the tissue, eventually being stored. Pharmacological agonism of PPARδ has been shown to increase the expression of several oxidative proteins including UCP3 and CPT1 [192]. However, unless combined with an endurance training program, no functional improvements are seen, implying that there must be an appropriate stimulus for any functional enhancements to manifest. Therefore the increases in mitochondrial respiratory capacity observed in the current investigation could be considered a compensatory response in anticipation of the increase in substrate flux to the mitochondria.

A second aim of this investigation was to characterise potential mechanisms responsible for the increase in mitochondrial function. The PPAR’s are a family of
ligand-activated nuclear transcription factors that sense and respond to dietary lipids and their metabolites [193]. Of the three isoforms, PPARα and PPARδ are the two preferentially expressed in skeletal muscle, with PPARδ being the predominant isoform in this tissue [194]. Both PPARα and PPARδ play key roles in FA metabolism [115], acting with the transcriptional co-activator and “master regulator” of mitochondrial biogenesis, PGC-1α [195, 196] to induce increases in both nuclear and mitochondrial target genes for FA catabolism (Fig. 1.5 [171, 195]). Previous studies have identified PPARδ and PGC-1α as important mediators of diet-induced increases in mitochondrial function [197]. Furthermore, PPARδ is known to directly interact with CPT1 [195], while HFD’s or pharmaceutical PPARδ agonists increase UCP3 mRNA [171, 195] and protein expression [110, 198]. Unlike previous studies that have investigated the effect of a HFD on mitochondrial signalling, large increases in circulating FFA’s were not seen following the WD. However, total serum triglycerides were almost two-fold greater (Table 2.2). Moreover, marked differences in the gene and/or protein expression of PPARδ or PGC-1α could not be demonstrated (Fig. 2.5-2.7), the two signalling proteins that are thought to be responsible for HFD-induced alterations to mitochondrial function [110]. Despite this, the possibility that post-translational regulation of protein activity may be a potential mechanism by which mitochondrial function is enhanced cannot be discounted. Indeed it is well known that the acetylation status of PGC-1α is important in regulating its activity [199] while PPARδ itself may post translationally modify PGC-1α [200].

Of note was that the expression of PPARδ’s down-stream target protein CPT1 remained unchanged, despite an increase in total CPT activity (Fig. 2.6 and 2.7). Previous studies have reported increases in UCP3 mRNA following a WD [171], and in line with this UCP3 protein expression was observed to increase in the current study (Fig. 2.6 and 2.7). Taken together, increased CPT activity and UCP3 protein expression could be indicative of an increase in PPARδ activity [201], despite no increase in PPARδ gene or protein expression. While UCP3 remains without an established function [202, 203], increases in UCP3 expression due to PPAR stimulation implicate this protein in an array of metabolic processes that involve substrate handling. Further investigations will need to be undertaken over prolonged intervention periods to
determine if PPARδ activity is indeed altered by a WD. However it is also plausible that the reduced percentage of fat in a WD may have less agonist potential than that of a HFD and as such, other mechanisms may be responsible for the observed increase in CPT activity and UCP3 expression.

In conclusion, the results of the present investigation demonstrate that an increase in mitochondrial respiratory capacity occurs in response to WD-induced obesity. This is likely a compensatory mechanism to cope with excess energy availability in the absence of a simultaneous increase in energy demand. Such a scenario would be expected to contribute to intracellular lipid deposition and overall adiposity when sustained for prolonged periods without a concomitant increase in physical activity.
Divergent skeletal muscle respiratory capacities in rats artificially selected for high and low running ability: a role for NOR1?


3.1 Introduction

The ability to deliver and utilize oxygen is an essential component of metabolic regulation, with an individual’s VO$_{2\text{max}}$ a good correlate of whole-body health status [38]. 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 [153]. However, VO$_{2\text{max}}$ 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 [38].

Through two-way artificial selection, Koch and Britton [160] have generated an animal model of low- or high-aerobic exercise capacity 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 differed substantially in aerobic exercise capacity (374% difference between LCR and HCR, P<0.0001), whilst simultaneously presenting with markedly different metabolic and cardiovascular disease risk factors [161].

Because skeletal muscle mitochondrial respiration is a limiting factor for VO$_{2\text{max}}$, and as skeletal muscle oxidative capacity correlates with whole-body insulin sensitivity [140], 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 assertion, HCR rats are better at utilizing O$_2$, an adaptive variation likely to be present at the level of the skeletal muscle [153]. Results from a series of studies in this model [161, 162, 165-168, 204, 205] provide direct evidence that skeletal muscle from HCR rats has superior substrate handling ability (i.e., ‘metabolic flexibility’) and mitochondrial enzyme activities compared to LCR rats. However, there is some contention as to whether this is due to increases in the mitochondrial reticulum [165] or an enhanced capacity of some or all of the oxidative enzymes [167, 168, 206]. In addition, Lessard et al. have shown that the differences in metabolic flexibility are linked to β-adrenergic signaling through the NR4A orphan nuclear receptor Neuron-derived clone 77 (Nur77) [162, 204]. The NR4A family of orphan nuclear receptors Nur77, Nuclear receptor related protein 1 and Neuron-derived orphan receptor 1 (NOR1) has recently emerged as a key player in the regulation of a number of important metabolic processes [162, 207-209]. All three have been linked to the regulation of skeletal muscle substrate handling and whole-body glucose homeostasis [162, 207-209], whereas NOR1 has been shown to be essential for oxidative metabolism [210] and may also be important in promoting several oxidative adaptations in the muscle [206].

Since previous studies have been inconclusive as to whether skeletal muscle respiratory capacity is due to an increased number of mitochondria [165] or increased mitochondrial activity [166, 167], the aim of this study was to determine the _ex vivo_ activity and protein expression of the key ETS respiratory complexes in muscle from LCR and HCR rats. It was hypothesized that skeletal muscle respiratory capacity would be greater in HCR rats compared to LCR rats, and that this difference would be due to a greater mitochondrial density in the muscle. Furthermore, a second hypothesis was that
the expression of NOR1 would be greater in the muscle of HCR rats compared to LCR rats.

### 3.2 Materials and 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 described previously by Koch and Britton [160]. Female offspring of either LCR ($n=12$, generation 27) or HCR ($n=12$, generation 27) rats were housed under a controlled 12:12 hr 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 commencement of any experimental procedures. This study was undertaken with approval from both the University of Michigan and RMIT University animal ethics committees.

**Blood analyses**

After a 5 hr fast ~15 µL blood was collected via tail cut and glucose concentration was measured on a hand-held glucometer (Roche Diagnostics, Castle Hill, NSW, Australia). 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 (ALPCO Immunoassays, Salem, NH).

**Glucose Tolerance Testing**

The protocol for the GTT is outlined in Chapter 2. Briefly, fasted rats received a single glucose bolus (2 g/kg body mass) via intra-peritoneal injection, and blood glucose concentration was monitored at 30 min intervals throughout the subsequent 120 min. Additional aliquots of blood were collected at 30, 60 and 120 min post-injection for the later determination of serum insulin concentrations in response to the glucose challenge.
Prior to monitoring, rats were familiarized with activity monitoring chambers comprising infrared beams in the X, Y and Z axes (MED Associates, inc. St Albans, VT) during three 10 min sessions in the week before testing. Spontaneous activity was monitored during both diurnal (1200 h) and nocturnal (0200 h) sessions. Horizontal, vertical and ambulatory locomotor activities were determined in 20 sec intervals over 2 x 10 min sessions.

Tissue collection procedures were conducted after a 5 hr fast in 11 wk old rats, which were weighed and anaesthetized with sodium pentobarbital (60 mg/kg body mass) and hind limb muscles immediately excised. Similar experiments were performed on soleus and EDL portions from the same leg (for reasons outlined in Chapter 2). The left soleus was placed in ice-cold BIOPS and prepared for respirometry experiments according to the protocol outlined in Chapter 2. The contra-lateral soleus muscle was carefully dissected into longitudinal strips from tendon to tendon (15-20 mg per strip) and placed in pre-gassed Krebs-Henseleit (KHB) buffer for the measurement of basal and insulin-stimulated glucose uptake. The remaining hind limb muscles were freeze-clamped in liquid N\textsubscript{2} and stored at -80 °C for later analyses. Periovarian 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 pre-oxygenated KHB (NaCl, 118.5 mM; NaHCO\textsubscript{3}, 25 mM; KCl, 4.74 mM; MgSO\textsubscript{4}.7H\textsubscript{2}O, 1.19 mM; KH\textsubscript{2}PO\textsubscript{4}, 1.18 mM; CaCl\textsubscript{2}, 2.5 mM; pH 7.4) containing mannitol, 32 mM; pyruvate, 2 mM; glucose, 8 mM; and fatty-acid free BSA, 0.1%. Vials were maintained at 30 °C in a shaking water bath throughout the experiment. Basal and insulin-stimulated glucose uptake was determined according to the method of Young et al. [211], with modifications [204, 212, 213]. Following 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 next washed for 10 min in KHB modified with mannitol, 40 mM; pyruvate, 2 mM; fatty-acid free BSA, 0.1% and insulin, 60 nM, if it was present in the previous incubation. Following glucose wash-out, strips were transferred to vials containing oxygenated KHB containing fatty-acid free BSA, 0.1%; pyruvate, 2 mM; 2-[1,2-\textsuperscript{3}H]-deoxyglucose, 1 mM (1 mCi/mL; Perkin Elmer); D-[1-\textsuperscript{14}C]-mannitol, 39 mM (0.1 μCi/mL; Perkin Elmer) and insulin, 60 nM, if it was present in the previous incubations. After 20 min, the reaction was stopped by washing muscle strips in KHB twice, blotting the tissue on filter paper, then freeze-clamping it with tongs pre-cooled in liquid N\textsubscript{2}. Muscle strips were weighed, homogenized in 10% trichloroacetic acid, and transferred to vials containing 5 mL of scintillation fluid (Ultima Gold XR, Perkin Elmer). Duplicate aliquots were counted in a liquid scintillation counter (Packard Tri-Carb, Perkin Elmer) set for simultaneous \textsuperscript{3}H and \textsuperscript{14}C counting. Rates of basal and insulin-stimulated skeletal muscle 2-[1,2-\textsuperscript{3}H]-deoxyglucose transport were calculated according to the protocol described by Lessard \textit{et al.} [176].

**HIGH-RESOLUTION RESPIROMETRY**

Approximately 10 mg of soleus (\(n=10\) per group) was prepared for respirometry experiments using the methods outlined in \textit{Chapter 2}. The remaining, non-permeabilized muscle was frozen in liquid N\textsubscript{2} for later analyses.

**SUBSTRATE-UNCOUPLER-INHIBITOR TITRATION PROTOCOL**

Malate (2 mM) was added and \(J_{O_2}\) (pmol.s\textsuperscript{-1}.mg\textsuperscript{-1} wet weight) stabilized for 5-10 min. Subsequently, tissue samples were subjected to a SUIT protocol that sequentially evaluates the electron-transferring flavoprotein (ETF; fat oxidation) in leak state through the addition of octanoylcarnitine, 0.2mM (TOCRIS Bioscience; state 2 respiration), oxidative phosphorylation with electron flux through the ETF, by titration of ADP (1 and 2.5 mM; state 3 respiration), oxidative phosphorylation with electron flux through the ETF and CI with the addition of glutamate (10 mM), oxidative phosphorylation with electron flux through the ETF, CI and CII with the addition of succinate (10 mM), maximal coupled oxidative phosphorylation 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), 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 ROX with the addition of antimycin A (2.5 µM). Additionally, CIV activity was determined following the addition of the artificial electron donor \(N,N,N,N',N'-\text{tetramethyl-p-phenylenediamine dihydrochloride}\) (0.5 mM) in the presence of ascorbate (2 mM). The reaction was terminated by the addition of \(\text{NaN}_3\) (50 mM), after which the chambers were re-oxygenated for the determination of auto-oxidation. Mass specific \(\text{O}_2\) flux was determined using DATLAB (Oroboros, Innsbruck; Austria) from steady state \(J_0\), normalized to tissue wet weight and adjusted for instrumental background, ROX and auto-oxidation. In addition, FCR’s were calculated to determine mitochondrial function independent of mitochondrial density.

**MITOCHONDRIAL ENZYME ACTIVITIES**

Muscle homogenates \((n=10\) per group\) were prepared over ice from freeze-clamped soleus and EDL muscles (10-20 mg) according to the same protocols outlined in Chapter 2. CS and \(\beta\)-HAD activities were determined according to the modified methods of Srere (CS) \([183]\) and Bergmeyer (\(\beta\)-HAD) \([184]\). These protocols are described in Chapter 2 \([214]\).

**NUCLEIC ACID EXTRACTION AND QUANTIFICATION**

DNA and RNA were each extracted according to the same methods outlined in Chapter 2. For each nucleic acid, an aliquot was taken from each sample \((n=7\) per group\) and pooled. The pooled RNA or DNA sample was then quantified and serially diluted to produce standard curves for inclusion in each PCR run.

**REVERSE TRANSCRIPTION AND REAL-TIME PCR**

First-strand cDNA synthesis was performed for RNA extracts using Superscript VILO (Invitrogen). Relative mRNA expression was determined using commercially available Taqman primer/probe sets (Applied Biosystems, Mulgrave, Australia) for PGC-1\(\alpha\) \((Ppargc1a\); cat. no. Rno0580241_m1\) and Fibronectin type III domain containing 5 \((Fndc5;\) cat. no. Rno1519161_m1\). Glyceraldehyde-3-phosphate dehydrogenase \((Gapdh;\) cat. no. Rno1775763_g1\) was included as a housekeeping gene.
to normalize $C_T$ values. Quantification was performed in duplicate using a 72-well Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 65°C for 60 s. Calculations were made using the $\Delta\Delta C_T$ method [190].

**Mitochondrial DNA copy number determination**

MtDNA copy number was determined in DNA extracts according to the protocol described in Chapter 2 [214], using Taqman primer/probe sets for mitochondrial-encoded NADH dehydrogenase-1 ($MT\text{-}ND1$; cat. no. Rn03296764_s1) and nuclear-encoded Gapdh. DNA samples were subjected to the same PCR conditions as described above. MtDNA copy number was calculated as described by Venegas & Halberg [189].

**Immunoblot analysis**

Approximately 50 mg of frozen soleus or EDL was homogenized in buffer according to the protocol described in Chapter 2 for immunoblot analysis [214]. Muscle lysates containing 10 µg protein from either EDL or soleus muscle were prepared in 4 x Laemlli buffer, subjected to SDS-PAGE, then transferred to PVDF membranes. Membranes were blocked (5% non-fat dry milk) for 1 hr at room temperature, then incubated overnight at 4 °C with primary antibodies specific for the following proteins: PGC-1 (≈100 KDa, Chemicon, #ab3242), FNDC5 (≈28 KDa, Abcam, #ab93373), Nur77 (≈48 KDa, Santa Cruz Biotechnology #sc5569), NOR1 (≈68 KDa, Abcam #92777), Glucose transport protein (GLUT4, ≈45 KDa, Abcam #ab654), Fatty acid translocase/CD 36 (FAT/CD36; ≈80KDa, Abcam #17044), Complexes I, II, III, IV (subunit 1) and V of the ETS (≈18, ≈25, ≈45, ≈37, ≈52 KDa, respectively, MitoSciences, #MA604), Complex IV subunit II (≈25 KDa, MitoSciences, #MS405), Complex IV subunit IV (≈15 KDa, MitoSciences, #MS407), CS (≈52 KDa, Abcam #ab96600) and UCP3 (≈35 KDa, Affinity BioReagents, #PA1-055). $\alpha$-Tubulin (≈50 KDa, Sigma, St. Louis, MO) 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 ± SE. A two-way ANOVA was used to analyze data from the GTT. Physical activity and glucose uptake data were analyzed using a one-way ANOVA with a Student Newman-Keuls post-hoc test. An unpaired t-test was used to compare groups for all other analyses unless otherwise specified. Significance is reported where \( P<0.05 \). Statistical analyses were performed using Graph Pad Prism software.

### 3.3 Results

**HCR and LCR rats have divergent metabolic health parameters.**

The metabolic health characteristics of each rat phenotype are displayed in Table 3.1. Although there were no differences in the ages of the animals, LCR were 28% heavier than HCR \( (P<0.0001) \) and had 48% more periovarian 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 compared to HCR \( (P=0.02) \). HCR were more glucose tolerant than LCR as determined by the area under the glucose curve during the GTT \( (\text{LCR}, 1267 \pm 47.4 \text{ and HCR}, 1100 \pm 27.8; P=0.01; \text{Fig. 3.1A and B}) \). Similarly, the insulin response to the GTT was 135% greater in LCR compared to HCR as determined by the area under the insulin curve during the GTT \( (\text{LCR}, 195.2 \pm 15.8 \text{ and HCR}, 83.6 \pm 11.3; P=0.001; \text{Fig. 3.1C and D}) \).

<table>
<thead>
<tr>
<th>Table 3.1. Body composition and metabolic parameters</th>
<th>LCR</th>
<th>HCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, d</td>
<td>80.9 ± 0.6</td>
<td>81.0 ± 0.6</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>214.1 ± 3.7</td>
<td>167.7 ± 4.2*</td>
</tr>
<tr>
<td>Periovarian 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>
</tbody>
</table>

LCR and HCR rats display divergent metabolic parameters. HCR, high capacity runner. LCR, low capacity runner. Values are means ± SE. \( n=8-12 \) per group. *\( P<0.05 \)
The glucose uptake response to insulin is enhanced in HCR rats

Rates of glucose uptake in the soleus are displayed in Fig. 3.2. There was no difference in the rate of basal glucose uptake between LCR and HCR (3.21 ± 0.35 and 3.57 ± 0.16 µM/g/h, respectively). Insulin stimulation increased glucose uptake in both groups (LCR, 5.16 ± 0.21, \( P < 0.0001 \) and HCR, 6.00 ± 0.23 µM/g/h, \( P < 0.0001 \)), although this increase was of a greater magnitude in HCR (14%; \( P = 0.02 \)).
HCR RATS ARE MORE ACTIVE THAN LCR RATS

Physical activity data for each group is presented in Table 3.2. During the diurnal period, HCR completed a greater number of horizontal beam breaks \( (P=0.0004) \), spent more time ambulatory \( (P=0.02) \), less time stationary \( (P=0.02) \) and traveled a greater total distance \( (P=0.04) \) compared with LCR rats. During the nocturnal phase, HCR 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 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.
Table 3.2. Physical activity levels of animals.

<table>
<thead>
<tr>
<th></th>
<th>Diurnal period</th>
<th>Nocturnal period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LCR</td>
<td>HCR</td>
</tr>
<tr>
<td>No movement, %</td>
<td>67.1 ± 0.7</td>
<td>61.9 ± 1.9</td>
</tr>
<tr>
<td>Stereotypic movement, %</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, count</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>
</tbody>
</table>

HCR, high capacity runner. LCR, low capacity runner. Values are means ± SE. n=8-12 per group. *P<0.05 different to LCR day. †P<0.05 different to LCR night. ‡P<0.05 different to HCR day.

Mitochondrial respiratory capacity is greater in HCR rats compared to 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. 3.3). State II respiration with electron leak through the ETF was not different between groups (LCR, 5.55 ± 0.42 and HCR, 6.61 ± 0.83 pmol.s⁻¹.mg wet wt⁻¹), however state III respiration with electron flux through the ETF was greater in HCR than LCR at 1 (17.6 ± 1.2 and 27.9 ± 1.7 pmol.s⁻¹.mg wet wt⁻¹ respectively) and 2.5mM ADP (30.68 ± 1.97 and 19.54 ± 1.35 pmol.s⁻¹.mg wet wt⁻¹ respectively). State III respiration with electron flux through both the ETF and CI was greater in HCR compared to LCR (41.27 ± 2.44 and 26.77 ± 1.59 pmol.s⁻¹.mg wet wt⁻¹ respectively), and when flux through the ETF was taken into account, CI values for HCR remained elevated (HCR, 11.73 ± 2.14 and LCR, 7.62 ± 0.22 pmol.s⁻¹.mg wet wt⁻¹; P=0.09). State III respiration with electron flux through the ETF, CI and CII was greater in HCR compared to LCR at sub maximal (80.56 ± 2.79 and 65.29 ± 3.10 pmol.s⁻¹.mg wet wt⁻¹ respectively) and maximal ADP saturation (95.52 ± 2.34 and 83.74 ± 2.34 pmol.s⁻¹.mg wet wt⁻¹ respectively). Uncoupled respiration with electron flux through the ETF and CII was greater in HCR compared to LCR (54.70 ± 2.19 and 46.72 ± 1.00 pmol.s⁻¹.mg wet wt⁻¹, respectively; P=0.007). Maximal uncoupled flux through the ETS (ETF, CI and CII) was greater in HCR compared to LCR (106.50 ± 3.54 and 87.86 ± 3.20 pmol.s⁻¹.mg wet wt⁻¹ respectively). CIV activity, as determined through the use of artificial electron donors, was not different between groups (LCR, 156.70 ± 6.7 and HCR, 169.7 ± 10.35 pmol.min⁻¹.mg wet wt⁻¹ respectively).
In order to assess $O_2$ flux independent of mitochondrial density, we calculated the FCR for each substrate combination. These are shown in Table 3.3.

Figure 3.3. Mass-specific $O_2$ consumption in the soleus of LCR □ and HCR ■ rats. CIV, respiratory complex IV. D1, [1 mM] adenosine diphosphate (ADP). D2.5, [2.5 mM] ADP. D5, [5 mM] ADP. ETF, Electron Transferring Flavoprotein. ETS, Electron Transfer system. G, Glutamate. HCR, high capacity runner. LCR, low capacity runner. M, Malate. Oct, Octanoylcarnitine. Soleus, $m$. soleus. S, Succinate. Values are means ± SE. n=11 per group. *$p<0.05$
Table 3.3. Mitochondrial flux control ratios

<table>
<thead>
<tr>
<th></th>
<th>LCR</th>
<th>HCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETF, Leak (State II)</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>ETF, OXPHOS (State III)</td>
<td>0.23 ± 0.01</td>
<td>0.29 ± 0.02*</td>
</tr>
<tr>
<td>ETF &amp; CI, OXPHOS (State III)</td>
<td>0.32 ± 0.01</td>
<td>0.39 ± 0.2*</td>
</tr>
<tr>
<td>ETF, CI &amp; CII, OXPHOS (State III, Submax)</td>
<td>0.77 ± 0.02</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>ETF, CI &amp; CII, OXPHOS (State III, Max)</td>
<td>0.96 ± 0.02</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>ETF &amp; CII, Uncoupled (State IV)</td>
<td>0.57 ± 0.01</td>
<td>0.51 ± 0.01*</td>
</tr>
</tbody>
</table>

CI, complex I. CII, complex II. ETF, electron transferring flavoprotein. HCR, high capacity runner. LCR, low capacity runner. OXPHOS, oxidative phosphorylation. Values are means ± SE. n=8-12 per group. *P<0.05.

INCREASED O_2 flux in muscle from HCR rats is associated with greater mitochondrial enzyme activity and increased MtDNA copy number

CS activity was greater in HCR compared to LCR rats in both the soleus (HCR, 21.64 ± 1.21 and LCR, 14.58 ± 1.23 µmol/min/g wet wt; P=0.009; Fig. 3.4A) and EDL muscles (HCR, 9.98 ± 0.62 and LCR, 7.06 ± 0.77 µmol/min/g wet wt; P=0.01; Fig. 3.4A). β-HAD activity was also greater in HCR compared to LCR in soleus (HCR, 5.62 ± 0.45 and LCR, 3.78 ± 0.31 µmol/min/g wet wt; P=0.003; Fig. 3.4B) and EDL (HCR, 1.29 ± 0.11 and LCR, 0.75 ± 0.066 µmol/min/g wet wt; P=0.0004; Fig. 3.4B). In the EDL, MtDNA copy number was elevated in HCR compared to LCR (11810 ± 1640 and 7584 ± 891, respectively; P=0.049; Fig. 3.4C). MtDNA copy number was 44% greater in the soleus of HCR (HCR, 11930 ± 3235 and LCR, 6665 ± 1015; P=0.16; Fig. 3.4C).

MUSCLE-SPECIFIC PROTEIN EXPRESSION OF PGC-1α AND FNDC5

The relative expression of PGC-1α mRNA was not different between groups for either soleus or EDL muscle (data not shown). PGC-1α protein expression was not different in the soleus (Fig. 3.5), although a small but significant increase was observed in the EDL of HCR rats (9%; Fig. 3.6; P=0.03). Expression of the PGC-1α-dependent FNDC5 gene was not different between groups in either muscle type (data not shown). Similarly, FNDC5 protein expression was not different in the soleus (Fig. 3.5). FNDC5 protein expression was greater in the EDL of the HCR rats (17%; Fig. 3.6; P=0.0002).
Figure 3.4. Enzyme activities and relative MtDNA copy number in both soleus and EDL of LCR □ and HCR ■ rats. (A) CS activity, \( n=10 \) per group. (B) \( \beta \)-HAD activity, \( n=10 \) per group. (C) MtDNA:nDNA, \( n=7 \) per group. \( \beta \)-HAD, \( \beta \)-hydroxyacyl-CoA dehydrogenase. CS, citrate synthase. EDL, m. extensor digitorum longus. HCR, high capacity runner. LCR, low capacity runner. MtDNA, mitochondrial DNA. Values are means ± SE. *\( P<0.05 \)
Figure 3.5. Protein content of soleus muscle as determined by immunoblotting. (A) Representative blots and (B) relative protein expression for LCR □ and HCR ■ rats. AU, arbitrary units. CI, complex I. CII, complex II. CIII, complex III. CIV, complex IV subunit I. CIV', complex IV subunit II. CIV'', complex IV subunit IV. CV, complex V. CS, citrate synthase. FAT/CD36, fatty acid translocase. FNDC5, Fibronectin domain containing 5. GLUT4, glucose transport protein 4. HCR, high capacity runner. LCR, low capacity runner. NOR1, Neuron-derived orphan receptor 1. Nur77, neuron-derived clone 77. PGC-1, peroxisome proliferator activated receptor-γ coactivator-1α. Soleus, m. soleus. UCP3, uncoupling protein 3. Values are means ± SE. n=8-12 per group. *P<0.05
Figure 3.6. Protein content of EDL muscle as determined by immunoblotting. (A) Representative blots and (B) relative protein expression for LCR □ and HCR ■ rats. AU, arbitrary units. CI, complex I; CII, complex II; CIII, complex III; CIV^I, complex IV subunit I; CIV^II, complex IV subunit II; CIV^IV, complex IV subunit IV; CV, complex V; CS, citrate synthase. EDL, m. extensor digitorum longus. FAT/CD36, fatty acid translocase. FNDC5, Fibronectin domain containing 5. GLUT4, glucose transport protein 4. HCR, high capacity runner. LCR, low capacity runner. NOR1, Neuron-derived orphan receptor 1. Nur77, neuron-derived clone 77. PGC-1, peroxisome proliferator activated receptor-γ coactivator-1α. UCP3, uncoupling protein 3. Values are means ± SE. n=8-12 per group. *P<0.05
MITOCHONDRIAL PROTEIN EXPRESSION IS GREATER IN HCR

In the soleus muscle, protein expression of all individual respiratory complexes was greater in HCR compared to LCR (Fig. 3.5; \( P < 0.05 \)), except for subunit I of CIV. Additionally, expression of CS was 14\% greater in HCR compared to LCR (Fig. 3.5; \( P = 0.03 \)). In the EDL, expression of all respiratory complexes was greater in HCR compared to LCR (Fig. 3.6; \( P < 0.05 \)), except for subunit I of CIV, which was not different. CS protein expression was 38\% greater in HCR (Fig. 3.6; \( P < 0.0001 \)).

NOR1 EXPRESSION IS GREATER IN HCR RATS

In both soleus and EDL muscles, protein expression of NOR1 was greater in HCR rats (soleus 21\%, \( P = 0.009 \); EDL 23\%, \( P = 0.016 \); Fig. 3.5 and 3.6). Expression of Nur77 (soleus 40\%, \( P = 0.02 \); EDL 25\%, \( P = 0.01 \)) and NR4A target proteins FAT/CD36 (soleus 4\%, \( P = 0.04 \); EDL 9\%, \( P = 0.02 \)) and UCP3 (soleus 32\%, \( P = 0.01 \); EDL 20\%, \( P = 0.0003 \)) were also increased in both muscle types from HCR (Fig. 3.5 and 3.6; both \( P < 0.05 \)).

3.4 Discussion

The motivation for developing the LCR/HCR model system originated after Koch and Britton noted the strong statistical association between low endurance exercise capacity and increased morbidity and mortality in humans. From this, the aerobic hypothesis was formulated, proposing that aerobic energy metabolism is a central mechanistic determinant of the divide between disease and health. As an unbiased test of this hypothesis divergent artificial selection for intrinsic low and high endurance running capacity was applied to rats. As predicted by this hypothesis disease risks and reduced longevity segregated strongly with low aerobic capacity [215]. This chapter presents evidence which describes features that can explain the intrinsic aerobic exercise capacity differences between the LCR and HCR rats 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 [216], there is a growing body of evidence to suggest that a substantial genetic component underlies many complex metabolic disorders [11, 12]. The current investigation demonstrates that skeletal muscle
respiratory capacity is greater in muscle from HCR compared with LCR rats (Fig. 3.3), and that this enhanced respiratory capacity is likely due to greater relative mitochondrial enzyme activities (particularly those with a role in fat oxidation (Fig. 3.3 and 3.4)) coupled with increased mitochondrial number (Fig. 3.4-6). This increase in muscle oxidative capacity is associated with an increase in the protein expression of the nuclear receptor NOR1 (Fig. 3.5 and 3.6), which has previously been linked to the adaptive response of skeletal muscle in response to exercise training, [206, 217] and the expression of several proteins involved in glucose metabolism [162, 207-209]. These novel findings complement previous reports that have shown that there are a greater number of mitochondria present in the glycolytic muscle from HCR rats [165], and that muscle substrate handling is superior in these animals [162, 176, 204].

Low aerobic capacity, independent of physical activity levels, is a key predictor of early mortality [13, 14]. Along with a reduced lifespan [215] LCR rats express a number of characteristics common to metabolic disease phenotypes, such as increased body mass and adiposity (Table 3.1 [153, 166, 205, 218]), hyperinsulinaemia (Table 3.1 [161, 205, 219]) and impaired glucose tolerance (Fig. 3.1 [165, 166, 205]). In contrast, HCR rats live 6-8 months longer [215] and present with superior metabolic health, characterized by resistance to weight gain [163, 166, 205] and an increased capacity for the uptake and oxidation of both glucose (Fig. 3.2 [165, 204]) and long-chain fatty acids [165, 166, 204, 205]. The differential expression of these characteristics appears to be linked to muscle oxidative capacity [205], 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 [153]. Previous reports that indicate that there is a greater number of mitochondria in the muscle of HCR rats [161, 165] are confounded by other reports that do not support this observation [166, 168], and instead conclude that the difference in oxidative capacity is not due to mitochondrial size or number [166-168], but a difference in mitochondrial enzyme activity.

To address this, respirometry experiments were performed, monitoring O₂ consumption through the mitochondria of permeabilized soleus muscle of LCR and HCR rats in response to specific substrate combinations. In contrast to individual
enzyme activity assays, this dynamic measurement reflects the integrated activities of β-oxidation, the TCA cycle and the ETS. During ADP-stimulated (state 3) respiration a greater O2 consumption with electron flux through the ETF, CI and CII was observed (Fig. 3.3). Additionally, maximal activity of the ETS was greater in muscle from HCR (Fig. 3.3). Previously, it has been shown that the oxidation of the long-chain FA palmitate is greater in in vitro soleus muscle incubations of HCR [204] and during hind-limb perfusion experiments [165]. Here it is confirmed that the capacity for total lipid oxidation is enhanced in the muscle of HCR, and that this is due to the increased activity of mitochondrial enzymes involved in the oxidation of fatty acids. Specifically, respiratory flux control ratios indicate that independent of mitochondrial volume, the relative contribution of the ETF to maximal flux is greater in HCR when the muscle is exposed to the medium-chain FA octanoylcarnitine (Table 3.3). Furthermore, β-HAD activity (the third enzyme of the β-oxidation pathway, Fig. 1.3A) was greater in muscle from HCR rats (Fig. 3.4B). 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 oxidative phosphorylation with CI and CII activity is similar for LCR and HCR (Table 3.3). 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 muscle from HCR rats.

Since respirometry and enzyme activity measurements are indirect determinants of mitochondrial content and must be supported by additional measurements, the MtDNA copy number (Fig. 3.4C), and the expression of a number of mitochondrial proteins (Fig. 3.5 and 3.6) in both soleus and EDL muscles are also reported. MtDNA copy number was found to be greater in both muscles under investigation, and moreover, the expression of a number of mitochondrial proteins was also increased in both muscle types, supporting the 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 (Fig. 3.5 and 3.6) along with elevated maximal activities (Fig. 3.4A) in both soleus and EDL muscles of HCR rats. This difference in skeletal muscle is no doubt a
major contributing factor to the overall phenotype of these rats. Additionally, the
greater capacity for lipid oxidation observed in HCR rats is likely to result in the
sparking of glycogen during exercise, thereby contributing to their overall phenotype.
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 [162, 165, 204], along with
an increased capacity for lipid oxidation and overall enhanced rates of oxidative
phosphorylation, 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 (Fig. 3.6). This protein has
recently been identified as PGC-1α-dependent membrane protein that is cleaved to
become the putative myokine irisin [220]. Although it was not possible to measure
circulating irisin concentrations from LCR and HCR rats, 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. Indeed, exercise training has
been shown to increase the concentration of irisin in both mice and humans [220], so it
is quite plausible that HCR have higher concentrations of circulating irisin than LCR
rats. Furthermore, the “browning” effect seen in white adipose tissue following irisin
administration [220] may not be restricted to this tissue, so the possibility that irisin
elicits a similar effect on glycolytic skeletal muscle cannot be discounted. 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 (Chapter 4).

Previously Nur77 and its downstream targets have been shown to be expressed to
a lesser extent in LCR rats and have been linked to impaired β-adrenergic signaling in
the skeletal muscle [162]. Another NR4A nuclear receptor, NOR1, shares high
sequence homology with Nur77 and has been shown to be induced under many of the
same conditions [207]. Pearen et al. [210] have 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 to their wild-type litter mates [221]. The current
study provides evidence that HCR rats have greater NOR1 protein levels in both the soleus and EDL muscles (Fig. 3.5 and 3.6) and it is suggested that NOR1 may serve as a possible mechanism for increasing the cells oxidative machinery. In support of a possible NOR1-mediated response, no differences in PGC-1α mRNA expression were observed between phenotypes. Furthermore, little (in the EDL; Fig. 3.6) or no difference (in the soleus; Fig. 3.5) in PGC-1 protein levels was observed, suggesting that an alternative program may be responsible for the greater respiratory capacity seen 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 in-depth 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, oxidative capacity and induces a rapid increase in NOR1 transcription [206, 217, 222]. Although both LCR and HCR animals are kept under identical housing and feeding regimens that exclude formal exercise training, the possibility that habitual activity levels may play a role in the development of their distinct phenotypes cannot be discounted. Therefore the activity of the rats was monitored during both diurnal and nocturnal periods. It was found that HCR rats were consistently more active than LCR rats (Table 3.2). This observation is in agreement with others [163, 219, 223] who report differences in the habitual activity levels of LCR and HCR rats from earlier generations. Furthermore, Novak et al. have identified different activity levels in another strain of obesity-resistant rats [224], while others have identified a type of ‘inactivity physiology’ in humans [225, 226]. 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 [223, 226-228], 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, it is possible that the differences in physical activity are is partially responsible for the divergent metabolic phenotype displayed by these two rat strains, particularly as Lessard et al. have previously shown that exercise training LCR rats reverses many of negative metabolic health traits observed in these animals [204, 229].
In summary, the investigation described in this chapter uses an animal model in which artificial selection for high- and low-running capacity (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 display 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 nuclear receptor NOR1 has been shown to play a key role in metabolic regulation and is necessary for oxidative metabolism, it is proposed that the differential expression of NOR1 observed in HCR and LCR rats contributes to their distinct phenotypes and overall metabolic regulation.
Exercise training enhances white adipose tissue metabolism in rats selectively bred for low- or high-endurance running capacity


4.1 Introduction

WAT mass is linked to metabolic health and plays a critical role in the maintenance of whole-body energy homeostasis [230, 231]. Increased WAT mass, especially in visceral storage depots, is associated with a greater risk of metabolic disease and mortality [232, 233], while enlarged adipocyte size due to increased lipid content is linked to intrinsic cellular metabolic defects [234, 235]. Conversely, small adipocytes may play a protective role against the increased risk of metabolic disease [236] because, compared to larger adipocytes, these cells have enhanced rates of glucose transport [78, 237-239]. Although excessive lipid storage in visceral WAT depots is linked to metabolic abnormalities such as insulin resistance and impaired lipolysis [240, 241], the metabolic characteristics of WAT in differing metabolic phenotypes has not been well characterized.

Compared to other metabolically active tissues the oxidative capacity of WAT is relatively low [83], but essential cellular activities such as adipogenesis, lipogenesis, lipolysis and FA oxidation require large amounts of ATP [86, 232, 242]. Given that WAT metabolism is altered in obesity and insulin resistance [82, 242] and the metabolic activity of most cells is highly dependent on mitochondrial content, impairments in the regulation of the adipocyte mitochondrial network may lead to dysfunctional WAT metabolism [231]. Indeed, diminished mitochondrial gene
expression has been observed in WAT from insulin resistant humans [81, 82] and rodents [73, 86, 87].

As described in Chapter 3, animal models of high- and low- aerobic treadmill running capacity have been generated (in the absence of exercise training) through two-way artificial selection [160]. Such selection has produced rats that simultaneously present with different metabolic and cardiovascular disease risk factors without the necessity for any environmental intervention [161]. It has been demonstrated that the metabolic characteristics of the skeletal muscle from these rats diverge substantially [165, 204, 243] and that exercise training ameliorates many of the adverse health features observed in the LCR rats [204]. Since exercise training is capable of reducing visceral WAT lipid content and adipocyte size [75, 78, 237, 239] while increasing the WAT expression of a number of mitochondrial proteins [72, 73], it is hypothesized that the oxidative profile of visceral WAT will be lower in untrained LCR compared to HCR rats, but that short-term exercise training will ameliorate this impairment. In line with this hypothesis, the aim of the current investigation is to determine if differences in intrinsic running capacity or training state would affect the expression and activity of a number of proteins with important roles in WAT metabolism.

### 4.2 Materials and methods

**Experimental animals**

This study was undertaken with the combined approval of animal ethics committees from the University of Michigan (Ann Arbor, MI) and California State University (Northridge, CA). Rat models for LCR and HCR were derived from genetically heterogeneous N:NIH stock rats by artificial selection for treadmill running capacity as described in Chapter 3 [160]. Rats were housed in pairs in a temperature controlled environment which provided a reverse 12:12 h light-dark cycle. Throughout the study, rats were given ad libitum access to standard rodent chow and water. Prior to the commencement of any experimental procedures, rats were allowed to acclimate to laboratory conditions for 1 wk.
**EXERCISE TRAINING PROTOCOL**

Age-matched pairs of ~20 wk old male LCR and HCR rats were randomly assigned to two groups: sedentary (LCR-SED, n=10 and HCR-SED, n=10) or exercise trained (LCR-EX, n=10 and HCR-EX, n=10). Rats assigned to undergo exercise training completed a 6-wk (four days/wk) incremental treadmill running protocol where all rats completed the same absolute cumulative running distance (~10 km) [204, 244]. Trained rats undertook their final exercise bout 48 h before the commencement of any experimental procedures.

**TISSUE COLLECTION AND ANALYSES**

Following a 5 h fast, blood samples were taken for the analysis of fasting blood glucose concentrations using a hand-held glucometer (Roche Diagnostics, Castle Hill, NSW, Australia). Serum was assessed for fasting insulin concentrations using a rat-specific ELISA (ALPCO Immunoassays, Salem, NH) and for non-esterified FA’s using a commercially available kit (WAKO Pure Chemical Industries, Osaka, Japan). Rats were weighed and anaesthetized using sodium pentobarbital (1 mL/kg body mass). Hind limb skeletal muscles and epididymal fat pads were surgically excised, weighed, freeze clamped in liquid nitrogen and stored at -80 °C for later analyses. Skeletal muscle analyses have been reported by Lessard et al. [204].

**CITRATE SYNTHASE ACTIVITY**

Approximately 100 mg of epididymal adipose tissue (n=8-10 per group) was visibly cleared of blood vessels and connective tissue, then mechanically homogenized in buffer [175 mM KCl and 2 mM EDTA (pH 7.4), 1:2 dilution] and centrifuged at 20,000 G for 15 min at 4 °C. The infranatant was collected and assayed for CS activity as according to the same protocol outlined in Chapter 2 [214]. Protein concentration of the infranatant was determined using the bicinchoninic method (Pierce, IL). Activity is expressed in nM/min/µg protein.

**IMMUNOBLOT ANALYSIS**

Approximately 250-300 mg of epididymal adipose tissue was visibly cleared of blood vessels and connective tissue and prepared according to the protocol outlined in Chapter 2 (1:2 dilution). Protein concentration of the infranatant was determined
using the bicinchoninic method (Pierce, IL). Adipose tissue lysates containing 10 µg protein were prepared in 4 x Laemmli buffer, subjected to SDS-PAGE, then transferred to PVDF membranes. A pooled lysate sample was prepared and included in each gel as an internal control for normalizing the data. Ponceau staining was used to confirm equal protein transfer. Membranes were then washed and blocked (5% non-fat dry milk or 5% BSA) for 1 hr at room temperature prior to incubation with the appropriate antibodies. Membranes were incubated overnight at 4 °C with primary antibodies specific for CS (~52 kDa; Abcam #ab96600), mitochondrial respiratory complexes I, II, III, IV (subunit 4; CIVIV) and V of the ETS (~18, ~25, ~45, ~15, ~52 kDa respectively; MitoSciences, #MA604 and #MS407), UCP1 (Santa Cruz Biotechnology #sc6529), PGC-1 (~100 kDa; Chemicon, #ab3242), hormone sensitive lipase (HSL; ~88 kDa; Cell Signaling, #4107), phospho-HSLSer660 [245], Adipose Triglyceride Lipase (ATGL; ~54 kDa; Cell Signaling, #2138), phospho-ATGLSer66 (~54 kDa [246]), β3-adrenergic receptor (β3-AR, ~68 kDa; Santa Cruz Biotechnology #sc50436), perilipin 1 (PLIN1 ~68 kDa; Sigma P1873), comparative gene identification-58 (CGI-58; ~42 kDa; Santa Cruz Biotechnology), Fatty Acid Binding Protein 4 (FABP4; ~15 kDa; Abcam #ab37458), NUR77 (~48 kDa; Santa Cruz Biotechnology #sc5569), NOR1 (~68 kDa; Abcam #92777), GLUT4 (~45 kDa; Abcam #ab654), AMPKα (~62 kDa; Cell Signaling #2532), AMPK-phospho-Thr172 (~62 kDa; Cell Signaling #2535), Extracellular Regulated Kinase 1/2 (ERK1/2; ~46 and ~42 kDa; Cell Signaling #9102), phospho-ERK1/2T202/Y204 (~46 and ~42 kDa; Cell Signaling #9102), p38 Mitotgen Activated Protein Kinase (p38 MAPK; ~44 kDa; Cell Signaling #9212), phospho-p38 MAPKT180/Y182 (~44 kDa; Cell Signaling #9211), c-JuN terminal Kinase 1/2 (JNK1/2; ~50 and ~46 kDa; Cell Signaling #9252) or phospho-JNK1/2T183/Y185 (~50 and ~46 kDa; Cell Signaling #9251). Membranes were also probed with anti-α-Tubulin (~50 kDa; Cell Signaling #2144) or β-Actin (~42 kDa; Sigma Aldrich) to confirm equal protein loading. After 1 h room-temperature incubation in the appropriate secondary antibody, protein expression was detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry.
**Statistical analysis**

All data is expressed as the group mean ± SE, unless otherwise specified. All data was analysed using a two-way ANOVA with running capacity and training state as fixed factors. Where main effects were considered significant, a Tukey test for multiple comparisons was conducted. Significance is reported where $P<0.05$. All statistical analyses were completed using GraphPad Prism software.

4.3 Results

**Physiological parameters**

Data for body mass and fat pad mass have previously been reported by Lessard *et al.* [204] with new statistical analyses presented here. Adipose tissue protein content is reported as an indirect marker of changes in adipose tissue cellularity. Intrinsic running capacity and training status were main effects for body mass (Fig. 4.1A; $P<0.0001$ and $P<0.0001$, respectively), fat pad mass (Fig. 4.1B; $P<0.0001$ and $P=0.0002$, respectively) and total tissue protein (Fig. 4.1C; $P=0.026$ and $P=0.024$, respectively). In addition, an interaction between running capacity and training status was observed for body mass ($P<0.0001$) and fat pad mass ($P=0.003$). LCR were heavier than HCR with (33.4%, $P<0.0001$) or without (26.6%, $P<0.0001$) exercise training. LCR-SED was heavier than LCR-EX (31.1%, $P<0.0001$), whereas there was no difference between HCR-SED and HCR-EX. Epididymal fat pad mass was greater in LCR than HCR with (62.5%, $P<0.0001$) or without exercise training (50%, $P<0.0001$). LCR-SED had heavier fat pads than LCR-EX (34.4%, $P<0.0001$), whereas there was no difference between HCR-SED and HCR-EX. In line with this, total protein of WAT was lower in LCR-SED compared to HCR-SED (22.4%, $P=0.052$), whereas adipose protein was 33.4% greater in LCR-EX compared to LCR-SED ($P=0.057$). There were no differences in adipose protein between HCR-SED and HCR-EX, or LCR-EX and HCR-EX.

Intrinsic running capacity improved fasting blood glucose (main effect, $P=0.0002$) and serum FFA concentrations (main effect, $P=0.02$; Table 4.1), but not fasting serum insulin levels. Compared to HCR-SED, LCR-SED had 8% higher blood glucose ($P=0.0008$) and 37% higher NEFA concentrations ($P=0.003$). Exercise training was a main effect for fasting serum FFA’s ($P<0.0001$) and insulin concentrations ($P<0.0001$),
while there was a tendency for LCR-EX to have lower blood glucose concentrations than LCR-SED (8%; $P=0.11$). Exercise training had little effect on blood glucose concentrations in HCR animals. Both LCR-SED and HCR-SED had higher serum insulin concentrations when compared to LCR-EX (37%, $P=0.0004$) and HCR-EX (38%, $P=0.0002$), respectively. LCR-SED had 57% higher serum NEFA concentrations compared to LCR-EX ($P<0.0001$), whereas training had little effect on FFA concentrations in HCR animals.

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<th>Table 4.1 Blood parameters</th>
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<td>Glucose [mM]</td>
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<td>NEFA [mM]$^+$</td>
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EX, exercise trained. HCR, high capacity runner. LCR, low capacity runner. NEFA, non-esterefied fatty acids. SED, sedentary. Values are means ± SE. n=7-10 per group. $^*P<0.05$ main effect for running capacity; $^+P<0.05$, main effect for training status; $^aP<0.05$, different to LCR-SED; $^bP<0.05$, different to HCR-SED; $^cP<0.05$, different to LCR-EX; $^dP<0.05$, different to HCR-EX.

**CITRATE SYNTHASE ACTIVITY**

CS activity was increased with exercise training (Fig. 4.2A; main effect $P=0.01$) and tended to be increased in rats with high intrinsic running capacity (main effect, $P=0.09$). CS activity was 58% higher in epididymal fat pads from LCR-EX compared to LCR-SED ($P=0.08$).

**MITOCONDRIAL PROTEIN CONTENT**

Under equal protein loading conditions (10 µg total protein), the contents of TCA cycle (CS), oxidative phosphorylation (ETS complexes I-V) and mitochondrial biogenesis (PGC-1α) proteins was similar between LCR and HCR rats independent of exercise training status (Fig. 4.2B). UCP1 was not detected in any sample (data not shown).
Figure 4.1 (A) Body mass, (B) Fat pad mass and (C) WAT total protein for LCR □ and HCR ■ rats with or without exercise training. EX, exercise trained. HCR, high capacity runner. LCR, low capacity runner. SED, sedentary. Values are means ± SE. n=7-10 per group. *P<0.05 main effect for running capacity. †P<0.05 main effect for training state. ‡P<0.05 interaction between running capacity and training state. aP<0.05, different to LCR-SED; bP<0.05, different to HCR-SED; cP<0.05, different to LCR-EX; dP<0.05, different to HCR-EX.
Figure 4.2. (A) CS activity and (B) representative immunoblots of mitochondrial protein expression in WAT from LCR □ and HCR ■ rats with or without exercise training. CI, complex I. CII, complex II. CIII, complex III. CIVIV, complex IV subunit IV. CV, complex V. CS, citrate synthase. EX, exercise trained. HCR, high capacity runner. LCR, low capacity runner. PGC-1α, peroxisome proliferator activated receptor-γ co-activator-1α. SED, sedentary. WAT, white adipose tissue. Values are means ± SE. n=8-12 per group. †P<0.05 main effect for training state. ‡P<0.05, different to LCR-SED; §P<0.05, different to HCR-EX.
**INTRACELLULAR REGULATORS OF LIPOLYSIS**

$\beta_3$-AR expression was increased with exercise training (Fig. 4.3A; main effect $P=0.03$), whereas running capacity showed a tendency to increase this parameter ($P=0.09$). There was a significant interaction between running capacity and exercise training ($P=0.006$). The expression of the $\beta_3$-AR was 17% greater in HCR-SED compared to LCR-SED ($P=0.006$) and 18% greater in LCR-EX compared to LCR-SED ($P=0.004$). The phosphorylation of ATGL at Ser$^{406}$ and HSL at Ser$^{660}$ was assessed as surrogate markers of their activity (3, 53). ATGL Ser$^{406}$ phosphorylation and total ATGL protein content were increased by both running capacity ($P=0.02$ and $P=0.003$, respectively) and exercise training status (Fig. 4.3B and C; $P=0.0005$ and $P=0.03$, respectively). Post-hoc analyses revealed a 25% increase in ATGL Ser$^{406}$ phosphorylation in HCR-EX compared to HCR-SED ($P=0.01$). Total ATGL expression was 17% greater in HCR-EX compared to LCR-EX (Fig. 4.3C; $P=0.04$) and the ratio of ATGL Ser$^{406}$ to total ATGL was not different. Total HSL protein remained similar for all groups, although there was a tendency for HSL Ser$^{660}$ phosphorylation to be decreased in both LCR-EX and HCR-EX with training (main effect $P=0.09$). There was no difference in the ratio of HSL Ser$^{660}$ phosphorylation to total HSL protein (data not shown). The content of PLIN1 (which controls lipolysis by regulating protein-protein interactions at the surface of lipid droplets, thereby facilitating access of lipases to their substrates) was increased by training (Fig. 4.3D; $P=0.02$) and an interaction was observed between training and running capacity ($P=0.003$). Post-hoc analyses revealed that PLIN1 expression was 19% greater in LCR-SED compared to HCR-SED ($P=0.03$) and 25% greater in HCR-EX compared to HCR-SED ($P=0.002$). The protein content of CGI-58 (which binds to and activates ATGL triglyceride lipase activity) was increased by 15% in HCR-SED compared to LCR-SED, and 15% greater in LCR-EX compared to LCR-SED, although neither of these values attained statistical significance (Fig. 4.3E; $P=0.08$ and $P=0.1$, respectively). There were main effects for both running capacity and exercise training for CGI-58 protein content (Fig. 4.3E; $P=0.04$ and $P=0.03$, respectively). No difference in FABP4 expression was observed between groups (data not shown).
Figure 4.3. Relative protein expression and representative immunoblots of the lipolytic proteins (A) β3-AR, (B) phospho-ATGLS406, (C) total ATGL, (D) PLIN1 and (E) CGI-58 in WAT from LCR □ and HCR ■ rats with or without exercise training. ATGL, adipose triglyceride lipase. AU, arbitrary units. β3-AR, β3-adrenergic receptor. CGI-58, comparative gene identification-58. EX, exercise trained. HCR, high capacity runner. LCR, low capacity runner. PLIN1, perilipin. SED, sedentary. WAT, white adipose tissue. Values are means ± SE. n=8-12 per group. *P<0.05 main effect for running capacity. †P<0.05 main effect for training state. aP<0.05, different to LCR-SED; bP<0.05, different to HCR-SED; cP<0.05, different to LCR-EX; dP<0.05, different to HCR-EX.
NOR1, NUR77 AND GLUT4 EXPRESSION

A main effect of exercise training was observed for NOR1 expression (Fig. 4.4A; \( P<0.0001 \)). Post-hoc analyses revealed that NOR1 expression was increased by 24% in LCR-SED compared to LCR-EX \( (P=0.008) \) and 21% in HCR-SED compared to HCR-EX \( (P=0.01) \). Intrinsic running capacity did not influence the expression pattern of NOR1, while the expression of NUR77 was similar in LCR and HCR rats with or without exercise training (results not shown). The expression of GLUT4 was elevated with high intrinsic running capacity (Fig. 4.4B; \( P<0.0001 \)) and with exercise training \( (P=0.007) \), with post-hoc analyses revealing that GLUT4 protein content was increased by 16% in HCR-EX compared to HCR-SED \( (P=0.01) \). Compared to LCR-EX, GLUT4 protein content was 22% greater in HCR-EX \( (P=0.0007) \).

STRESS KINASE ACTIVATION

Stress-activated kinase activation was investigated to determine their involvement in the adaptive response of WAT metabolism to exercise training. Total p38 MAPK expression was reduced with training (Fig. 4.5A; \( P=0.03 \)), while there was a tendency for phosphorylation of p38 MAPK on the Thr\(^{180} \) and Tyr\(^{182} \) residues to be reduced by training \( (P=0.07) \). There was a main effect of exercise training on the ratio of phospho-p38 MAPK\(^{T180/Y182} \) to total p38 MAPK \( (P=0.03) \). No differences were observed in total JNK1/2 expression, however training increased phospho-JNK1/2\(^{T183/Y185} \) \( (P=0.02) \) and the ratio of phospho-JNK1/2\(^{T183/Y185} \) to total JNK1/2 (Fig. 4.5B; \( P=0.002 \)). No differences were observed in total ERK1/2 expression (Fig. 4.5C), although a significant main effect of running capacity was observed for phospho-ERK1/2\(^{T202/Y204} \) \( (P=0.03) \). There was also a tendency for the ratio of phospho-ERK1/2\(^{T202/Y204} \) to total ERK1/2 to be affected by running capacity (LCR>HCR; \( P=0.06 \)). No differences were observed in total or phospho-AMPK\(^{T172} \) expression, or the ratio of phospho-AMPK\(^{T172} \) to total AMPK expression (data not shown).
Figure 4.4. Relative protein expression and representative immunoblots of (A) NOR1 and (B) GLUT4 in WAT from LCR □ and HCR ■ rats with or without exercise training. AU, arbitrary units. EX, exercise trained. GLUT4, glucose transport protein 4. HCR, high capacity runner. LCR, low capacity runner. NOR1, neuron-derived orphan receptor 1. SED, sedentary. WAT, white adipose tissue. Values are means ± SE. n=8-12 per group. *P<0.05 main effect for running capacity. †P<0.05 main effect for training state. aP<0.05, different to LCR-SED; bP<0.05, different to HCR-SED; cP<0.05, different to LCR-EX; dP<0.05, different to HCR-EX.
Figure 4.5. Relative phosphorylation and representative immunoblots of (A) p38 MAPK, (B) JNK1/2 and (C) ERK1/2 in WAT from LCR □ and HCR ■ rats with or without exercise training. AU, arbitrary units. ERK1/2, extracellular regulated kinase 1/2. EX, exercise trained. HCR, high capacity runner. JNK1/2, c-JuN terminal kinase 1/2. LCR, low capacity runner. p38 MAPK, p38 mitogen-activated protein kinase. SED, sedentary. WAT, white adipose tissue. Values are means ± SE. n=8-12 per group. †P<0.05 main effect for training state. *P<0.05, different to LCR-SED; †P<0.05, different to LCR-EX; ‡P<0.05, different to HCR-EX.
4.4 Discussion

Using a rat model of divergent running capacity, this chapter describes novel findings that show 1) the mitochondrial protein content of visceral WAT is not related to intrinsic exercise capacity, 2) a short-term (6 wk) program of endurance exercise training does not modulate visceral WAT mitochondrial protein expression (despite training-induced increases in citrate synthase activity), 3) intrinsic running capacity and training status is associated with the differential WAT expression of several key lipolytic proteins, and 4) irrespective of intrinsic running capacity, exercise training induces alterations in the activity and expression of a number of proteins essential to the intracellular regulation of WAT lipid metabolism.

In humans, low aerobic capacity is a strong predictor of early mortality [13, 14]. This important clinical association suggests that the capacity for oxygen metabolism is the underlying determinant of the divide between complex disease and health (Aerobic Hypothesis; Koch & Britton, 2008 [247]). Artificial selection for aerobic capacity has been used as an unbiased test of this theory. Along with cardiovascular disease risk [161] and reduced lifespan [215], LCR rats express a number of characteristics common to metabolic disease phenotypes, such as increased body mass and adiposity [205, 243], hyperinsulinaemia [161, 205, 243] and impaired glucose tolerance [165, 205, 243]. In contrast, HCR rats live 6-8 months longer [215] and present with superior metabolic health, characterized by resistance to weight gain in the face of a high-fat diet [163, 205] and an increased capacity for the uptake and oxidation of glucose [165, 204, 243] and FA’s [165, 204, 205, 243]. While the differential expression of these key characteristics appears to be linked to the oxidative capacity of the skeletal muscle (see Chapter 3 for details [243]), less is known about the metabolic characteristics of the adipose tissue from these divergent aerobic phenotypes.

Chapter 3 presented evidence demonstrating that the skeletal muscle of LCR rats contains fewer mitochondria than HCR rats [165, 243]. Furthermore, impaired β-adrenergic signaling and lipolysis has been observed in the muscle of LCR rats [162, 204]. Given the divergent metabolic characteristics observed in LCR and HCR rats and the importance of WAT in regulating circulating concentrations of FA’s and glucose [230, 248, 249], this study sought to determine if differences similar to those seen in
muscle could also be identified in the WAT. Unlike previous investigations that report reduced expression of a selection of mitochondrial genes and proteins in the visceral WAT of insulin resistant rodents [86, 87] and humans with type 2 diabetes [82], differences in the expression of the key proteins involved in oxidative phosphorylation and the TCA cycle could not be detected in visceral WAT from LCR and HCR phenotypes (Fig. 4.2B). Although the possibility that differences in mitochondrial protein expression were masked due to equal amounts of protein being analyzed in each experiment cannot be ruled out (particularly since LCR-SED have less protein per gram of adipose tissue compared to the other three groups, Fig. 4.1C), the level of expression of all mitochondrial proteins measured was consistent for both sedentary and exercise trained cohorts, suggesting that the abundance of oxidative enzymes in visceral WAT may not be an important factor for determining running capacity and the associated phenotypes of the LCR-HCR rat model system.

Chronic β-adrenergic stimulation as a result of increased physical activity [72, 250] or pharmacological activation [251] has been shown to enhance the oxidative capacity of WAT by up-regulating the expression of genes involved in oxidative phosphorylation and fat oxidation. Although the training program employed in this study was successful in ameliorating many of the metabolic differences observed in the skeletal muscle of LCR compared to HCR rats [204], including a reduction in body mass and adiposity (Fig. 4.1), it had little effect on oxidative enzyme expression in the WAT of either phenotype, an observation that is consistent with findings in human subcutaneous WAT [252]. Although mitochondrial protein content was not different, a marked training-induced increase in citrate synthase activity was observed in both LCR and HCR phenotypes (Fig 4.2A). While this might appear to be in contrast to the earlier findings (especially given that citrate synthase protein expression was unchanged), it is important to note that citrate is essential for replenishing the extra-mitochondrial pool of acetyl-CoA, a substrate essential to de novo lipogenesis [253]. Furthermore, lipogenic pathways are energetically costly processes (Fig. 1.2). Therefore, although training may not have altered the amount of mitochondrial protein, changes in cellular energy needs in response to physiological demand are likely to have induced tighter allosteric control and a number of post-translational changes that increase the
maximal capacity of mitochondrial enzymes involved in the different energy producing pathways [254]. A limitation of this study is the absence of additional measures representing the coordinated activities of β-oxidation, the TCA cycle and oxidative phosphorylation.

Glucose incorporation into triglyceride (TG) is an essential component of lipid synthesis and intracellular glucose availability in adipocytes is dependent on plasma membrane glucose transport efficiency (a process that is directly associated with the intracellular pool of GLUT4 [78, 255, 256], training-induced increases in WAT GLUT4 expression may be associated with an increased capacity for WAT TG synthesis. Indeed, adipose-specific GLUT4 over expression induces an increase in the capacity for TG synthesis via both re-esterification and de novo lipogenic pathways leading to an increase in total adipose mass in sedentary animals [255, 257]. Although it was not possible to measure lipogenic activity in the present study, both running capacity and training status are shown to affect GLUT4 content in visceral WAT (Fig. 4.4B), with LCR rats having reduced WAT GLUT4 content compared with HCR rats. Whether or not other adipose depots show similar changes remains to be determined. Either way, differences in adipose GLUT4 content in both sedentary and trained animals could have important implications on whole-body adiposity in these divergent rat phenotypes [230]. It is also worth noting that exercise training has been reported to increase subcutaneous WAT GLUT4 expression in type 2 diabetic humans [258]. Whether the same changes are observed in human visceral WAT is unclear, since subcutaneous and visceral adipose depots may have distinct responses to exercise [75]. Similarly, it is possible that the different visceral adipose depots may respond disparately to training. Thus, another limitation of this study is limiting the analyses to the epididymal WAT depot.

TG synthesis is tightly coupled with TG catabolism, therefore it is not surprising that exercise training induces an increase in the capacity for both basal and catecholamine-stimulated WAT lipolysis [259]. This is predominantly attributed to repeated transient elevations in circulating catecholamines acting via β-adrenergic signaling pathways [260]. The β3-AR plays an important role in regulating energy balance, particularly in WAT [261]. The expression of the β3-AR was reduced in LCR-
SED compared to HCR-SED (Fig. 4.3A), which agrees with studies in both obese rodents [262, 263] and humans [264] that report impaired β-adrenergic signaling as an important factor in obesity development. Indeed, functional β-adrenergic signaling is essential for obesity resistance [265]. In line with this, exercise training ‘rescued’ the reduction in β3-AR in LCR-EX rats (Fig. 4.3A), while concomitantly reducing fat pad and total body mass (Fig. 4.1). Conversely, training did not effect β3-AR levels in HCR-EX (Fig. 4.3A), nor did it effect fat pad or total body mass (Fig. 4.1). These findings are similar to previous observations of impaired β-adrenergic signaling in the skeletal muscle of LCR compared to HCR rats [162], an impairment that is also ameliorated with exercise training [204].

β3-AR stimulation by catecholamines increases lipolysis by activating protein kinase A (PKA), which phosphorylates both ATGL [246] and HSL [266] to increase lipase activity. Furthermore, PKA phosphorylation of PLIN1 facilitates the dissociation of CGI-58 from PLIN1, thereby allowing CGI-58 to interact with ATGL to maximally activate lipolysis [267]. Given that the primary hypotheses of the current study were mitochondrial-focused, lipolysis could not be measured directly. Instead, the phosphorylation state of ATGL and HSL at key activating serine residues was determined, as well as the protein abundance of other key lipolytic proteins. ATGL Ser\(^{406}\) phosphorylation and total ATGL content were increased in HCR vs. LCR rats and by exercise training in both groups (Fig. 4.3B and C). Similarly, CGI-58 was increased in HCR vs. LCR rats and increased by exercise training in both groups (Fig. 4.3E). Based on knowledge of protein function and results from knockout mice studies [268], the changes reported herein would predict increased lipolysis in high capacity runners and following exercise training. Such a response would match fatty acid availability with the increased fatty acid oxidation capacity/rates observed in HCR compared with LCR rats [162, 165, 243] and is consistent with an endurance trained individuals reliance on fatty acid as an energy source [33].

Previous investigations have reported links between β-adrenergic signaling, the expression of the orphan nuclear receptor NOR1 and whole-body lipid and carbohydrate metabolism [207]. Although its targets are largely unknown, NOR1 is purported to play an important role in regulating oxidative metabolism and glucose
transport in a number of tissues [207]. Notably, NOR1 is a cAMP-dependent target of PKA that is up-regulated upon HSL and ATGL inhibition [269, 270]. Chapter 3 demonstrates that NOR1 expression is reduced in the skeletal muscle of LCR compared to HCR rats, a finding that suggests that the abundance of NOR1 may be linked to the oxidative capacity of the tissue [243]. In this chapter (Chapter 4) the difference in NOR1 expression was shown to be tissue-specific, as LCR-SED and HCR-SED displayed a similar abundance of NOR1 in WAT. This finding is incongruous with human studies that demonstrate that NOR1 is more highly expressed in the WAT of obese compared to healthy humans [271]. However, following weight loss the expression of NOR1 in human WAT is ‘normalized’ to levels similar to healthy control subjects [271]. Similarly, a reduction in NOR1 expression of a similar magnitude was observed for both LCR and HCR rats following exercise training (24% and 21% respectively, Fig. 4.4A). Taken collectively, this observation of reduced NOR1 expression following exercise training in both LCR and HCR rats suggests a principal role for NOR1 in the metabolic regulation of WAT.

Given the role of β-adrenergic signaling in WAT metabolism and the finding that the expression of several proteins downstream of β-adrenergic stimulation are dependent on running capacity and/or training status (Fig. 4.5), the next logical step was to characterize the phosphorylation of several stress kinases that are purported to interact with β-adrenergic signaling pathways to influence intracellular metabolism [254, 272, 273]. Based on these experiments, it can now be reported that a short-term exercise training program induces distinct changes in the activity of the stress kinases p38 MAPK and JNK1/2 in visceral WAT (Fig. 4.5A and B). Both p38 MAPK and JNK1/2 are activated by FFA’s [274, 275] and both are increased in response to elevated lipolytic activity [276]. Importantly, the β-adrenergic activation of p38 MAPK has been shown to occur through cAMP-dependent mechanisms involving PKA [277] and both p38 MAPK and JNK1/2 phosphorylation are known to be elevated in visceral WAT during human obesity [278]. This chapter describes a modest reduction in the phosphorylation of p38 MAPK in visceral WAT following exercise training (Fig. 4.5A), a finding that is consistent with observations in human and rodent skeletal muscle, where training-induced attenuation of resting p38 MAPK activity is associated with the
adaptive response to exercise training [279, 280]. It is also worth noting that p38 MAPK is a known activator of PGC-1α (and therefore mitochondrial biogenesis) [281, 282] and as such, training-induced reductions in p38 MAPK activity may partially explain why changes in mitochondrial protein expression were not observed in the current study. In contrast to the findings for p38 MAPK, phosphorylation of JNK1/2 was elevated as a result of exercise training (Fig. 4.5B). This is also consistent with findings in skeletal muscle [280], but inconsistent with reports that diet-induced elevations in JNK1/2 phosphorylation are attenuated by swim training in obese rats [283, 284]. Although the individual roles of JNK and p38 MAPK in adipocyte metabolism are no doubt complex, these findings indicate that in WAT, both kinases are exercise-responsive (in a manner similar to findings in skeletal muscle), thus implicating them in the array of signaling responses associated with changes in energy demand.

In summary, this chapter investigates the relationship between exercise capacity and WAT metabolism in regards to whole-body metabolic health. Novel data is presented, showing that the content of selected mitochondrial proteins in visceral WAT does not differ in relation to intrinsic exercise capacity or exercise training status and, as such, it can be posited that tighter allosteric control and/or post-translational regulation of oxidative enzymes may be important for coordinating aerobic energy metabolism in WAT. Running capacity and training state were also shown to regulate the expression and activity of a number of proteins down-stream of β-adrenergic signaling pathways, particularly those that are essential to WAT lipolysis. It is suggested that training-induced changes in the expression of the orphan nuclear receptor NOR1 and the activities of the stress kinases p38 MAPK and JNK1/2 may be important factors for determining the expression and activity of metabolic proteins in WAT following exercise training. Further studies are necessary to provide mechanistic support for these purported relationships.
Chapter 5

Perspective and significance

The work undertaken for this thesis comprised a series of independent but related studies aimed at enhancing our understanding of the relationship between genetic factors and environmental stimuli in determining the capacity for aerobic energy production in skeletal muscle and white adipose tissue. Rodent models of divergent intrinsic running capacity (and, by association, metabolic health profile) and environmental interventions (i.e., diet and exercise) were employed in order to explore some of the mechanisms that determine the capacity for mitochondrial energy production in these two insulin-responsive tissues.

The main findings were the identification of several novel mechanisms by which mitochondrial adaptations to environmental stimuli can influence the capacity for energy substrate metabolism in skeletal muscle and white adipose tissue. It was shown that physical activity was a powerful environmental stimulus capable of improving or, in some instances, completely overriding metabolic pathways that have been genetically predisposed to metabolic inflexibility and disease-risk.

The first investigation (described in detail in Chapter 2) addressed the question of whether an obesogenic rodent diet (typical of a human Western diet, which is moderately high in fat but also high in carbohydrate) could induce changes in skeletal muscle respiratory capacity similar to that observed with rodent high-fat feeding (typically very high in fat but low in carbohydrate). The finding that mitochondrial respiratory capacity was indeed increased in response to Western diet feeding, and that it was likely due to an increase in mitochondrial content, raises the possibility that diet-induced mitochondrial biogenesis is an adaptive response designed to help cope with the increased flux of energy substrates to metabolically active tissues. Indeed, as
contraction-induced mitochondrial biogenesis is triggered by disturbances in the metabolic state of the muscle (i.e., changes to the ATP:ADP ratio, elevated [Ca$$^{2+}$$], rate of NAD$$^+$$ cycling, etc.), it seems reasonable to suggest that a persistent oversupply and flux of energy substrate to the muscle would also cause metabolic perturbations capable of inducing mitochondrial biogenesis. In support of this notion, Western diet-induced elevations in mitochondrial protein expression were more pronounced in the primarily glycolytic *m. extensor digitorum longus*, wherein an increase in mitochondrial machinery may be necessary to alleviate some of the stress endured by the primarily oxidative muscle groups in response to lipid overload. The finding that the intramyocellular triglyceride content was elevated in *m. soleus* but not *m. extensor digitorum longus* further supports this hypothesis.

Another important finding from this investigation was that, despite a significant increase in adiposity, rats consuming a Western diet did not show signs of impaired glucose tolerance. Furthermore, the fasting insulin levels of these rats were only moderately elevated (30%). These findings are indicative of early-stage insulin resistance and are more reflective of the pathogenesis of human insulin resistance than the rapid impairments typically seen after feeding rats an extreme high-fat diet (60-80% energy from fat versus ~40% in a typical Western diet). These disparate effects of diets differing in macronutrient composition on insulin action have important implications for future rodent studies investigating obesity and its comorbidities, in that progression of these pathologies occurs much more slowly in humans (and in rodents fed a Western diet) and therefore may be associated with a number of functional adaptations prior to the presentation of overt metabolic disease. Given that diet-induced mitochondrial biogenesis may be one (transient) adaptation, a time-course of disease progression in rats fed a Western diet would help to clarify the relationship between obesity, insulin resistance and skeletal muscle mitochondrial function. Understanding when and where these purported functional adaptations take place could allow for early intervention strategies aimed at slowing (or even reversing) cardio-metabolic disease progression.

Although environmental factors (such as diet and level of habitual physical activity) are important contributors to metabolic health, evidence also points to a strong genetic underpinning to many metabolic diseases. Therefore, the aim of the
second investigation (Chapter 3) was to identify whether or not skeletal muscle mitochondrial function and/or density was related to intrinsic running capacity (and subsequently, metabolic disease risk), and to characterize some of the potential mechanisms underlying these differences. The first major finding from this study was that skeletal muscle from rats with inherent high running capacity (and superior metabolic health; HCR) has an increased mitochondrial content and, in addition, an elevated capacity for lipid oxidation compared to muscle from rats with intrinsically low running capacity (LCR). This finding is consistent with the observation that endurance trained individuals have a greater reliance on lipid-based fuels as an energy source, and explains, in part, how intrinsically determined skeletal muscle metabolism contributes to the phenotype of running capacity and its correlated traits. However, this interpretation is not without its limitations. Importantly, despite originating from the same genetically heterogeneous background and being exposed to identical environmental conditions, LCR rats are less active than their HCR counterparts. Given that habitual physical activity is a powerful environmental influence on skeletal muscle metabolism, the disparity in spontaneous activity between these two divergent phenotypes makes it difficult to ascertain whether or not part (or all) of the differences in muscle respiratory capacity can be attributed to differing physical activity levels or, if differing levels of habitual activity are an indirect consequence of low (or high) inherent skeletal muscle oxidative capacity. The degree to which habitual physical activity contributes to skeletal muscle mitochondrial content and lipid metabolism in this rodent model system is an important question to be addressed in future studies.

The second major finding of this investigation (Chapter 3) was that the orphan nuclear receptor NOR1 was more highly expressed in the muscle of HCR rats compared to LCR rats. Previous studies have implicated this receptor in a plethora of metabolic processes, most notably for its role in promoting oxidative adaptations in skeletal muscle in both rodents and humans. At the center of this paradigm is an apparent relationship between β-adrenergic signaling, PKA- and p38 MAPK-dependent NOR1 activation and the oxidative capacity of skeletal muscle (particularly rates of lipid oxidation). Indeed knock-down of NOR1 in myotubes suppresses the expression of peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α)
and partitions energy metabolism toward O$_2$-independant processes. Taken together, these findings implicate NOR1 as playing a major role in determining skeletal muscle mitochondrial content. However, the functional role and primary targets of NOR1 must be first be characterized in order to determine the precise mechanisms involved. Tissue culture and animal studies that take a gain/loss of function approach will go a long way to providing such information.

Recent advances in our understanding of white adipose tissue metabolism have demonstrated that it is more than the inert lipid storage facility it was once thought to be. White adipose tissue plays a central role in regulating whole-body lipid metabolism, with the co-ordination of the rates of lipolysis and lipogenesis/fatty acid re-esterification being a crucial factor for substrate metabolism in other peripheral tissues (such as skeletal muscle). As these are all energetically costly processes, impaired mitochondrial function in white adipose tissue has been posited to play a role in the pathogenesis of obesity and insulin resistance. Thus, the primary aim of the third investigation (Chapter 4) was to characterize the expression and activity of mitochondrial proteins important to the tricarboxylic acid cycle and oxidative phosphorylation in white adipose tissue from the LCR and HCR rat phenotypes. Additionally, since exercise training has recently been shown to alter important aspects of white adipose tissue energy metabolism (such as improved insulin action and elevated expression of PGC-1$\alpha$ and uncoupling protein 1), the effect of a short-term treadmill running protocol on white adipose tissue from LCR and HCR rats was also investigated.

The first important finding from this study was that the abundance of selected mitochondrial proteins under investigation was not different between LCR and HCR rats, nor did they change in response to exercise training, despite training-induced increases in oxidative enzyme activity (i.e., citrate synthase). This raises the possibility that post-translational changes and/or tighter allosteric control are of greater importance to white adipose tissue metabolism than mitochondrial enzyme content per se. In skeletal muscle, training-induced mitochondrial biogenesis is dependent on both exercise intensity and volume. However, less is known about the effects of different exercise protocols on mitochondrial biogenesis in white adipose tissue. Given that
previous studies have reported training-induced increases in the white adipose tissue content of selected mitochondrial enzymes (respiratory complexes II, IV and cytochrome C), the possibility that a more intense training protocol to the one employed in this study could induce increased mitochondrial enzyme content cannot be discounted. Nevertheless, the improvements in whole-body metabolism that were observed in both LCR and HCR rats following training indicate that the mitochondrial content of visceral white adipose tissue is not likely to be as important as other points of metabolic control (i.e., the activity of lipolytic and lipogenic proteins). Indeed, the findings presented in Chapter 4 demonstrate that the visceral white adipose tissue of LCR and HCR rats is exercise-responsive in a way that alters its capacity for lipid metabolism. In line with this notion, the observation of training-induced increases in the abundance and activating phosphorylation of lipolytic proteins, as well as differences between LCR and HCR rats, is consistent with an endurance trained individuals greater reliance on fatty acids as an energy source, as previously noted. Such a finding is also consistent with observations from the muscle of LCR and HCR rats after the same training protocol. Thus, in untrained HCR rats, a greater capacity for lipolysis in both white adipose tissue and skeletal muscle, as well as an increased capacity for lipid oxidation in muscle, are likely to be important factors underlying the distinct phenotypes.

The mechanisms behind training-induced changes in white adipose tissue metabolism are not well defined. However, the results from this investigation (Chapter 4) demonstrate that exercise training down-regulates the expression of NOR1 and the activating phosphorylation of p38 MAPK. Given that both NOR1 and p38 MAPK are cAMP-dependent targets of PKA, and that exercise training resulted in an increase in expression of several important lipolytic proteins, this implicates the β-adrenergic signaling cascade in the adaptive response of white adipose tissue to exercise training. The precise mechanisms that control the actions and/or interactions of each of the exercise-responsive proteins are complex. As such, this is an exciting new area of research to pursue. Mechanistic studies using tissue culture, ex vivo incubations and transgenic models will aid our understanding of the actions of these proteins while contributing to our overall understanding of whole-body metabolism.
In conclusion, the findings presented in this thesis suggest that although there is a genetic predisposition towards obesity and its comorbidities, this may, in part, be mediated by a predisposition toward physically inactive behaviors. Whether differences in habitual activity are a cause or a consequence of differing capacities for skeletal muscle and/or white adipose tissue metabolism is a question that remains unanswered. Regardless, the results from the studies undertaken for this thesis demonstrate that diet and exercise are powerful environmental stimuli capable of altering metabolic pathways that have otherwise been (genetically) programmed toward metabolic health or disease-risk.
References


Appendix
Skeletal muscle respiratory capacity is enhanced in rats consuming an obesogenic Western diet

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1School of Medical Sciences, Royal Melbourne Institute of Technology (RMIT) University, Bundoora, Australia; 2Health Innovations Research Institute, RMIT University, Bundoora, Australia; 3Department of Physical Education, Daegu University, Daegu, South Korea; and 1Institute of Sport Exercise and Active Living, Victoria University, Melbourne, Australia

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Stephenson EJ, Camera DM, Jenkins TA, Kosari S, Lee JS, Hawley JA, Stepto NK. Skeletal muscle respiratory capacity is enhanced in rats consuming an obesogenic Western diet. Am J Physiol Endocrinol Metab 302:E1541–E1549, 2012. First published April 10, 2012; doi:10.1152/ajpendo.00590.2011.—Obesity-induced lipid over-supply promotes skeletal muscle mitochondrial biogenesis. Previous investigations have utilized extreme high-fat diets (HFD) to induce such mitochondrial perturbations despite their disparity from human obesogenic diets. Here, we evaluate the effects of Western diet (WD)-induced obesity on skeletal muscle mitochondrial function. Long-Evans rats were given ad libitum access to either a WD [40% energy (E) from fat, 17% protein, and 43% carbohydrate (30% sucrose); n = 12] or a control diet (CON; 16% of E from fat, 21% protein, and 63% carbohydrate; n = 12) for 12 wk. Rats fed the WD consumed 23% more E than CON (P = 0.0001), which was associated with greater increases in body mass (23%, P = 0.0002) and adiposity (17%, P = 0.03). There were no differences in fasting blood glucose concentration or glucose tolerance between diets, although fasting insulin was increased by 40% (P = 0.007). Fasting serum triglycerides were also elevated in WD (86%, P = 0.001). The maximal capacity of the electron transfer system was greater following WD (37%, P = 0.02), as were the maximal activities of several mitochondrial enzymes (citrate synthase, β-hydroxyacyl-CoA dehydrogenase, carnitine palmitoyltransferase). Protein expression of citrate synthase, UCP3, and individual respiratory complexes was greater after WD (P < 0.05) despite no differences in the expression of peroxisome proliferator-activated receptor (PPAR)α, PPARγ, or PPARγ coactivator-1 mRNA or protein abundance. We conclude that the respiratory capacity of skeletal muscle is enhanced in response to the excess energy supplied by a WD. This is likely due to an increase in mitochondrial density, which at least in the short term, and in the absence of increased energy demand, may protect the tissue from lipid-induced impairments in glycemic control.

Skeletal muscle is a major site for both fatty acid (FA) and glucose disposal (28, 46). In obesity, inappropriate lipid deposition in nonadipose tissues (such as skeletal muscle) and a concomitant reduction in the ability of cells to completely oxidize lipids has been described frequently (3, 31, 44). Given that the mitochondria are entirely responsible for FA β-oxidation, it is not surprising that mitochondrial functional defects and deficiencies have been observed in the skeletal muscle of obese rodents (7, 37, 48) and humans (30, 42). These findings have led to the notion that lipid-induced abnormalities in mitochondrial metabolism are a causative factor in the pathogenesis of obesity and its comorbidities (1, 11, 30, 31, 39, 41, 48, 53).

The issue of whether mitochondrial “deficiency and/or dys-function” contributes to the obesity sequelae has been questioned in light of recent reports of high-fat diet (HFD)-induced increases in mitochondrial enzyme activities and protein expression (13, 21, 25, 51, 52). Indeed, such diets cause an increase in circulating free fatty acids (FFA) (19–21, 53), which, acting as ligands to peroxisome proliferator-activated receptors (PPARs) (18), are thought to increase mitochondrial biogenesis via the PPARγ-coactivator-1α (PGC-1α) pathway (18, 21, 26, 45). The results of these studies, as well as previous work from our laboratory (15), suggest that certain aspects of mitochondrial function (i.e., β-oxidation and oxidative phosphorylation) may be upregulated following a HFD.

High-fat fed rodents rapidly gain weight while concomitantly developing insulin resistance (32, 33, 57). As such, this model is used frequently to investigate the underlying mechanisms of obesity and its associated conditions. Investigations are often undertaken based on the assumption that these pathologies develop similarly in rodents as they do in humans. However, the typical human obesogenic diet, i.e., a Western diet (WD), is moderately high in fat (≈40–45% of total energy [E]) yet still comprises a substantial proportion of E from carbohydrate (particularly simple sugars) and protein (43). In contrast, a typical rodent HFD usually contains extreme levels of fat (≈80% of total available E) and very low carbohydrate (9, 43). Given the role diet composition plays in the mechanistic progression of obesity and insulin resistance, the disparity between the macronutrient breakdown of a rodent HFD and a typical human obesogenic diet is an important factor to consider when interpreting results (9, 56). Whereas diets high in fat and diets high in sugar may result in similar outcomes, such as rapid weight gain (9, 56), increased circulating lipids (9, 56), and insulin resistance (9), the mechanisms underlying these changes are likely to be markedly different (9).
In the current investigation, we fed rats a commercially available diet that closely mimics a human WD (a diet both high in fat and in sucrose) and measured the mitochondrial respiratory capacity of skeletal muscle using high-resolution respirometry. We hypothesized that 12 wk of a WD would increase the respiratory capacity of skeletal muscle mitochondria via the upregulation of PPARγ and PGC-1α.

METHODS

Animals. Eight-week-old male Long-Evans rats (n = 24) were obtained from Monash Animal Services (Monash, Australia) and housed under a controlled 12:12-h light-dark cycle at a constant temperature of 22°C. Animals had ad libitum access to food and water and were allowed to acclimate to the Royal Melbourne Institute of Technology (RMIT) Animal Facility for 1 wk prior to commencement of the study. All procedures were approved by the RMIT Animal Ethics Committee.

Experimental groups. Following the acclimation period rats were randomly assigned to one of two 12-wk dietary interventions: a control diet (CON; n = 12, 16% of E from fat, 21% from protein, and 63% from carbohydrate, 16.1 kJ/g; Specialty Feeds, Glen Forrest, Western Australia, Australia) or a commercially available WD (n = 12, 40% E from fat, 17% from protein, and 43% from carbohydrate, 19.4 kJ/g; Specialty Feeds). The macronutrient breakdown of each diet is presented in Table 1. Body mass and food intake were monitored twice weekly.

Intraperitoneal glucose tolerance tests. After 11 wk, all rats were subjected to a glucose challenge to assess glucose clearance. Following an overnight fast (14 h), ~15 μl of blood was collected via tail cut, and glucose concentration was measured on a hand-held glucometer (Roche Diagnostics, Castle Hill, New South Wales, Australia). Rats then received a single glucose bolus (2 g/kg body wt) via intraperitoneal injection, and blood glucose concentration was monitored at 30-min intervals over 120 min postinjection.

Tissue collection and analyses. After 12 wk, animals were decapitated and hindlimb muscles immediately excised. The left soleus (~87% type I and ~13% type IIA fibers) was placed in ice-cold muscle preservation medium [BIOPS; 50 mM K+—MES, 20 mM taurine, 0.5 mM dithiothreitol, 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; Ca-EGTA buffer (10 mM; 2.77 mM CaK+—EGTA + 7.33 mM K+—EGTA, 0.1 μM free calcium)] (38, 47, 54), whereas the contralateral soleus and extensor digitorum longus (EDL; ~2% type I, ~42% type IIA, and ~56% type IIB fibers) muscles were snap-frozen in liquid N2 for later analyses. Epidymal fat pads were removed and weighed as indicators of adiposity. All reagents used were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia) unless otherwise specified.

High-resolution respirometry. High-resolution respirometry provides a direct measurement of mitochondrial respiratory capacity ex vivo from which we can obtain information about specific components in a working system (38). Soleus (n = 6 diet groups) was separated mechanically over ice using fine forceps (38) 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 medium (MiR05; 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM MgCl2, 20 mM taurine, 10 mM KHPO4, 20 mM HEPES; adjusted to pH 7.1 with KOH at 37°C, and 1 g/l fatty acid-free BSA; ACROS Organics) (38). Duplicate tissue samples (2–3.5 mg) were transferred to the chambers of an O2K-Oxygraph high-resolution respirometer (Oroboros, Innsbruck, Austria) containing 2 ml of MiR06 (MiR05 + 280 IU/ml catalase) and calibrated to air saturation. Individual chambers were oxygenated to ~485 nmol/ml with pure O2 (BOC Australia).

Substrate uncoupler inhibitor titration protocol. Malate (2 mM) was added to the chamber and the mass specific oxygen flux (J2O2; pmol·s−1·mg−1 wet wt) stabilized for 5–10 min. The tissue was then subjected to a substrate uncoupler inhibitor titration (SUIT) protocol that sequentially evaluates complex (C)I leak state (the addition of 5 mM pyruvate and 10 mM glutamate; state 2 respiration), oxidative phosphorylation with electron flux through CI by titration (the addition of 0.25, 0.75, 2.5, and 5 mM ADP; state 3 respiration), maximal capacity of the electron transport system (ETS) by uncoupling with a stepped titration (the addition of 0.5 and 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone), uncoupled oxidative phosphorylation with electron flux through CI by titration (with the addition of 0.5 μM of the CI inhibitor rotenone), and residual O2 consumption (ROX, with the addition of NaN3, 200 μM). Chambers were maintained at 37°C, and O2 saturation was maintained between 300 and 450 nmol/ml via regular titrations of H2O2. Mass-specific O2 flux was determined using DatLab (Oroboros) from steady-state J2O2 normalized to tissue wet weight and adjusted for instrumental background. The ETS (mitochondrial function independent of mitochondrial density (38)) was calculated to determine mitochondrial function independent of mitochondrial density (38).

Mitochondrial enzyme activities. Muscle homogenates (n = 10/group) were prepared over ice from snap-frozen soleus or EDL (10–20 mg for each) in buffer (175 mM KCl and 2 mM EDTA, pH 7.4, 1:50 or 1:100 dilution) and then subjected to three freeze-thaw cycles. Circate synthase (CS), β-hydroxyacyl-CoA dehydrogenase (β-HAD), and total carnitine palmitoyltransferase (CPT) activities were determined using the methods of Sere (49), Bergmeyer (5), and Bieber et al. (6), respectively, with the following modifications. Briefly, aliquots of homogenate were added to the appropriate wells of a 96-well microplate with a working solution, with final concentrations of either 72.5 mM Tris·HCl, 0.45 mM acetyl-CoA, 0.1 mM DTNB (for CS), 50 mM Tris·HCl, 2 mM EDTA, 250 μM NADH (for β-HAD), and 116 mM Tris·HCl or 1 mM EDTA, 1.1 mM t-carnitine, and 100 μM DTNB (for CPT). After the wells were monitored for background activity, reactions were initiated via the addition of 0.5 mM oxaloacetic acid (for CS) and 100 μM acetacetyl-CoA (for β-HAD) or 1.9 mM palmitoyl-CoA (for CPT). Enzyme activities were determined by monitoring A528 absorbance at 412 (for CS and CPT) and 355 nm (for β-HAD) for 3 min at 25°C. Extinction coefficients of 13.6 (for CS and CPT) and 6.22 μmol·cm−1·mol−1·s−1·mg−1 wet wt. Intramyocellular triglyceride determination. Intramyocellular triglyceride (IMTG) concentration was determined as described previously (48). Briefly, freeze-dried soleus and EDL (n = 8/group for each muscle) were powdered and cleaned of all visible blood and connective tissue. Total lipids in muscle fragments (5–10 mg of dry muscle) were extracted from Sigma-Aldrich (Castle Hill, New South Wales, Australia).
were extracted in chloroform-methanol (13). The organic portion was then evaporated under a stream of N2 and reconstituted in chloroform. The resultant supernatant was evaporated and saponified in ethanolic KOH, and glycerol content was measured using a NanoDrop 1000 spectrophotometer. Muscle lysates containing either 5 or 20 μg of protein were prepared in 4× Laemmlie buffer, subjected to SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. Membranes were blocked (5% nonfat dry milk) for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies specific for signaling proteins [PPARδ (no. PAI-823A; Thermo Scientific), PPARα (no. ab24509; Abcam), and PGC-1 (no. ab3242; Chemicon)] and their targets CPT I-M (no. sc20670; Santa Cruz Biotechnology) and UCP3 (no. PAI-055; Affinity BioReagents), for mitochondrial proteins [complexes I, II, III, IV (subunit I), and V of the electron transfer system (no. MA604; MitoSciences), complex IV (subunit II, no. MS405; MitoSciences), complex IV (subunit IV, no. MS407; MitoSciences), CS (no. ab96600; Abcam)], and as a protein loading control (α-tubulin; Sigma, St. Louis, MO).

Protein was detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry.

### Statistical analyses
All values expressed as mean ± SE. An unpaired t-test was used to compare groups for all analyses except the intraperitoneal glucose tolerance test and the stepped titration of ADP, where a two-way analysis of variance was used. All statistical analyses were performed using Graph Pad Prism software. Significance is reported where P < 0.05.

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**Results**

**WDs are obesogenic.** Rats fed the WD maintained a greater energy intake than CON throughout the study (335 ± 6 and 274 ± 4 kJ/day, respectively; P < 0.0001; Table 1). From 6 wk, WD rats were heavier than CON (379.1 ± 7.1 and 356.6 ± 3.9 g, respectively; P = 0.01) and remained heavier throughout the intervention (Fig. 1A). As illustrated in Fig. 1B, epididymal fat pads were heavier in WD compared with CON (8.1 ± 0.4 and 6.9 ± 0.3 g, respectively; P = 0.03). IMTGs were elevated 37% in WD compared with CON in the soleus (42.8 ± 5.1 and 31.2 ± 4.3 μmol/g dry wt, respectively), although values did not attain statistical significance (P = 0.10; Table 2). In the EDL, IMTGs were not different between groups (P > 0.05).

**Table 2. Physiological parameters in blood and skeletal muscle of fasted animals**

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<tr>
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<th>Control</th>
<th>WD</th>
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<tr>
<td>Fasting blood glucose, mM</td>
<td>5.70 ± 0.24</td>
<td>5.10 ± 0.18</td>
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<tr>
<td>Fasting serum insulin, μg/ml</td>
<td>1.70 ± 0.12</td>
<td>2.39 ± 0.20*</td>
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<tr>
<td>Fasting serum cholesterol, mM</td>
<td>2.99 ± 0.17</td>
<td>3.53 ± 0.19</td>
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<tr>
<td>Fasting serum triglycerides, mM</td>
<td>1.66 ± 0.13</td>
<td>3.09 ± 0.36*</td>
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<tr>
<td>Fasting serum free fatty acids, mM</td>
<td>0.61 ± 0.02</td>
<td>0.73 ± 0.07</td>
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<td>Intramyocellular triglycerides, μmol/g dry wt</td>
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<tr>
<td>Soleus</td>
<td>31.17 ± 4.27</td>
<td>42.78 ± 2.54</td>
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<tr>
<td>EDL</td>
<td>5.93 ± 1.16</td>
<td>5.97 ± 1.26</td>
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Values represent means ± SE, as determined after 12 wk; n = 8–12 animals/group. EDL, extensor digitorum longus. *P < 0.05.
In oxidative muscle the capacity of the ETS was greater following consumption of a WD. To evaluate the capacity of the ETS and thus determine maximal O$_2$ flux through the mitochondria, we measured O$_2$ flux in the soleus during a SUIT protocol with substrates for CI and CII (Fig. 2A). State II respiration with electron leak through CI was greater in WD compared with CON (WD 9.9 ± 0.6 pmol·s$^{-1}$·mg wet wt$^{-1}$ and CON 5.8 ± 0.6 pmol·s$^{-1}$·mg wet wt$^{-1}$, *P < 0.001). A similar trend was observed for state III respiration with flux through CI, although this difference did not attain statistical significance (WD 89.7 ± 8.9 pmol·s$^{-1}$·mg wet wt$^{-1}$ and CON 70.6 ± 6.8 pmol·s$^{-1}$·mg wet wt$^{-1}$, *P = 0.11). However, greater sensitivity to ADP was observed (*P < 0.05; Fig. 2B). State III respiration with flux through both CI and CII together (WD 137.2 ± 11.8 pmol·s$^{-1}$·mg wet wt$^{-1}$ and CON 101.7 ± 9.8 pmol·s$^{-1}$·mg wet wt$^{-1}$) and noncoupled respiration with flux through CII (WD 73.9 ± 5.3 pmol·s$^{-1}$·mg wet wt$^{-1}$ and CON 56.5 ± 4.5 pmol·s$^{-1}$·mg wet wt$^{-1}$) were greater in WD (*P = 0.04 and *P = 0.03, respectively). Similarly, uncoupled/maximal flux through the ETS was greater in WD compared with CON (152.0 ± 11.3 pmol·s$^{-1}$·mg wet wt$^{-1}$ and 110.8 ± 9.8 pmol·s$^{-1}$·mg wet wt$^{-1}$, respectively, *P = 0.02).

To assess O$_2$ flux independently of mitochondrial density, we calculated the flux control ratios for each substrate combination (Table 3). There were no differences between dietary groups.

**Consumption of a WD increases maximal activity of mitochondrial enzymes.** Both CS (WD 23.5 ± 1.5 μM·min$^{-1}$·g wet wt$^{-1}$ and CON 19.4 ± 1.0 μM·min$^{-1}$·g wet wt$^{-1}$, *P = 0.03; Fig. 3A) and total CPT activity (WD 0.8 ± 0.1 μM·min$^{-1}$·g wet wt$^{-1}$ and CON 0.4 ± 0.1 μM·min$^{-1}$·g wet wt$^{-1}$, *P = 0.01; Fig. 3B) were higher in the soleus after WD compared with CON. There were no differences between dietary groups for β-HAD activity in the soleus (WD 4.9 ± 0.2 μM·min$^{-1}$·g wet wt$^{-1}$ and CON 4.1 ± 0.3 μM·min$^{-1}$·g wet wt$^{-1}$, *P = 0.11; Fig. 3B). In the EDL, CS (WD 10.1 ± 0.5 μM·min$^{-1}$·g wet wt$^{-1}$ and CON 8.3 ± 0.5 μM·min$^{-1}$·g wet wt$^{-1}$, *P = 0.01; Fig. 3A), β-HAD (WD 2.6 ± 0.1 μM·min$^{-1}$·g wet wt$^{-1}$ and CON 2.2 ± 0.2 μM·min$^{-1}$·g wet wt$^{-1}$, *P = 0.04; Fig. 3B), and CPT (WD 0.13 ± 0.01 μM·min$^{-1}$·g wet wt$^{-1}$ and CON 0.09 ± 0.01 μM·min$^{-1}$·g wet wt$^{-1}$, *P = 0.02; Fig. 3C) activities were all greater following the WD.

**Enhanced respiratory capacity was not associated with changes in PPARβ or PGC-1 expression in the soleus.** No differences were observed in the gene expression of PPARβ or PGC-1a in either soleus or EDL muscles (Fig. 4). There were also no differences in the protein expression of PGC-1, PPARβ, or CPT I between dietary treatments for the soleus. Small but significant increases were observed in the protein expression of UCP3 in both soleus (3%, *P = 0.04; Fig. 5) and EDL muscle (6%, *P = 0.002, Fig. 6). In the EDL, PGC-1

<table>
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<th>Table 3. Flux control ratios</th>
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<tr>
<td>Complex I, leak</td>
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<tr>
<td>Complex I, OXPHOS (state III)</td>
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<tr>
<td>Complex I, OXPHOS (state III)</td>
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<tr>
<td>Complex II, OxPHOS (state III)</td>
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<tr>
<td>Complex II, uncoupled</td>
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Values represent means ± SE; n = 6 animals/group. OXPHOS, oxidative phosphorylation. Flux control ratios are the respirometry data normalized to maximal O$_2$ consumption in uncoupled states (electron transport system).
protein expression was 4% greater following the WD ($P = 0.005$; Fig. 6), and there was a trend toward increased expression of PPARα ($P = 0.07$; Fig. 6). Similarly to the soleus, there was a trend toward increased mtDNA copy number with the WD (42%, $P = 0.08$; Fig. 4B), although this did not attain statistical significance.

**DISCUSSION**

The mitochondrion has long been implicated in the pathogenesis of obesity and metabolic dysfunction (30, 34). However, the extent of mitochondrial involvement and whether these changes are secondary to lifestyle factors (i.e., physical inactivity) are a matter of current debate. Previously, it has been shown that a mitochondrial deficiency exists in obese humans (30) and rodents (11, 48). However, the results of recent studies suggest that there is dissociation between mitochondrial dysfunction and the progression of obesity (4, 19, 21, 51). Indeed, accumulating evidence indicates that HFDs induce increases in the expression (13, 19, 21, 51) and activity (1, 51)
of key mitochondrial enzymes and that this process is likely to be driven by an increase in circulating FFAs (21). Our data support this hypothesis and demonstrate for the first time that skeletal muscle respiratory capacity is enhanced after 12 wk on an obesogenic (high-fat, high-sucrose) WD.

We employed a method of high-resolution respirometry that allows the direct monitoring of \( \text{O}_2 \) consumption by the ETS in permeabilized muscle fibers (38) and reflects the integrated activity of the tricarboxylic acid cycle and electron transfer system, providing a dynamic measurement of the oxidative capacity of tissue ex vivo (38). We show that 12 wk of a WD induces an increase in the activity of the ETS in skeletal muscle with a high oxidative capacity. When the data was normalized to allow interpretation of function independent of mitochondrial volume, this increase was attenuated, suggesting that mitochondrial biogenesis, rather than increased activity, is likely to be responsible for the increase in respiratory capacity.

This finding is supported by the increased expression and activity of the mitochondrial enzyme CS, an enzyme long used as a marker enzyme for mitochondrial content. Additional mitochondrial enzyme activity and protein measurements in the EDL muscle (composed mainly of primarily glycolytic fibers) further support this notion.

Previously, it has been shown in WD- (56) and HFD-fed rodents (19, 21, 51) that skeletal muscle has an increased capacity for fat oxidation after as little as 4 wk. In the current study, we observed increased activity of total CPT (CPT I and CPT II), which was indicative of an increase in the delivery of long-chain fatty acids into the mitochondria. Although we did not measure fat oxidation directly, HFD-induced increases in the activity of CPT I have previously been associated with greater rates of fat oxidation (10, 51). Interestingly, in the soleus we did not observe any differences in the activity of \( \beta-HAD, \) the third enzyme of the \( \beta-oxidation \) pathway, imply-
ing that the rate of fatty acid delivery to the mitochondria may be discordant with the capacity for β-oxidation in muscle with high oxidative capacity. However, it is important to note that these data are of maximal enzyme activity ex vivo and are not indicative of in vivo demand.

Although we can only speculate whether or not this mismatch is likely to contribute to cellular lipid deposition (IMTGs were elevated 37% in the soleus but remained similar in the EDL, where β-HAD activity was elevated), the notion that the β-oxidation and tricarboxylic acid cycle activities are disproportionate and contribute to lipid deposition in diet-induced obesity is not a new concept. Previously, Hoehn et al. (24) demonstrated that simply driving metabolism toward increased fat oxidation is not sufficient to increase energy expenditure, whereas Koves et al. (31) suggested that incomplete fatty acid oxidation and accumulation of lipid species occur when the import of fatty acid into the mitochondria exceeds metabolic demand. Moreover, human studies have shown that, despite increases in IMTG storage, rates of fatty acid oxidation per se are unlikely to be responsible for the accumulation of lipid species during obesity but rather the reduction in physical activity. Our findings suggest that chronic feeding of a WD increases the cellular machinery necessary to cope with increases in energy expenditure, and yet without the appropriate energy demand substrates will accumulate in the tissue, eventually being stored. Pharmacological agonism of PPARδ has been shown previously to increase the expression of several oxidative proteins, including UCP3 and CPT I (36). However, unless combined with an endurance training program, no functional improvements are seen, implying that there must be an appropriate stimulus for any functional enhancements to manifest. We propose that the increases in mitochondrial respiratory capacity observed in the current investigation are a compensatory response in anticipation of a potential increase in substrate flux to the mitochondria.

A second aim of this investigation was to characterize potential mechanisms responsible for the increase in mitochondrial function. The PPARs are a family of ligand-activated nuclear transcription factors that sense and respond to dietary lipids and their metabolites (16). Of the three isoforms, PPARα and PPARδ are the two expressed preferentially in skeletal muscle, with PPARγ being the predominant isoform in this tissue (8). Both PPARα and PPARδ play key roles in fatty acid metabolism (18), acting with the transcriptional coactivator and “master regulator” of mitochondrial biogenesis, PGC-1α (40, 50), to induce increases in both nuclear and mitochondrial target genes for fatty acid catabolism (50, 56). Previous studies have identified PPARδ and PGC-1α as important mediators of diet-induced increases in mitochondrial function (29). Furthermore, PPARδ is known to interact directly with CPT I (50), whereas HFDs or pharmaceutical PPARγ agonists increase UCP3 mRNA (50, 56) and protein expression (22, 51). Unlike previous studies that have investigated the effect of a HFD on mitochondrial signaling, we did not find a large increase in circulating FFAs following the WD. However, total serum triglycerides were almost twofold greater. Moreover, we were unable to demonstrate marked differences in the gene and/or protein expression of PPARγ or PGC-1α, the two signaling proteins that are thought to be responsible for HFD-induced alterations to mitochondrial function (51). Despite this, we cannot discount the possibility that posttranslational regulation of protein activity may be a potential mechanism by which mitochondrial function is enhanced. Indeed, it is well known that the acetylation status of PGC-1α is important in regulating its activity (14), whereas PPARγ itself may posttranslationally modify PGC-1α (15).

Of note was that the expression of PPARγ’s downstream target protein CPT I remained unchanged despite an increase in total CPT activity. Previous studies have reported increases in UCP3 mRNA following a WD (56), and in line with this we show increases in UCP3 protein expression. Taken together, increased CPT activity and UCP3 protein expression could be indicative of an increase in PPARδ activity (17) despite no increase in PPARγ gene or protein expression. Although UCP3 remains without an established function (2, 35), increases in UCP3 expression due to PPAR stimulation implicates this protein in an array of metabolic processes that involve substrate handling. Further investigations will need to be undertaken over prolonged intervention periods to determine whether PPARγ activity is indeed altered by a WD. However, it is also plausible that the reduced percentage of fat in a WD may have less agonist potential than that of a HFD, and as such, other mechanisms may be responsible for the observed increase in CPT activity and UCP3 expression.

In conclusion, the results of the present investigation demonstrate that an increase in mitochondrial respiratory capacity occurs in response to WD-induced obesity. This is likely a compensatory mechanism to cope with excess energy availability in the absence of a simultaneous increase in energy demand. Such a scenario would be expected to contribute to intracellular lipid deposition and overall adiposity when sustained for prolonged periods. Further investigations are required to identify the precise mechanisms that lead to this adaptation.

GRANTS
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DISCLOSURES
The authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS
E.J.S., T.A.J., S.K., J.A.H., and N.K.S. contributed to the conception and design of the research; E.J.S., D.M.C., T.A.J., J.S.L., S.K., and N.K.S. performed the experiments; E.J.S., D.M.C., J.S.L., and N.K.S. analyzed the data; E.J.S. interpreted the results of the experiments; E.J.S. prepared the figures; E.J.S. drafted the manuscript; E.J.S., J.A.H., and N.K.S. edited and revised the manuscript; E.J.S., D.M.C., J.A.H., and N.K.S. approved the final version of the manuscript.

REFERENCES
E1548 WESTERN DIET-INDUCED MITOCHONDRIAL BIOGENESIS


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

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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 inborn 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 = 0.0009) and β-hydroxyl-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.

The ability to deliver and utilize O2 is an essential component of metabolic regulation, with an individual’s maximal aerobic power [maximal O2 consumption (VO2 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, VO2 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 VO2 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 O2, 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 (Nor)1 (16, 17). The NR4A family of orphan NRs [Nor77, Nur11, and neuron-derived orphan receptor (Nor)1] 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, Castle Hill, NSW, Australia). 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 Immunoasays).

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. Additional 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 K-β-MES, 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); Ca⁺⁺-EGTA buffer (10 mM; 2.77 mM CaK₂EGTA + 7.73 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/stripe) and placed in pregassed Krebs-Henselte buffer (KHB) for measurements of basal and insulin-stimulated glucose uptake. The remaining hindlimb muscles were frozen 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 mM glucose, 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 with 10 min in KHB modified with 40 mM mannitol, 2 mM pyruvate, 0.1% fatty-acid free BSA, and 60 nM 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-[1,2-3H]deoxyglucose (1 μCi/ml, Perkin-Elmer), 39 mM n-tetradecanoate (0.1 μCi/ml, Perkin-Elmer), and 60 nM 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 freezing 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-[1,2-3H]deoxyglucose transport were calculated as previously described (15).

High-resolution respirometry. Approximately 10 mg of the soleus (or EDL) soleus muscle was excised and individual muscles 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 [MIR05; 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-Oxigraph high-resolution respirometer (Oroboros, Innsbruck, Austria) containing 2 ml MIR05 (pH range 280/140 ml catalase) and calibrated to air saturation. Individual chambers were oxygenated to ~485 mmol/ml with pure O₂ (BOC Australia). Chambers were maintained at a temperature of 37°C, and O₂ saturation was kept between 450 and 300 mmol/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 octanoylcarnitine (ETF; fat oxidation) in leak state through the addition of octanoylcarnitine (etc) and then on the ETF (ETC) complexes.
Intrinsic Running Capacity and Mitochondrial Respiration • Stephenson EJ et al.

nitine (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 CI 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′-tetramethyl-p-phenylenediamine dihydrochloride (0.5 mM) in the presence of ascorbate (2 mM). The reaction was terminated by the addition of Na2SO4 (50 mM), after which the chambers were reoxygenated for the determination of auto-oxidation. Mass-specific O2 flux was determined using DATLAB (Oroboros) 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 NaCl, 25 mM NaH2PO4, 10 mM HEPES, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 1 mM benzamidine, and 1 mM PMSF; 1:8 dilution) and centrifuged at 20,000 g for 30 min at 4°C. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce). Muscle lysates containing 10 µg protein were prepared in 4× Laemmli buffer, subjected to SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. Membranes were blocked (5% nonfat dry milk) for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies specific for the following proteins: PGC-1 (−100 kDa, no. ab3242, Chemicon), FNDC5 (−28 kDa, no. ab93373, Abcam), Nur77 (−48 kDa, no. sc5569, Santa Cruz Biotechnology), Nri1 (−68 kDa, no. 92777, Abcam), glyceral transporter 4 (−47 kDa, no. ab654, Abcam), fatty acid translocase/CD36 (−80 kDa, no. 17044, Abcam), CI (−18 kDa), CII (−25 kDa), complex III (CIII; −45 kDa), CIV subunit I (−37 kDa), and complex V (CV; −52 kDa) of the ETS (no. MA604, MitoSciences), CIV subunit II (−25 kDa, no. MS405, MitoSciences), CIV subunit IV (−15 kDa, no. MS407, MitoSciences), CS (−52 kDa, no. ab69600, 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. Two-way ANOVA was used to analyze data from the intraperitoneal glucose tolerance test (IP-GTT). Physical activity and glucose uptake data were analyzed using one-way ANOVA with a Student-Newman-Keuls post hoc test. An unpaired t-test was used to compare groups for all other analyses unless otherwise specified. Statistical analyses were performed using Graph Pad Prism software. Significance is reported where P < 0.05.

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 mass. 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 insulin curve during the IP-GTT (LCR rats: 196.2 ± 47.4 and HCR rats: 83.6 ± 11.3, P = 0.01; Fig. 1, C and D).

Table 1. Body composition and metabolic parameters

<table>
<thead>
<tr>
<th></th>
<th>LCR Rats</th>
<th>HCR Rats</th>
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<tbody>
<tr>
<td>Age, days</td>
<td>80.9 ± 0.6</td>
<td>81.0 ± 0.6</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>
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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.
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-
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>No movement, %</td>
<td>67.1 ± 0.7‡</td>
<td>61.9 ± 1.9†</td>
</tr>
<tr>
<td>Stereotypic movement, %</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 ± 79.31</td>
<td>1721.0 ± 187.9‡†</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>
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<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>
</tr>
<tr>
<td>ETF, CI, and OXPHOS (state 3)</td>
<td>0.32 ± 0.01</td>
<td>0.39 ± 0.02*</td>
</tr>
<tr>
<td>ETF, CI, CII, and OXPHOS (state 3, submaximal)</td>
<td>0.77 ± 0.02</td>
<td>0.76 ± 0.03</td>
</tr>
<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 and OXPHOS (uncoupled, state 4)</td>
<td>0.57 ± 0.01</td>
<td>0.51 ± 0.01*</td>
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</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 ($P < 0.05$; Fig. 5) except for CIV subunit I. Additionally, expression of CS was $14\%$ greater in HCR rats compared with LCR rats ($P = 0.03$; Fig. 5). In the EDL muscle, expression of all individual ETS complexes was greater in HCR rats compared with LCR rats ($P < 0.05$; Fig. 6) except for CIV subunit I, which was not different. CS protein expression was $38\%$ greater in HCR rats ($P < 0.0001$; 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 (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 (Figs. 4–6). This increase in muscle oxidative capacity is associated with an increase in the protein expression of the NR Nor1 (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 (Table 1) (10, 24, 25, 39), hyperinsulinemia (Table 1) (25, 40, 48), and impaired glucose tolerance (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 (Fig. 2) (17, 35) and long-chain fatty acids (17, 24, 25, 35). The differential expression of these characteristics appears to be
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 \( \text{O}_2 \) 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 \( \text{O}_2 \) 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 \( \beta \)-oxidation, the TCA cycle and the ETS. During ADP-stimulated (state 3) respiration, we observed greater \( \text{O}_2 \) 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 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, \( \beta \)-HAD activity (the third enzyme of the \( \beta \)-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 \( \text{O}_2 \) 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-\( \gamma \) 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 \( \pm \) 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. Indeed, 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 consistently 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-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

Author contributions: E.J.S., N.K.S., L.G.K., S.L.B., and J.A.H. conception and design of research; E.J.S. performed experiments; E.J.S. analyzed data; E.J.S. interpreted results of experiments; E.J.S., prepared figures; E.J.S., drafted manuscript; E.J.S., N.K.S., L.G.K., S.L.B., and J.A.H. edited and revised manuscript; E.J.S., N.K.S., L.G.K., S.L.B., and J.A.H. approved final version of manuscript.

REFERENCES


30. Pea...
Exercise training enhances white adipose tissue metabolism in rats selectively bred for low- or high-endurance running capacity

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Impaired visceral white adipose tissue (WAT) metabolism has been implicated in the pathogenesis of several lifestyle-related disease states, with diminished expression of several WAT mitochondrial genes reported in both insulin-resistant humans and rodents. We have used rat models selectively bred for low- (LCR) or high-intrinsic running capacity (HCR) that present simultaneously with divergent metabolic phenotypes to test the hypothesis that oxidative enzyme expression is diminished in WAT from LCR animals. Based on this assumption, we further hypothesized that short-term exercise training (6 wk of treadmill running) would ameliorate this deficit. Approximately 22-wk-old rats (generation 22) were studied. In untrained rats, the abundance of mitochondrial respiratory complexes I–V, citrate synthase (CS), and PGC-1 was similar for both phenotypes, although CS activity was greater than 50% in HCR (P = 0.09). Exercise training increased CS activity in both phenotypes but did not alter mitochondrial protein content. Training increased the expression and phosphorylation of proteins with roles in β-adrenergic signaling, including β3-adrenergic receptor (16% LCR, P < 0.05), NOR1 (24% LCR, 21% HCR, P < 0.05), phospho-ATGL (25% HCR, P < 0.05), perilipin (25% HCR, P < 0.05), CIG-58 (15% LCR, P < 0.05), and GLUT4 (16% HCR, P < 0.0001). A training effect was also observed for phospho-P38 MAPK (12% LCR, <20% HCR, P < 0.05) and phospho-INK (29% LCR, <20% HCR, P < 0.05). We conclude that in the LCR-HCR model system, mitochondrial protein expression in WAT is not affected by intrinsic running capacity or exercise training. However, training does induce alterations in the activity and expression of several proteins that are essential to the intracellular regulation of WAT metabolism.

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**MATERIALS AND METHODS**

*Animal model.* This study was undertaken with the combined approval of the animal ethics committees from both the University of Michigan (Ann Arbor, MI) and California State University (Northridge, CA). Rat models for LCR and HCR were derived from genetically heterogeneous N:NIH stock (National Institutes of Health) rats by artificial selection for treadmill running capacity, as described previously (31). Rats were housed in pairs in a temperature-controlled environment that provided a reverse 12:12 light-dark cycle. Throughout the study, rats were given ad libitum access to standard rodent chow and water. Prior to the commencement of any experimental procedures, rats were allowed to acclimate to laboratory conditions for 1 wk.

*Experimental design.* Age-matched pairs of 20–25 wk-old male LCR and HCR rats were randomly assigned to two groups: sedentary (LCR-SED (n = 10) and HCR-SED (n = 10)) or exercise trained (LCR-EX (n = 10) and HCR-EX (n = 10)). Rats assigned to undergo exercise training completed a 6-wk (4 days/wk) incremental treadmill running protocol where all rats completed the same absolute cumulative running distance (~10 km) (32, 39). Trained rats undertook their final exercise bout 48 h before the commencement of any experimental procedures.

*Tissue collection and blood analyses.* Following a 5-h fast, blood samples were taken for the analysis of fasting blood glucose concentrations using a hand-held glucometer (Roche Diagnostics, Castle Hill, New South Wales, Australia). Serum was assessed for fasting insulin concentrations using a rat-specific ELISA (ALPCO diagnostics, Salem, NH) and for nonesterified FAs (NEFA) using a commercially available kit (WAKO Pure Diagnostics, Osaka, Japan). Rats were weighted and anesthetized using pentobarbital sodium (1 mL/kg body mass). Hindlimb skeletal muscles and epididymal fat pads were surgically excised, weighed, freeze-clamped in liquid nitrogen, and stored at −80°C for later analyses. Muscle data has been reported previously (39).

*Citrate synthase activity.* Approximately 100 mg of epididymal adipose tissue was visibly cleared of blood vessels and connective tissue and then mechanically homogenized in buffer [175 mM KCl and 2 mM EDTA (pH 7.4), 1:2 dilution] and centrifuged at 20,000 g for 15 min at 4°C. The infranatant was collected and assayed for citrate synthase activity, as described previously (61). Protein concentration of the infranatant was determined using the bicinchoninic method (Pierce). Activity is expressed in nM·min⁻¹·mg protein⁻¹.

*Immunoblotting.* Approximately 250–300 mg of epididymal adipose tissue was visibly cleared of blood vessels and connective tissue and then mechanically homogenized in buffer (50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, 1 mM benzamidine, 1 mM phenylenediamine sulfonate fluoride, 1:8 dilution) and centrifuged at 20,000 g for 30 min at 4°C. Protein concentration of the infranatant was determined using the bicinchoninic method (Pierce). Adipose tissue lysates containing 10 μg of protein were prepared in 4× Laemmli buffer, subjected to SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. A pooled lysate sample was prepared and included in each gel as an internal control for normalizing the data. Ponceau staining was used to confirm equal protein transfer. Membranes were then washed and blocked (5% nonfat dry milk or 5% BSA) for 1 h at room temperature prior to incubation with the appropriate antibodies. Membranes were incubated overnight at 4°C with primary antibodies specific for citrate synthase (CS; ~52 kDa; Abcam, ab94650), mitochondrial respiratory complexes I, II, III, IV (ubiquinol:cytochrome c oxidoreductase (subunit 4; COX-IV), and V of the electron transport chain (~18, ~25, ~45, ~15, and ~52 kDa respectively; MitoSciences, nos. MA604 and MS407), uncoupling protein 1 (UCP1; Santa Cruz Biotechnology, no. sc6529), peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1; ~100 kDa; Chemicon, no. ab3242), hormone-sensitive lipase (HSL; ~88 kDa; Cell Signaling Technology, no. 4107), phospho-HSL (Ser560) (71), adipose triglyceride lipase (ATGL; ~54 kDa; Cell Signaling Technology, no. 2138), phospho-ATGL (Ser466; ~54 kDa (53)), β3-adrenergic receptor (β3-AR; ~68 kDa; Santa Cruz Biotechnology, no. sc30436), perilipin 1 (PLIN1; ~68 kDa; Sigma, P1873), pancreatic-specific gene identification (CGI-58; ~42 kDa), FA-binding protein 4 (FABP4; ~15 kDa; Abcam, no. ab73458), neuron-derived clone 77 (NUR77; ~48 kDa; Santa Cruz Biotechnology, no. sc5569), neuron-derived orphan receptor 1 (NOR1; ~68 kDa; Abcam, no. 92777), GLUT4 (~45 kDa; Abcam, no. ab6543), AMP-activated protein kinase (AMPK; ~62 kDa; Cell Signaling Technology, no. 2532), AMPK phospho-Thr172 (~62 kDa; Cell Signaling Technology, no. 2535), extracellular regulated kinase 1/2 (ERK1/2; ~46 and ~42 kDa; Cell Signaling Technology, no. 9102), phospho-ERK1/2 (Thr202/Tyr204); (~46 and ~42 kDa; Cell Signaling Technology, no. 9101), p38 mitogen-activated protein kinase (p38 MAPK; ~44 kDa; Cell Signaling Technology, no. 9212), phospho-p38 MAPK (Thr180/ Tyr182; ~44 kDa; Cell Signaling Technology, no. 9211), c-Jun NH2-terminal kinase 1/2 (JNK1/2; ~50 and ~46 kDa; Cell Signaling Technology, no. 9252), or phospho-JNK1/2 (Thr183/Tyr185; ~50 and ~46 kDa; Cell Signaling Technology, no. 9251). Membranes were also probed with anti-α-tubulin (~50 kDa; Cell Signaling Technology, no. 2144) or β-actin (~42 kDa; Sigma-Aldrich) to confirm equal protein loading. After 1-h room temperature incubation in the appropriate secondary antibody, protein expression was detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry.

*Statistical analyses.* All data are expressed as group means ± SE, unless specified otherwise. All data were analyzed using a two-way ANOVA, with running capacity and training state as fixed factors. Where main effects were considered significant, a Tukey test for multiple comparisons was conducted. Significance is reported where P < 0.05. All statistical analyses were completed using GraphPad Prism software.

**RESULTS**

*Physiological parameters.* Data for body mass (BM) and fat pad mass have been reported previously (39), with new statistical analyses presented here. Adipose tissue protein content is reported as an indirect marker of changes in adipocyte cellularity. Intrinsic running capacity and training status were main effects for BM (P < 0.0001 and P < 0.0001, respectively; Fig. 1A), fat pad mass (P < 0.0001 and P = 0.0002, respectively; Fig. 1B), and total tissue protein (P = 0.026 and P = 0.024, respectively; Fig. 1C). In addition, an interaction between running capacity and training status was observed for BM (P < 0.0001) and fat pad mass (P = 0.003). LCR rats were heavier than HCR rats with (33.4%, P < 0.0001) or without (26.6%, P < 0.0001) exercise training. The LCR-SED group was heavier than the LCR-EX group (31.1%, P < 0.0001), whereas there was no difference between HCR-SED and HCR-EX. Epididymal fat pad mass was greater in LCR than HCR rats with (62.5%, P < 0.0001) or without exercise training (50%, P < 0.0001). LCR-SED rats had heavier fat pads than LCR-EX rats (34.4%, P < 0.0001), whereas there was no difference between HCR-SED and HCR-EX rats. In line with this, total protein of WAT was lower in LCR-EX compared with LCR-SED rats (34.4%, P < 0.0001) and LCR-EX compared with HCR-SED rats (P = 0.057). There were no differences in adipocyte protein between HCR-SED and HCR-EX or LCR-EX and HCR-EX rats.

Intrinsic running capacity improved fasting blood glucose (main effect, P = 0.0002) and serum NEFA concentrations...
LCR-SED and HCR-SED rats had higher serum insulin concentrations compared with LCR-EX (37%, \( P = 0.0004 \)) and HCR-EX rats (38%, \( P = 0.0002 \)), respectively. LCR-SED rats had 57% higher serum NEFAs concentrations compared with LCR-EX rats (\( P < 0.0001 \)), whereas training had little effect on NEFA concentrations in HCR animals.

**CS activity.** CS activity was increased with exercise training (main effect, \( P = 0.01 \); Fig. 2A) and tended to be increased \( P < 0.05 \) in rats with HCR (main effect, \( P = 0.09 \)). CS activity was 58% higher in epididymal fat pads from LCR-EX compared with LCR-SED rats (\( P = 0.08 \)).

**Mitochondrial protein content.** Under equal protein loading conditions (10 \( \mu \)g of total protein), the contents of tricarboxylic acid cycle (CS), oxidative phosphorylation (electron transfer complexes I–V), and mitochondrial biogenesis (PGC-1) proteins were similar between LCR and HCR rats independent of exercise training status (Fig. 2B). UCP1 was not detected in any sample (data not shown).

### Intracellular regulators of lipolysis. **\( \beta \)-AR expression** was increased with exercise training (main effect, \( P = 0.03 \); Fig. 3A), whereas running capacity showed a tendency to increase this parameter (\( P = 0.09 \)). There was a significant interaction between running capacity and exercise training (\( P = 0.006 \)). The expression of the **\( \beta \)-AR** was 17% greater in HCR-SED compared with LCR-SED rats (\( P = 0.006 \)) and 18% greater in LCR-EX compared with LCR-SED rats (\( P = 0.004 \)). The phosphorylation of ATGL at Ser\(^{406} \) and HSL at Ser\(^{660} \) was assessed as surrogate markers of their activity (3, 53). ATGL Ser\(^{406} \) phosphorylation and total ATGL protein content were increased by both running capacity (\( P = 0.02 \) and \( P = 0.003 \), respectively) and exercise training status (\( P = 0.0005 \) and \( P = 0.03 \), respectively; Fig. 3, B and C). Post hoc analyses revealed a 25% increase in ATGL Ser\(^{406} \) phosphorylation in HCR-EX compared with LCR-SED rats (\( P = 0.01 \); Fig. 3B). Total ATGL expression was 17% greater in HCR-EX compared with LCR-EX rats (\( P = 0.04 \); Fig. 3C) and the ratio of ATGL Ser\(^{406} \) to total ATGL was not different. Total HSL protein remained similar for all groups, although there was a tendency for HSL Ser\(^{660} \) phosphorylation to be decreased in both LCR-EX and HCR-EX rats with training (main effect, \( P = 0.09 \)). There was no difference in the ratio of HSL Ser\(^{660} \) phosphorylation to total HSL protein (data not shown). The content of PLIN1 (which controls lipolysis by regulating protein-protein interactions at the surface of lipid droplets, thereby facilitating access of lipases to their substrates) was increased by training (\( P = 0.02 \); Fig. 3D), and an interaction was observed between training and running capacity (\( P = 0.003 \)). Post hoc analyses revealed that PLIN1 expression was 19% greater in LCR-SED than LCR-SED rats (main effect, \( P = 0.02 \); Table 1), but not fasting serum insulin levels. Compared with HCR-SED, LCR-SED rats had 8% higher blood glucose (\( P = 0.0008 \)) and 37% higher NEFA concentrations (\( P = 0.003 \)). Exercise training was a main effect for fasting serum NEFAs (\( P < 0.0001 \)) and insulin concentrations (\( P < 0.0001 \)), whereas there was a tendency for LCR-EX rats to have lower blood glucose concentrations than LCR-SED rats (8%; \( P = 0.11 \)). Exercise training had little effect on blood glucose concentrations in HCR animals. Both

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**Table 1. Blood parameters**

<table>
<thead>
<tr>
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<th>LCR-SED</th>
<th>HCR-SED</th>
<th>LCR-EX</th>
<th>HCR-EX</th>
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<tr>
<td>Glucose, mM*</td>
<td>6.2 ± 0.1a</td>
<td>5.3 ± 0.1b</td>
<td>5.7 ± 0.2</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.5 ± 0.04a, b</td>
<td>0.5 ± 0.02a, b</td>
<td>0.3 ± 0.06a, b</td>
<td>0.3 ± 0.07a, b</td>
</tr>
<tr>
<td>NEFA, mM**</td>
<td>0.38 ± 0.03a, b</td>
<td>0.24 ± 0.02c</td>
<td>0.16 ± 0.03a, b</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 7–10 \) rats/group. LCR, low-capacity running rats; HCR, high-capacity running rats; SED, sedentary; EX, exercise trained; NEFA, nonesterified fatty acids; \( \ast P < 0.05 \), main effect for running capacity; \( \ast P < 0.05 \), main effect for training status; \( \ast P < 0.05 \), different from LCR-SED; \( \ast P < 0.05 \), different from HCR-SED; \( \ast P < 0.05 \), different from LCR-EX; \( \ast P < 0.05 \), different from HCR-EX.
compared with HCR-SED rats ($P = 0.03$) and $25\%$ greater in HCR-EX compared with HCR-SED rats ($P = 0.002$). The protein content of CGI-58 (which binds to and activates ATGL triglyceride lipase activity) was increased by $15\%$ in HCR-SED compared with LCR-SED rats and $15\%$ greater in LCR-EX compared with LCR-SED rats, although neither of these values attained statistical significance ($P = 0.08$ and $P = 0.1$, respectively; Fig. 3E). There were main effects for both running capacity and exercise training for CGI-58 protein content ($P = 0.04$ and $P = 0.03$, respectively; Fig. 3E). No difference in FABP4 expression was observed between groups (data not shown).

**NOR1, NUR77, and GLUT4 expression.** A main effect of exercise training was observed for NOR1 expression ($P < 0.0001$; Fig. 4A). Post hoc analyses revealed that NOR1 expression was increased by $24\%$ in LCR-SED compared with LCR-EX rats ($P = 0.0008$) and $21\%$ in HCR-EX compared with HCR-SED rats ($P = 0.01$). Intrinsic running capacity did not influence the expression pattern of NUR77, whereas the expression of NUR77 was similar in LCR and HCR rats with or without exercise training (results not shown). The expression of GLUT4 was elevated with high intrinsic running capacity ($P > 0.0001$; Fig. 4B) and with exercise training ($P = 0.007$), with post hoc analyses revealing that GLUT4 protein content was increased by $16\%$ in HCR-EX compared with HCR-SED rats ($P = 0.01$). Compared with LCR-EX rats, GLUT4 protein content was $22\%$ greater in HCR-EX rats ($P = 0.0007$).

**Stress kinase activation.** We investigated several stress-activated kinases to determine their involvement in the adaptive response of WAT metabolism to exercise training. Total p38 MAPK expression was reduced with training ($P = 0.03$; Fig. 5A), whereas there was a tendency for phosphorylation of p38 MAPK on the Thr$^{180}$ and Tyr$^{182}$ residues to be reduced by training ($P = 0.07$). There was a main effect of exercise training on the ratio of phospho-p38 MAPK$^{Thr^{180}/Y^{182}}$ to total p38 MAPK ($P = 0.03$). No differences were observed in total JNK1/2 expression; however, training increased phospho-JNK1/2$^{Thr^{183}/Y^{185}}$ ($P = 0.02$) and the ratio of phospho-JNK1/2$^{Thr^{183}/Y^{185}}$ to total JNK1/2 ($P = 0.002$; Fig. 5B). No differences were observed in total ERK1/2 expression (Fig. 5C), although a significant main effect of running capacity was observed for phospho-ERK1/2$^{Thr^{183}/Y^{185}}$ to total ERK1/2 ($P = 0.03$). There was also a tendency for the ratio of phospho-ERK1/2$^{Thr^{183}/Y^{185}}$ to total ERK1/2 to be affected by running capacity (LCR $>$ HCR, $P = 0.06$). No differences were observed in total or phospho-AMPK$^{Thr^{172}}$ expression, or the ratio of phospho-AMPK$^{Thr^{172}}$ to total AMPK expression (data not shown).

**DISCUSSION**

Using a rat model of divergent running ability, we present novel data demonstrating that 1) the mitochondrial protein content of visceral WAT is not related to intrinsic exercise capacity, 2) a short-term (6 wk) program of endurance exercise training does not modulate visceral WAT mitochondrial protein expression (despite training-induced increases in citrate synthase activity), 3) intrinsic running capacity and training status are associated with the differential WAT expression of several key lipolytic proteins, and 4) irrespective of intrinsic running capacity, exercise training induces alterations in the activity and expression of a number of proteins essential to the intracellular regulation of WAT lipid metabolism.

In humans, low aerobic capacity is a strong predictor of early mortality (48, 73). This important clinical association suggests that the capacity for oxygen metabolism is the underlying determinant of the divide between complex disease and health (aerobic hypothesis; see Ref. 31). We used artificial selection for aerobic capacity as an unbiased test of this theory. Along with cardiovascular disease risk (75) and reduced lifespan (33), LCR rats express a number of characteristics common to metabolic disease phenotypes, such as increased body mass and adiposity (49, 61), hyperinsulinemia (49, 61, 75), and impaired glucose tolerance (49, 56, 61). In contrast, HCR rats live 6–8 mo longer (33) and present with superior metabolic health characterized by resistance to weight gain in the face of a high-fat diet (49, 50) and an increased capacity for the uptake and oxidation of glucose (39, 56, 61) and FAs (39, 49, 56, 61).
Fig. 3. Representative immunoblots and relative protein expression of β3-adrenergic receptor (β3-AR; A), phospho-ATGL S406 (B), ATGL (C), perilipin 1 (PLIN1; D), and comparative gene identification-58 (CGI-58; E) in epididymal WAT from LCR (open bars) and HCR rats (filled bars) with or without exercise training. Values are means ± SE; n = 8–10/group. Significance is reported where P < 0.05. *Main effect for running capacity; †main effect for training; ‡interaction between running capacity and training; ^different from LCR-SED; a different from HCR-SED; c different from LCR-EX; d different from HCR-EX. AU, arbitrary units.
Although the differential expression of these key characteristics appears to be linked to the oxidative capacity of the skeletal muscle (61), less is known about the metabolic characteristics of the adipose tissue from these divergent aerobic phenotypes.

We have shown previously that the skeletal muscle of LCR rats contains fewer mitochondria than HCR rats (56, 61). Furthermore, we have also observed impaired \( \beta \)-adrenergic signaling and lipolysis in the muscle of LCR rats (38, 39). Given the divergent metabolic characteristics observed in LCR and HCR rats and the importance of WAT in regulating circulating concentrations of FAs and glucose (1, 27, 76), we sought to determine whether differences similar to those seen

![Fig. 4. Representative immunoblots and relative protein expression of neuron-derived orphan receptor 1 (NOR1; A) and glucose transporter 4 (GLUT4; B) in epididymal WAT from LCR (open bars) and HCR rats (filled bars) with or without exercise training. Values are means ± SE; \( n = 8–10 \) group. Significance is reported where \( P < 0.05 \). *Main effect for running capacity; †main effect for training; a different from LCR-SED; b different from HCR-SED; c different from LCR-EX; d different from HCR-EX.](image)

Although the differential expression of these key characteristics appears to be linked to the oxidative capacity of the skeletal muscle (61), less is known about the metabolic characteristics of the adipose tissue from these divergent aerobic phenotypes.

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![Fig. 5. Representative immunoblots and relative protein expression of phospho-p38 MAPK\(^{T180/Y182}\) and total p38 MAPK (A), phospho-JNK1/2\(^{T183/Y185}\) and total JNK1/2 (B), and phospho-ERK1/2\(^{T202/Y204}\) and total ERK1/2 in epididymal WAT (C) from LCR (open bars) and HCR rats (filled bars) with or without exercise training. Values are means ± SE; \( n = 8–10 \) group. Significance is reported where \( P < 0.05 \). Main effect for training; a different from LCR-SED; c different from LCR-EX; d different from HCR-EX.](image)
in muscle could also be identified in the WAT. Unlike previous investigations that reported reduced expression of a selection of mitochondrial genes and proteins in the visceral WAT of insulin-resistant rodents (58, 63) and humans with type 2 diabetes (70), we did not detect differences in the expression of the key proteins involved in oxidative phosphorylation and the tricarboxylic acid cycle in visceral WAT from LCR and HCR phenotypes (Fig. 2B). Although we cannot rule out the possibility that differences in mitochondrial protein expression were masked due to equal amounts of protein being analyzed in each experiment (particularly since LCR-SED rats have less protein per gram of adipose tissue compared with the other 3 groups; Fig. 1C), the level of expression of all mitochondrial proteins measured was consistent for both sedentary and exercise-trained cohorts, suggesting that the abundance of oxidative enzymes in visceral WAT may not be an important factor for determining running capacity and the associated phenotypes of the LCR-HCR rat model system.

Chronic β-adrenergic stimulation as a result of increased physical activity (9, 62) or pharmacological activation (20) has been shown to enhance the oxidative capacity of WAT by upregulating the expression of genes involved in oxidative phosphorylation and fat oxidation. Although the training program employed in this study was successful in ameliorating many of the metabolic differences observed in the skeletal muscle of LCR compared with HCR rats (39), including a reduction in body mass and adiposity (Fig. 1), it had little effect on oxidative enzyme expression in the WAT of either phenotype, an observation that is consistent with previous findings from our laboratory in human subcutaneous WAT (8). Although mitochondrial protein content was not different, we observed a marked training-induced increase in citrate synthase activity in both LCR and HCR phenotypes (Fig. 2A). Although this might appear to be in contrast to our earlier findings (especially given that citrate synthase protein expression was unchanged), it is important to note that citrate is essential for replenishing the extramitochondrial pool of acetyl-CoA, a substrate essential to de novo lipogenesis (44). Furthermore, lipogenic pathways are energetically costly processes. Therefore, although training may not have altered the amount of mitochondrial protein, changes in cellular energy needs in response to physiological demand are likely to have induced tighter allosteric control and a number of posttranslational changes that increase the maximal capacity of mitochondrial enzymes involved in the different energy-producing pathways (26). A limitation of the current study is the absence of additional measures representing the coordinated activities of β-oxidation, the TCA cycle, and oxidative phosphorylation.

Glucose incorporation into triglycerides (TG) is an essential component of lipid synthesis, and intracellular glucose availability in adipocytes is dependent on plasma membrane glucose transport efficiency [a process that is associated directly with the intracellular pool of GLUT4 (22, 59, 64)]; training-induced increases in WAT GLUT4 expression may be associated with an increased capacity for WAT TG synthesis. Indeed, adipose-specific GLUT4 overexpression induces an increase in the capacity for TG synthesis via both reesterification and de novo lipogenic pathways, leading to an increase in total adipose mass in sedentary animals (59, 67). Although we were unable to measure lipogenic activity in the present study, we demonstrate that both running capacity and training status affect GLUT4 content in visceral WAT (Fig. 4B), with LCR rats having reduced WAT GLUT4 content compared with HCR rats. Whether or not other adipose depots show similar changes remains to be determined. Either way, differences in adipose GLUT4 content in both sedentary and trained animals could have important implications on whole body adiposity in these divergent rat phenotypes (1). It is also worth noting that exercise training has been reported to increase subcutaneous WAT GLUT4 expression in type 2 diabetic humans (25). Whether the same changes are observed in human visceral WAT is unclear, since subcutaneous and visceral adipose depots may have distinct responses to exercise (18). Similarly, it is possible that the different visceral adipose depots may respond disparately to training. Thus, another limitation of the current study is limiting our analyses to the epididymal WAT depot.

TG synthesis is tightly coupled with TG catabolism; therefore, it is not surprising that exercise training induces an increase in the capacity of both basal and catecholamine-stimulated WAT lipolysis (51). This is attributed predominantly to repeated transient elevations in circulating catecholamines acting via β-adrenergic signaling pathways (66). The β3-AR plays an important role in regulating energy balance, particularly in WAT (35). The expression of the β3-AR was reduced in LCR-SED compared with HCR-SED rats (Fig. 3A), which agrees with studies in both obese rodents (12, 45) and humans (30) that report impaired β3-adrenergic signaling as an important factor in obesity development. Indeed, functional β3-adrenergic signaling is essential for obesity resistance (29). In line with this, exercise training “rescued” the reduction in β3-AR in LCR-EX rats (Fig. 3A) while concomitantly reducing fat pad and total body mass (Fig. 1). Conversely, training did not affect β3-AR levels in HCR-EX rats (Fig. 3A), nor did it affect fat pad or total body mass (Fig. 1). These findings are similar to our previous observation of impaired β3-adrenergic signaling in the skeletal muscle of LCR compared with HCR rats (38), an impairment that is also ameliorated with exercise training (39).

β3-AR stimulation by catecholamines increases lipolysis by activating protein kinase A (PKA), which phosphorylates both ATGL (53) and HSL (3) to increase lipase activity. Furthermore, PKA phosphorylation of PLIN1 facilitates the dissociation of CGI-58 from PLIN1, thereby allowing CGI-58 to interact with ATGL to maximally activate lipolysis (72). Given that the primary hypotheses of the current study were mitochondrial focused, we did not measure lipolysis directly. Instead, we determined the phosphorylation state of ATGL and HSL at key activating serine residues as well as the protein abundance of other key lipolytic proteins. ATGL Ser406 phosphorylation and total ATGL content were increased in HCR vs. LCR rats and by exercise training in both groups (Fig. 3B and C). Similarly, CGI-58 was increased in HCR vs. LCR rats and by exercise training in both groups (Fig. 3E). Based on our knowledge of protein function and results from knockout mice studies (24), the changes reported herein would predict increased lipolysis in high-capacity runners and following exercise training. Such a response would match fatty acid availability with the increased fatty acid oxidation capacity/ rates observed in HCR compared with LCR rats (38, 56, 61) and is consistent with an endurance-trained individual’s reliance on FA as an energy source (28).
Previous investigations have reported links between β-adrenergic signaling, the expression of the orphan nuclear receptor NOR1, and whole body lipid and carbohydrate metabolism (54). Although its targets are largely unknown, NOR1 is purported to play an important role in regulating oxidative metabolism and glucose transport in a number of tissues (54). Notably, NOR1 is a cAMP-dependent target of PKA that is upregulated upon HSL and ATGL inhibition (36, 46). We have shown recently that NOR1 expression is reduced in the skeletal muscle of LCR compared with HCR rats and have suggested that this may be linked to the oxidative capacity of the tissue (61). Here, we demonstrate that the difference in NOR1 expression is tissue specific, as LCR-SED and HCR-SED rats displayed a similar abundance of NOR1 in WAT. This is incongruous with human studies that demonstrate that NOR1 is more highly expressed in the WAT of obese compared with healthy humans (69). However, following weight loss the expression of NOR1 in human WAT is “normalized” to levels similar to healthy control subjects (69). Similarly, we have observed a reduction in NOR1 expression of a similar magnitude for both LCR and HCR rats following exercise training (24 and 21% respectively; Fig. 4A). Taken collectively, our observation of reduced NOR1 expression following exercise training in both LCR and HCR rats suggests a principal role for NOR1 in the metabolic regulation of WAT.

Given the role of β-adrenergic signaling in WAT metabolism and our finding that the expression of several proteins downstream of β-adrenergic stimulation is dependent on running capacity and/or training status (Fig. 5), we sought to characterize the phosphorylation of several stress kinases that are purported to interact with β-adrenergic signaling pathways to influence intracellular metabolism (7, 26, 68). To our knowledge, we are the first to report that a short-term exercise training program induces distinct changes in the activity of the stress kinases p38 MAPK and JNK in visceral WAT. Both p38 MAPK and JNK are activated by FAs (11, 21), and both are increased in response to elevated lipolytic activity (47). Importantly, the β-adrenergic activation of p38 MAPK has been shown to occur through cAMP-dependent mechanisms involving PKA (10), and both p38 MAPK and JNK phosphorylation are known to be elevated in visceral WAT during human obesity (5). Here, we have observed a modest reduction in the phosphorylation of p38 MAPK in visceral WAT following exercise training (Fig. 5A), a finding that is consistent with observations in human and rodent skeletal muscle, where training-induced attenuation of resting p38 MAPK activity is associated with the adaptive response to exercise training (41, 42). It is also worth noting that p38 MAPK is a known activator of PGC-1α (and therefore mitochondrial biogenesis) (2, 77), and as such, training-induced reductions in p38 MAPK activity may partially explain why we did not see changes in mitochondrial protein expression in the current study. In contrast to the findings for p38 MAPK, phosphorylation of JNK was elevated as a result of exercise training (Fig. 5B). This is also consistent with findings in skeletal muscle (42) but inconsistent with reports that diet-induced elevations in JNK phosphorylation are attenuated by swim training in obese rats (13, 52). Although the individual roles of JNK and p38 MAPK in adipocyte metabolism are no doubt complex, our findings indicate that in WAT both kinases are exercise responsive (in a manner similar to findings in skeletal muscle), thus implicat-


