The impact of lifestyle factors on whole body lipid handling and skeletal muscle mitochondrial content and function

By

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ABSTRACT

The impact of lifestyle factors on whole body lipid handling and skeletal muscle mitochondrial content and function

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University of Guelph, 2015

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The worldwide incidence of obesity has reached pandemic proportions, and is associated with a high risk of developing type 2 diabetes (T2D). Distinct lifestyle choices including sedentary behavior and surplus caloric intake are recognized contributors to obesity development. Lifestyle interventions that target physical activity and dietary habits, including alterations in macronutrient composition, have been shown to improve health status. Importantly, lifestyle interventions are valuable approaches to study the intricate biochemical changes that occur in diverse metabolic tissues under healthy and pathological conditions. Studies in this thesis utilized exercise and dietary essential fatty acid (EFAs) interventions to advance our understanding of how lifestyle factors improve metabolic disturbances within major insulin responsive tissues (i.e., skeletal muscle, white adipose tissue (WAT), and liver) associated with obesity and T2D risk.

Skeletal muscle is important given its mass and high capacity for insulin-stimulated glucose transport. Within skeletal muscle, whole-body insulin resistance (IR) has been strongly linked to mitochondrial dysfunction (i.e., reductions in content and/or function). This thesis investigated the posttranscriptional events during exercise-induced mitochondrial biogenesis in lean mice. Results demonstrated that increases in mitochondrial protein content following chronic exercise training occurred in a cellular
environment promoting the destabilization of mRNA. These findings provide insight on the regulation of mitochondrial biogenesis in skeletal muscle.

This thesis subsequently explored diets enriched with EFAs (α-linolenic (ALA) and linoleic (LA) acids), which prevented whole-body IR and preserved muscle-specific insulin signaling in obese rats; however, their divergent impacts on mitochondrial bioenergetics suggested the involvement of a different mechanism of action.

Therefore, it was investigated if reactive lipid species (diacylglycerol (DAG) and ceramide) accumulation was reduced in major insulin-responsive tissues. However, results demonstrated unaltered DAG and ceramide levels in skeletal muscle, WAT and the liver. However, ALA and LA distinctly remodeled the FA composition of major lipid fractions (triglycerides, DAGs and phospholipids) within these diverse tissues, which may underline their protective effects. Together, these results extend our understanding of the biological effects of ALA and LA.

Overall, this thesis highlights the therapeutic value of lifestyle factors and their wide-ranging effects in major insulin responsive tissues implicated in the pathogenesis of T2D.
ACKNOWLEDGEMENTS

To my advisors Dr. Graham Holloway and Dr. David Mutch, thank you for your tremendous mentorship throughout the duration of my doctoral studies. I have benefited greatly from your unique perspectives on otherwise similar subject matter. You have both helped advance my laboratory skillset, and challenged me to push the boundaries of my critical thinking, and I am grateful for the opportunity to work under your guidance.

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<td>3'-UTR</td>
<td>3' untranslated region</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-Hydroxyxenonenal</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acyl-CoA synthetase</td>
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<td>ALA</td>
<td>Alpha-linolenic acid</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocase</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>AS160</td>
<td>AKT substrate of 160 kDa</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AUF1</td>
<td>ARE-binding factor 1</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>CaMK</td>
<td>Calcium-calmodulin kinase</td>
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<td>CPT-1</td>
<td>Carnitine palmitoyltransferase 1</td>
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<td>CUG-BP1</td>
<td>CUG-binding protein 1</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<td>DNL</td>
<td>De novo lipogenesis</td>
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<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<td>ETC</td>
<td>Electron transport chain</td>
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<td>EWAT</td>
<td>Epidydmal white adipose tissue</td>
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<td>FA</td>
<td>Fatty acid</td>
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<td>FABPc</td>
<td>Cytosolic fatty acid binding protein</td>
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<td>FABPpm</td>
<td>Plasma membrane associated fatty acid binding protein</td>
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<td>FADH2</td>
<td>Flavin adenine dinucleotide</td>
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<td>FAT/CD36</td>
<td>Fatty acid translocase/cluster of differentiation 36</td>
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<td>FATP</td>
<td>Fatty acid transport protein</td>
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<td>FFA</td>
<td>Free fatty acids</td>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBA$_{1c}$</td>
<td>Glycosylated haemoglobin</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
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<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
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<tr>
<td>HuR</td>
<td>Human antigen R</td>
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<tr>
<td>IKK</td>
<td>I kappa β kinase</td>
</tr>
<tr>
<td>IMF</td>
<td>Intermyofibrillar mitochondria</td>
</tr>
<tr>
<td>IMM/OMM</td>
<td>Inner/outer mitochondrial membrane</td>
</tr>
<tr>
<td>IMTG</td>
<td>Intramuscular triglyceride</td>
</tr>
<tr>
<td>IPGTT/IPITT</td>
<td>Intraperitoneal glucose/insulin tolerance test</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>LA</td>
<td>Linoleic acid</td>
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<td>Abbreviation</td>
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<tr>
<td>LCFA</td>
<td>Long-chain fatty acid</td>
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<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>miRNA</td>
<td>Micro RNA</td>
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<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mtTERF</td>
<td>Mitochondrial transcriptional termination factor</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>n-3/n-6</td>
<td>Omega-3/omega-6</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NDUFB8</td>
<td>Complex 1 subunit NADH dehydrogenase 1 beta subcomplex 8</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>P-CoA</td>
<td>Palmitoyl-CoA</td>
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<td>p38 MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
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<tr>
<td>PDK</td>
<td>Phosphoinositide-dependent protein kinase</td>
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<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma co-activator 1 alpha</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
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<tr>
<td>Polyγ</td>
<td>Mitochondrial RNA polymerase γ</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<tr>
<td>PTPIB</td>
<td>Protein-tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
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<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA binding protein</td>
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<tr>
<td>RCR</td>
<td>Respiratory control ratio</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
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<td>SS</td>
<td>Subsarcolemmal mitochondria</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TAG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TCA</td>
<td>Citric acid cycle</td>
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<tr>
<td>TFAM</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>TIM/TOM</td>
<td>Translocase of the inner/outer mitochondrial membrane</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UCP3</td>
<td>Uncoupling protein 3</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
</tr>
<tr>
<td>ΔG°</td>
<td>Free energy</td>
</tr>
<tr>
<td>ΔΨ</td>
<td>Electric potential</td>
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CHAPTER 1
REVIEW OF THE LITERATURE
1.1 Introduction and overview

The physiology of energy metabolism is well orchestrated, with oxidative phosphorylation (aerobic), glycolysis (anaerobic) and creatine phosphate comprising the three major pathways of simultaneous adenosine triphosphate (ATP) production. Oxidative phosphorylation occurs within mitochondria and relies on the catabolism of lipids and carbohydrates, and produces the majority of ATP at rest and during steady-state exercise. Mitochondria are organelles found in all tissues within the human body, except for red blood cells (430), and consequently contribute to cellular homeostasis and life in general. Importantly, the oxidative capacity of cells is highly responsive to homeostatic perturbations, and various stimuli including exercise, nutrition and diseases (e.g. obesity and insulin resistance (IR)) can influence mitochondrial content in diverse tissues (398). Therefore, understanding the basic mechanisms regulating mitochondrial physiology has broad implications for general health and disease. Two ‘mitochondrial centric’ working models of IR have been proposed: 1) a dysfunction in mitochondrial fatty acid (FA) oxidation stemming from a reduction in intrinsic mitochondrial function and/or content, and 2) increased mitochondrial reactive oxygen species (ROS) production and oxidative stress. Both concepts are premised on intrinsic changes with mitochondria as key events in disease progression, and excess caloric intake in the absence of similar metabolic demand for ATP production as an underpinning concept. Within this framework, the current thesis has focused on the impact of modifying lifestyle factors in the regulation of skeletal muscle mitochondrial content and function, whole-body lipid handling and the implications for obesity and IR. In addition to skeletal muscle, this work
has also explored white adipose tissue (WAT) and the liver, comprising the three major insulin-responsive tissues involved in whole-body lipid metabolism.

1.2 Skeletal muscle lipid metabolism

Globally, over one billion individuals are overweight or obese (Body mass index; BMI ≥ 25.0 kg/m²) according to World Health Organization estimates (224). In Canada, Public Health Ontario reported in 2013 that over 25% of adults are obese (BMI ≥ 30.0 kg/m²), and at high risk of developing IR and/or Type 2 diabetes (T2D) (289). Considerable research has focused on identifying metabolic disturbances in obesity, and how they might link with the development of IR. In this regard, normal lipid metabolism (outlined in Figure 1.1) appears to be affected by chronic nutrient overload in the absence of an elevated demand.
Figure 1.1 – Lipid metabolism and delivery to skeletal muscle. The delivery of free fatty acids (FFA) to working skeletal muscle to generate adenosine triphosphate (ATP) involves a cooperative arrangement between multiple tissues. The major steps and regulatory points may include, but are not limited to; 1) Transport of dietary lipids absorbed in the gastrointestinal tract as part of chylomicrons, 2) FFA liberation from adipose tissue lipolysis, 3) Transport of lipids from the liver via lipoproteins (i.e. LDL), 4) Lipoprotein and FFA (with albumin) entry into blood for delivery to muscle, 5) Liberation of FFA from lipoproteins via lipoprotein lipase (LPL) and transport of FFA from capillary endothelium into the interstitium, 6) Transport across the sarcolemmal membrane into muscle, 7) Local supply of FFA from intramuscular triglyceride (IMTG) droplets, 8) Activation of FFA (with FABPc), to FFA-COA, 9) Transport of FFA-COA across mitochondrial membrane, 10) Beta-oxidation of FFA-COA, 11) Electron transport chain (ETC) regulation, and 12) ATP synthesis. LDL; low-density lipoprotein. FABPc, cytosolic fatty acid binding protein. TAG; triglyceride. (363)

1.2.1 Overview of insulin signaling and lipid metabolism

Lipids play an essential role in whole-body energy metabolism by regulating cell membrane composition, transducing cell signals, controlling gene transcription, and serving as substrates for mitochondrial oxidation in major insulin-responsive tissues (397). Skeletal muscle is a primary site of FA utilization, where dietary lipids can be oxidized for ATP production, or stored in intramuscular triacylglycerol (IMTG) droplets (176).
A number of sources contribute to the pool of lipids in circulation that can be taken up into skeletal muscle (outlined in Figure 1.1). Dietary lipids, typically in the form of TAGs, can enter the blood stream as part of chylomicrons, while free FAs (FFAs) liberated from WAT enter the blood stream as part of a complex with the binding protein albumin (393). Also, lipids endogenously synthesized within the liver can also enter circulation, exported through the formation of lipoprotein complexes (i.e. very low density lipoproteins; VLDL). FFAs from LDL complexes can be liberated by interaction with lipase enzymes such as lipoprotein lipase (LPL). The protein-mediated transport of FFAs into skeletal muscle represents a key regulatory point in lipid metabolism (32), though IMTGs also represent an important source of endogenous FFAs within skeletal muscle. Both exogenous and endogenous FFAs are bound by cytosolic fatty acid binding proteins (FABPc), and are then accepted by FA translocase (FAT/CD36) at the outer mitochondrial membrane. This leads to the activation of FFAs to FFA-CoA by the enzyme acyl-CoA synthetase (ACS). Entry of FFA-CoA into the mitochondrial matrix is enabled by CPT-I, an important regulatory point in mitochondrial FA oxidation. Within the mitochondrial matrix, FAs are catabolized through β-oxidation to generate acetyl-CoA used in oxidative phosphorylation. This includes the citric acid cycle (TCA) and electron transport chain (ETC), where acetyl-CoA is utilized to produce reducing equivalents for ATP synthesis. Although several points regulating muscle FA utilization/storage involve mitochondria, WAT and the liver influence the systemic supply of lipids delivered to muscle. Therefore, including WAT and the liver in studies examining whole-body lipid metabolism may provide a more complete picture compared to skeletal muscle alone.
Endocrine signals (i.e. insulin) and energetic demands during exercise (contraction) appear to independently stimulate muscle FA and glucose uptake, and thus can additively combine to increase transport (171). Skeletal muscle is responsible for approximately 70-80% of insulin-stimulated glucose clearance postprandially (91), and is therefore the major peripheral target of insulin action. An elevation in blood glucose stimulates insulin secretion by pancreatic β-cells (332) and triggers a complex intracellular signaling cascade in skeletal muscle, outlined in Figure 1.2.

Insulin signaling begins with the interaction between insulin and the insulin receptor, a cell surface receptor comprised of two α and two β subunits (339). Although insulin receptors are expressed in numerous tissues, they are most abundant in the major insulin responsive tissues (i.e., skeletal muscle, WAT and liver) (379). The binding of insulin to its receptor triggers a series of transphosphorylation events that leads to the recruitment of adaptor proteins including the insulin receptor substrate (IRS) family (379). IRS proteins act as multi-site docking proteins, and upon phosphorylation at specific tyrosine residues, promote the binding and activation of phosphatidylinositol 3-kinase (PI3K) (339). Next, phosphatidylinositol-4,5-bisphosphate, a major substrate of PI3K is phosphorylated to produce phosphatidylinositol-3,4,5-triphosphate, which subsequently attracts phosphoinositide-dependent protein kinase (PDK) and leads to the phosphorylation of protein kinase B (PKB; also called Akt) (339). Finally, Akt can phosphorylate the Akt substrate of 160 kDa (AS160), thereby enabling GLUT4 translocation towards the plasma membrane. Insulin also promotes lipid uptake into muscle by stimulating the translocation of FA transporters from their intracellular pools to the plasma membrane (171). These FA transporters include FAT/CD36, FA transport
proteins (FATPs), and plasma membrane associated FABP (FABPpm) (see reviews by Thong et al. (380), Boucher et al. (37), and Bonen et al. (32) for further details).

**Figure 1.2 – Skeletal muscle insulin signaling.**
The binding of insulin to its receptor leads to tyrosine phosphorylation activity that results in phosphorylation of the insulin receptor substrate (IRS). This is followed by recruitment and activation of phosphoinositide 3-kinase (PI3K). PI3K activation leads to phosphoinositide-dependent protein kinase-1 (PDK1) attraction and subsequent phosphorylation of AKT/PKB. Next, AKT/PKB phosphorylates the AKT substrate of 160 kDa (AS160), promoting GLUT4 translocation to the plasma membrane for glucose uptake. Insulin also signals the translocation of fatty acid transporters (FAT/CD36, FABPpm and FATP) from the intracellular pool to the plasma membrane for LCFA uptake. GLUT, glucose transporter; FAT/CD36, fatty acid translocase/cluster of differentiation 36; FABPpm, plasma membrane fatty acid binding protein; FATP, fatty acid transport protein; LCFA, long-chain fatty acid;

Whole-body IR in obesity can be traced to disturbances in normal insulin signaling within major insulin responsive tissues. Although the molecular details have not been fully elucidated, reductions in mitochondrial function and/or content are thought to contribute by reducing the capacity for FA utilization. This is problematic given that obesity is generally associated with a surplus of lipid availability, and therefore, mitochondrial dysfunction may exacerbate lipid accumulation within skeletal muscle. Initial evidence suggested that elevated IMTG content correlated with IR in obesity (294, 307). However, more recent work has extended this concept to implicate specific lipid mediators of IR, namely, diacylglycerols (DAGs) (169) and ceramides (2). In addition,
mitochondrial ROS production is linked with stress-response mechanisms that interfere with insulin signaling. In obesity, this results from increased flux through the mitochondrial electron transport chain (ETC) that is traced to nutritional overload and the provision of excess reducing equivalents (381). Further work is required to delineate the mechanism(s) of obesity-related IR, and our current understanding of these concepts will be discussed below.

### 1.2.2 Dysfunctional lipid metabolism: Obesity and skeletal muscle IR

Excess energy intake in obesity increases systemic FFA availability and uptake into muscle, and favors partitioning toward storage in lipid droplets versus oxidation given the absence of increased metabolic demand. Muscle samples from obese Zucker rats (243) and obese (4, 33) and non-obese T2D individuals (4) revealed that a permanent relocation of FAT/CD36 to the plasma membrane underlies lipid accumulation in muscle.

Several lines of evidence led to the classical view that skeletal muscle lipid accumulation, and therefore elevated IMTG content, caused muscle IR. Electron microscopy demonstrated that both the size and number of IMTG droplets was increased in IR obese rats (210). Furthermore, a high caloric intake in humans was linked with increased IMTG content and impaired whole-body glucose tolerance (340). Conversely, caloric restriction reduced IMTGs and improved insulin sensitivity in rats (352), and a low-calorie diet showed similar benefits in T2D subjects (174). Despite these correlations, other studies have divorced the link between IMTG and skeletal muscle IR. Increased IMTG content in skeletal muscle from DGAT1 overexpressing mice protected against HFD-induced IR (239). Also, elevated IMTG content in exercise trained
individuals was associated with greater insulin sensitivity (129). In addition to total IMTG content, Nielsen et al. suggested that the subcellular redistribution of IMTG to the subsarcolemmal (SS) region contributed to IR in obese individuals (282). In support, obese T2D subjects had a 3-fold higher SS lipid content compared to non-diabetic obese and endurance-trained subjects. Following exercise training, SS lipid content was reduced by ~50% in obese T2D subjects, resembling non-diabetic obese, and overall, a strong inverse association between SS lipid content and insulin sensitivity was observed (282). However, subsequent work showed that increased SS lipid content is not required for IR in obese Zucker rats, as SS regions in glycolytic muscle were devoid of IMTG droplets despite being IR (210). Furthermore, SS lipid content actually increased in muscle of obese Zucker rats following chronic muscle stimulation, in parallel with increased insulin-stimulated glucose transport (151). Altogether, total and SS-localized IMTG may simply serve as markers of altered lipid metabolism in obesity. Instead, increased IMTG content is typically accompanied by the accumulation of more biologically active and harmful reactive lipids intermediates (e.g. DAGs and ceramides) that can interfere with skeletal muscle insulin signaling, as illustrated in Figure 1.3
Excess lipid uptake can increase mitochondrial reactive oxygen species (ROS) production, which activates stress kinases such as nuclear factor kappa B (NfκB) that serine phosphorylate and inhibit the insulin receptor substrate (IRS). Increased diacylglycerol (DAG) can activate PKC isoforms that also inhibit IRS activity. Ceramides can activate protein phosphatase 2A (PP2A) to dephosphorylate AKT. Saturated LCFA and/or tumor necrosis factor-alpha (TNF-α) levels can inhibit IRS activity through JNK activation. These events can promote an insulin resistance by interfering with GLUT4 translocation to the plasma membrane. Long-chain fatty acids, LCFA; Phosphoinositide 3-kinase, PI3K; phosphoinositide-dependent protein kinase-1, PDK; nuclear factor kappa B, NfκB; protein kinase C, PKC; c-Jun N-terminal kinases, JNK; Akt substrate of 160 kDa, AS160.Figure adapted from (362).

1.2.3 Reactive lipid species and skeletal muscle insulin resistance

DAG can accumulate within skeletal muscle (151, 388, 389) during TAG synthesis, phospholipid breakdown (353), de novo synthesis (382), or when IMTG storage is impaired (106). DAG accumulation can prevent GLUT4 translocation and glucose uptake, by interfering with the insulin-signaling cascade through phosphorylation of serine residues in IRS proteins (267). Proteomic work has identified nearly 70 serine residue consensus sites, of which 20+ are phosphorylated by insulin (422). More than 16 different kinases reversibly phosphorylate these serine residues, including the PKC-θ and -δ isoforms which are known to be activated by DAGs (39). In rodents, a 60% reduction in skeletal muscle DAG-kinase activity was associated with DAG accumulation and glucose intolerance (71). By contrast, exercise training in humans, and chronic muscle
stimulation in rats improved muscle DAG content (47, 151). In some instances, however, total muscle DAG content has not correlated with IR. Examples include increased muscle DAG content in lean insulin sensitive, trained individuals (8), and no change in muscle DAG content in obese IR individuals (15, 79, 265, 348). Instead, more recent studies indicate that only membrane-localized DAG and specific DAG species (i.e. 16:0 and 18:1) may be associated with IR (26, 388). However, in obese Zucker rats, chronic muscle stimulation increased insulin-stimulated glucose uptake but did not change 16:0 DAG, while 18:1 DAG increased and elevated the DAG desaturation index (18:1/18:0) (151). These results suggest that the relationship between IR and total DAG content may be more complicated and requires a thorough breakdown of DAG species in future work. However, the fact that DAG content is elevated in trained athletes (8) and remains unchanged in lean healthy rats despite increased insulin-stimulated glucose uptake (151) suggests that DAG does not always cause IR.

Ceramides are another major reactive lipid species linked to skeletal muscle IR (267). Ceramides are an important subclass of sphingolipid derivatives that arise from several pathways including de novo synthesis using SFA lipid precursors (i.e. palmitic acid, 16:0), and the hydrolysis of sphingomyelin (28). Ceramide formation increases in response to stress signals including oxidative stress and inflammation, which are known features of the obese phenotype (28). Indeed, ceramide content was also elevated in muscle from obese Zucker rats (151, 386). Ceramides can activate protein phosphatases (i.e. PP2A) that interfere with insulin signal transduction (28, 267) through inhibition of Akt activation (see Figure 1.3) (347). Also, ceramides can strongly activate JNK and IKK (403, 405), and further supports a link between obesity and IR (169). Adams et al.
demonstrated that in obese IR humans, reduced insulin-stimulated Akt phosphorylation was attributed to a two-fold higher muscle ceramide content (2). Lipid infusion in healthy, insulin-sensitive subjects also increased muscle ceramide content and reduced insulin sensitivity (373). Furthermore, lean offspring of T2D subjects exhibit higher muscle ceramide content and lower insulin sensitivity (372). Conversely, preventing ceramide accumulation in mice overexpressing sphingosine kinase 1 protected against HFD-induced JNK activation and IR (46). Also, pharmacologically preventing ceramide synthesis protected against obesity-related IR in mice (143). Interestingly, this study concluded that ceramide is a key intermediate in only saturated FA (SFA)-induced IR, whereas, polyunsaturated FAs are not substrates for ceramide synthesis. Mimicking exercise through chronic muscle stimulation in rats improved muscle ceramide content (151). Therefore, targeting dietary lipid profiles and physical activity may represent therapeutic strategies protecting against ceramide-induced IR. However, ceramide content does not always associate with IR. For instance, ceramide content was elevated in insulin sensitive mice overexpressing perilipin 2 (36). Alternatively, specific ceramide species are linked with IR, such as 18:0 ceramides (388). Yet, 18:0 ceramide was similar between trained athletes and IR subjects (360), and reducing 18:0 ceramide in obese Zucker rats through chronic muscle stimulation did not improve insulin-stimulated glucose uptake (151). Clearly more precise molecular approaches are required to understand the exact role of individual ceramide species. Obese individuals often exhibit insulin signaling abnormalities unrelated to ceramide action. This includes defects in IRS-1 tyrosine phosphorylation, and reduced IRS-1-associated PI3K activity (85), indicating that ceramides are not solely responsible for lipid-induced IR. Nevertheless,
reducing ceramide accumulation by altering dietary lipid consumption (i.e. increased PUFAs), or through exercise may offer protection against the ceramide-specific inhibition of muscle insulin signaling. Importantly, ceramides form barrel-like channels within the outer mitochondrial membrane (OMM) that disrupt the regulation of membrane transport and ATP synthesis, and can induce apoptosis by releasing proteins from the intermembrane space (82). Therefore, ceramide accumulation may contribute to mitochondrial dysfunction linked with obesity and IR.

1.3 Skeletal muscle mitochondrial bioenergetics

1.3.1 Overview of mitochondrial structure and function

Mitochondria are commonly considered the “powerhouse” of cells, a reference attributed to both their high ATP regenerative capacity and the key pathways of lipid metabolism that occur within mitochondria. A number of unique structural features are important to the bioenergetics functions of this organelle. Mitochondria contain an outer and inner (IMM) membrane. The OMM is equally composed of protein and lipids, and is permeable to most molecules up to 5 kDa in size (19). The IMM is composed of nearly 80% protein, housing the ETC complexes I-IV, ATP synthase, and has a folded shape to form cristae that maximize surface area. The IMM is generally impermeable to most ions and molecules unless specific transporters exist. Between the OMM and IMM is the intermembrane space, where important bioenergetics proteins like mitochondrial creatine kinase and cytochrome c are located. The highly regulated reticular structure of mitochondria permits elongation and shortening, through fusion and fission processes, and is important during mitochondrial biogenesis. Skeletal muscle mitochondria exist in
two spatially distinct populations. Subsarcolemmal (SS) mitochondria are found near the sarcolemmal membrane to provide ATP for membrane-associated processes (i.e. ion transport) and account for ~20% of skeletal muscle mitochondria (222). Intermyofibrillar (IMF) mitochondria comprise the remaining ~80% and are embedded deep within muscle fibers, providing ATP for muscle contraction (91). SS and IMF mitochondria are morphologically distinct, and respond distinctly to changes in diet composition (69, 234), and perturbations associated with IR and obesity (74, 150). Therefore, it is important to separate SS and IMF mitochondria for analysis. Despite the unique properties of SS and IMF mitochondria, both subpopulations contain enzymes involved in the TCA cycle and β-oxidation, as well as mitochondrial DNA within their matrices (see Figure 1.4).

**Figure 1.4 - Overview of mitochondrial energy metabolism.**
Reducing equivalents (NADH, FADH$_2$; boxes with dashed lines) generated from the catabolism of carbohydrates and lipids contain electrons for the electron transport chain (ETC). The reducing equivalents are reduced and the electrons transfer (dashed line) through protein complexes (I-IV) embedded in the inner mitochondrial membrane (IMM). Water (H$_2$O) is formed when molecular oxygen (O$_2$), the final electron acceptor, is reduced at complex IV. In complex I, III and IV, changes in free energy from electron transfer drives proton (H$^+$) translocation from the matrix to the intermembrane space. The resultant proton gradient across the IMM can be utilized for ATP synthesis at complex V. NADH; nicotinamide adenine dinucleotide, FADH$_2$; flavin adenine dinucleotide, TCA; tricarboxylic acid cycle. PDH; pyruvate dehydrogenase. Cyto c; cytochrome c.
The TCA cycle is a point of convergence for substrates undergoing oxidative metabolism. This eight-step cycle oxidizes acetyl-CoA, derived from pyruvate produced via glycolysis in the cytosol, or FA β-oxidation, to yield three NADH and one FADH$_2$ reducing equivalent molecules (397). NADH and FADH$_2$ contain electrons that undergo a series of transfers thorough the ETC to ultimately produce ATP. Electrons from NADH enter the ETC at complex I, while FADH$_2$ feeds electrons into complex II. Ubiquinone (also called coenzyme Q) is a hydrophobic molecule that accepts two electrons and facilitates electron transfer to complex III. Thereafter, complex III catalyzes the transfer of electrons from ubiquinol to cytochrome c, a small heme-containing protein located on the outer leaflet of the IMM. At complex IV, oxygen accepts electrons from reduced cytochrome c and is reduced to water, serving as the final electron. The transfer of electrons through complexes I, III and IV, releases enough free energy (ΔG$^\circ$) to drive proton movement from the mitochondrial matrix to the intermembrane space. This generates a “proton motive force” across the IMM resulting from differences in pH (ΔpH) and electric potential (ΔΨ). When protons move back into the mitochondrial matrix through the ATP synthase complex, the energy released drives the phosphorylation of ADP to produce ATP. This “chemiosmotic hypothesis” was originally published by Dr. Peter Mitchell in 1961, and is the accepted model for oxidative phosphorylation (262).

The TCA cycle and ETC are tightly linked together and the flux of metabolic substrates is influenced by energy demand. When ATP demand is low, reducing equivalents can continue to increase mitochondrial membrane potential, which gradually builds a “back pressure” to slow electron movement through the ETC (116). Since
electrons are often transferred individually, the risk of electron slippage onto oxygen to form superoxide anions is dramatically elevated. Uncoupling proteins embedded in the IMM can dissipate membrane potential as a defense mechanism, permitting proton movement into the matrix independent of ATP synthesis (96). ROS can activate uncoupling proteins through de-glutathiolation (247), and lower complex I activity to reduce electron flux into the ETC (164). Furthermore, magnesium superoxide dismutase (MnSOD) is a highly active antioxidant enzyme that quenches superoxide, producing hydrogen peroxide (H$_2$O$_2$). Other antioxidant enzymes such as glutathione and catalase can reduce H$_2$O$_2$ to H$_2$O and O$_2$ to protect against oxidative damage (142). Since H$_2$O$_2$ permeates the mitochondrial membrane, it is measured as a marker of the balance between energy supply and demand (303). Therefore, ROS can exert harmful effects when not tightly regulated, a situation observed with chronic nutritional overload in obesity (161). Otherwise, ROS can support insulin action by inhibiting the protein-tyrosine phosphatase 1B (PTP1B), which dephosphorylates tyrosine residues in IRS1 to inhibit insulin signaling (102). ROS also act as signaling molecules to mediate the redox regulation of cellular events including the biogenesis of mitochondria (423).

1.3.2 Overview of skeletal muscle mitochondrial biogenesis

Mitochondrial content fluctuates in response to the energetic state of the cell. Exercise training increases mitochondrial content in skeletal muscle, while sedentary behavior and obesity are associated with reduced mitochondrial content (257, 329). Therefore, it is important to understand the physiological cues that regulate mitochondrial content under normal and pathological contexts. Mitochondrial biogenesis is the
processes by which pre-existing mitochondria undergo growth and division to increase cellular content (122, 395). Mitochondria possess their own genome, a small double-stranded circular DNA consisting of ~16.5 kilobases (kb). Mitochondrial DNA (mtDNA) contains 37 genes coding for 13 ETC complex subunits (complexes I, III, IV and ATP synthase), 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (395). Comparatively, the nuclear genome encodes the majority of the ~1600 mitochondrial proteins. Therefore, mitochondrial biogenesis is a complex that process requires an intricate and tightly regulated crosstalk between these two genomes (122). The major events in mitochondrial biogenesis are illustrated in Figure 1.5.

**Figure 1.5 - Schematic representation of mitochondrial biogenesis.**

Peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) activates various nuclear transcription factors (NTFs) to stimulate transcription of nuclear-encoded mitochondrial proteins in addition to mitochondrial transcription factor (TFAM). TFAM is required for initiation of mitochondrial DNA (mtDNA) transcription and replication. Nuclear-encoded mitochondrial precursor proteins are imported into mitochondria through transport machinery for the outer- (TOM) and inner- (TIM) mitochondrial membranes. Both mitochondrial proteins encoded by nuclear and mitochondrial genomes are then assembled, including subunits of the electron transport chain (ETC) complexes. Mitochondrial fission of the outer- and inner- mitochondrial membranes is facilitated by dynamin-related protein 1 (DRP1) and optical atrophy 1 (OPA1) respectively. Meanwhile, mitofusins (MFNs) control mitochondrial fusion. Both mitochondrial fusion and fission are required for proper organization of the mitochondrial network. Adapted from (395)
The transcription of nuclear-encoded mitochondrial genes is initiated by transcription factors binding to the promoter region. The nuclear respiratory factors 1 and 2 (NRF-1 and -2) were among the first transcription factors characterized. NRFs act on genes encoding cytochrome c, ETC subunits (189), and the mitochondrial transcription factors -A (TFAM), -B1 (TFB1M), and -B2 (TFB2M) (127). Moreover, the estrogen-related receptors (ERRs) α, β and γ can drive virtually all aspects of mitochondrial biogenesis, regulating genes involved in FAO, TCA cycle, ETC complexes and oxidative phosphorylation (101). Finally, the peroxisome proliferator-activated receptor (PPAR) alpha (PPARα) was shown to co-ordinately regulate nuclear genes encoding mitochondrial FAO enzymes (245, 344). PPARs can be activated by lipid-based ligands (i.e. dietary FAs) and form heterodimers with retinoid X receptors that bind to DNA response elements. Mechanistically, this links lipid metabolism and mitochondrial gene expression, highlighting the influence of dietary FAs in mitochondrial biogenesis (344).

Broader coactivator proteins have also emerged in the literature, and regulate mitochondrial biogenesis through the simultaneous induction of multiple nuclear transcription factors. The discovery of PPARγ-coactivator-1α (PGC-1α) and its related isoform PGC-1β was considered a major advancement for our understanding of mitochondrial biogenesis (419). PGC-1α and β share extensive sequence homology, particularly in the N-terminal activation domain, central regulatory domain and C-terminal RNA binding domain (233). Accordingly, both isoforms exhibit considerable functional overlap within skeletal muscle (427). The PGC-1 proteins are master regulators of mitochondrial biogenesis, stimulating gene targets of the TCA cycle, electron transport, oxidative phosphorylation and mitochondrial membrane organization
Once phosphorylated, PGC-1α translocates into the nucleus to co-activate and induce the expression of nuclear-encoded mitochondrial gene targets (394). Among these gene targets is TFAM, which initially suggested an indirect role for PGC-1α in promoting nuclear-mitochondrial genomic crosstalk during biogenesis. However, PGC-1α also translocates to the mitochondrial matrix to stimulate mtDNA transcription, in an AMPK-dependent manner (364) by forming a complex with TFAM at the regulatory D-loop region (11, 337). Collectively, these findings led to the view that PGC-1α was required for mitochondrial biogenesis. For instance, stimulating PGC-1α expression in rat skeletal muscle (25) and cultured cardiac myocytes (230) showed that PGC-1α coactivates nuclear targets NRF-1/-2, PPARs and ERRs (115). Conversely, PGC-1α knockdown in mouse skeletal muscle reduced mitochondrial gene expression (12), mitochondrial enzyme content and activity (3). However, PGC-1α is not required for mitochondrial biogenesis. In whole-body PGC-1α-knockout mice, five weeks of exercise training still increased cytochrome c and complex IV subunit 1 protein content in muscle (231). In addition, running endurance was similar between muscle-specific PGC-1α-knockout and wild-type mice, which exhibited comparable mitochondrial density, expression of oxidative phosphorylation gene targets, and ETC complex activity after 2 weeks of wheel running (334). Zechner et al. generated a muscle-specific PGC-1α/β double knockout mouse model to test the overall importance of PGC-1 proteins in mitochondrial metabolism (427). Compared to single PGC-1α or β knockouts, these mice exhibited substantially lower running capacity, a 2-3-fold reduction in mitochondrial gene expression, and pronounced defects in mitochondrial structure and respiratory function (427). Taken together, at least one functional PGC-1 gene is required to maintain
mitochondrial structure and function in skeletal muscle. However, several PGC-1α splice variants have been identified in skeletal muscle (318), and appear to be uniquely induced through different promoters on the PGC-1α gene. This undoubtedly adds an additional layer of complexity in understanding the exact role of PGC-1α in mitochondrial biogenesis. Nevertheless, PGC-1 proteins are among the most versatile transcriptional coactivators directly implicated in the nuclear and mitochondrial genomic crosstalk during mitochondrial biogenesis.

Post-transcriptional mechanisms also influence mitochondrial protein synthesis during biogenesis by regulating mRNA transcript localization, interaction with micro-RNAs (miRNAs), and mRNA stability (22). RNA binding proteins (RBPs) control the steady-state concentration of mitochondrial mRNA species and therefore the synthesis of their corresponding proteins. This is achieved by altering rates of mRNA synthesis and decay, and represents a post-transcriptional mechanism regulating mitochondrial biogenesis. Approximately 8-10% of human mRNA transcripts contain AU-rich elements (AREs) within the 3’ untranslated region (3’-UTR). This region is characterized by the presence of one or more AUUUA pentamers (22). Two RBPs that interact with AREs to regulate mRNA stability are the stabilizing human antigen R (HuR) and destabilizing ARE-binding factor 1 (AUF1) proteins. Additionally, CUG-binding protein 1 (CUG-BP1) promotes mRNA destabilization by binding to conserved GU-rich elements (GREs) present in the 3’-UTR of some mRNA transcripts (396). RBPs regulate mRNA interaction with miRNAs; small non-coding RNAs that promote mRNA degradation. By tethering various protein factors to the 3’-UTR, miRNAs also inhibit protein synthesis to silence gene expression (162). HuR, AUF1 and CUG-BP1 can act independently or
compete for RNA binding domains to influence mRNA stability, and ultimately mitochondrial protein synthesis during biogenesis (156).

Successful completion of mitochondrial biogenesis requires the targeting and transport of nuclear-encoded mitochondrial proteins to various mitochondrial compartments. This permits assembly with mtDNA-encoded proteins, and is crucial for organizing the mitochondrial network during biogenesis (395). Molecular chaperones escort proteins to mitochondria, and must be unfolded for import into mitochondria. The protein import machinery is comprised of translocase complexes of the OMM (TOM) and IMM (TIM). TOM imports proteins through a general import pore roughly 400 kDa in size (156). The TIM machinery aids in targeting proteins to the intermembrane space, IMM, or mitochondrial matrix (156). Importantly, factors required to initiate mtDNA transcription and replication are among the precursor proteins targeted to the mitochondrial matrix.

Transcription and replication of mtDNA is initiated entirely by nuclear-encoded mitochondrial transcription factors such as TFAM, TFB1M and TFB2M. TFAM is required to initiate mtDNA transcription, and stimulates assembly of the transcription initiation complex composed of the mitochondrial RNA polymerase γ (Polγ) (111) and a transcriptional termination factor (mtTERF) to conclude transcription (112). PGC-1α was also shown to directly interact with TFAM at the D-loop region to promote mtDNA transcription (10). The replication of mtDNA involves a displacement loop that arises from the asynchronous and bidirectional transcription of the light and heavy mtDNA strands originating in the D-loop region (111). This enhances mtDNA copy number and is required for mitochondrial fission to increase the number of individual mitochondria.
Fission and fusion processes are important during mitochondrial biogenesis, enabling mitochondria to divide and remain structurally organized (395). Dynamin-related proteins (DRPs) like DRP1 and optic atrophy 1 (OPA1) are GTPases that regulate fission events and IMM remodeling (17), while mitofusins (MFNs) regulate fusion events. DRPs (123), OPA1 (66), and MFNs (288) are important for successful biogenesis and mitochondrial dynamics, but are not essential. For example, in mice, only the loss of both mfn1 and mfn2 gene expression resulted in severe mitochondrial structural and functional defects, loss of mtDNA stability, a 50-70% reduction in body weight, and death between 6-8 weeks of age (67). However, retaining one functional mitofusin gene maintained a relatively normal phenotype. Together, these results show that mitochondrial dynamics is required to prevent organelle dysfunction, but does not rely solely upon a single protein target. Obesity is linked with an imbalance in mitochondrial dynamics, favoring greater mitochondrial fission and an IR phenotype. Mitochondria were smaller and fragmented in C2C12 cells treated with the SFA palmitate, and in HF-fed IR ob/ob mice. Greater DRP1 expression, impaired mitochondrial function, and reduced insulin-stimulated glucose uptake was also observed (177). Conversely, obese and T2D humans had lower MFN2 protein content in muscle biopsies, which increased following bariatric surgery that ameliorated IR (433). Overall, an imbalance in mitochondrial dynamics in obesity can interfere with normal mitochondrial content and function, and may link mitochondrial dysfunction with the development of IR.
1.3.3 Skeletal muscle mitochondrial dysfunction in obesity and IR

The link between mitochondrial dysfunction and IR was first observed in skeletal muscle during the late 1990s, where obese and IR individuals were shown to have reduced mitochondrial oxidative capacity (185, 187). Although this deficiency appeared to uniquely affect SS mitochondria (329), reduced IMF content has also been reported in IR humans (74). Electron microscopy analysis showed smaller mitochondrial size in muscle from obese individuals (184), while mtDNA content was also reduced in a separate investigation (329). Gene microarray studies demonstrated a coordinated downregulation of oxidative phosphorylation and mitochondrial biogenesis gene targets in humans with T2D and non-diabetics with a family history of T2D (269, 298). Proteomic analysis of skeletal muscle revealed reductions in several mitochondrial proteins in obese and T2D individuals (165). Further evidence has continued to accumulate in recent years through the use of cell culture (139) and rodent models (138, 362) that support a link between mitochondrial dysfunction and IR.

Despite mounting evidence, this topic has been heavily debated in the literature as a result of contradicting evidence. Multiple studies show that an IR-inducing HFD can actually elevate muscle mitochondrial oxidative capacity in rodents (134, 368). Paradoxically, mice overexpressing muscle-specific PGC-1α became IR after HF feeding, despite a 2.4-fold increase in mitochondrial density, and 60% increase in ATP synthesis capacity (72). Instead, the inhibition of insulin signaling was attributed to DAG-induced PKCθ activation, which led to serine phosphorylation of IRS-1 (72). Moreover, muscle from IR obese humans and rodents can have normal mitochondrial content (117, 362), and respiratory function (40, 154, 229, 362). These findings clearly show that
mitochondrial dysfunction is not always linked with IR. However, increasing mitochondrial content can be protective; obese Zucker rats were protected against diet-induced IR following Pgc-1α transfection in muscle (24).

An important consideration is that while subjects with T2D exhibit a 30% reduction in muscle mitochondrial content (269), the capacity for substrate oxidation can still increase ~40-fold during vigorous exercise (216). Thus, the remaining mitochondria are functionally normal (as shown in (40, 154)) and substrate oxidation should not be limited by 30% lower mitochondrial content under resting conditions. The conditions under which mitochondrial function is assessed may contribute to the discrepant findings in the literature. Mitochondrial function is traditionally evaluated as a maximal capacity measurement, utilizing saturating ADP concentrations that promote a maximal rate of respiration (40, 263). However, under most physiological conditions, ATP turnover and ADP availability limit respiration (409), and therefore, may not reflect in vivo conditions. As such, using submaximal ADP concentrations may better represent physiological conditions. Indeed, Smith et al. showed that submaximal ADP-stimulated respiration was reduced in obese Zucker rats despite having a similar mitochondrial content as lean controls (362). In this study, resveratrol supplementation increased submaximal ADP respiration and increased ANT2 content, an important mitochondrial ADP transport protein (362). The important methodological considerations in this study permitted the assessment of more subtle changes in mitochondrial function that are physiologically relevant to obesity and IR. Subsequent work has shown that dietary n-3 PUFA (e.g., EPA and DHA) supplementation can increase mitochondrial ADP sensitivity (lower Km value) in healthy subjects, in the absence of changes in mitochondrial content and ANT1/2
content (137). This suggests that ADP sensitivity may be altered through post-translational modifications of ANT proteins. Importantly, the binding of ADP to the ATP synthase complex reduces membrane potential, suggesting that improving ADP sensitivity, and thus ADP availability, can lower the propensity of mitochondrial ROS emission, which has been causatively linked with IR and T2D (9, 161, 229).

The link between mitochondrial ROS and T2D has been known since the 1930s. The pharmacological agent dinitrophenol (DNP) was shown to have anti-obesity effects by increasing energy expenditure through mitochondrial uncoupling (376). Mitochondrial uncoupling provides a defense mechanism to prevent excess mitochondrial ROS emission. Chronic elevations in mitochondrial ROS emission (i.e. H$_2$O$_2$) gradually overwhelms cellular redox buffering capacity, and signals the activation of stress kinases such as JNK and IKK that can inhibit insulin signaling (i.e. serine phosphorylation of IRS-1) (116). Treatment of L6 myotubes with palmitate reduced insulin-stimulated glucose uptake, increased superoxide production and mtDNA damage (425, 426). Direct treatment with H$_2$O$_2$ also reduced glucose uptake in cultured myotubes (135). However, pharmacological inhibition of IKK protected SFA-treated L6 myotubes from IR (359). The overexpression of MnSOD in skeletal muscle (31), and catalase in mitochondria both protected rodents against IR from HFDs (225). Furthermore, Anderson et al. normalized mitochondrial H$_2$O$_2$ emission using the mitochondrial-targeting antioxidant SS31, and protected rats against HFD-induced IR (9). However, not all studies support this hypothesis; HF-fed mice treated with SkQ (Skulachev ion), a mitochondrial-specific antioxidant, reduced ROS and oxidative stress within skeletal muscle but failed to protect against IR (292). Therefore, mitochondrial ROS and oxidative stress can contribute, but
are not a prerequisite, to the development of IR in obesity. From a temporal standpoint, elevations in mitochondrial ROS that precede IR, may be a favorable adaptation in the short-term given that ROS can stimulate insulin secretion (309) and mitochondrial biogenesis (423). Theoretically, this is advantageous for maintaining blood glucose levels, and mitigating a high mitochondrial membrane potential caused by an increased flux of reducing equivalents. Importantly, this demonstrates how mitochondrial ROS production serves as a barometer of the balance between energy supply and demand. Chronic elevations in mitochondrial ROS production are instead detrimental, being linked with oxidative damage of pancreatic β-cells (309) and mitochondrial components (34), both of which contribute to IR and risk for T2D. As such, preventing chronic elevations in mitochondrial ROS may protect against diet-induced IR.

Similar to assessing mitochondrial respiration, ROS emission is typically assessed under conditions of maximal stimulation, using saturating concentrations of succinate and oligomycin to drive reverse electron flow. Therefore, this method may not reflect in vivo conditions, where submaximal changes (i.e. ADP sensitivity) can influence ROS production. Smith et al. demonstrated this as resveratrol treatment improved ADP sensitivity, reduced maximal H$_2$O$_2$ emission and enhanced insulin-stimulated glucose uptake in obese Zucker rats (362). Increased ADP transport into the mitochondrial matrix likely reduced ROS production by dissipating membrane potential via ATP synthase. Recently, this model was extended to include a role for reactive lipids (palmitoyl-CoA; P-CoA) in mediating reductions in ADP-sensitivity. Ludzki et al. showed that although exercise training improved submaximal ADP sensitivity, whole-body glucose tolerance, and skeletal muscle insulin sensitivity in obese adults, a pre-incubation of permeabilized
muscle fibres with P-CoA significantly increased mitochondrial H$_2$O$_2$ emission in the presence of 100 µM ADP (242). Importantly, these studies merge reactive lipid and ROS-based hypotheses of IR at the point of impaired mitochondrial ADP sensitivity, and show that both diet and exercise improve mitochondrial ADP sensitivity in healthy (137) and obese states (242, 362).

1.3.4 Lifestyle strategies to improve mitochondrial dysfunction

Obesity is a multifactorial metabolic state influenced by ethnic, genetic and socioeconomic factors (55, 289, 406); however, lifestyle choices represent the predominant modifiable factors (324). Therefore, research focused on implementing lifestyle interventions continues to garner much attention in the prevention and/or improvement of negative health outcomes linked with obesity. Exercise and diet-induced increases in mitochondrial content are thought to create a favorable cellular environment that enhances the capacity for ATP synthesis and fat oxidation. In turn, this may reduce the elevated burden posed on cells from exposure to saturated FAs in obesity, thereby supporting insulin action and its hormonal regulation of energy metabolism.

The beneficial effects of exercise have been well documented in the literature; however, our understanding of these benefits at the molecular level continues to grow. The onset of contractile activity induces a number of rapid events, including ATP turnover, calcium cycling, oxygen consumption and ROS production. These biochemical changes activate kinases that evoke post-translational modifications to nuclear transcription factors and coactivators involved in mitochondrial biogenesis (338). Among the kinases activated are AMP-activated protein kinase (AMPK), calcium-calmodulin
kinase II (CaMK), PKB, and mitogen-activated protein kinase p38 (p38 MAPK) (156). Therefore, it is evident that exercise can enhance mitochondrial content and function through a number of signaling mechanisms (5, 284, 286, 414, 416, 432).

ATP utilization during exercise increases cellular ratios of ADP:ATP and AMP:ATP, activating the energy-sensing enzyme AMPK. AMPK is a heterotrimer, of which the α2 catalytic subunit is strongly activated during exercise (156). AMPK activation increases PGC-1α promoter activity, mRNA and protein content, thereby, stimulating mitochondrial biogenesis (168). However, PGC-1α mRNA and protein content are respectively increased in AMPK-α2 knockout mice following acute treadmill running (180), and in muscle-specific AMPK-α2 inhibited mice following 6 weeks of voluntary wheel running (331). While the possibility remains that residual AMPK activity enabled such increases, it is likely pathways independent of AMPK may be involved in mitochondrial adaptations following exercise. For example, bursts in Ca2+ concentrations evoked by muscle contraction enhances CaMK activity and stimulates mitochondrial biogenesis in a PGC-1α-dependent manner. The effects of CaMK were shown to be mediated by p38 MAPK (414) by enhancing PGC-1α promoter activity (156), and PGC-1α protein activity through phosphorylation (86). ROS can also activate signaling pathways involved in mitochondrial metabolism. Non-mitochondrial sources of ROS driven by exercise have been identified, such as the flavoprotein oxidoreductase system of the plasma membrane (299). ROS-induced increases in mtDNA copy number and mitochondrial content (306) occur in an AMPK-dependent manner, enhancing NRF-1 and PGC-1α expression (167). Evidence from these studies suggests that tightly
regulated ROS production can, in part, mediate the beneficial effects of exercise training on mitochondrial adaptations.

Collectively, numerous signaling mechanisms activated by exercise training converge at PGC-1α to enhance transcriptional events associated with mitochondrial biogenesis; however, the posttranscriptional events regulating mitochondrial biogenesis are not well understood. In particular, the steady-state concentration of mitochondrial mRNA transcripts influences the synthesis of mitochondrial proteins during biogenesis. To this end, a role for RBPs in dictating the stability of mRNA transcripts during mitochondrial biogenesis has been suggested. For instance, an increased cytoplasmic expression AUF1, an mRNA destabilizing protein, coincided with the reduced stability of PGC-1α, TFAM and NRF-2 mRNA transcripts (87). Interestingly, the rate of mRNA transcript decay varied among these gene targets, and it is thought that the abundance of AREs and GREs within the 3’-UTR determines the propensity for interaction with RBPs and therefore rates of mRNA decay (87). This suggests a model where increased mRNA stability prolongs its steady-state concentration and leads to greater protein synthesis. Recent work examining myocyte differentiation revealed that greater TFAM mRNA stability increased mRNA concentration throughout differentiation, in parallel with TFAM protein content and mitochondrial biogenesis (81). This broadly suggests that the stabilizing/destabilizing effect of RBPs can contribute to the posttranscriptional regulation of mitochondrial biogenesis. Therefore, the activity of RBPs during exercise-induced mitochondrial biogenesis warrants further examination.

In addition to exercise, alterations in dietary habits can stimulate mitochondrial biogenesis. Excess energy intake was associated with reduced expression of PGC-1α and
mitochondrial dysfunction in skeletal muscle linked with the development of obesity and IR (394). Whereas, caloric restriction was shown to enhance PGC-1α expression and increase oxidative capacity through AMPK activation (58). Caloric restriction represents an approach that lowers total caloric intake, including total dietary lipid content. However, nutritional approaches that alter dietary lipid composition may also increase mitochondrial content.

For instance, long-chain n-3 PUFAs act as direct ligands for PPARs and can stimulate FA oxidation, reduce lipid biosynthesis and enhance mitochondrial content (60, 366, 394, 419). In mice, 10 weeks of fish-oil treatment (3.4% kcals from EPA/DHA) increased muscle PPARα/γ, PGC-1α, TFAM and NRF-1 expression, and markers of mitochondrial content (214). The individual effects of EPA and DHA remain unclear given the absence of a normal-fat diet group (~10% kcals from fat); fish-oil was paired with a HFD, but a HFD alone evoked similar results. This emphasizes that evaluating individual PUFA species can highlight unique biological effects. Recently, the effects of EPA and DHA on mitochondrial dysfunction were examined individually using a normal-fat background diet (179). While both n-3 PUFAs exerted similar biological effects, only EPA enrichment in muscle was suggested to prevent the loss of mitochondrial function (179). Long chain n-3 PUFAs have also been implicated negatively in mitochondrial ROS formation (see review (6) for further details), raising questions of potential adverse effects. Whether long-chain n-3 PUFA-derived mitochondrial ROS is detrimental to mitochondrial membrane dynamics, permeability, mtDNA integrity, or is tightly regulated and promotes favorable adaptations to mitochondrial content remains unknown. To this end, very little is known about alpha-
linolenic acid (ALA), the n-3 precursor to EPA and DHA, which is an essential FA (EFA) required in the diet. Moreover, omega-6 (n-6) PUFAs are consumed in a far greater proportion than n-3 PUFAs, and have been negatively implicated in the pathophysiology of obesity and IR. However, this notion remains controversial (119) given that i) the dietary consumption of the n-6 EFA, linoleic acid (LA), is necessary, ii) a lack of research in this area has left the relationship between LA and skeletal muscle mitochondrial dysfunction poorly characterized, and iii) whether the quantity of LA consumed, or its conversion to longer-chain n-6 PUFAs such as arachidonic acid (AA), drive any potential negative effects is unclear. Therefore, a number of avenues must still be explored before more definitive claims can be made regarding any therapeutic value of dietary PUFAs, particularly with regards to EFAs.

1.4 General overview of essential fatty acid metabolism

1.4.1 Dietary sources of EFAs

ALA and LA are the two EFAs that cannot be synthesized in humans and must therefore be consumed in the diet. Common dietary sources of ALA include: soybeans, eggs, chia seeds, flaxseeds, walnuts, canola oil, flaxseed oil and soybean oil (52). Dietary sources of LA include: pecans, pine nuts, sunflower seeds, and common cooking oils such as canola, safflower and corn oils (52). Canadian dietary reference intakes (DRIs) suggest that 0.6-1.2% of total daily energy come from ALA and 5-10% from LA (53). In more absolute quantities, the respective adequate intake (AI) of ALA and LA is 1.1-1.6 and 11-17 g/day for Canadian adults (LA:ALA ratio of ~10:1) (53). However, the 2012 Canadian community health survey (CCHS) found that the majority of adults exceeded
the daily percent energy consumption for ALA, but fell short for LA (LA:ALA ratio of ~7:1), and that ~25% of adults exceeded the recommended total fat intake (54). This highlights the current gap in our understanding of EFA requirements; on one hand it is thought that an excess of n-6 PUFA in Western diets is linked with the high incidence of metabolic disease (355), yet Canadian adults statistically fell short of the AI values for LA. Importantly, AI values are used when insufficient data is available to establish a recommended dietary allowance (RDA) (53) and therefore, strongly emphasizes the need for further research.

1.4.2 ALA and insulin resistance

ALA is the most abundant n-3 PUFA in the Western diet, and is required to support normal functioning of the brain, central nervous system and vision. Long-term studies report the benefits of ALA consumption on T2D incidence. In the Singapore Chinese health study (prospective cohort of > 43,000 subjects aged 45-74 years-old), those in the highest quintile of ALA consumption (≥1.1 g/day) exhibited a 21% lower risk of developing T2D after 10 years, compared to the lowest ALA consumption quintile (≤0.27 g/day), as assessed by self-reporting and follow-up measures of glycosylated haemoglobin (HBA\textsubscript{1C}) (44). Similar results were observed from a prospective longitudinal cohort study in the US, in which the association between plasma phospholipid ALA, and the risk of T2D in 3088 older adults was examined (97). It was found that subjects in the highest quartile of plasma ALA (>0.18% of total plasma FAs; also consumed the most ALA (1.41 g/day)) had a 43% lower risk for developing T2D over 10 years, as assessed by the use of insulin or hypoglycemic agents, and plasma
glucose measures. The populations examined in the Singapore and US-based studies exhibited considerable variability in ethnicity, genetic make-up and amount of ALA consumed, yet demonstrated consistent findings regarding the beneficial effects of ALA. However, not all human trials report beneficial effects. In a short-term study, where a healthy mixed-gendered population consumed 6.0 g/day of ALA for 3 weeks, no effect was found on T2D risk as assessed by serum glucose, HBA$_{1C}$ and HOMA-IR (100). This indicates either a longer study intervention period was required or high ALA intake has minimal health impacts in a healthy population. Lastly, a recent meta-analysis reported a non-significant 7% reduced risk for T2D with ALA consumption (n=6 cohorts), and 10% with circulating ALA (n=7 cohorts), but had population heterogeneity and low statistical power, suggesting more research is required (417).

Animal-based studies have shown that ALA can offer protection against IR and T2D. In rats, 12 weeks of ALA supplementation (Linseed oil, 1.1 g/100 g diet; 2:1 LA:ALA ratio), protected against whole-body IR as determined with fasting plasma insulin, and insulin area under the curve (AUC) following an oral glucose challenge (126). Also, dietary enrichment of flaxseed oil (1.0 versus 6.2 g/100 g diet) corresponded to a lower LA:ALA ratio (60:1 versus 9:1) and protected mice against non-alcoholic fatty liver disease, high fasting plasma glucose and maintained plasma insulin comparable to healthy control animals after 8 weeks (188). Lastly, in ob/ob mice, 4 weeks of flaxseed oil supplementation was sufficient to protect against whole-body IR, as assessed by AUC values following an oral glucose challenge (276). These data show that ALA supplementation from flaxseed oil for as little as 4 weeks can protect against whole-body IR, and thus represents a favourable dietary intervention.
The use of ALA supplementation in conjunction with a HFD can provide insights on lifestyle strategies to prevent obesity-related complications. This concept was explored in animals fed 40% HFDs varying in LA:ALA ratios (1:1, 5:1, 10:1 and 20:1; from corn, soybean, coconut and flaxseed oils) (105). Although all HFD groups became IR, the 1:1 ratio HFD conferred some protection by lowering circulating lipid and WAT inflammation markers (105). Of importance, the ALA dose from flaxseed in the 1:1 HFD corresponded to 2.2 g/100 g, and may have been insufficient to offset the harmful effects of the HFD. In support of this, a separate investigation where a HFD contained ALA (~6 g/100 g ALA dose; 1:2 LA:ALA ratio) led to improved glucose tolerance, reduced markers of WAT inflammation and reduced abdominal adiposity (322). This suggests that at higher doses, ALA may mitigate the detrimental effects of a HFD. The study was also amongst the few to examine ALA, EPA and DHA individually, and showed distinct FA effects. EPA and DHA reduced HFD-induced weight gain to a greater extent, but ALA improved whole-body glucose tolerance more robustly than EPA and DHA (322). Altogether, this indicates that different n-3 PUFAs act through distinct mechanisms to confer their protective effects.

Mechanistic insights on the protective effects of ALA on insulin sensitivity have recently emerged using an in vitro model. In C2C12 muscle cells, ALA treatment reversed impairments in insulin-stimulated glucose uptake caused by the SFA, palmitic acid (296). These improvements were linked with increased Akt phosphorylation, suggesting that insulin signalling remained intact. Moreover, while palmitic acid increased the activation of the serine/threonine kinases JNK-1/2 and PKCθ, ALA treatment was consistent with the dephosphorylation of JNK-1/2 and PKCθ (296). Thus,
ALA protected insulin signalling by reducing the inhibitory effect of JNK and PKC on IRS-1. This work also demonstrated that ALA increased AMPK activation, suggesting that glucose uptake was also enhanced through an insulin-independent mechanism (296). The increased AMPK activity lead to the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) (296), which reduced allosteric inhibition of CPT-1 by malonyl-CoA, thereby stimulating FAO (255). Therefore, ALA may protect glucose homeostasis by lowering the intracellular accumulation of lipid metabolites such as DAGs and ceramides by stimulating FA oxidation. This aligns with recent findings that ALA treatment increased expression of mitochondrial targets CPT-1 and UCP3 in C2C12 muscle cells through PPARδ activation (385). Altogether, initial mechanistic evidence to explain the potential insulin-preserving effects ALA have begun to emerge, and whether these in vitro findings can be verified using in vivo models remains to be shown.

Promising results have emerged from the few studies examining the biological effects of ALA in obesity, IR and T2D. However, very few studies have simultaneously examined the effects of dietary ALA on multiple insulin-responsive tissues. As such, a basic characterization of the tissue-specific enrichment of ALA is still lacking, but may be useful in understanding whether obesity and IR are associated with a deficiency in ALA. Furthermore, future work examining major tissue lipid fractions, particularly those implicated in IR such as DAGs and ceramides may provide a more precise understanding of the metabolic benefits of ALA. Further mechanistic studies examining how ALA may preserve insulin sensitivity in the face of obesity is needed. Recently, fish oil supplementation (EPA/DHA) was shown to alter mitochondrial membrane composition and respiratory function in young, healthy adults (137). Whether ALA exerts similar
effects on mitochondrial dysfunction observed in obese and IR states remains to be shown. In conclusion, there is initial evidence to suggest that ALA may not only be protective in the context of obesity and IR, but may also have functions that are distinct to that of EPA/DHA. Collectively, this suggests that studies investigating ALA are warranted.

1.4.3 LA and insulin resistance

LA is a major cellular membrane constituent and is the most abundant PUFA in the modern Western diet (23). The classic view that LA is harmful for insulin sensitivity warrants careful consideration, as observed with recent human-based clinical trials suggesting that LA may even be protective against T2D risk. In the Singapore Chinese health study, the subjects with the lowest risk for T2D were in the highest quintile of dietary omega-6 PUFA intake (12.9 g/day total n-6 PUFA), compared with subjects at highest risk (4.2 g/day total n-6 PUFA) (44). Importantly, this challenges the link between high LA consumption and risk for T2D (23). Given that LA is the most abundant dietary n-6 PUFA (23), the quantity of LA likely consumed by the lowest risk population would fall within the current Health Canada AI guidelines. Moreover, examining LA consumption in a Japanese male population revealed that the quartile of men with the highest LA intake had a 47% lower serum concentration of C-reactive protein (CRP), a marker of inflammation commonly associated with obesity-related co-morbidities (319). The LA consumption of these subjects corresponded to ~5% of total dietary energy intake, and importantly, aligns with Health Canada’s guidelines (53). This indirectly suggests that current guidelines for LA intake may not link to the high incidence of
metabolic disease in Western society as strongly as previously suggested (355). To this end, circulating LA levels were also inversely correlated with CRP levels in a healthy, young adult, mix-gendered Canadian population (301). Moreover, a recent prospective cohort study in Finland (n = 1,302 middle aged male subjects) found that LA content in plasma phospholipids, cholesterol esters and TAGs was inversely correlated with the development of T2D (assessed using AUC of OGTT and HBA1C values) over the 5.9-year follow-up period (213). Lastly, a recent 2015 meta-analysis examining PUFA content of total plasma phospholipids and T2D risk demonstrated that LA content is lower in obese subjects compared to normal-weight (9 cohorts, n = 628 subjects) (110). Taken together, emerging literature has shown that higher LA consumption often correlates with a lower risk for T2D, whereas, obesity may be linked to a deficiency in LA that elevates T2D risk.

More broadly, human and animal-based studies indicate that reduced PUFA content in skeletal muscle phospholipids associates with lower insulin action (35, 371). In obesity, reduced PUFA content is often accompanied by increased SFA content, which is believed to increase membrane rigidity and inhibit insulin action by either lowering the number of insulin receptors or the binding affinity of insulin to its receptor (35, 89). LA consumption may support insulin action through increased PUFA incorporation into plasma membranes, ultimately enhancing membrane fluidity (89). This relationship also extends to skeletal muscle mitochondrial membranes, where a greater unsaturation index (influenced mainly by LA and AA) was positively correlated with rates of FA oxidation (149). Altogether, a growing body of observational data challenges the classical view that LA is harmful for insulin sensitivity, given that this n-6 EFA has been associated with
reduced T2D risk; however, the supporting mechanistic framework requires further elucidation.

Studies targeting skeletal muscle have begun to demonstrate potential mechanisms underlying the biological effects of LA. Pre-treatment of C2C12 muscle cells with LA maintained insulin-stimulated activation of Akt and glucose uptake in comparison to pre-treatment with the SFA, palmitate (347). Similar results were reported using rat L6 myotube cultures, where LA maintained insulin-stimulated glucose uptake compared to palmitate (228). Importantly, it was demonstrated that LA did not increase DAG or ceramide levels compared to palmitate, highlighting a potential mechanism by which LA protects insulin sensitivity (228). However, this finding is not uniform, as incubation of rat soleus muscle with LA significantly increased DAG content after 6 hours, but did not change ceramide accumulation (144). Conversely, in rats fed HFDs (53% from either SFA-rich lard or LA-rich safflower oil), it was found that the LA-HFD protected against whole-body IR (OGTT and HOMA-IR) and had lower muscle DAG and ceramide content compared to SFA-HFD (228). Methodological differences may account for these discrepant findings, but collectively these studies show that LA can protect insulin sensitivity; however, the molecular mechanisms preventing the increases in DAG and/or ceramide levels remain unknown. One potential mechanism is that LA preferentially diverts FA-CoAs toward TAG synthesis, as demonstrated in rats fed HFDs composed of either SFAs (lard), oleic acid (oleic sunflower oil) or LA (sunflower oil) (251). In this study, rats fed the LA-HFD had significantly greater TAG content in muscle compared to rats fed the other HFDs (251); however, food consumption was not matched between the different diet groups. This is an important caveat given that the LA-
HFD was consumed in far greater amounts than the other diets. Another possibility is that LA consumption confers protection by enhancing FA oxidation. For example, feeding rats a LA-HFD for 4 weeks increased muscle content of FATP1 (251), a transport protein believed to channel FAs toward oxidation. Indeed, elevated FATP1 protein content, through fatp1 transfection in rats, led to enhanced palmitate transport and oxidation in muscle, but not TAG esterification (153). Therefore, by enhancing TAG synthesis and/or FA oxidation, LA may protect against diet-induced IR.

Many fundamental biological effects of dietary LA remain unknown despite being an EFA. A number of studies using a range of experimental models (i.e., primary cell culture, animal and human cohorts) present data that conflict with the notion that LA consumption is positively associated with the growing incidence of obesity and IR. From these studies it is apparent that LA can reduce the muscle content of DAGs and ceramides, and improves mitochondrial membrane structure, function and biogenesis. Altogether, LA appears to improve a number of major mechanisms linked with the development of IR in obesity, thereby demonstrating its potential therapeutic value.

1.5 Considerations in other insulin-responsive peripheral tissues

During periods of positive energy balance (e.g. after a meal or with obesity), WAT serves as the primary location for storage of lipids. Thus, WAT provides a long-term fuel reserve that can be mobilized for use in other tissues during times of need, such as in skeletal muscle and the liver.

The liver can be viewed as a chemical processing factory, the functions of which are optimized by its strategic anatomical location, receiving nutrients and chemicals from
blood that circulate through the stomach, pancreas and the small and large intestines amongst other organs. As such, the liver significantly contributes to glucose and lipid homeostasis at the whole-body level.

Insulin regulates the metabolic functions of WAT and the liver to maintain blood glucose values within a narrow range during fasting and fed states. Similar to skeletal muscle, the tissue-specific regulation evoked by insulin becomes compromised by excess energy intake in obesity.

### 1.5.1 White adipose tissue (WAT)

WAT exists in a number of subcutaneous and visceral depots and primarily stores lipids. WAT accounts for roughly 10-15% of insulin-stimulated postprandial glucose clearance from the blood (333). Comparatively, this is less than skeletal muscle (~30%); however, WAT IR is still considered a major contributor to IR at the whole-body level.

Temporally, WAT IR was shown to precede the development of IR in the liver and skeletal muscle in mice (388). Compared to liver and skeletal muscle, WAT responds to much lower levels of circulating insulin, highlighting the importance of tightly regulated lipolysis and lipogenesis (59). Impairments in the capacity of WAT to store excess lipids combined with the gradual decline in the ability of insulin to suppress lipolysis ultimately contribute to IR and ectopic lipid accumulation within the liver and skeletal muscle. The development of WAT IR appears to be mechanistically similar to that of skeletal muscle (as shown in Figure 1.3).

The link between mitochondrial dysfunction and WAT IR has gained interest in recent years. Adipocytes generally contain few mitochondria, but these mitochondria
serve a crucial role in metabolic homeostasis, participating in adipogenesis, lipogenesis, lipolysis and fatty acid esterification (38). Obesity has been linked with reduced WAT mitochondrial gene expression (413), protein content, mitochondrial function (62), and increased ROS emission associated with oxidative stress and IR (62, 291). Exercise has been shown to induce mitochondrial biogenesis in WAT through PGC-1α, involving signaling pathways similar to that in skeletal muscle (e.g. AMPK, p38 MAPK) (124, 196, 375, 401). Increasing WAT mitochondrial content and function, as well as regulating ROS emission, may protect against IR by reducing FA release into circulation (i.e. increased FA re-esterification) or by enhancing FAO that can help reduce lipid accumulation.

Ceramide accumulation within WAT has been shown to blunt insulin-stimulated Akt phosphorylation and glucose uptake, causing WAT IR (64). In humans, both obesity and T2D were positively correlated with WAT ceramide content (30), particularly 16:0-rich ceramide species (389). However, depot-specific measurements in humans revealed that only visceral WAT shows increased ceramide content (125). Interestingly, pro-inflammatory cytokines have been shown to promote the synthesis and accumulation of SFA-rich ceramides (133). Given that dietary SFAs can induce pro-inflammatory stress and act as substrates for ceramide synthesis, altering dietary lipid composition as a form of protection against WAT IR has gained interest.

Dietary EFAs may exert protective effects against IR in obese WAT. In male and female humans, it was recently shown that subjects with the lowest prevalence of metabolic syndrome and fasting blood glucose were in the highest quartile of ALA content in subcutaneous WAT biopsies (61). In mice, a 45% HFD enriched with 10%
ALA (total energy) from soybean oil, led to increased expression of β-oxidation gene-targets, PGC-1α protein and copies of mtDNA (431). These changes were associated with reduced visceral adiposity, decreased adipocyte size and repressed lipogenesis (431). This indicates that enhanced mitochondrial content and function was associated with improved markers of adiposity. AMPK appeared to mediate these insulin-sensitizing anti-adipogenic effects, as AMPK-α1/2 knockout mice were not protected from the HFD containing ALA (431). Taken together, EFAs may exert protective effects in WAT in conditions of obesity and/or nutrient overload.

1.5.2 Liver

The liver is an endocrine organ that metabolizes lipids that are stored, excreted to other tissues, or endogenously metabolized within hepatocytes (166, 397). Insulin has a central role in this context by promoting FA and TAG synthesis; a process generally referred to as de novo lipogenesis (191). TAGs can either be stored within hepatic lipid droplets or exported as VLDLs and delivered to WAT or muscle for storage and/or oxidation (397). However, obesity is associated with hepatic lipid overload, and a number of pathophysiological disturbances impair insulin action similar to that seen in skeletal muscle and WAT.

A major source of hepatic lipid overload comes from circulating FFA, which are systemically elevated by IR in WAT (21). Excess FA uptake into the liver reduces insulin clearance rates, thereby contributing directly to systemic hyperinsulinemia (182). Insulin-mediated suppression of hepatic glucose output is also diminished, leading to hyperglycemia. Hyperglycemia promotes de novo lipogenesis (DNL), which utilizes
glucose as a precursor for FA synthesis (27). Collectively, uptake and endogenous synthesis of FAs are two principle mechanisms by which lipids accumulate in the liver.

In this regard, DAG and ceramide accumulation within the liver has been well characterized, and these reactive lipids have been shown to inhibit insulin signaling at the levels of IRS-1 (181) and Akt (63, 290) similar to that seen in other insulin-responsive tissues. However, conflicting reports have shown that increasing hepatic DAG and ceramide content does not always result in hepatic or whole-body level IR (45, 56, 260). Therefore, discerning a role for reactive lipid metabolites is challenging and complex, and studies showing reductions in these lipids are often confounded by concomitant changes in adiposity and alterations in hepatic lipid composition. Nevertheless, strategies to reduce hepatic lipid content are still optimal to protect against the development of IR.

Mitochondrial FA oxidation represents an avenue towards reducing hepatic lipid accumulation. Mitochondrial dysfunction and ROS emission have been well characterized in obese and IR states (121, 249, 277, 280, 300), yet compensatory increases in hepatic FAO have been shown (76, 141, 166, 223), suggesting the involvement of mitochondrial “uncoupling” (351). As such, compensatory elevations in mitochondrial FAO are thought to precede the eventual dysfunction that results from chronic lipid oversupply.

Therapeutic interventions, including the use of dietary EFAs may reduce lipid accumulation and synthesis, or conversely, increase utilization (e.g. mitochondrial FAO) thereby protecting against hepatic and whole-body IR. Supplementing diets with 5% ALA-rich chia seeds for 8 weeks significantly improved whole-body glucose tolerance and hepatic lipid content associated with a HFD alone (321). One month of dietary ALA
enrichment (4% energy by weight) enhanced hepatic FAO and prevented NAFLD in obese Zucker rats (272). However, increasing hepatic FAO by supplementing a 45% HFD with perilla oil (15% total energy from ALA) did not protect rats from whole-body IR (428). The discrepant findings are likely accounted for by methodological differences; 10% versus 45% fat diets, 4 versus 16 weeks feeding period, and rat species. In mice, manipulation of the LA:ALA ratio (1:1 to 20:1) in a 40% HFD did not improve mitochondrial content or oxidative stress after 20 weeks and lead to NAFLD (104). Importantly, this study provides proof of principle that LA, even at a 20:1 LA:ALA ratio in a HFD, was no more harmful than at a 1:1 ratio. Overall, despite conflicting findings, there is some indication that ALA and LA have protective effects in the liver.

1.6 Summary

In diverse metabolic tissues, the content and function of mitochondria is considered important in maintaining energy homeostasis. As such, dysfunction within the mitochondrial population of diverse tissues is believed to contribute to various metabolic disturbances associated with disease development. Exercise and diet represent lifestyle factors that can enhance mitochondrial bioenergetics and influence whole-body lipid metabolism.

Exercise-based studies have greatly contributed to our current mechanistic understanding of mitochondrial biogenesis; however regulation at the posttranscriptional level represents a gap in this framework that warrants further study. In addition, altering dietary lipid profiles through changes in the type and total quantity of fat consumed has been linked with disease incidence. ALA and LA are EFAs proposed to have beneficial
effects with respect to obesity and IR. Initial evidence has linked these benefits to changes in mitochondrial bioenergetics and lipid metabolism within diverse tissues. Thus, examining whether these EFAs can protect against the development of diet-induced IR presents an attractive avenue of future research. Overall, exercise and nutritional interventions are valuable tools in elucidating the mechanisms underlying improvements in mitochondria and whole-body lipid metabolism and may lead to novel therapeutic strategies aimed at preventing diet-induced IR.
CHAPTER 2

AIMS OF THE THESIS
2.1 Overall rationale

Lifestyle choices such as exercise and diet are two modifiable factors widely documented to improve obesity-related health complications such as IR; however, the mechanisms underlying these improvements remain poorly defined. IR can manifest from an imbalance between energy supply and demand, which stems from an excess energy intake in the absence of increased demand. Current data suggests that exercise and dietary interventions that lead to improvements in cellular energy homeostasis and insulin sensitivity likely involve adaptations within mitochondria and lipid metabolism. Therefore, the overall objective of this thesis was to advance our knowledge of how lifestyle factors can regulate skeletal muscle mitochondrial content and function, and whole-body lipid metabolism. Accordingly, three independent studies were conducted to address this objective.

2.2 Specific objectives and hypotheses

Study #1

A well-characterized adaptation to exercise training is an increase in the content of skeletal muscle mitochondria. However, a fundamental understanding of the posttranscriptional events regulating exercise-induced mitochondrial biogenesis is still lacking. The overall objective of this study was to determine whether RNA binding proteins known to influence mRNA stability contribute to the posttranscriptional regulation of exercise-induced mitochondrial biogenesis.
The specific objectives of study #1 were to:

1. Examine the effects of acute (1 day) and chronic (1 week and 4 weeks) exercise training on markers of skeletal muscle mitochondrial content in mice.
2. Examine the role of RNA binding proteins (RBPs) on the posttranscriptional regulation of exercise-induced mitochondrial biogenesis within skeletal muscle.

Based on the objectives of study #1, I hypothesized the following:

1. Only 4 weeks of exercise training duration would increase markers of skeletal muscle mitochondrial content, while training durations of 1 day and 1 week would not.
2. Exercise training would decrease the content of destabilizing RBPs (AUF1 and CUG-BP1) within skeletal muscle, an important event that precedes the induction of mitochondrial biogenesis.
3. Exercise-induced mitochondrial biogenesis requires a cellular environment promoting mRNA stability.

Study #2

Mitochondrial dysfunction is a common feature of obese and IR states. Dietary long-chain n-3 PUFAs have been shown to exert lipid lowering and insulin-sensitizing effects, while n-6 PUFAs are generally considered harmful for insulin sensitivity. However, very little is known about the individual effects of dietary ALA and LA with respect to skeletal muscle mitochondrial content and function. The overall objective of this study was to determine whether ALA and LA supplementation could alter obesity-related IR by regulating aspects of mitochondrial dysfunction.
The specific objectives of study #2 were to:

1. Investigate whether diets enriched with either ALA or LA would protect against the development of whole-body glucose intolerance in the obese Zucker rat model.
2. Examine the effects of ALA and LA on skeletal muscle-specific insulin signaling mechanism.
3. Examine the effects of ALA and LA on skeletal muscle mitochondrial content and function in obesity.

Based on the objectives of study #2, I hypothesized the following:

1. A diet enriched with ALA would protect against the development of impaired whole-body glucose homeostasis in obesity, and would be linked to the preservation of skeletal muscle insulin signaling.
2. A diet enriched with LA would exacerbate obesity-related impairments in whole-body glucose homeostasis, and would be linked with the inhibition of skeletal muscle insulin signaling.
3. Dietary ALA, but not LA, would improve skeletal muscle mitochondrial dysfunction in obesity, serving as a potential mechanism of protection against muscle-specific and whole-body insulin resistance.

Study #3

The accumulation of reactive DAG and ceramide lipid species within major insulin-responsive tissues, specifically, skeletal muscle, WAT and the liver, has been linked to obesity-related IR. The results from Study #2 demonstrated that both ALA and
LA protected against the development of IR in obese Zucker rats; however, these protective effects occurred independent of changes in mitochondrial content and/or function. Therefore, the overall objective of this study was to characterize the lipid profiles of skeletal muscle, WAT and liver, to determine if reductions in DAG and/or ceramide accumulation mediated the protective effects of ALA and LA.

The specific objectives of study #3 were to:

1. Characterize the effects of ALA and LA on the lipid profiles of the major insulin-responsive peripheral tissues skeletal muscle, WAT and the liver.
2. Examine if ALA and LA supplementation affected the accumulation of DAG and ceramide reactive lipid species within skeletal muscle, WAT and the liver.

Based on the objectives of study #3, I hypothesized the following:

1. ALA and LA supplementation will lead to a distinct remodeling of tissue PUFA content. Specifically, ALA will increase content of n-3 PUFA species, and LA will increase n-6 PUFA content.
2. ALA and LA supplementation will reduce the accumulation of DAG and ceramide species within skeletal muscle, white adipose tissue and the liver, serving as potential mechanism underlying the protective effects of ALA and LA.
CHAPTER 3:

EXERCISE TRAINING INCREASES THE EXPRESSION AND NUCLEAR LOCALIZATION OF MRNA DESTABILIZING PROTEINS IN SKELETAL MUSCLE

Presented as published:


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3.1 Abstract

While a paucity of information exists regarding post-transcriptional mechanisms influencing mitochondrial biogenesis, in resting muscle the stability of peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) mRNA has been linked to mitochondrial content. Therefore, in the current study we have examined if exercise promotes mRNA accumulation through the induction of proteins affiliated with mRNA stabilization (HuR) or conversely by decreasing the expression of mRNA destabilizing proteins (AUF1 and CUG-BP1). A single bout of exercise increased (P<0.05) the mRNA content of the transcriptional co-activator PGC-1α ~3.5 fold without affecting mRNA content for HuR, CUG-BP1 or AUF1. One week of treadmill exercise training did not alter markers of mitochondrial content, the mRNA stabilizing protein HuR, or the mRNA destabilizing protein AUF1. In contrast, the mRNA destabilizing protein CUG-BP1 increased ~40%. Four weeks of treadmill training increased the content of subunits of the electron transport chain ~50%, suggesting induction of mitochondrial biogenesis. Expression levels for HuR and CUG-BP1 were not altered with chronic training; however AUF1 expression was increased post-training. Specifically, training increased (P<0.05) total muscle expression of two of the four AUF1 isoforms ~50% (AUF1p37, AUF1p40). Interestingly, these two isoforms were not detected in isolated nuclei; however, a large band representing the other two isoforms (AUF1p42, AUF1p45) was present in nuclei and increased ~35% following chronic training. Altogether the current data provides evidence that mitochondrial biogenesis occurs in the presence of increased CUG-BP1 and AUF1 following, suggesting that reductions in known mRNA destabilizing proteins likely does not contribute to exercise-induced mitochondrial biogenesis.
3.2 Introduction

A well-characterized observation following chronic exercise training is an increased mitochondrial content in skeletal muscle (145, 147, 157). The increased mitochondrial content is thought to result from transient increases in mRNA content following bouts of exercise (304, 311, 408). A key component of mitochondrial biogenesis is the coordination between the mitochondrial and nuclear genomes (as reviewed in (156)). While the molecular mechanisms regulating mitochondrial biogenesis have not been fully elucidated, in recent years it has become apparent that the nuclear encoded transcriptional co-activator peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) is a key regulator of this process (127, 155, 189, 325, 342, 343, 419).

Over the past decade numerous signals provoked by environmental stimuli and/or cellular stress have been identified as potential mechanisms that induce mitochondrial biogenesis (as reviewed in (156)). In skeletal muscle, the molecular mechanisms stimulating mitochondrial biogenesis are activated immediately after a single bout of exercise (283, 304, 312), a process that appears to be regulated by calcium-calmodulin kinase (CaMK) activated signalling (414), p-38 mitogen-activated protein kinase (p38 MAPK) (414) and AMP activated protein kinase (AMPK) (170). These signalling events are thought to regulate the sub-cellular location of PGC-1α as well as the transcriptional rate of genes co-activated by PGC-1α, resulting in an accumulation of mitochondrial proteins, and therefore the induction of mitochondrial biogenesis.

While much research has focused on the events regulating gene transcription following exercise, scarce information exists regarding post-transcriptional mechanisms.
that may influence mitochondrial biogenesis. However, the stability of mRNA, and therefore the steady-state concentration of mRNA species, has been directly linked to the expression of a gene (103, 130). In eukaryotic cells a variety of pathways involving RNA binding proteins (RBPs) have been identified that regulate mRNA stability (160). RBPs use either AU or GU-rich elements within the 3’-untranslated region of mRNA to influence the half-life of mRNA species (160), which represents the balance between stabilizing and destabilizing proteins. Several RBPs have been identified; however, the best characterized include: Human Antigen R (HuR) which utilizes AU-rich elements to promote mRNA stability (109), AU-rich binding factor 1 (AUF1) which promotes mRNA destabilization (94), and CUG binding protein 1 (CUG-BP1) which utilizes GU-rich elements to promote mRNA destabilization (227). Little is known about the regulatory roles of these proteins in mature mammalian muscle, however a recent report using biological tissue variation in mitochondrial content found that the ratio of AUF1:HuR, and therefore mRNA destabilization, was associated with in vitro rates of PGC-1α mRNA decay (87).

The response of RBPs following chronic aerobic exercise training remains to be determined, but a shift towards an environment promoting mRNA stabilization (i.e. an increase in HuR relative to either CUG-BP1 or AUF1) may provide an early molecular mechanism favouring mitochondrial biogenesis. Therefore, in the present study we have examined the mRNA content of PGC-1α, HuR, AUF1 and CUG-BP1 in the quadriceps muscle of mice following an acute bout of exercise, as well as protein changes following 1 and 4 weeks of treadmill training. In addition, we examined the potential for acute and chronic exercise to influence nuclear levels of HuR, AUF1 and CUG-BP1. Contrary to
our hypothesis, exercise did not increase the mRNA stabilizer HuR, while we provide evidence that exercise increased, not decreased, the expression of CUG-BP1 and AUF1 in a temporally distinct manner.

3.3 Materials and Methods

3.3.1 Animals

C57BL/6 mice were bred at the University of Guelph and housed in an animal facility with free access to standard chow and water with a 12:12 hour light-dark cycle (n=40). At ~12 weeks of age mice were randomly selected for either; 1) acute exercise and recovery experiments (n=6 per time point), 2) one week of exercise training (n=5 control and trained) or 3) for a four week training program (n=6 control and trained). All experiments were performed in accordance with procedures approved by the University of Guelph Animal Care Committee.

3.3.2 Acclimatization

All mice participated in a three-day acclimatization program in order to familiarize them with a motorized rodent treadmill with brushes at the rear of the treadmill for encouragement. The mice were run at 15m/min 0% grade, 20m/min 0% grade and 20m/min 5% grade respectively for a total of ten minutes each day. Mice were allowed to recover for three days before experiments commenced, the quadriceps muscle was harvested for all experiments.

3.3.3 Acute Exercise and mRNA determinants

For acute exercise studies muscle was harvested from different animals prior to exercise (rest), immediately after exercise (Post), and 3 hours after completing exercise
(3hr Post). The treadmill exercise consisted of running mice for 90 minutes on a motorized treadmill at 15m/min and a 5% grade. RNA was isolated using a combination of homogenization in TRIzol reagent (Invitrogen) and purification using an RNeasy mini kit (Qiagen), as we have previously reported (35). Individual detection of AUF1 isoform mRNA species was not possible, so primers were designed to detect all 4 AUF1 isoforms (as confirmed with the NCBI BLAST tool). Dissociation curves were run on all plates to ensure a single oligonucleotide sequence was amplified, and primer efficiency was ensured before commencing experiments. The following primer sets were used for PGC-1α: 18S Forward 5’-GTTGGTTTTCGGAACTGAGGC-3’, 18S Reverse ‘5’-GTCGGCATCGTTTATGGTCG-3’; PGC-1α Forward 5’-CAATGAGCCCGCAACATAT-3’, PGC-1α Reverse 5’-CAATCCGTCTTTCATCCACC-3’; HuR Forward 5’-AGCAATCAGCACACTGAACG-3’, HuR Reverse 5’-TTGCGCGAGCATATGACA-3’; CUG-BP1 Forward 5’-GCTGGTCTGAACACACTTGG-3’, CUG-BP1 Reverse 5’-GTTCCCAGAGGAGGCAGTC-3’; AUF1 Forward 5’-CCAACAGGTGGTGATAAGCAG-3’, AUF1 Reverse 5’-TCCAATTCAGGAACCTTGATAGAAAA-3’.

3.3.4 Exercise training program

The training program consisted of training five days per week to volitional fatigue each day. Five animals completed 1 week of training, while six animals completed 4 weeks of training. The speed and grade of the treadmill were increased every week. The training consisted of running at the following intensities: week 1 at 22m/min 15% grade, week 2 at 23m/min 20% grade, week 3 at 27m/min 20% grade and week 4 at 31m/min
20% grade. In all weeks, the speed was increased by 5m/min after the first hour of running to ensure animals consistently ran for ~75 minutes. Animals were allowed to recover for 48 hours and then animals were anaesthetized with pentobarbitol (6mg/kg body weight) and the vastus lateralis was removed and immediately snap frozen in liquid nitrogen. Tissue was stored at -80°C until needed.

3.3.5 Western Blot Analysis

Tissue was homogenized (1mL per sample for ~50mg of tissue) for 60 seconds at 4m/s (MediCorp) in cell lysis buffer consisting of 1% Triton X, 50 mM Tris-HCl, 1mM EDTA, 1mM EGTA, 50 mM NaF, 10mM sodium β-glycerol phosphate, 5mM sodium pyrophosphate, 2mM DTT, 1mM Na orthovanadate, 1mM PMSF and 10 µg/mL of each aprotinin, leupeptin, pepstatin A (pH 7.5). Samples were then spun at 1500xg for 15 minutes, and the supernatant recovered for Western blotting. Bradford Protein Assay (BioRad) was used to determine protein concentrations, and 30 µg of protein was loaded for all Westerns. Samples were run on a SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. Five markers of the electron transport chain (C-I-20, C-II-30, C-III-Core 2, C-IV-I and C-V-a) were detected with use of the MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (MitoSciences, 1:1000). The following antibodies were also used; Complex IV subunit 4 (Invitrogen; 1:30,000). PGC-1α (Calbiochem (1:1000) and Millipore (1:1000)), AUF1 (Millipore; 1:500), HuR (Santa Cruz; 1:2000), CUG-BP1 (Santa Cruz; 1:1000) and SIRT1 (Upstate; 1:2000). The bands were visualized with enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer), using a FluorChem HD2 Alpha Innotech imager. Quantification of the relative protein expression was completed using the provided software. Gels were cut
prior to transferring to enable multiple proteins to be detected from the same Western blot, as well as to ensure all samples were transferred and detected on a single membrane to limit variability, and Ponceau staining was used to verify constant loading.

3.3.6 Isolation of nuclear extracts

Nuclear extraction was performed using a commercial kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s specifications, with minor modification as we have previously reported (150).

3.3.7 Statistics

All data is presented as the mean ± standard error of the mean (SEM). A nonparametric Mann-Whitney U-Test was used throughout, and statistical significance was recognized at $P < 0.05$. 
3.4 Results

3.4.1 Acute mRNA responses to exercise

Immediately following exercise, PGC-1α mRNA was increased ~3 fold (P<0.05; Fig 1), and remained unchanged following three hours of recovery, similar to previous reports (9). In contrast, exercise did not alter the mRNA abundance of HuR, CUG-BP1 or AUF1 (Fig. 3.1).

Figure 3.1 - The effects of acute exercise and recovery on the mRNA expression of HuR, CUG-BP1 and AUF1.
Exercise consisted of mice running at a speed of 15m/min at 5% grade for 90 minutes. The quadriceps muscle was taken from animals at rest, immediately after exercise (Post), or from mice allowed to recover for 3 hours (3hr Post). Data is expressed as a fold change from the resting control mice. Primers were designed in order to detect all four isoforms of AUF1. N=6 for all independent experiments. Data are expressed as mean ± S.E.M. *p<0.05, significantly different from resting control.

3.4.2 mRNA stabilizing/destabilizing proteins responses to 1 week of training

To examine the potential of mRNA stabilizing/destabilizing proteins to regulate mitochondrial biogenesis, we next examined responses following 1 week of treadmill training before pronounced adaptations in mitochondrial content occurred. As expected, treadmill training for this duration did not increase the total cellular content of various markers of mitochondrial content (Fig. 3.2 A-F), nor did it alter the content of PGC-1α protein (Fig. 3.2 G, H) or SIRT1 protein (Fig. 3.2 I).
Figure 3.2 - Markers of mitochondrial content, PGC-1α and SIRT following 1 week of training. Compared to before training (control; C), 1 week of treadmill training (1wk Trained; T) did not change markers of mitochondrial content, including Complex 1 subunit NDUFB8 (A); complex II subunit 30 kDa (B); complex III subunit Core 2 (C); complex IV subunit 1 (D); complex IV subunit 4 (E); ATP synthase subunit α (F); peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) using either the Calbiochem (G) and Millipore (H) antibodies, nor did it affect the content of sirtuin 1 (SIRT1; I). N=5 for all independent experiments. Data are expressed as mean ± S.E.M.

Therefore, we sought to characterize potential increases in HuR, or decreases in either CUG-BP1 or AUF1, as potential mechanisms contributing to early mitochondrial biogenesis. However, contrary to our hypothesis, HuR (Fig. 3.3A) and the AUF1 isoforms quantified (37kDa, 40kDa and 45kDa; Fig. 3C-F) were not altered. In contrast, the mRNA destabilizer CUG-BP1 was increased (P<0.05; Fig. 3.3B) following 1 week of training.
Figure 3.3 - HuR, CUG-BP1 and AUF1 total cellular proteins following 1 week of training.
Compared to before training (control; C), 1 week of treadmill training (1wk Trained; T) did not change the content of Human Antigen R (HuR; A) or any of the AU-rich binding factor 1 (AUF1) isoforms quantified (D-F). In contrast, 1 week of training increase the content of CUG binding protein 1 (CUG-BP1; B). The 42 kDa AUF1 isoform was too faint to reliably quantify in mice (C), however all four isoforms were easily detected in HeLa cells (C). N=5 for all independent experiments. Data are expressed as mean ± S.E.M. *p<0.05, significantly different from control.

3.4.3 mRNA stabilizing/destabilizing proteins responses to 4 weeks of training

We next aimed to determine chronic changes in HuR, CUG-BP1 and AUF1 proteins following 4 weeks of training. Treadmill training increased (P<0.05) the total cellular content of various markers of mitochondrial content (Fig. 3.4 A-F). Specifically, training increased the content of complex 1 subunit NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8 (NDUFB8), ~200% (Fig. 3.4 A); complex II subunit 30 kDa ~50% (Fig. 3.4 B); complex III subunit Core 2 ~50% (Fig. 3.4 C); complex IV subunit 1 ~50% (Fig. 3.4 D); complex IV subunit 4 ~50% (Fig. 3.4 E) and ATP synthase subunit a ~50%
(Fig. 3.4 F). In addition, there was a trend for an increase (P>0.05) in the total cellular content the PGC-1α protein (Fig. 3.4 G, H) as determined with two commercially available antibodies, while treadmill training did not alter the total cellular content of SIRT1 protein (Fig. 3.4 I).

Figure 3.4 - Markers of mitochondrial content, PGC-1α and SIRT1 following 4 weeks of training. Compared to before training (control; C), 4 weeks of treadmill training (4wk Trained; T) increased markers of mitochondrial content, including Complex 1 subunit NDUFB8 (A); complex II subunit 30 kDa (B); complex III subunit Core 2 (C); complex IV subunit 1 (D); complex IV subunit 4 (E); ATP synthase subunit α (F). In addition, 4 weeks of treadmill training resulted in a strong trend for an increased content of peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) using either the Calbiochem (G) and Millipore (H) antibodies. Training did not affect the content of sirtuin 1 (SIRT1; I). N=6 for all independent experiments. Data are expressed as mean ± S.E.M. *p<0.05, significantly different from control.

Given the recent suggestion that the ratio of stabilizing/destabilizing proteins affects rates of PGC-1α mRNA decay (87), and the trends for increased PGC-1α protein in the current study, we next examined the content of HuR, CUG-BP1 and AUF1 proteins
following 4 weeks of training. There were no significant changes (P > 0.05) in the mRNA stabilizing protein HuR (Fig. 3.5 A) or the destabilizing protein CUG-BP1 (Fig. 3.5 B; P=0.13) post-training. In contrast, the mRNA destabilizing protein AUF1 increased (P<0.05) post-training. Specifically, training increased the expression of two of the three AUF1 isoforms quantified (37kDa and 40kDa; Fig. 3.5 C-F).

**Figure 3.5 - HuR, CUG-BP1 and AUF1 total cellular proteins following 4 weeks of training.** Compared to before training (control; C), 4 weeks of treadmill training (4wk Trained; T) did not change the content of Human Antigen R (HuR; A) or CUG binding protein 1 (CUG-BP1; B). In contrast, training increased two of the three AU-rich binding factor 1 (AUF1) isoforms quantified (D-F). N=6 for all independent experiments. Data are expressed as mean ± S.E.M. *p<0.05, significantly different from control.

As a result, both 1 week and 4 weeks of treadmill training promote an mRNA destabilizing environment, as indicated by a reduced ratio of HuR/ CUG-BP1 (Fig. 3.6 A) and HuR/AUF1 (Fig. 3.6 B), respectively. In addition, these ratios displayed negative Pearson correlation coefficients (P<0.05 for HuR/AUF) with PGC-1α protein, suggesting
that PGC-1α protein levels are highest in an environment with the lowest HuR/AUF ratio (Fig. 3.6 C and D), and therefore the least stable mRNA.

Figure 3.6 - Training promotes an mRNA destabilizing environment.
The ratio of HuR / CUG-BP1 was decreased following 1wk of training (A), while the ratio of HuR / AUF1 was decreased following 4wk of training (B). All AUF1 bands were summed for the calculation of this ratio. Pearson correlations between PGC-1α and both HuR/CUG-BP1 (C) and HuR/AUF1 (D) suggest that PGC-1α protein is the highest when these ratios are the lowest. N=5-6 for all independent experiments. Data are expressed as mean ± S.E.M. *p<0.05, significantly different from control.

3.4.4 Nuclear abundance of mRNA stabilizing/destabilizing proteins in response to acute and chronic exercise

Given the unexpected finding that chronic exercise increased the apparent ‘destabilizing’ environment, we further sought to investigate the sub-cellular location of HuR, CUG-BP1 and AUF1 following exercise by determining the nuclear content of these proteins. As an initial experiment we used resting muscle to show that in nuclei
devoid of cytosolic contamination (ie. LDH), HuR, CUG-BP1 and AUF1 could all be detected (Fig. 3.7 A). In particular, AUF1\textsuperscript{p37} and AUF1\textsuperscript{p40} were not detected in nuclear extracts, while a large band that spanned 42-45 kDa was present (Fig. 3.7 A). Given the presence of all proteins within the nucleus, we next determined the effect of acute exercise and recovery on the nuclear content of these targets. Similar to a lack of an acute exercise-induced increase in mRNA, acute exercise and recovery did not alter the nuclear content of HuR (Fig. 3.7B), CUG-BP1 (Fig. 3.7 C) or AUF1 (Fig. 3.7 D). In addition, chronic exercise training for 4 weeks did not alter the nuclear content of HuR or CUG-BP1 (Fig. 3.7 E and F, respectively). In contrast, the 42-45 kDa band corresponding to AUF1 was increased ~35% in isolate nuclei following training (Fig. 3.7 G), similar to the observed increase in total cellular AUF1 protein.
Figure 3.7 - Nuclear localization of HuR, CUG-BP1 and AUF1 following acute and chronic exercise. HuR, CUG-BP1 and AUF1 were all detected in nuclei devoid of lactate dehydrogenase (A). Acute exercise (post) and 3 hours of recovery (3Hr post) did not increase the nuclear content of HuR (B), CUG-BP1 (C) or AUF1 (D). 4 weeks of treadmill training (4 Wk trained) also did not increase the nuclear content of HuR (E) or CUG-BP1 (F). In contrast, AUF1 was increased following chronic training (G). N=6 for all independent experiments. Data are expressed as mean ± S.E.M. *p<0.05, significantly different from resting control.

3.5 Discussion

Altogether the current data provides evidence that exercise-induced mitochondrial biogenesis occurs in the absence of total cellular or nuclear increases in the mRNA stabilizer HuR. In contrast, mitochondrial biogenesis occurs in the presence of increased expression (CUG-BP1 and AUF1) and nuclear content (AUF1) of known mRNA destabilizers.
Post-translational modifications, and specifically the previous finding that rates of mRNA decay influences steady-state mRNA concentrations and ultimately the expression of a gene (103, 130), provides additional regulation that may influence the expansion of mitochondrial volume observed with training. Despite the apparent importance of mRNA stability, a paucity of literature exists regarding the malleability of this system in response to exercise. However a recent report has suggested that the *in vivo* ratio of HuR/AUF1 directly regulates the *in vitro* stability of PGC-1α mRNA (87). Both HuR and AUF1 utilize AU rich binding motifs, and have been shown to antagonize each other to coordinate mRNA stability (209). While HuR and CUG-BP1 utilize different binding regions, HuR has previously been shown to antagonize miRNA-mediated repression, despite miRNA binding motifs being proximal to HuR binding sites (271). Therefore, the apparent relationship previously suggested for HuR/AUF1 in the *in vitro* regulation of PGC-1α mRNA stability (87) may also extend to HuR/CUG-BP1, as the overall stability of mRNA likely reflects the competitive and non-competitive balance of several stabilizing related proteins. In the context of exercise-induced mitochondrial biogenesis, an increase in these ratios, due to either increases in HuR or decreases in AUF1 or CUG-BP1 proteins, could provide a novel level of regulation. However, the data in the current study does not support a strong relationship between these mRNA stabilizing ‘ratios’ and PGC-1α protein or mitochondrial content. This is exemplified by the finding that the ratio of HuR/CUG-BP1 and HuR/AUF1 display negative Pearson correlation coefficients with PGC-1α protein following training. HuR was constant throughout the exercise-training period, and therefore the negative correlation was entirely dependent on the increased content of CUG-BP1 and AUF1. Therefore, despite
training-induced mitochondrial biogenesis, exercise creates a cellular environment promoting mRNA destabilization (increased the relative abundance of CUG-BP1 and AUF1\textsuperscript{p37} and AUF1\textsuperscript{p40} following 1 and 4 weeks of training, respectively). Altogether, increased expression of HuR, or decreased expression of CUG-BP1 or AUF1, are not required for the induction of mitochondrial biogenesis, and therefore likely do not contribute to exercise-induced increases in PGC-1\(\alpha\) protein. However, the increase in AUF1 protein following training may account for the previous observation of reduced \textit{in vitro} PGC-1\(\alpha\) mRNA stability following chronic muscle contraction (208). This could result from AUF1 directly interacting with PGC1\(\alpha\) mRNA, or through augmenting dicer content/activity (1).

The current data suggests CUG-BP1 protein does not regulate PGC-1\(\alpha\) protein accumulation; however, it is possible that CUG-BP1 influences exercise-induced adaptations through alternative mechanisms. While the exact physiological role of CUG-BP1 remains poorly characterized, a recent study used immunoprecipitation in concert with oligonucleotide microarrays to identify 613 putative CUG-BP1 targets primarily related to cell growth and apoptosis (327). Therefore, the early increase in CUG-BP1 could potentially regulate these pathways, as opposed to mitochondrial biogenesis. However, it should be pointed out that NADH dehydrogenase (ubiquinone) Fe-S protein 2, a mitochondrial electron transport chain protein, has been suggested to be a target of CUG-BP1 (327), and therefore the increase in CUG-BP1 before mitochondrial biogenesis remains surprising. We also provide evidence that chronic training promotes an environment affiliated with mRNA decay, as 4 weeks of training increased two isoforms of AUF1 (AUF1\textsuperscript{p37} and AUF1\textsuperscript{p40}). Similar to CUG-BP1, the functional role of AUF1
remains to be fully elucidated, but given the divergent temporal response to exercise training, it is likely that CUG-BP1 and AUFI regulate different cellular responses. While over-expression and knockdown of AUFI in HeLa cells led to expected changes in the mRNA content of known targets (decreased and increased, respectively), supporting AUFI’s purported role as an mRNA destabilizer, these two approaches did not affect the same targets (254). These differences may reflect alterations in the relative isoform expression of AUFI following over-and-under expression experiments, however this remains to be determined. The two AUFI isoforms that were increased in the current study at the total cellular level following 4 weeks of training (AUFI_p37 and AUFI_p40) are located in both the nuclei and cytosol in various cell lines; however, in the current study we provide evidence that AUFI_p37/p40 are only located in the cytosol of mature mammalian muscle. In contrast, the two higher molecular weight isoforms (AUFI_p42 and AUFI_p45) are localized exclusively within the nuclei (13, 410, 429), and in the current study were increased following chronic training. Given the divergent cellular localization of the various AUFI isoforms it is likely that the higher and lower molecular weight isoforms are regulated by distinct mechanisms. This is plausible given that both AUFI_p37 and AUFI_p40 isoforms are lacking a large domain in the C terminus encoded by exon 7 (434). Moreover, it should be noted that AUFI_p40 contains two phosphorylation sites within a region encoded by exon 2 that are not present within AUFI_p37 (410).

Following transcription, AUFI is spliced into four isoforms (399), of which it has been hypothesized that the AUFI_p42 isoform possesses the greatest biological effect (70). Unfortunately in the current study we were unable to reliably quantify the AUFI_p42 band at the total cellular level as it was extremely faint. This is unlikely a methodological
limitation, as the antibody detected all four isoforms with HeLa positive control extracts, of which the AUF1\textsuperscript{p42} band was the most prominent (Fig. 4C). Further, AUF1\textsuperscript{p42} was detected in isolated nuclei from muscle. Previous research in rats has found a prominent AUF1\textsuperscript{p42} band in skeletal muscle using the same antibody (87); therefore it is currently unclear if this represents species variability in the relative expression of each isoform. Future research examining the functional consequence and biological importance of each isoform within mature mammalian muscle is clearly warranted.

In the current study we report typical increases in markers of mitochondrial content with training (147), and these changes were affiliated with strong trends for an increase in PGC-1\textalpha content; fitting with previous reports in human skeletal muscle (49, 158, 206, 304, 335). SIRT1 is an NAD\textsuperscript+ dependent type III deacetylase, which although is not required for exercise-induced mitochondrial biogenesis (308), has nevertheless been proposed to regulate exercise induced activation of PGC-1\textalpha (57). In the current study we found no change in SIRT1 protein, supporting our previous work divorcing the relationship between total SIRT1 protein and the induction of mitochondrial biogenesis following chronic AICAR administration and chronic-low frequency stimulation in rats (132). In addition, others have previously shown that mitochondrial biogenesis ensues following training in human skeletal muscle despite unaltered SIRT1 protein (131); however this is not uniformly supported (237).

3.5.1 Perspectives and Significance

The present study provides evidence that exercise training increases the protein content of CUG-BP1 and various AUF1 isoforms, suggesting mitochondrial biogenesis occurs in an environment with an increased capacity for mRNA destabilization.
Therefore, two potential explanations exist, 1) mRNA stabilizers/destabilizers are externally regulated to influence mRNA stability and rates of mitochondrial biogenesis, or 2) exercise induces mRNA destabilization as an initial signal to induce cellular remodeling. To date, direct evidence that HuR, CUG-BP1 and AUF1 are externally regulated, while probable, does not exist. However, it was shown in the mid 1990’s that continuous muscle contraction caused a decrease in a mRNA-protein interaction that occurred in the 3’-UTR region of cytochrome c (420). Interestingly, the molecular weight detected coincides with AUF1 (420). Although speculative, these data may suggest that continuous muscle contraction initially (5 days; (420)) decreases the interaction between AUF1 and mRNA to promote mRNA stability during exercise. It is likely that this regulation involves covalent modification, as opposed to changes in subcellular location, as the current data provides evidence that alterations in the nuclear content of HuR, CUG-BP1 and AUF1 do not represent a major regulatory point in the induction of mitochondrial biogenesis. In support of the second possibility, it has previously been shown using several models (i.e. denervation and muscle contraction/increased load) that protein degradation is involved in cellular remodelling and protein synthesis (219). Indeed, in the late 1970’s Millward and colleagues showed that muscle contraction increased protein degradation throughout a 4-week intervention and suggested that increased protein degradation was required to cause protein synthesis (220). In this respect, a decrease in mRNA stability, as a result of increased CUG-BP1 or AUF1, could alter the balance towards protein breakdown as an initial signal to induce mitochondrial biogenesis. Clearly the role of mRNA stabilizers/destabilizers in skeletal muscle remodeling requires further investigation.
CHAPTER 4:

LINOLEIC AND α-LINOLENIC ACID BOTH PREVENT INSULIN RESISTANCE BUT HAVE DIVERGENT IMPACTS ON SKELETAL MUSCLE MITOCHONDRIAL BIOENERGETICS IN OBESE ZUCKER RATS

Presented as published:

4.1 ABSTRACT

The therapeutic use of polyunsaturated fatty acids (PUFA) in preserving insulin sensitivity has gained interest in recent decades; however, the roles of linoleic acid (LA) and α-linolenic acid (ALA) remain poorly understood. We investigated the efficacy of diets enriched with either LA or ALA on attenuating the development of insulin resistance (IR) in obesity. Following a twelve-week intervention, LA and ALA both prevented the shift towards an IR phenotype and maintained muscle-specific insulin sensitivity otherwise lost in obese control animals. The beneficial effects of ALA were independent of changes in skeletal muscle mitochondrial content and oxidative capacity, as obese control and ALA treated rats showed similar increases in these parameters. However, ALA increased the propensity for mitochondrial H$_2$O$_2$ emission and catalase content within whole-muscle, and reduced markers of oxidative stress (4-HNE and carbonyl content). In contrast, LA prevented changes in markers of mitochondrial content, respiratory function, H$_2$O$_2$ emission and oxidative stress in obese animals, thereby resembling levels seen in lean animals. Together, our data suggests that LA and ALA are efficacious in preventing IR but have divergent impacts on skeletal muscle mitochondrial content and function. Moreover, we propose that LA has value in preserving insulin sensitivity in the development of obesity; thereby challenging the classical view that n-6 PUFAs are detrimental.
4.2 INTRODUCTION

Skeletal muscle, given its mass and capacity for insulin-stimulated glucose uptake, has been implicated in the development of IR in obesity. The use of PUFA as a treatment modality has gained considerable interest, with a particular emphasis on the insulin-sensitizing effects of long-chain n-3 PUFA [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] (214, 281, 370). EPA and DHA are proposed to improve insulin sensitivity through remodeling of mitochondrial membrane phospholipid composition (192), reduced intramuscular accumulation of reactive lipid intermediates (214), and increased transcription of gene targets involved in mitochondrial biogenesis and fatty acid oxidation (FAO) (175, 214, 281). Together, the prominent theory surrounding n-3 PUFA and insulin sensitivity suggests improvements in mitochondrial bioenergetics.

Mitochondrial dysfunction is a central hypothesis in the progression of skeletal muscle IR and is traditionally characterized by reduced content or impairment of function affecting rates of FAO (186). However, given that increased mitochondrial content can parallel the development of IR (134, 150, 387) and that the capacity of ATP production far exceeds reductions in content (146, 163), the relationship between mitochondrial dysfunction and IR remains largely unresolved. Recently, alterations in mitochondrial bioenergetics have also been associated with increased reactive oxygen species (ROS) emission-induced IR (9), while pharmacological and genetic approaches that increase antioxidants prevent diet-induced IR (31, 226). Therefore, therapies that improve mitochondrial oxidative phosphorylation or reduce mitochondrial ROS emission may be particularly advantageous. While n-3 PUFA appear ideal in mitigating IR, improved
insulin sensitivity with EPA and DHA occurs independent of improvements in mitochondrial content or function (214), but is known to increase mitochondrial susceptibility to oxidative damage (i.e. lipid peroxidation) and propensity to emit ROS (214). Clearly the mechanistic relationship between long-chain n-3 PUFA and insulin sensitivity remains to be fully delineated.

In comparison to EPA and DHA, little is known about the relationship between IR and α-linolenic acid (ALA). Although ALA can be endogenously converted into EPA/DHA, tracer studies have revealed that the conversion efficiency is low (< 8%) (320, 407). Therefore, it is conceivable that ALA and EPA/DHA have divergent effects on insulin sensitivity, although this remains to be shown. In contrast, n-6 PUFA have traditionally been viewed as detrimental to insulin sensitivity, in part, because they serve as precursors for the production of pro-inflammatory eicosanoids (119); however, this view has been challenged as accumulating evidence suggests not all n-6 PUFA are pro-inflammatory (178). Interestingly, LA may also influence mitochondrial function as it is the predominant fatty acyl moiety in the mitochondrial-specific phospholipid species cardiolipin (192). Current estimates suggest that n-6 PUFA are consumed in 5 to 20-fold greater amounts than n-3 PUFA (51); however, the health benefits of LA supplementation remain ambiguous. This highlights the need to study LA and the mechanisms by which it may influence IR in obesity.

We therefore investigated in young obese Zucker rats if LA and ALA enriched diets could prevent the expected age-related decline in glucose homeostasis. Skeletal muscle mitochondria exist in two spatially distinct subpopulations known as subsarcolemmal (SS) and the predominant intermyofibrillar (IMF) mitochondria. These
subpopulations possess unique characteristics (29, 148, 264, 293) and respond differently to various metabolic perturbations in obesity and type 2 diabetes (T2D) (74, 150, 329), as well as changes in diet composition (69, 234). We therefore also determined subpopulation-specific responses of SS and IMF mitochondria to LA and ALA enriched diets, and the necessity of adaptations within these mitochondria in mitigating IR. Altogether our data suggest that both LA and ALA prevented impairments in whole-body glucose homeostasis consistently seen with obese Zucker rats, and have differential effects on SS mitochondrial content and function.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Five-week old male lean (n=48) and obese (n=48) Zucker rats were purchased from Charles River. Animals were housed in a temperature-regulated room on a 12:12 hr light-dark cycle with water available ad libitum. Control animals were given unrestricted access to control diet while treated animals within each genotype were pair-fed to match for caloric content. After twelve-weeks animals were randomly assigned to either determine whole body and muscle specific insulin sensitivity (n=6) or for assessments of mitochondrial bioenergetics (n=10). Anesthesia (60 mg/kg sodium pentobarbital injection), animal care, and housing procedures were approved by the University of Guelph Animal Care Committee.

4.3.2 Diets and Feeding

All diets used in the present study were purchased through Research Diets (New Brunswick, NJ, US). Daily food consumption of lean and obese rats fed the control diet
(#AIN-93G; 20% protein, 64% carbohydrate and 16% fat) was recorded by weight in order to pair-feed rats given LA (#AIN-93G + 10% safflower oil; 20% protein, 54% carbohydrate and 26% fat) and ALA (#AIN-93G + 10% flaxseed oil; 20% protein, 54% carbohydrate and 26% fat) supplemented diets. Diet fatty acid composition was confirmed by gas-chromatography.

4.3.3 Whole-body glucose and insulin tolerance

Four-hour fasted animals underwent an intraperitoneal glucose (IPGTT, 2 g/kg) and insulin (IPITT, 1.0 U/kg) tolerance test separated by 48 hours, as previously described (173).

4.3.4 Muscle specific insulin signaling

To determine the phosphorylation of proteins involved in insulin-mediated signaling by Western blotting (described below) muscle was excised before and 15 minutes after an intraperitoneal insulin injection (1.0 U/kg), and rapidly frozen in liquid nitrogen.

4.3.5 Skeletal muscle mitochondrial isolation

Isolation of SS and IMF mitochondria was achieved by differential centrifugation. The respective speeds of centrifugation at each step were adapted from previous work (80), as well as the chemical composition of isolation buffer (383). The exact protocols used in the present study were previously reported (211).

4.3.6 Mitochondrial Bioenergetics

Rates of mitochondrial oxygen consumption and mitochondrial hydrogen peroxide (H$_2$O$_2$) emission were measured, as previously reported (211). In addition, separate experiments were performed to measure rates of oxygen consumption in the
presence of 25 µM palmitoyl-CoA (P-CoA) + 2 mM malate + 750 µM L-carnitine. A submaximal (100 µM) ADP concentration was used to determine P/O ratios and a saturating ADP concentration (5 mM) to determine maximal P-CoA driven respiration.

4.3.7 Western blotting

Whole-muscle homogenate (n=6) as well as isolated SS and IMF mitochondrial samples were separated by electrophoresis using SDS-PAGE, transferred to polyvinylidene difluoride membranes, and quantified, as previously reported (211). The following commercially available antibodies were used: total and phosphorylated (Thr308 and Ser473) Akt (Cell Signalling), total and phosphorylated (Thr642) AS160 (Cell Signalling), MitoProfile Total OXPHOS antibody cocktail (MitoSciences), adenine nucleotide translocase 1 (ANT1, MitoSciences), ANT2 (Abcam, Cambridge, MA), manganese-superoxide dismutase (SOD2; Abcam), uncoupling protein 3 (UCP3, Abcam), and 4-hydroxynonenal (Alpha Diagnostics). All samples were detected from the same Western blot by cutting gels and transferring onto a single membrane to limit variability. Equal loading of protein was verified using Ponceau staining.

4.3.8 Protein Carbonylation

The commercially available Oxyblot Protein Oxidation Detection Kit (Millipore; Billerica, MA) was used to assess protein carbonylation, as previously described (292).

4.3.9 Statistics

A one-way ANOVA, followed by a Newman-Keuls Multiple Comparison post-hoc analysis was used to determine the effects of LA and ALA supplementation within genotypes. It was determined that diets did not affect markers of interest in lean animals, thus permitting the use of an unpaired Student’s t-test to compare diet-matched lean and
obese Zucker rats for subsequent analyses (Figures 1-7). A \( p \leq 0.05 \) was considered statistically significant.

### 4.4 RESULTS

#### 4.4.1 LA and ALA maintain whole-body glucose homeostasis

LA and ALA did not alter glucose or insulin tolerance in lean animals (Figure 4.1A, C). In contrast, obese control rats had elevated fasting blood glucose compared to control lean rats (13.9±2.1 mM vs. 5.0±0.2 mM), which resulted in an increased AUC during both glucose and insulin intolerance tests. However, when the baseline values where adjusted to take into consideration the obesity related increase in fasting blood glucose, consumption of both ALA and LA prevented glucose and insulin intolerance in obese animals (Figure 4.1C-F). Specifically, the baseline value during the IPGTT was constrained as the lowest individual glucose concentration within each genotype (lean = 3.9 mM and obese = 5.5 mM) (Figure 1E), while during the IPITT individual baseline values were set as the lowest blood glucose value of each animal. This method adjusts for the elevated basal glycaemia of obese control rats, allowing for a more concrete assessment of glucose and insulin action independent of fasting blood glucose levels. Accordingly, we report that obese control rats exhibit a substantially greater AUC during both glucose (+70%) and insulin challenges (+84%) relative to lean controls, while no differences were observed between diet-match animals fed LA and ALA (Figure 4.1E and F). Altogether, these data suggest that both LA and ALA prevented the development of insulin resistance in obese Zucker rats.
Figure 4.1 – Intraperitoneal glucose (IPGTT) and insulin (IPITT) tolerance tests for (A, C, respectively) lean and (B, D, respectively) obese rats. Black circles = control diet; black triangles = ALA diet; black squares = LA diet. Area under the curve (AUC) values for (E) IPGTT and (F) IPITT. Data expressed as means ± SEM. n = 6 for each measure. + significantly different from obese control (P < 0.05). * significantly different from diet-matched lean animals (P < 0.05).
4.4.2 LA and ALA preserve skeletal muscle insulin signaling

Several parameters can influence whole body glucose and insulin tolerance independent of skeletal muscle insulin sensitivity (e.g. glucose/insulin actions within adipose tissue, liver and pancreas). Therefore it was important to specifically determine skeletal muscle insulin sensitivity in obese animals following LA and ALA supplementation. To determine this we next investigated the ability of insulin to induce phosphorylation of proteins involved in the canonical insulin-signaling cascade. Within lean and obese animals there were no differences in total content of Akt and AS160 protein (Figure 4.2A). In obese control animals, insulin failed to stimulate phosphorylation of Akt at serine 473 (Figure 4.2B) and threonine 308 (Figure 2C), as well as AS160 at threonine 642 (Figure 4.2D), above basal levels (Figure 4.2A). In contrast, obese rats supplemented with ALA maintained insulin-induced phosphorylation of Akt Ser473 (+100%) and Thr308 (+75%), as well as AS160 Thr642 (+40%) (Figures 2B-D). While LA evoked similar improvements in Akt phosphorylation at both sites, no changes were seen with AS160 (Thr642). These data, in combination with the IPITT results, suggest both LA and ALA maintain skeletal muscle insulin signaling in obese Zucker rats.
Figure 4.2 – Skeletal muscle insulin-signaling proteins in basal state and following insulin injection.
(A) Representative blots of total and phosphorylated Akt (B, serine 473; C, threonine 308) and (D) AS160 threonine 642. Data for insulin-stimulated phosphorylation are expressed as means ± SEM of the percentage change from the basal state. n = 6 for each measure. * significantly different from basal state of same animal (P < 0.05).

4.4.3 ALA preferentially increases ETC proteins in SS mitochondria

The accumulation of OXPHOS proteins in whole-muscle extracts was not different following ALA or LA supplementation compared to the control (Figure 4.3 A-F).
Figure 4.3 – Skeletal muscle homogenate mitochondrial OXPHOS proteins.
(A) Representative blots reveal no changes in (B) complex I subunit NDUFB8; (C) complex II subunit 30 kDa; (D) complex III subunit Core 2; (E) complex IV subunit 4; (F) ATP synthase subunit α (F) of obese animals in comparison to diet-matched lean animals. n = 6 for each measure. Data expressed as mean ± SEM.

We therefore re-examined OXPHOS protein content in purified SS and IMF mitochondrial fractions. Compared to lean controls, obese control rats showed a significant increase (+100%) in ATP synthase content in SS mitochondria only (Figure 4.4 A, F). Interestingly, SS mitochondria from obese ALA rats showed significant increases in complex I subunit NUDFB8 (+100%), complex III subunit core 2 (+80%), and ATP synthase (+150%) as observed in obese control animals (Figures 4.4 A, B, D, F). In contrast, the content of electron transport chain (ETC) markers in mitochondria
isolated from LA supplemented rats appeared identical to diet-matched leans (Figures 4.4 A-F), suggesting an absence of compensatory adaptations. Finally, IMF mitochondria remained constant for all OXPHOS protein targets measured across all groups (Figure 4.4 A-F), likely accounting for the inability to detect SS mitochondrial adaptations at the whole-muscle level.

Figure 4.4 – Changes in OXPHOS proteins of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria.
(A) Representative blots for (B) complex 1 subunit NDUFB8; (C) complex II subunit 30 kDa; (D) complex III subunit Core 2; (E) complex IV subunit 4; (F) ATP synthase subunit α (F). n = 10 for each measure. Data are expressed as mean ± SEM. * significantly different from diet-matched lean animals (P < 0.05).
4.4.4 LA prevents compensatory bioenergetic adaptations in obesity

We next determined if mitochondrial bioenergetics were altered by measuring rates of oxygen consumption and \( \text{H}_2\text{O}_2 \) emission in isolated SS and IMF mitochondria. To confirm the integrity of our isolation protocol, mitochondrial ADP:O and respiratory control ratios (RCR), as well as absolute values of state 3 and 4 respiration, are presented in Table 4.1. Following analysis it was determined that mean values for all lean animals did not vary significantly; therefore, for simplicity, we present subsequent analyses as a percent change corresponding to diet-matched lean animals.

**Table 4.1 – Mitochondrial respiratory characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Lean Control</th>
<th>Lean ALA</th>
<th>Lean LA</th>
<th>Obese Control</th>
<th>Obese ALA</th>
<th>Obese LA</th>
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<tr>
<td><strong>SS Mitochondria – Pyruvate Respiration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P/O Ratio</td>
<td>2.47 ± 0.07</td>
<td>2.56 ± 0.08</td>
<td>2.53 ± 0.06</td>
<td>2.57 ± 0.08</td>
<td>2.49 ± 0.09</td>
<td>2.56 ± 0.05</td>
</tr>
<tr>
<td>RCR</td>
<td>10.9 ± 1.21</td>
<td>9.6 ± 0.92</td>
<td>10.5 ± 1.21</td>
<td>13.2 ± 1.27</td>
<td>10.4 ± 1.11</td>
<td>12.9 ± 1.35</td>
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<tr>
<td>State 4</td>
<td>24.5 ± 3.28</td>
<td>21.1 ± 1.68</td>
<td>25.1 ± 3.38</td>
<td>26.6 ± 3.79</td>
<td>30.5 ± 3.21</td>
<td>18.9 ± 1.60*</td>
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<tr>
<td>State 3</td>
<td>247 ± 39.8</td>
<td>184 ± 13.9</td>
<td>262 ± 39.5</td>
<td>327 ± 41.6</td>
<td>297 ± 42.7</td>
<td>234 ± 23.0</td>
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<tr>
<td><strong>IMF Mitochondria – Pyruvate Respiration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/O Ratio</td>
<td>2.48 ± 0.08</td>
<td>2.49 ± 0.07</td>
<td>2.35 ± 0.07</td>
<td>2.42 ± 0.07</td>
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<td>RCR</td>
<td>20.5 ± 3.44</td>
<td>16.2 ± 2.62</td>
<td>15.7 ± 3.03</td>
<td>17.8 ± 3.15</td>
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<td>State 4</td>
<td>26.8 ± 55.2</td>
<td>28.9 ± 52.2</td>
<td>25.8 ± 39.6</td>
<td>23.9 ± 33.5</td>
<td>22.8 ± 49.1</td>
<td>18.2 ± 31.7</td>
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<td>State 3</td>
<td>426 ± 41.2</td>
<td>389 ± 45.1</td>
<td>348 ± 34.5</td>
<td>389 ± 49.3</td>
<td>364 ± 32.7</td>
<td>352 ± 52.5</td>
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<tr>
<td><strong>SS Mitochondria – Palmitoyl-CoA Respiration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/O Ratio</td>
<td>2.27 ± 0.11</td>
<td>2.33 ± 0.1</td>
<td>2.26 ± 0.17</td>
<td>2.49 ± 0.12</td>
<td>2.28 ± 0.12</td>
<td>2.52 ± 0.1</td>
</tr>
<tr>
<td>RCR</td>
<td>9.11 ± 1.02</td>
<td>6.3 ± 0.58</td>
<td>5.75 ± 0.55</td>
<td>7.13 ± 0.7</td>
<td>9.34 ± 1.21</td>
<td>8.23 ± 0.72</td>
</tr>
<tr>
<td>State 4</td>
<td>14.2 ± 1.99</td>
<td>16.8 ± 1.85</td>
<td>22.9 ± 4.52</td>
<td>24.2 ± 2.97</td>
<td>29.7 ± 4.9</td>
<td>28.1 ± 5.7</td>
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<td>State 3</td>
<td>116 ± 11.8</td>
<td>101 ± 13.6</td>
<td>142 ± 30.9</td>
<td>196 ± 34.4</td>
<td>238 ± 31.9</td>
<td>185 ± 33.9</td>
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<tr>
<td><strong>IMF Mitochondria – Palmitoyl-CoA Respiration</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P/O Ratio</td>
<td>2.18 ± 0.22</td>
<td>2.26 ± 0.13</td>
<td>2.16 ± 0.14</td>
<td>2.65 ± 0.13</td>
<td>2.32 ± 0.13</td>
<td>2.33 ± 0.18</td>
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<tr>
<td>RCR</td>
<td>12.8 ± 2.47</td>
<td>11.8 ± 2.29</td>
<td>8.65 ± 1.75</td>
<td>10.2 ± 1.86</td>
<td>14.6 ± 3.11</td>
<td>10.5 ± 1.1</td>
</tr>
<tr>
<td>State 4</td>
<td>19.6 ± 6.91</td>
<td>25.5 ± 3.01</td>
<td>20 ± 16.1</td>
<td>21.7 ± 2.42</td>
<td>20.1 ± 3.94</td>
<td>16.9 ± 2.17</td>
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<td>State 3</td>
<td>187 ± 28.5</td>
<td>158 ± 28.9</td>
<td>173 ± 21.4</td>
<td>220 ± 42.4</td>
<td>229 ± 33.7</td>
<td>178 ± 32.2</td>
</tr>
</tbody>
</table>

Absolute rates of oxygen consumption in the presence (state 3) and absence (state 4) of ADP for isolated subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. Diets groups are control, \( \alpha \)-linolenic acid (ALA) or linoleic acid (LA). State 3 and 4 values are expressed as nmol/min/mg mitochondrial protein. Respiratory control ratios...
(RCR; state 3/state 4) and ADP consumed per unit oxygen (P/O ratio) reflect mitochondrial integrity and coupling. Data are presented as the mean ± SEM. n = 10 for each measure. * Significantly different than obese ALA (p<0.05)

We measured mitochondrial pyruvate and palmitoyl-CoA supported respiration as a primary assessment of respiratory function. In comparison to lean animals, obese controls exhibited significant increases in maximal ETC capacity (complex I and complex I+II) of SS mitochondria only (Figure 4.5 B-F), analogous to that observed in ATP synthase protein content (Figure 4.4). Similar adaptations were seen in obese ALA rats compared to their lean counterparts, including a significant increase in pyruvate-supported state 4 respiration (Figure 4.5 A). In contrast, respiration of SS mitochondria from obese LA rats was identical to lean animals; thus fitting with the observed expression of OXPHOS proteins. Rates of oxygen consumption in IMF mitochondria were similar in all groups and diets. Altogether, it appears that the improvements in whole-body and muscle-specific insulin sensitivity conferred by ALA and LA are associated with distinct impacts on mitochondrial content and function.
4.4.5 Mitochondrial H$_2$O$_2$ emission and markers of oxidative stress

We next determined if PUFA supplementation was associated with a reduced mitochondrial H$_2$O$_2$ emission and oxidative stress. Obese control rats did not display increased SS or IMF mitochondrial H$_2$O$_2$ emission (Figure 4.6 A), while ALA increased maximal H$_2$O$_2$ emission rates in SS mitochondria by 80% (Figure 4.6 A). When
expressed relative to absolute state 4 respiration values (Table 4.1), diet-specific differences in H$_2$O$_2$ emission were abolished and resembled that of lean animals. Interestingly, a recent study showed that EPA/DHA supplementation in mice on a high fat diet increased ROS emission in isolated mitochondria using a similar approach (214). Therefore, we sought to rule out the contribution EPA/DHA-derived lipid radicals, which could potentially interact with amplex red to artificially increase background fluorescence. Using purified EPA and DHA at concentrations known to exist in rat mitochondria, we showed that increased H$_2$O$_2$ emission following ALA supplementation was not a methodological artifact (data not shown). In addition, LA supplementation did not alter maximal H$_2$O$_2$ emission in obese animals (Figure 4.6 A). To assess the implications of the change in maximal H$_2$O$_2$ emission, we quantified protein carbonyls and 4-hydroxynonenal (4-HNE) content (a marker of lipid peroxidation) in whole muscle extracts and in isolated mitochondria. Protein oxidation was not significantly altered by genotype or by diet in whole muscle extracts (Figure 4.6 B) or in isolated mitochondrial fractions (Figure 4.6 C). In contrast, these analyses revealed that, despite unaltered rates of mitochondrial H$_2$O$_2$ emission, obese control animals have a significant increase in total muscle lipid peroxidation (+30%), suggesting the presence of oxidative stress (Figure 4.6 D). Furthermore, 4HNE was increased ~70% within IMF mitochondria of obese controls, but was reduced in SS mitochondria (Figure 4.6 E). Overall, in obesity LA prevented changes in 4HNE content within whole muscle (Figure 4.6 D) and SS/IMF mitochondria (Figure 4.6 E), resembling lean healthy animals. ALA supplementation also prevented increases in 4HNE content within whole muscle (Figure 4.6 D) and IMF mitochondria (Figure 4.6 E) seen in obese control rats.
Figure 4.6 – Markers of oxidative stress.
(A) Maximal succinate H$_2$O$_2$ emission in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria.
(B) Protein oxidation in muscle homogenate and (C) SS and IMF mitochondria. (D) 4-hydroxynonenal (4-HNE) content in muscle homogenate and (E) SS and IMF mitochondria indicates lipid peroxidation. Representative blots shown in respective panels. $n = 10$ for each measure. Data expressed as mean ± SEM. * significantly different from diet-matched lean animals ($P < 0.05$).
4.4.6 Mitochondrial ADP transport, uncoupling, and antioxidant proteins

Given the apparent discrepancy between maximal *in vitro* mitochondrial H$_2$O$_2$ emission rates and *in vivo* markers of oxidative stress, we next examined the expression of proteins known to influence mitochondrial H$_2$O$_2$ emission. ANT1 content did not change in SS mitochondria across all groups and diets (Figure 4.7 A). In contrast, ANT1 content in IMF mitochondria from obese control rats increased significantly (+100%), but were comparable to a lean phenotype in obese LA and ALA groups (Figure 4.7 A). Relative to lean healthy animals, ANT2 was decreased 50% in SS mitochondria of obese controls; however, this change was prevented with LA and ALA supplementation (Figure 4.7 B). In obese rats the abundance of UCP3 (Figure 4.7 C) in SS mitochondria was increased (p<0.05) across all diet groups (control +250%; ALA +300% and LA +500%). Finally, SOD2 content in SS mitochondria of obese controls showed a trending increase (p=0.07) and was significantly elevated in IMF mitochondria (+50%). These changes were prevented by LA and ALA in obese rats (Figure 4.7 D), as SOD2 content was similar to lean animals. In contrast, catalase content in muscle homogenate was not altered in either obese control or LA groups, but was significantly increased (+60%) in obese rats fed ALA (Figure 4.7 E).
Figure 4.7 – Mitochondrial uncoupling and antioxidant defense.
Changes in (A) adenine nucleotide translocase 1 (ANT1), (B) ANT2, and (C) mitochondrial uncoupling protein 3 (UCP3), (D) manganese superoxide dismutase (SOD2) in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. For data shown in (A-D), n = 10 for each measure. (E) Catalase content in muscle homogenate; n = 6 for all groups. Data expressed as mean ± SEM. * significantly different from diet-matched lean animals (P < 0.05).
4.5 DISCUSSION

The current study shows that the development of IR in obesity can be prevented by dietary supplementation with LA and ALA. Strikingly, a moderate 10% isocaloric increase in either of these PUFA species was efficient in attenuating the impaired glucose homeostasis documented in a common genetic model of obesity and insulin resistance. These findings were associated with the conservation of skeletal muscle insulin signaling and oxidative stress relative to lean healthy animals. Examining aspects of mitochondrial dysfunction revealed that LA and ALA have markedly different impacts on SS mitochondrial ETC content and bioenergetics compared to IMF. This was further supported by ALA-specific increases in maximal H$_2$O$_2$ emission in SS mitochondria, as well as the expression of catalase. Overall, the current data supports a beneficial link between ALA and insulin sensitivity, and provides novel evidence that LA can prevent impairments in glucose homeostasis and skeletal muscle insulin sensitivity in a model of severe genetic obesity.

4.5.1 ALA attenuates the development of whole-body glucose homeostasis

Obese Zucker rats display normal glucose and insulin tolerance at 5 weeks of age (435), when the current dietary intervention was initiated, however rapidly display genetic obesity, hyperinsulinemia, hyperlipidemia and peripheral insulin resistance. Therefore, obese Zucker rats represent an attractive model to investigate nutritional approaches that prevent the development of IR (190). While the benefits of fish oil on insulin sensitivity are supported extensively in the literature (205, 214, 281, 349, 370), we provide evidence that ALA, the precursor of EPA and DHA, may also be efficacious in improving insulin sensitivity. Recent work suggests that fish oil supplementation
promotes the expression of OXPHOS proteins in ameliorating IR (214). The current data supports this interpretation, as we observed similar increases in H$_2$O$_2$ emission, OXPHOS proteins and catalase with ALA. We extend this model to show responses exclusive to SS mitochondria, which given the proximity to nuclei, may represent a local signal to drive gene transcription. These results are in agreement with our previous work (25, 150) and work from others (198, 234, 377); supporting the notion that significant changes within SS mitochondria can impact lipid homeostasis and insulin sensitivity.

### 4.5.2 LA prevents the decline of whole-body glucose homeostasis

In comparison to n-3s, n-6 PUFA have garnered a more negative reputation due to their links to oxidative stress, IR, and inflammation. However, multiple studies have challenged this view (18, 114, 119, 317, 350). Indeed, the current data shows that LA prevented the development of whole-body glucose intolerance, maintained muscle specific insulin sensitivity, and 4HNE content in obese animals. In contrast to ALA, these improvements were independent of changes in H$_2$O$_2$ emission, OXPHOS proteins and antioxidant enzyme expression; raising the possibility of a divergent mechanism for improving insulin sensitivity. However, our findings do not exclude the possibility that LA remodels the membrane cardiolipin profile within mitochondria (192), which is known to impact mitochondrial function (149, 192). Although previous reports have linked obesity with changes that would likely promote mitochondrial H$_2$O$_2$ emission, including increased ETC sensitivity to reducing equivalents (218) and diminished sensitivity to ADP (365), we found no changes in maximal ADP-stimulated respiration following LA supplementation. Therefore, it remains possible that LA may alter the
dynamic response of mitochondria to submaximal substrate concentrations. Regardless of the elusive mechanism-of-action, the current data provides convincing evidence that LA prevented IR in obese Zucker rats.

4.5.3 Mitochondrial H$_2$O$_2$ emission, uncoupling and antioxidant proteins

The current study only found increased ETC content within SS mitochondria, which represents ~20% of total cellular mitochondrial volume (113, 159), accounting for the absence of changes in whole muscle measurements. The current study cannot explain mechanistically why SS mitochondria preferentially respond, although this appears to be a conserved observation across cellular stresses (150, 234). However, the increased expression of ETC proteins within the SS mitochondria likely contributed to the increase in maximal mitochondrial H$_2$O$_2$ emission, as normalization of emission rates to state IV respiration negated all differences. The increase in ETC subunits following ALA supplementation may therefore be construed as a negative adaptation, as mitochondrial lipid uptake and ROS emission has been causally linked to IR (9), possibly through ROS-mediated activation of the NF-κB/κB/IKKβ pathway, attenuating insulin signaling by serine phosphorylation of the insulin receptor substrate 1 (IRS1) (359, 424). However, this working model remains controversial, as chronic mitochondrial antioxidant treatment that improves cellular redox balance does not improve insulin sensitivity following a high-fat diet (292). Perhaps subtle increases in mitochondrial ROS emission are required for the transcriptional adaptations necessary to regulate metabolic homeostasis during a high fat challenge. Our data indirectly supports this model as ALA supplementation increased maximal H$_2$O$_2$ emission and OXPHOS proteins in SS mitochondria, as well as
muscle catalase content. Our results are consistent with the suggestion that incorporating n-3 PUFA into mitochondrial membranes increases the propensity for ROS production (6, 214). Furthermore, in vivo markers of oxidative stress (4HNE and carbonyl content) suggest that despite an increase in maximal H$_2$O$_2$ emission, ALA supplementation conserved redox balance in whole-muscle and mitochondrial samples. Given that ROS have several intracellular functions, including participation in the complex signaling network involved in mitochondrial biogenesis (172, 235), perhaps the increased OXPHOS expression within SS mitochondria in the obese ALA group is, in part, mediated through tightly regulated ROS signaling.

In contrast, we found no differences in maximal H$_2$O$_2$ emission or catalase content in lean and obese animals fed LA. Unlike the changes seen with obese control and ALA groups, mitochondria from the obese LA group were identical to their lean counterparts, suggesting that compensatory bioenergetic adaptations are not necessary to preserve glucose homeostasis and muscle insulin signaling while consuming LA. Previous work showed that arachidonic acid increased mitochondrial ROS emission and was linked to mitochondrial dysfunction (78). Surprisingly, the precursor LA did not alter rates of mitochondrial H$_2$O$_2$ emission, and therefore prevention of IR occurs through a mechanism not investigated in the current study. Although speculative, LA was shown to drive a lipoxygenase-mediated eicosanoid response, leading to production of the PPAR-α activating 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE) (197). Therefore, if LA evokes a PPAR-mediated improvement in glucose homeostasis, the mechanism by which it acts may differ from that of n-3 PUFA. Alternatively, LA consumption may involve primary adaptations within the liver, pancreas and white adipose tissue and
secondary responses within muscle, a possibility that has not been explored in the current study. Regardless, the current study provides evidence that LA is beneficial at preventing the development of insulin resistance.

4.5.4 Perspectives and Limitations

The current study provides insight on the link between ALA and insulin sensitivity, and evidence that LA supplementation represents additional therapeutic potential. Although aspects of mitochondrial dysfunction were very similar between obese control and ALA supplemented rats, the preservation of skeletal muscle insulin signaling and whole-body glucose homeostasis highlights the value of this n-3 PUFA. The precise mechanism(s) by which LA and ALA exert their preserving effects requires further elucidation, and may involve changes in liver given the enhanced glucose tolerance. Indeed, EPA/DHA may augment hepatic IR and lipotoxicity by increasing FAO, inhibiting \emph{de novo} lipogenesis and reducing proinflammatory cytokine production (315). Furthermore, within white adipose tissue, these n-3 PUFAs are known to improve factors influencing IR such as adipocyte morphology, rates of endogenous FAO, adipokine secretory profiles as well as immuno-metabolic status (as recently reviewed (315)). Whether LA and ALA exert their effects through similar mechanisms remains to be shown.

In the current study we were unable to uncouple the effects of ALA from EPA and DHA; therefore, it is possible that the effects seen with ALA are due to its conversion (albeit limited) into EPA/DHA. Future work using animal models that prevent the conversion of ALA into EPA/DHA will enable us to more definitively describe the independent role of ALA on skeletal muscle insulin signaling. Also, in the current study
the macronutrient composition of the LA and ALA diets were out of necessity different than control diets, having higher fat (26% vs. 16% in control diet), and by default decreased carbohydrate content (54% vs 64% in control diet). Therefore, future studies should also determine if the modest 10% increase/decrease in dietary fat/carbohydrate could over-ride the strong genetic predisposition for an IR phenotype.

More importantly, our data challenges the traditional view that LA is harmful, and welcomes the reassessment of its use as a therapeutic strategy for preserving insulin sensitivity. Despite observing no changes in mitochondrial content, function and maximal H$_2$O$_2$ emission in isolated mitochondria, LA maintained skeletal muscle insulin signaling similar to ALA. Future investigations should focus on changes in membrane phospholipid composition, as both n-3 and n-6 PUFA are known to compete in the remodeling of membranes including mitochondria, and may also preferentially accumulate in different tissues. The impacts of LA and ALA on IR may transcend the boundaries of skeletal muscle and mitochondria, but nevertheless, represent valuable therapeutic strategies for preventing the development of an insulin-resistant phenotype in obesity.
CHAPTER 5:

LA AND ALA PREVENT GLUCOSE INTOLERANCE IN OBESE MALE RATS WITHOUT REDUCING REACTIVE LIPID CONTENT, BUT CAUSE TISSUE-SPECIFIC CHANGES IN FATTY ACID COMPOSITION

Presented as published:

5.1 Abstract

While the cause of type 2 diabetes remains poorly defined, the accumulation of reactive lipids within white adipose tissue, skeletal muscle and liver have been repeatedly implicated as underlying mechanisms. The ability of polyunsaturated fatty acids (PUFAs) to prevent the development of insulin resistance has gained considerable interest in recent years; however, the mechanisms of action remain poorly described. Therefore, we determined the efficacy of diets supplemented with either linoleic acid (LA) or α-linolenic acid (ALA) in preventing insulin resistance and reactive lipid accumulation in key metabolic tissues of the obese Zucker rat. Obese Zucker rats displayed impaired glucose homeostasis and reduced n-3 and n-6 PUFA content in the liver and epididymal white adipose tissue (EWAT). After the 12-wk feeding intervention, both LA- and ALA-supplemented diets prevented whole body glucose and insulin intolerance, however ALA had a more pronounced effect. These changes occurred in association with n-3 and n-6 accumulation in all tissues studied, albeit to different extents (EWAT>liver>muscle). Triacylglycerol (TAG), diacylglycerol (DAG), ceramide and sphingolipid accumulation were not attenuated in obese animals supplemented with either LA or ALA, suggesting that preservation of glucose homeostasis occurred independent of changes in reactive lipid content. However, PUFA-supplemented diets differentially altered the fatty acid composition of TAGs, DAGs, and PLs in a tissue-specific manner, suggesting essential fatty acid metabolism differs between tissues. Together, our results implicate that remodeling of the fatty acid composition of various lipid fractions may contribute to the improved glucose tolerance observed in obese rats fed PUFA-supplemented diets.
5.2 Introduction

While the etiology of type 2 diabetes is poorly understood, metabolic dysfunction within white adipose tissue (WAT), skeletal muscle and liver have been repeatedly implicated in the development of insulin resistance (84, 201, 388), which represents a strong risk factor for type 2 diabetes (136, 287). A primary working model suggests that fatty acid oversupply to peripheral tissues results in insulin resistance (354, 374, 388). Exactly how ectopic lipid accumulation causes insulin resistance in peripheral tissues remains debatable; however, a strong association exists with reactive lipid intermediates. Specifically, diacylglycerol (DAG) accumulation (73, 238, 246, 382, 388, 415) has been proposed to antagonize insulin signaling by mediating protein kinase C (PKC) serine phosphorylation of the insulin receptor substrate (IRS), thereby inhibiting the insulin signaling cascade (169). In addition to DAGs, ceramides have also been associated with the induction of insulin resistance in a variety of tissues (2, 46, 374, 391, 415), as ceramide accumulation reduces the activation of AKT (347) to attenuate GLUT4-mediated glucose uptake (241). Further, reactive lipids have been linked to inflammation, including facilitating the effects of TNFα (367) and inducing JNK signaling (46), which is thought to result in serine phosphorylation-mediated inhibition of the insulin receptor substrate (IRS). In addition, activation of inflammation has been linked to IL-1β mediated reductions in IRS protein, further attenuating the biological effects of insulin (170). Collectively, these studies suggest a causal link between reactive lipid accumulation and insulin resistance in peripheral tissues. Therefore, treatment strategies that reduce reactive lipid species in these tissues may be beneficial in managing and/or preventing insulin resistance.
Diets supplemented with omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have been shown to improve glucose tolerance (214, 252, 281, 370); however, the majority of these past studies have examined eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Very little is known regarding the effects of α-linolenic acid (ALA, 18:3n-3); however, we have previously shown that a diet supplemented with flaxseed (i.e., rich in ALA) prevents obesity-induced insulin resistance (252). The effect of ALA on reactive lipids in diverse tissues is unknown; however, carnitine palmitoyltransferase (CPTI) has a high sensitivity for ALA-CoA moieties (77), and PUFAs in general, and ALA in particular has a high rate of oxidation within skeletal muscle (93). It is therefore possible that preferential oxidation of ALA within mitochondria may reduce the accumulation of reactive lipid species in diverse tissues.

In contrast to the recognized benefits of n-3 PUFAs, diets enriched with omega-6 (n-6) PUFAs have traditionally been viewed as detrimental primarily because they are precursors for pro-inflammatory eicosanoids (119). However, evidence suggests that this notion is overly simplified (178). Additionally, we recently reported that a diet supplemented with safflower (i.e., rich in linoleic acid (LA, 18:2n-6)) protected against obesity-induced insulin resistance (252). The mechanism-of-action involved in LA-mediated improvements in insulin sensitivity, and the potential alterations in reactive lipid profiles in key insulin sensitizing tissues following LA consumption, remain unknown. However, similar to ALA, CPTI, a key rate limiting enzyme for mitochondrial fatty acid oxidation, has a high sensitivity to LA (77) and this PUFA is oxidized to a greater degree in muscle than saturated fatty acids (e.g. stearate) (93). Therefore, in the
current study we aimed to determine if supplementing diets with ALA and LA could improve the reactive lipid profiles of skeletal muscle, liver and epididymal WAT (EWAT) of obese Zucker rats. It was hypothesized that both PUFA-supplemented diets would reduce reactive lipids in all tissues studied.

5.3 Materials and methods

5.3.1 Animals

Five-week old male lean (n=8) and obese (n=24) Zucker rats were purchased from Charles River (St. Constant, Quebec, Canada). Animals were housed in a temperature-regulated room on a 12:12 hr light-dark cycle with water available ad libitum. At 6 weeks of age animals commenced a twelve-week dietary intervention (n=8 per group). Lean and obese control animals were given unrestricted access to control diet, while obese animals fed PUFA-supplemented diets were pair-fed to match for caloric intake of obese controls (see below for details). Thereafter, animals were anesthetized with an injection of sodium pentobarbital (60 mg/kg) and the red tibialis anterior muscle, EWAT and liver were excised and immediately placed in liquid nitrogen. The University of Guelph Animal Care Committee approved all procedures.

5.3.2 Diets and Feeding

All diets used in the present study were purchased through Research Diets (New Brunswick, NJ, US). Daily food consumption of lean and obese rats fed the control diet (#AIN-93G; 20% protein, 64% carbohydrate and 16% fat; 4.00 kcal/gram) was recorded by weight in order to pair-feed rats given LA (#AIN-93G + 10% safflower oil; 20% protein, 54% carbohydrate and 26% fat; 4.24 kcal/gram) and ALA (#AIN-93G + 10%
flaxseed oil; 20% protein, 54% carbohydrate and 26% fat; 4.24 kcal/gram) supplemented diets to ensure calorie consumption was similar across diets. Fatty acid composition of the diets was confirmed by gas-chromatography (Table 5.1).

Table 5.1 – Fatty acid composition of diets

<table>
<thead>
<tr>
<th>Relative (% of total lipid detected)</th>
<th>AIN-93G</th>
<th>AIN-93G+flaxseed oil</th>
<th>AIN-93G+safflower oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>15:0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>10.7</td>
<td>5.2</td>
<td>6.5</td>
</tr>
<tr>
<td>16:1c9</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>17:1c10</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>3.9</td>
<td>3.4</td>
<td>2.5</td>
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<td>15.7</td>
<td>15.5</td>
</tr>
<tr>
<td>18:1c11</td>
<td>1.4</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>18:2n6</td>
<td>53.2</td>
<td>15.6</td>
<td>71.9</td>
</tr>
<tr>
<td>18:2tt</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
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<td>0.4</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>18:1c7</td>
<td>7.3</td>
<td>58.0</td>
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</tr>
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</tr>
<tr>
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<td>0.4</td>
</tr>
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<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
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<td>0.1</td>
<td></td>
<td>0.1</td>
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<tr>
<td>20:2n6</td>
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<tr>
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</tr>
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</tr>
<tr>
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<td>0.1</td>
<td></td>
<td>0.1</td>
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<tr>
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<td>0.1</td>
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</tr>
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<td>0.2</td>
</tr>
<tr>
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<td>0.1</td>
<td></td>
<td>0.1</td>
</tr>
</tbody>
</table>

Gas chromatography was utilized to determine the fatty acid composition of the purchased research diets. ALA; α-linolenic acid, LA; linoleic acid.

5.3.3 Whole-body glucose tolerance

Four-hour fasted animals underwent an intraperitoneal glucose (IPGTT, 2 g/kg) and insulin (IPITT, 1.0 U/kg) tolerance test separated by 48 hr. Blood was collected from a tail vein at various time points and blood glucose was determine using a glucometer (Freestyle Lite, Abbott Laboratories, St. Laurent, QC, Canada). To determine the area under the curve (AUC) during the insulin tolerance test, the baseline values were adjusted to examine the ‘response’ of the insulin injected. Specifically, the AUC baseline was
adjusted to the final glucose value during the ITT in order to negate any starting differences in fasting blood glucose concentrations.

5.3.4 Analysis of tissue lipid content

Lipids were extracted using a modified Folch method (118). Frozen samples were cleaned, freeze-dried and powdered in an aluminium mortar with a stainless steel pestle, pre-cooled in liquid nitrogen. Powdered samples were transferred into glass tubes containing 2 ml of methanol containing an antioxidant (0.01% BHT) at −20°C, followed by a 4 ml addition of chloroform, 1.5 mL of water. To correct for the extraction and assay losses, 100µl of internal standard mixture composed of tri-heptadecanoate (C17:0 TAG), di-heptadecanoate (C17:0 DAG) and di-heptadecanoic phosphatidylcholine (C17:0 PC) was added. Lipids within the chloroform layer were fractionated via thin-layer chromatography (TLC; silica plate 60, 0.25 mm, Merck) using heptane, isopropyl ether and acetic acid (60:40:3 ratio v/v/v) for TAG, DAG and PL separation. Dried plates were sprayed with 0.2% solution of 2’7’-dichlorofluorscein in methanol and briefly exposed to ammonia vapours, while bands were visualized under UV light. Lipid bands were identified according to the standards (Sigma) and scraped off the plates. Separated lipids were methylated in 14% boron trifluoride-methanol (270), while fatty acid methyl esters were extracted with pentane (392). Samples were dissolved in hexane and analysed using a Hewlett-Packard 5890 Series II gas chromatograph, an Agilent J&W CP-Sil 88 capillary column (50 m × 0.25mm I.D.) and flame-ionization detector. The oven temperature was programmed from 130°C to 220°C at 5°C/min and held at 220°C for 32 minutes. Subsequently, retention times of standards and individual fatty acids were quantified. Total amount of each fraction for TAG, DAG and PL were counted as a sum
of identified long chain fatty acids. The following fatty acid species were identified and quantified: myristic (14:0), palmitic (16:0), palmitoleic (16:1n-7), stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), α-linolenic (18:3n-3), arachidic (20:0), arachidonic (20:4n-6), eicosapentaenoic (20:5n-3), behenic (22:0), docosahexaenoic (22:6n-3) and nervonic (24:1n-9) acids.

Ceramide and sphingolipids were measured by HPLC, as previously described (152). Briefly, a small volume (50 µL) of the chloroform phase containing lipids extracted, as described above, was transferred to a fresh tube containing 40 pmol of N-palmitoyl-D-erythro-sphingosine (C17 base) as an internal standard. The samples were evaporated under a nitrogen stream, dissolved in 1.2 mL of 1M KOH in 90% methanol and heated at 90°C for 60 min to convert ceramide into sphingosine. This digestion procedure does not convert complex sphingolipids, such as sphingomyelin, galactosylceramide or glucosylceramide, into free sphingoid bases (259). Samples were then partitioned by the addition of chloroform and water. The upper phase was discarded and the lower phase was evaporated under nitrogen. The content of free sphingosine liberated from ceramide was then analyzed using HPLC as described above. The calibration curve was prepared using N-palmitoylsphingosine (Avanti Polar Lipids) as a standard. The chloroform extract used for the analysis of ceramide contains small amounts of free sphingoid bases. Therefore, the concentration of ceramide was corrected for the level of free sphingosine measured in the same sample.
5.3.5 Western blotting

Whole-muscle homogenate (10 µg) was separated by electrophoresis using SDS-PAGE and transferred to polyvinylidene difluoride membranes. The following commercially available antibodies were used: total and phosphorylated JNK1/2 (Cell Signalling) and total IRS (Millipore). Bands were visualized using enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer), a FluorChem HD2 Alpha Innotech imager and quantified using the software provided. Multiple proteins were detected from the same Western blot by cutting gels before transferring onto a single membrane to minimize variability. As a result, Ponceau staining was used to confirm consistent loading.

5.3.6 Statistics

All statistical analyses were conducted using Prism software (GraphPad Software, Inc. La Jolla, CA, USA). A one-way analysis of variance (ANOVA) was used to determine the effects of LA and ALA supplementation, followed by a Tukey post-hoc test when appropriate. A p < 0.05 was considered statistically significant. For fatty acid analyses in each lipid fraction within a tissue, we also accounted for multiple comparisons using a Bonferroni correction. In these instances significance was accepted as p < 0.0035 (i.e., 0.05 / 14 fatty acids = 0.0035). Values are reported as means ± S.E.M. throughout the manuscript.
5.4 Results

5.4.1 Effects of LA and ALA on whole body glucose homeostasis

Obese animals displayed elevated body weight; however, differences within obese animals were not observed as a result of pair-feeding (Figure 5.1A). Despite a lack of difference in body weight, ALA and LA supplementation attenuated the elevation in fasting blood glucose observed in obese animals (lean: 5.7±0.9 mM; obese: 14.2±1.0 mM; obese ALA: 9.4±1.1 mM; obese LA: 10.0±1.3 mM; p<0.05). In addition, while obese control animals displayed impaired glucose and insulin intolerance (Figures 5.1B-E), the AUCs during glucose and insulin tolerance tests indicated that obese animals supplemented with either ALA or LA were not significantly different from lean animals (Figures 5.1B-E). However, only ALA supplemented animals displayed decreased AUC relative to obese controls (Figures 5.1B-E), suggesting ALA is more efficient in preventing the obese Zucker phenotype.
Figure 5.1 – Glucose and insulin tolerance tests.

The effect of α-linolenic (ALA) and linoleic (LA) acid on body weight (A) intraperitoneal glucose (B) and insulin (D) tolerance tests. Area under the curve (AUC) values for glucose (C) and insulin (E) tolerance tests. Data expressed as means ± SEM. n = 8 for body weight and n=5 for glucose and insulin tolerance tests. Letters indicate similarities between groups. Where letters are absent, significance with an ANOVA was not attained. Body weight was higher in obese animals at all ages although not represented on the figure. Control; Ctrl
5.4.2 Effects of LA and ALA on global tissue lipid profiles

The observed differences in whole body glucose and insulin tolerance between obese animals were not associated with reductions in fasting serum free fatty acid levels (lean; 259±22, obese; 847±170, obese ALA; 957±174, obese LA; 700±65 µM: p<0.05, all obese higher than lean). We therefore next aimed to determine if muscle, liver and EWAT, three tissues known to influence whole body glucose homeostasis, displayed deficiencies in either total n-3 or n-6 PUFAs that could be rectified with ALA or LA supplementation. To accomplish this we summed the n-3 (ALA, EPA and DHA) and n-6 (LA and AA) of the various TAG, DAG and PL lipid fractions within each tissue. This approach revealed that the skeletal muscle of obese control animals exhibited increased n-3 and n-6 PUFAs compared to lean animals (Figures 5.2A, B). In obese animals supplemented with either ALA or LA, muscle tissue levels of n-3 and n-6 PUFAs were further increased, respectively, compared to the obese control. In contrast to muscle, the liver and EWAT of obese animals showed significant depletions in both n-3 and n-6 PUFAs compared to lean animals (Figure 5.2A, B) that were recovered following consumption of the respective diets. Therefore, ALA and LA diets increased n-3 and n-6 PUFAs in all three tissues; however, only liver and EWAT originally showed reductions in these fatty acids in obese animals.
Figure 5.2 – Changes in total tissue content of n-3 and n-6 PUFAs.
The effect of α-linolenic (ALA) and linoleic (LA) acid on total n-3 (A) and n-6 (B) polyunsaturated fatty acids. Values are reported in muscle, liver and epididymal white adipose tissue (EWAT), and are expressed as means ± SEM. n = 8 for each measure. Letters indicate similarities between groups. Where letters are absent, significance with an ANOVA was not attained.
Given the observation that ALA and LA supplementation improved glucose homeostasis and affected peripheral tissues, we next examined the tissue-specific effects on individual lipid fractions. In general, while the obese phenotype increased TAG, DAG and ceramides, neither ALA nor LA altered these responses in the muscle, liver or EWAT (Figures 5.3A-E). The exceptions were that ALA increased TAG within EWAT (Figure 5.3A), increased ceramide within muscle (Figure 5.3D) and decreased sphingolipids within the liver (Figure 5.3E). While total PL content was increased in muscle of obese animals, and decreased in liver and EWAT, neither ALA nor LA altered the total PL content in any of the tissues (Figure 5.3C). Altogether, these data suggest that ALA and LA do not dramatically alter the total tissue levels for TAGs, DAGs, PLs, ceramides or sphingolipids in association with improvements in insulin sensitivity. Therefore, we next examined the fatty acid composition of these lipid fractions within each tissue.
Figure 5.3 – Effect of ALA and LA on major lipid fractions in insulin-responsive tissues.

The effect of α-linolenic (ALA) and linoleic (LA) acid on total triacylglycerol (TAG; A), diacylglycerol (DAG; B), phospholipids (C), ceramides (D) and sphingolipids (E). Values are reported in muscle, liver and epididymal white adipose tissue (EWAT), and are expressed as means ± SEM. n = 8 for each measure. Letters indicate similarities between groups. Where letters are absent, significance with an ANOVA was not attained.
5.4.3 Changes in TAG composition

Within all tissues, consumption of the ALA diet enriched ALA and EPA n-3 PUFAs and concomitantly decreased n-6 PUFAs (LA and AA; Figure 4A-C). The opposite pattern of enrichment was observed following LA supplementation, where n-6 PUFAs were generally increased relative to control animals at the expense of n-3 PUFAs (Figure 5.4A-C). However, there were tissue specific differences in various fatty acids, which are highlighted with boxes in Figure 4. Specifically, the ALA diet increased TAG-DHA within the liver (Figure 5.4B) and did not reduce TAG-LA within EWAT, compared to the obese control (Figure 5.4C). The LA diet systematically reduced TAG-ALA and TAG-DHA in all three tissues, while TAG-EPA was reduced in both liver and EWAT, but not muscle. In addition to the dietary effects on PUFA profiles, ALA and LA also altered the levels of specific monounsaturated (MUFA) and saturated (SFA) fatty acids in the TAG fraction. This is potentially important, as palmitoleic, palmitic and stearic acids have been associated with insulin resistance (152, 388). In the current study neither palmitic acid nor stearic acid in the TAG fraction were altered in any tissue in response to the dietary interventions (Figure 5.4A-C), while across all tissues ALA supplementation decreased TAG behenic acid. In contrast to these consistent tissue responses, LA and ALA decreased TAG palmitoleic acid in the liver only (Figure 5.4A-C). Finally, LA increased TAG nervonic acid only within EWAT (Figure 5.4C). Collectively, these results highlight the relatively consistent responses in TAG composition following LA and ALA supplementation between muscle, liver and EWAT.
Figure 5.4 – Effect of ALA and LA on TAG fatty acid composition.

The effect of α-linolenic (ALA) and linoleic (LA) acid on triacylglycerol (TAG) fatty acid composition. Values for saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids are reported in muscle (A), liver (B) and epididymal white adipose tissue (EWAT; C). Values represent means ± SEM, expressed as a percentage of lean control (dotted line). n = 8 for each measure. Letters indicate similarities between groups. Where letters are absent, significance with an ANOVA and Bonferroni post-hoc test was not attained. Control; Ctrl.
5.4.4 Changes in DAG composition

While the response of DAG-ALA in the three tissues was relatively consistent between the two diets (Figure 5.5A-C), the responses of LA, AA, EPA and DHA were not (highlighted with boxes). Specifically, DAG-AA was only increased in EWAT following LA consumption compared to the obese control. ALA consumption had no effect on DAG-LA levels in the liver, but did reduce DAG-LA in the muscle and EWAT. Further, ALA consumption increased DAG-EPA in only the liver, and increased DAG-DHA within EWAT (Figures 5.5A-C). Overall, the PUFA diets had little effect on the SFA and MUFA composition of DAG; however, both diets reduced DAG myristic and stearic acids in muscle (Figure 5.5A) and increased DAG oleic acid in the liver. Collectively, these results highlight the ability of ALA and LA to decrease the SFA content of DAGs within muscle.
Figure 5.5 – Effect of ALA and LA on DAG fatty acid composition.
The effect of α-linolenic (ALA) and linoleic (LA) acid on diacylglycerol (DAG) fatty acid composition. Values for saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids are reported in muscle (A), liver (B) and epididymal white adipose tissue (EWAT; C). Values represent means ± SEM, expressed as a percentage of lean control (dotted line). n = 8 for each measure. Letters indicate similarities between groups. Where letters are absent, significance with an ANOVA and Bonferroni post-hoc test was not attained. Control; Ctrl.
5.4.5 Changes in total PL composition

In contrast to the liver specific increases observed for DAG-EPA following ALA consumption (Figure 5.5B), all three tissues showed strong increases in PL-EPA with this diet (Figures 5.6A-C). Overall, the changes in PL-LA, PL-ALA and PL-EPA were relatively consistent across tissues. However, in contrast, PL-AA was only increased in muscle following LA consumption, while PL-DHA was increased in liver following ALA consumption (Figures 5.6B, C). The dietary responses of SFA and MUFAs were relatively constant across all tissues studied, with the exceptions that PL palmitoleic and oleic acids were only increased in muscle following ALA supplementation (Figure 5.6A). Collectively, these results highlight the ability of muscle to incorporate AA and DHA into PLs, as well as the ability of EPA to be enriched into the PL fraction in all three tissues.
Figure 5.6 – Effect of ALA and LA on phospholipid fatty acid composition.
The effect of α-linolenic (ALA) and linoleic (LA) acid on phospholipid (PL) fatty acid composition. Values for saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids are reported in muscle (A), liver (B) and epididymal white adipose tissue (EWAT; C). Values represent means ± SEM, expressed as a percentage of lean control (dotted line). n = 8 for each measure. Letters indicate similarities between groups. Where letters are absent, significance with an ANOVA and Bonferroni post-hoc test was not attained. Control; Ctrl.
5.4.6 Changes in markers of tissue inflammation

Given the known link between reactive lipids and inflammation, we next examined JNK phosphorylation, a common marker of inflammation. Only the liver displayed an increase in JNK phosphorylation with obesity, but neither ALA nor LA altered this response (Figure 5.7A-F). In addition, activation of inflammation has been shown to reduce IRS1 protein content (170). In the current study obesity was associated with reductions in IRS1 protein in the muscle (Figure 5.7G) and liver (Figure 5.7H), but not in EWAT (Figure 5.7I). Neither ALA nor LA altered these responses. Combined, these data further substantiate the lack of improvements in reactive lipids observed with ALA and LA supplementation.
Figure 5.7 – Effect of ALA and LA on markers of inflammation and insulin signaling. The effect of α-linolenic (ALA) and linoleic (LA) acid on JNK1 (A-C) and JNK2 (D-F) phosphorylation and IRS1 content (G-I). Values represent means ± SEM, expressed as a percentage of lean control (dotted line). n = 8 for each measure. Letters indicate similarities between groups. Where letters are absent, significance with an ANOVA was not attained. Control; Ctrl.
5.5 Discussion

The current study has investigated the effects of diets supplemented with either ALA or LA on the lipid profiles of key metabolic tissues influencing whole body glucose homeostasis in a rodent model of obesity. Our data show that both ALA and LA prevent glucose intolerance, although ALA is more efficient. However, an attenuation of reactive lipid accumulation (i.e., DAGs and ceramides) is not required for the prevention of whole body glucose intolerance seen with increased consumption of either essential PUFA. Furthermore, the PUFA-supplemented diets differentially altered the fatty acid composition of TAGs, DAGs, and PLs in a tissue-specific manner. Together, our results suggest that remodeling the fatty acid composition of various lipid fractions may contribute to the improved glucose homeostasis observed in obese rats fed PUFA-supplemented diets.

While TAG is now widely considered an inert storage depot (238, 346), reactive lipids have gained considerable interest as a mechanism to cause insulin resistance in diverse tissues (2, 46, 73, 238, 246, 374, 382, 388, 391, 415). However, in the current study, the prevention in whole body glucose and insulin intolerance following PUFA supplementation was not associated with reductions in DAG or ceramide content in any tissue studied, with the exception of ALA reducing hepatic sphingolipids, a lipid fraction strongly associated with WAT insulin resistance (20, 388). The controversy surrounding which lipid species, if any, independently mediate the induction of insulin resistance extends beyond the current observations. Indeed, increases in DAG content can occur without the expected induction of insulin resistance within both muscle (8, 348) and liver (265), suggesting individual lipid species may have a more important role. For example,
DAGs containing stearic acid have been linked to liver and muscle insulin resistance (152, 388). While neither ALA nor LA had any effect on DAG-stearic acid levels within the liver, both diets reduced muscle DAG-stearic acid to levels equivalent to lean controls, which may contribute to the improved glucose homeostasis.

In the current study, despite similar glucose/insulin tolerance, the ceramide content of muscle in ALA supplemented animals was higher than lean controls. Since the ALA diet does not contain higher palmitate concentrations, these data may suggest ALA supplementation altered the metabolism of the liver or EWAT in a manner that increased the delivery of saturated fatty acids to muscle, although this remains speculative. Regardless, the ceramide content of obese animals, in all tissues studied, cannot explain to the prevention of glucose intolerance following ALA and LA supplementation.

The prevention in whole body glucose intolerance in the current study may also extend from rectifying the apparent derangements in essential n-3 and n-6 PUFA content in obese liver and EWAT. The accumulation of PUFAs within the tissues studied may indirectly, through alterations in gene transcription and/or metabolism, affect lipid-sensitive signaling pathways associated with insulin sensitivity. This latter point is particularly noteworthy given that we observed increases in EPA, DHA, and AA levels in the three tissues. Both rodents and humans have the ability to convert ALA into EPA and DHA, as well as LA into AA, through a series of desaturation and elongation steps (known as the FADS pathway) (15, 16, 48). Interestingly, a relationship between reduced FADS pathway activity and impaired insulin action and increased adiposity has been previously reported (295, 404). The FADS pathway is highly active in the liver (279) and we have recently reported that this pathway is also functional in adipocytes (326). Our
fatty acid data clearly shows that inter-conversion of ALA and LA into their longer chain counterparts occurred; however, we our unable to ascertain in which tissue(s) this inter-conversion occurred. Therefore, it is not possible to definitively conclude whether the improvements in glucose homeostasis seen in obese rats supplemented with flaxseed stem from ALA or EPA / DHA or a combination of all three fatty acids. This is pertinent given that Lanza et al. recently reported that whole-body glucose tolerance was improved in mice fed a high-fat diet supplemented with menhaden oil (214), thereby demonstrating that EPA and DHA are capable of improving metabolic health independent of ALA. Future studies in which the FADS pathway is inhibited are required in order to uncouple the inter-conversion of ALA to EPA / DHA, and better delineate the relationship of specific PUFAs and insulin sensitivity.

5.5.1 Perspectives and significance

The present data highlights the ability of both LA and ALA to prevent glucose intolerance in a rodent model of obesity, although the effect is greater following ALA supplementation. The current dietary approach did not replace saturated fatty acids with PUFAs, but rather increased the total fat composition of the diet, suggesting supplementation of essential PUFAs in the diet prevents glucose intolerance in the absence of reductions in either caloric intake or total ingestion of fat. While caution is warranted in extrapolating rodent data to humans, the genetic mutation within the obese Zucker rat (leptin deficiency) results in hyperphagia-induced obesity and insulin resistance, and therefore follows a similar disease progression normally described in humans. While the current results support the idea that PUFA supplementation can improve glucose homeostasis, the underlying mechanism(s) remain debatable. A major
working model for diet-induced insulin resistance is focused on reactive lipids, however neither ALA nor LA reduced total tissue levels of DAGs or ceramides, thereby challenging this long-standing hypothesis. While specific reactive lipid subspecies have been suggested to be more important in the induction of insulin resistance, there was no consistency between tissues. These data either challenge the necessity of increasing reactive lipids in mediating insulin resistance, or suggest tissue specificity exists with respect to which reactive lipids attenuate insulin responsiveness. With respect to this latter point, accumulation of the downstream products of ALA and LA metabolism (i.e., EPA, DHA and AA) within tissues appears to be unique, and future research should focus on elucidating the mechanisms-of-action for this observation.

Unfortunately, the current study does not delineate which tissue(s) contribute to the observed improvement in whole body glucose homeostasis. However, we have previously reported in these same animals that skeletal muscle insulin-stimulated Akt phosphorylation is improved following both ALA and LA supplemented diets, and therefore muscle is likely contributing to the observed responses (252). However, DAG was not reduced in muscle, and ALA actually increased ceramide content. Therefore, it is clear that a reduction in total DAG and ceramide content is not required for improvements in skeletal muscle insulin signaling. In addition, given the more robust accumulation of PUFAs in WAT and the liver, as well as the known large contribution of the liver to post-prandial glucose homeostasis (268), it is likely that these tissues also display an improved response to insulin, although this remains to be determined. Future research utilizing hyperinsulinemic euglycemic clamps with tracers are required to unravel the tissue specific alterations in insulin sensitivity following ALA and LA
supplementation in order to better understand the observations of the current study. Regardless of these gaps in our knowledge, the present data highlights the ability of LA and ALA to prevent the induction of glucose intolerance, and challenges the importance of reactive lipids in mediating these responses.
CHAPTER 6:

INTEGRATIVE DISCUSSION
6.1 Discussion

The focus of this thesis was to examine the impact of lifestyle factors on skeletal muscle mitochondrial bioenergetics and whole-body lipid handling. Although exercise training has long been known to stimulate mitochondrial biogenesis (145), the posttranscriptional regulation remains largely unexplored. This represents an important gap in our understanding given that nuclear gene expression is influenced at the posttranscriptional level (i.e. through changes in mRNA stability), and that mitochondrial biogenesis predominantly requires the expression of nuclear-encoded gene targets. Moreover, the content and function of mitochondria within skeletal muscle can also be altered in pathological contexts such as obesity, where reductions have been linked with the development of IR (267). Nutritional interventions that utilize essential dietary PUFAs (ALA and LA) are proposed to have insulin-sensitizing effects; however, it is unclear whether this is linked to improvements in mitochondrial content and/or function. Accordingly, using lifestyle interventions, this thesis aimed to improve our understanding of the regulation of exercise-induced mitochondrial biogenesis within skeletal muscle. In addition, it was determined if essential dietary PUFAs targeted skeletal muscle mitochondria, or possibly reactive lipid content, within multiple insulin-responsive tissues in the route of protecting against obesity-related IR.

In chapter 3 of this thesis, a common exercise training protocol was used to characterize changes in the expression of RBPs that influence mRNA stability. It was found that mitochondrial biogenesis coincides with increases in the content of RBPs that promote destabilization of mRNA. These results have extended our fundamental understanding of how posttranscriptional events regulate mitochondrial adaptations to
exercise training. In chapter 4, it was demonstrated that ALA and LA supplementation prevented IR in the obese Zucker rat, and investigated whether protection coincided with improvements in mitochondrial bioenergetics within skeletal muscle. However, the protective effects of ALA and LA did not correlate with improvements in mitochondrial bioenergetics, and thus, an alternate hypothesis was explored in Chapter 5. With a principle focus on the accumulation of reactive lipids linked with IR, the lipid profiles of the major insulin-responsive tissues were characterized to elucidate changes in whole-body lipid handling. Importantly, ALA and LA did not alter the total content of DAGs and ceramides. Instead, the observed enrichment of PUFA content within these tissues, including altered FA composition of TAG, DAG and PL lipid fractions, may account for the protective effects of ALA and LA, but requires further exploration. Altogether, studies in this thesis revealed the influence of lifestyle factors in both healthy and pathological conditions, and the implications of these findings will be integrated and discussed in a larger context.

6.2 Obesity and mitochondrial bioenergetics

Chapter 1 presented two working “mitochondrial centric” models of IR. The first model is premised on mitochondrial dysfunction (reduced function and/or content) that diminishes capacity for FAO and thereby promotes ectopic lipid accumulation. The second model describes a state whereby chronic nutrient overload promotes elevated mitochondrial ROS production and oxidative stress. In this regard, data presented in chapters 4 and 5 provide further insight as to whether the protection conferred by ALA
and LA against obesity-related IR occurred through prevention of changes described by either, if not, both of these working models.

### 6.2.1 Considering a role for skeletal muscle mitochondrial dysfunction

Initial evidence supporting a link between skeletal muscle mitochondrial dysfunction and the development of IR emerged in the late 1990s (185, 187). Although mitochondrial dysfunction has since been assessed using a multitude of methods, this model postulates that the accumulation of lipids within skeletal muscle is the result of a reduction in the capacity of mitochondria to oxidize FAs (92, 139, 165, 269, 298, 329, 362). In the context of this thesis, if mitochondrial dysfunction was linked to IR then obese control rats would have a reduction in muscle mitochondrial content and/or function relative to their age and diet-matched lean counterparts. In addition, any protective effects of ALA and/or LA against IR would be expected to prevent such reductions. However, the evidence presented in chapter 4 revealed that, within the conditions of this study, mitochondrial dysfunction was not associated with the development of IR in obese Zucker rats. This aligns with several previous reports showing either no differences or increases in various parameters of mitochondrial bioenergetics, using different models of obesity including Zucker rats and HF fed rodents (90, 134, 148, 153, 172, 285, 368, 387).

Measuring total ETC protein content (complexes I-V) provides an index of changes in mitochondrial content (137, 153, 172, 217, 242, 292, 362). In chapter 4, total ETC protein content in SS and IMF mitochondria was not found to be different between lean control and all obese diet groups, which eliminates a role for reductions in
mitochondrial content. Although not utilized in this thesis, mitochondrial content can also be examined through transmission electron microscopy (TEM) (150, 153, 184, 194, 211, 310, 329). This method provides a means of visualizing SS and IMF mitochondria, quantifying changes in mitochondrial size, density, and whether abnormal morphological features (i.e. swelling, reduced cristae density) are present in pathophysiological conditions. As mitochondria constantly undergo fusion and fission, morphological abnormalities may be linked to impairments in mitochondrial dynamics and bioenergetics (194, 240, 361, 433). In addition, Dahl et al., recently used confocal microscopy and 3-Dimensional reconstruction to visualize mitochondrial networks within muscle. In healthy middle-aged subjects, SS and IMF mitochondria exhibited tubular, highly interconnected profusion networks, compared to a patient with T2D showing more discontinuous profission mitochondrial networks (88). Therefore, techniques that permit visualization of mitochondria may provide additional insight on alterations in mitochondrial function compared to measurements of ETC protein content alone.

Reductions in the intrinsic function of mitochondrial proteins (per unit mitochondria) may also contribute to an overall decrease in mitochondrial oxidative capacity. Using isolated mitochondria from muscle is a common in vitro approach that permits assessment of mitochondrial respiratory capacity (for methods review see (303)). In chapter 4, isolated mitochondria were used to assess function by stimulating respiration using either pyruvate or P-CoA as substrates; however, the maximal respiratory capacity of both SS and IMF mitochondria was not reduced at any point. An advantage of using isolated mitochondria is that SS and IMF mitochondria can be assessed separately to elucidate differences in these subpopulations. However, this
requires large tissue samples (≥ 150 mg) and the differential centrifugation process used can alter functional properties by disrupting interaction between subpopulations, and with other cellular components occurring in vivo (303). Nevertheless, while IR has been linked to deficiencies in the oxidative capacity of either SS mitochondria (69, 234, 329) or IMF mitochondria (74), results from this thesis are consistent with a large body of evidence that has divorced this relationship (40, 134, 154, 229, 285, 362, 368, 387).

It is important to acknowledge that in chapter 4, mitochondrial function was assessed using saturating concentrations of ADP to promote a maximal rate of respiration. However, as described earlier, ADP availability can limit respiration under most physiological conditions (409), and thus may not reflect in vivo conditions. Instead, using submaximal ADP concentrations can provide an index of mitochondrial function that is more reflective of the in vivo state. Although not assessed in this thesis, it is conceivable that ALA and LA protected against IR by preserving mitochondrial ADP sensitivity, and therefore warrants consideration.

6.2.2 ALA and LA protect against IR by maintaining mitochondrial ADP sensitivity

Findings presented in chapter 4 are among the first to examine the independent effects of ALA and LA on mitochondrial respiratory function in obesity. Changes in submaximal ADP sensitivity were not assessed using isolated mitochondria, however, results in chapters 4 and 5 intriguingly suggest a possible impact of ALA and LA supplementation. In chapter 5 it was reported that 12 weeks of ALA or LA supplementation respectively increased their total content within skeletal muscle, WAT and liver. While mitochondrial membrane composition was not measured in this thesis,
previous work has demonstrated that dietary PUFA consumption can lead to increases of these FAs in mitochondrial membranes. In rats, 9 weeks of LA supplementation (20% safflower oil w/w) increased LA content in cardiolipin (~2 fold increase; no change with ALA), a lipid fraction present in the IMM (107). Also, 12 weeks of fish oil supplementation (EPA/DHA) in human subjects increased n-3 PUFA content in isolated mitochondrial membranes (137). In this study it was proposed that PUFA-enriched mitochondrial membranes may directly alter ADP sensitivity, or indirectly by modifying the content and/or function of proteins involved in ADP transport and ATP synthesis (137). In chapter 4, ALA and LA prevented reductions in ANT2 content in SS mitochondria, which was observed in obese control rats. This is important because ANT2 has a high transport efficiency and capacity for ADP/ATP transport across the IMM (98) and reductions may therefore impact mitochondrial function. Whether preservation of ANT2 content in ALA and LA fed rats affected submaximal ADP sensitivity remains to be shown. However, fish oil (EPA/DHA) was previously shown to increase submaximal ADP sensitivity in the absence of changes in ANT1/2 and ATP synthase protein content, despite increasing maximal H$_2$O$_2$ emission (137). Recent studies suggest that post-translational modification to ANT isoforms may contribute to improved submaximal ADP sensitivity and insulin sensitivity (137, 242, 362). For example, acetylation of human ANT1 at lysine 23 inhibits its ADP/ATP transport function; however, acute exercise reduces acetylation at lysine 23, thereby alleviating ANT1 inhibition (258). Exercise training also enhances ADP sensitivity by increasing mitochondrial creatine kinase activity, an important enzyme that regulates energy transfer between mitochondrial and cytoplasmic compartments through phosphate shuttling.
Therefore, it appears lifestyle factors that improve insulin sensitivity can influence ADP sensitivity by targeting its transport; however, further work is needed to determine whether ALA and LA protected against IR through a similar mechanism.

Work presented in chapter 4 utilized an isolated mitochondrial preparation to assess bioenergetic function, allowing for separation of SS and IMF subpopulations. However, a limitation with respect to measuring ADP sensitivity is that $K_m$ values for ADP (11-20 µM at rest, as previously reported (383)) are not physiologically accurate. In relation to estimated free ADP concentrations (7-20 µM), mitochondrial respiration would be at 50% of maximum ($V_{max}$) at rest in isolated mitochondria (400). Whereas, in permeabilized muscle fibers, the apparent $K_m$ for ADP is more physiologically representative, exceeding that of isolated mitochondrial by one order of magnitude (207). Therefore, future work can assess the impact of EFA supplementation on ADP sensitivity using an in situ permeabilized muscle fiber preparation, as reported in other studies (137, 242, 362). However, an objective in chapter 4 of this thesis was to assess mitochondrial coupling, for which isolated mitochondria are better suited and thus widely used.

Alterations in mitochondrial ADP sensitivity can influence ROS emission as the binding of ADP to the ATP synthase complex dissipates mitochondrial membrane potential to lower the propensity for ROS emission. Data presented in chapters 4 and 5 may link compellingly with a role for mitochondrial ROS and oxidative stress in the development of IR.
6.2.3 Considering a role for mitochondrial ROS and oxidative stress

The second “mitochondrial centric” model of IR presented in this thesis implicates a role for elevations in mitochondrial ROS and oxidative stress (116, 381). In obesity, the provision of excess reducing equivalents in the absence of increased ATP demand leads to a cellular energetic imbalance. The resulting increase in mitochondrial membrane potential creates a “back pressure” on electron transport through the ETC and increases the propensity of ROS production (116, 199, 267, 381). Chronic elevations in mitochondrial ROS may gradually overwhelm antioxidant defense mechanisms, and promote oxidative damage to cellular components. For example, L6 myotubes treated with either palmitate or exogenous H$_2$O$_2$, and rodents fed a HFD, exhibit elevated markers of oxidative damage and IR (135, 172, 253, 425, 426). Whereas, targeting antioxidants to mitochondria, such as treatment with SS31 or catalase within mitochondria, protects against IR in mice (9, 291), although SOD2 overexpression did not confer similar improvements (215). Structural components of mitochondria, such as membrane phospholipids are also susceptible to oxidative damage by ROS, driving the formation of reactive lipid aldehydes such as 4HNE (199, 314). Importantly, 4HNE attacks nucleophillic groups within an array of cellular components, including proteins, to form covalent adducts that cause functional impairments (345). The accumulation of 4HNE adducts has therefore emerged as an index of lipid peroxide induced oxidative stress that is elevated in the IR state (172, 242, 314, 323, 345). In chapter 4, the content of 4HNE was significantly increased in the muscle of IR obese control rats relative to their lean counterparts, even though a corresponding increase in maximal H$_2$O$_2$ emission was not observed. This has previously been observed in exercise trained obese humans, where
muscle 4HNE content was reduced in the absence of changes in maximal H$_2$O$_2$ emission capacity (242). Importantly, maximal H$_2$O$_2$ emission capacity is commonly measured in the absence of ADP, using Oligomycin A, an inhibitor of ATP synthase. This highlights a potential disconnect between measures of maximal capacity versus the in vivo state, particularly ADP sensitivity. It is possible that mitochondrial H$_2$O$_2$ emission in the presence of a submaximal ADP dose (i.e. 100 µM ADP) could have been elevated instead in chapter 4, and has been previously observed in untreated ZDF rats (362). This could also explain why the content of 4HNE in IMF mitochondria was significantly elevated in the obese control group despite no change in maximal H$_2$O$_2$ emission. Whereas, ALA and LA enriched diets prevented elevations in whole muscle and IMF mitochondria 4HNE content, which coincided with preservation of glucose tolerance. As a result, data from this thesis places 4HNE as an important candidate linked to the development of IR.

In support, a comparison between the muscle 4HNE content of all obese diet groups and AUC values from the IPGTT reveals a strong positive Pearson correlation coefficient (Figure 6.1A). This suggests that glucose intolerance is greatest in those animals with the highest level of lipid peroxide-induced stress within muscle. Previous work has shown that 4HNE content is elevated in muscle from obese IR subjects, and that improvements in insulin sensitivity following exercise training are associated with reductions in 4HNE (242, 336). In this thesis, the maintenance of whole-body glucose tolerance in obese ALA and LA diet groups corresponded to lower muscle 4HNE content (comparable to lean animals), and preservation of insulin-stimulated Akt and AS160 activation. Both Akt and AS160 are important targets within the insulin-signaling cascade due to their roles promoting GLUT4 translocation to the plasma membrane to facilitate
glucose uptake (7, 128, 202, 221). When correlated with 4HNE content, Akt phosphorylation exhibits a negative relationship in Figure 6.1B, although this was a non-significant trend. Whereas, AS160 phosphorylation was significantly and negatively correlated (Figure 6.1C) with 4HNE muscle content. Collectively, this suggests that higher levels of 4HNE accumulation within muscle associate with a greater degree of insulin signaling inhibition, and may therefore provide an explanation for the prevention of IR with ALA and LA supplementation.
Oxidative stress correlates with insulin resistance in obesity.

Pearson correlations between 4HNE content in muscle homogenate and (A) intraperitoneal glucose tolerance test (IPGTT) area under the curve (AUC) values, (B) Akt phosphorylation (% of basal activation), (C) Akt substrate of 160kDA (AS160) (% of basal activation) suggest that glucose intolerance is highest and insulin signaling is most inhibited when 4HNE content in muscle is highest. Lastly, the degree of whole body glucose intolerance shows a significantly positive Pearson correlation coefficient with (D) 4HNE content within muscle IMF mitochondria. Data are expressed as means ± SEM. Statistical significance is p<0.05.
Altered subcellular distribution of lipids within muscle may distinguish the IR phenotype of obese control rats compared to their protected counterparts fed ALA and LA. As an absolute fraction, lipid droplet accumulation within the IMF region has previously been shown to account for the majority of total lipid content in muscle of IR obese Zucker rats (150, 212). This could explain the significant increase in 4HNE content in IMF versus SS mitochondria found in obese control animals (Figure 4.6E). Whether such changes in lipid distribution were prevented with ALA and LA supplementation to protect against oxidative stress and IR remains unknown. Nevertheless, 4HNE content within IMF mitochondria exhibits a significant positive correlation (Figure 6.1D) with AUC values from the IPGTT. A mechanistic link between elevated IMF 4HNE content and IR is plausible given that 4HNE has been shown to activate JNK, a stress kinase known to interfere with insulin signaling (313, 357, 390, 421). However, in Chapter 5, the activation of JNK1/2 in muscle was not found to be significantly different between all obese diet groups, which likely eliminates a role for JNK. Alternatively, 4HNE may directly interfere with insulin signaling, thereby promoting the IR phenotype in obese control rats. Recently, it was shown that in the presence of 50µM 4HNE, insulin-stimulated glucose uptake, IRS1 protein expression, and phosphorylation of Akt^{Ser473} and AS160^{Thr642} was significantly reduced in skeletal muscle of lean Zucker rats after 2-4 hours (323). These findings align with IR phenotype of the obese control group, as characterized in Chapters 4 and 5. A proposed mechanism is outlined in Figure 6.2 that links elevated 4HNE content in IMF mitochondria to the IR phenotype of obese control rats.
1) Superoxide anions (•O$_2^-$) generated as a by-product of electron transfer through the electron transport chain (ETC) complexes are reduced to 2) hydrogen peroxide (H$_2$O$_2$) by the manganese-dependent mitochondrial isoform of superoxide dismutase (SOD2) (99, 314). The conversion of H$_2$O$_2$ to 3) hydroxyl radicals (•OH) occurs via a Fenton reaction (99). Hydroxyl radicals initiate free radical chain reactions, particularly targeting membrane polyunsaturated fatty acids, to 4) produce 4-hydroxynonenal (4HNE), a reactive aldehyde capable of directly interfering with 5) multiple insulin signaling intermediates including the insulin receptor substrate (IRS), Akt/PKB and AS160 (313, 323). Alternatively, 6) H$_2$O$_2$ may activate stress-response kinases (JNK/IKK) that serine phosphorylate IRS1. These events promote insulin resistance by interfering with insulin-stimulated GLUT4 translocation to the plasma membrane and subsequent glucose uptake. Catalase and glutathione S-transferases represent major antioxidant enzymes that detoxify H$_2$O$_2$ and 4HNE respectively (circle with hatched line) (313). Akt substrate of 160 kDa, AS160; JNK, c-Jun N-terminal kinase; IKK, IκB kinase.

Superoxide anions generated as a by-product of electron flux through the ETC are reduced to H$_2$O$_2$ by the mitochondrial isoform of superoxide dismutase (SOD2). SOD2 content within IMF mitochondria from IR obese control rats was significantly increased (Figure 4.7D), which may have inadvertently accelerated hydroxyl radical formation.
through enhanced $\text{H}_2\text{O}_2$ production. The production of hydroxyl radicals from $\text{H}_2\text{O}_2$ is catalyzed by free transition-metal ions in a Fenton reaction (99). Few hydroxyl radicals are required to trigger damaging chain reactions that principally target PUFAs to form 4HNE (99, 313). As mentioned above, 4HNE has been shown to directly interfere with insulin-stimulated glucose transport and insulin signal transduction by reducing IRS1 protein content and tyrosine phosphorylation, as well as Akt and AS160 phosphorylation (95, 313, 323). Using immunoprecipitation and Western blotting, Demozay et al., found that 4HNE forms adducts with IRS1 in 3T3-L1 adipocytes, and proposed this direct interaction to account for the reductions in IRS1 protein content and insulin-stimulated tyrosine phosphorylation (95). More recently Pillon et al., attempted to recapitulate these findings in muscle samples from CD-1 mice; however, 4HNE was not found to form adducts with IRS1 (313). Therefore, it remains unclear if IR results from direct interference by 4HNE, or from $\text{H}_2\text{O}_2$-mediated activation of JNK or IKK (14, 381, 403, 405, 424), where 4HNE instead reflects a chronic state of oxidative stress. Furthermore, 4HNE is believed to upregulate UCP3 activity in a feedback mechanism that would reduce mitochondrial ROS production by lowering membrane potential and local $\text{O}_2$ concentration (reduced to $\text{H}_2\text{O}$ at complex IV) (83, 99). UCP3 activity can also be upregulated by ROS-induced de-glutathionylation of specific cysteine residues (247). In chapter 4, UCP3 activity was not measured, however protein content was increased in IMF mitochondria in the obese control group only. However, it is unclear if this affected overall UCP3 activity given that $\text{H}_2\text{O}_2$ emission and ‘leak respiration’ did not change. By comparison, ALA supplementation increased catalase content within whole muscle, though likely in response to the increase in maximal $\text{H}_2\text{O}_2$ emission from SS
mitochondria. Nevertheless, catalase is an antioxidant enzyme that quenches toxic levels of H$_2$O$_2$ (328) and may therefore have protected against oxidative stress-induced IR by restricting hydroxyl radical and 4HNE formation. These adaptations were likely unnecessary with LA supplementation as bioenergetic parameters and indices of oxidative stress were comparable to lean healthy animals. It is also possible that both ALA and LA upregulated the activity of glutathione S-transferase enzymes that represent a major detoxification system of 4HNE lipid aldehydes (314, 358). Enhancement of the glutathione antioxidant system would prevent increases in 4HNE content, and therefore inhibition of insulin signalling. This is pertinent given that an enrichment of mitochondrial membranes with n-3/n-6 PUFAs following ALA/LA supplementation may increase susceptibility for 4HNE formation. Previous studies have shown that diets deficient in ALA reduced glutathione antioxidant activity in liver samples from both young and old rats (75). In addition, hepatic expression of glutathione S-transferase enzymes was increased in rats fed an LA rich corn oil diet for 6 weeks (68). Therefore, while it is plausible that ALA and LA protected against IR by enhancing glutathione-mediated detoxification of 4HNE in muscle, it remains the subject of future investigation.

Enhancing the delivery of ADP to the mitochondrial matrix, which would dissipate membrane potential through ATP synthesis, can reduce mitochondrial ROS production. Within skeletal muscle, the two ANT isoforms, ANT1 and ANT2, counter-transport ADP and ATP across the IMM into the mitochondrial matrix (195). Therefore, changes in the content of these protein isoforms may influence the capacity of ADP transport, mitochondrial ROS production and skeletal muscle insulin sensitivity. The protein content of ANT1 within IMF mitochondria was robustly increased in muscle
samples from obese control rats only. Whether this was to increase mitochondrial uncoupling, or to enhance the capacity of ADP delivery into the mitochondrial matrix is unclear, but may be a compensatory increase to reduce mitochondrial ROS production. In addition, while ANT2 content remained unaltered in ALA and LA diet groups, it was reduced by 50% in SS mitochondria from obese control rats. Together, these findings suggest that mitochondrial ADP sensitivity may have been impaired in IR obese control rats, but preserved with ALA and LA supplementation.

It is unknown what causes or prevents the reductions in content of mitochondrial proteins such as ANT that ultimately influence mitochondrial function. Changes at the posttranscriptional level can either favour the synthesis or degradation of proteins by altering mRNA stability dynamics. As explored in chapter 3, increases in mitochondrial protein content following exercise training are associated with distinct alterations in RBPs that regulate mRNA stability.

6.3 Posttranscriptional regulation of mitochondrial biogenesis in healthy and obese conditions

6.3.1 Role of mRNA stability during exercise-induced mitochondrial biogenesis

Mitochondrial biogenesis involves the synthesis of proteins destined for import, folding, assembly and incorporation into the mitochondrial reticulum (122, 395). The rate of protein synthesis is largely dictated by the steady-state levels of corresponding mRNA. In turn, mRNA content is a function of its overall stability, the balance between rates of transcription and degradation, and is therefore considered an important parameter regulating gene expression (22, 42, 418). Currently, very little is understood about this posttranscriptional regulation with respect to exercise-induced mitochondrial biogenesis,
and was the focus of investigation in chapter 3. A principle finding was that the nuclear abundance of AUF1
\textsuperscript{p42/p45}, an mRNA destabilizing RBP, was increased in parallel with mitochondrial ETC protein content after 4 weeks of exercise training. Of significance, this may provide evidence of a posttranscriptional mechanism that contributes to a “plateau effect” in training adaptations that would gradually prevent further increases in muscle mitochondrial content. For example, Perry \textit{et al.} assessed the time-course response of mRNA after seven successive training sessions over two weeks. PGC-1\textalpha
mRNA and protein content peaked after the first training session, while citrate synthase and \(\beta\)-hydroxyacyl CoA dehydrogenase mRNA peaked after the third session. Thereafter, the transcriptional response of these targets was reduced following each successive bout of training, and led to the hypothesis that a plateau effect in training adaptations accounted for these reductions (236, 305). When considered with results from this thesis, the proposed plateau effect may be mediated by an accelerated rate of mRNA decay, and therefore, RBP-mediated regulation of mitochondrial biogenesis warrants consideration.

An important question is whether targets of AUF1 include mitochondrial mRNAs. Recently, a signature motif present in AUF1-target mRNAs was identified, composed of 29-39 nucleotides rich in AU bases (254). Mitochondrial mRNAs containing this motif include those encoding for mitochondrial protein import (TIM17A), protein synthesis (MRPL3), \(\beta\)-oxidation proteins (HADH), and ETC components (complex I, NDUFB5; cytochrome c, CYC; Complex IV, COX7A2L) (254). RBPs regulate mRNA stability by binding to AU-rich elements (AREs) in the 3’-UTR region of target transcripts (22, 193). When bound by AUF1, mRNA decay occurs in the cytoplasm and involves removal of the 5’ cap structure, 3’ deadenylation, and exonuclease-mediated degradation (22, 418).
However, chapter 3 showed that the nuclear content of AUF1 was increased. Within the nucleus, AUF1 is instead thought to increase mRNA stability for two reasons. First, AUF1 is absent from the cytoplasm where mRNA decay is regulated (65, 254). Second, AUF1 is thought to aid in nuclear pre-mRNA processing including splicing, 5’ capping, 3’ polyadenylation, and cytoplasmic export (254). Crucially, it was demonstrated that nuclear import and RNA-binding is a functional pre-requisite for AUF1 to destabilize mRNA in the cytoplasm (65, 254). Blocking nuclear import of AUF1 revealed that it could no longer influence mRNA stability in the cytoplasm (65). Therefore, the increased nuclear AUF1\textsuperscript{p42/p45} content in response to chronic exercise reported in chapter 3 can be viewed as an important step in the destabilization of mitochondrial mRNA and coordinated downregulation of mitochondrial biogenesis as outlined in Figure 6.3.
Figure 6.3 – Proposed mechanism of AUF1-mediated regulation of mitochondrial biogenesis.

A) Schematic of four AUF1 isoforms highlighting RNA-binding domains (RBD), exons 2 and 7, and the continuous (p37/p40 isoforms) and interrupted (p42/p45 isoforms) carboxy-terminal domains (CTD). B) Chronic exercise training results in a plateau effect characterized by a diminished transcriptional response of mitochondrial gene expression. 1) AMPK activation during exercise stimulates 2) importin α/β activity and association with 3) the nuclear import signal (NIS) of the AUF1 multiprotein complex. Upon nuclear import via the nuclear pore complex (NPC), the released AUF1 protein (p42/p45 isoforms) can influence 5) mitochondrial gene expression by first aiding in 6) pre-mRNA processing and then 7) binding AU-rich elements (AREs) within mature target mRNAs. Once exported to the cytoplasm, AUF1 can 8) destabilize target mRNA transcripts that ultimately results in 9) decay of mitochondrial mRNA species. NES, nuclear export signal; NTFs, nuclear transcription factors; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1 alpha.
Unique structures of the four AUF1 isoforms shown in Figure 6.3A distinguish their subcellular localization. AUF1\(^{p37/p40}\) isoforms contain an uninterrupted carboxy-terminal domain (CTD) that promotes nuclear localization, whereas the CTD is interrupted by exon 7 in the AUF1\(^{p42/p45}\) isoforms and promotes cytoplasmic localization (341). Accordingly, the nuclear import of AUF1\(^{p42/p45}\) requires the formation of a multiprotein complex with at least one AUF1\(^{p37/p40}\) isoform (341), and likely explains how nuclear AUF1\(^{p42/p45}\) content was increased in chapter 3. The import process (Figure 6.3B) begins with AMPK activation during exercise, which mobilizes importin \(\alpha\) through acetylation and phosphorylation (120, 402). Importin \(\alpha\) heterodimerizes with importin \(\beta\) (120, 402) and binds to the nuclear import signal in AUF1\(^{p37/p40}\) (341). In the nucleus this multiprotein complex dissociates, and the free AUF1\(^{p42/p45}\) proteins can aid in pre-mRNA processing events; although AUF1 can bind nuclear pre-mRNA (254), it remains unknown whether pre-mRNA degradation occurs, but mechanisms have been proposed, such as the addition of short poly(A) tails that trigger degradation by the nuclear exosome complex (41, 232). Once target mRNAs are processed and mature, AUF1\(^{p42/p45}\) can signal nuclear export and can destabilize mRNA in the cytoplasm. Ultimately, this would reduce steady-state levels of mRNA available for mitochondrial protein synthesis, and demonstrates how an increase in nuclear AUF1\(^{p42/p45}\) content following chronic exercise training may regulate mitochondrial biogenesis. Taken together, findings from this thesis have contributed to a greater understanding of the posttranscriptional regulation of mitochondrial biogenesis. Whether mRNA stability plays a role in obesity-related alterations in mitochondrial content also presents an avenue for future research.
6.3.2 Role of mRNA stability during mitochondrial biogenesis in obesity

Although obesity is associated with mitochondrial dysfunction and a reduction in skeletal muscle oxidative capacity, many studies have shown that mitochondrial content is actually increased in a number of models of obesity (90, 148, 172, 285, 387). Recent work by Jain et al. has extended our mechanistic understanding of this process. Briefly, obesity increases mitochondrial ROS emission that causes sarcoplasmic reticulum (SR) calcium ($\text{Ca}^{2+}$) leak, and subsequent activation of CaMKII to induce mitochondrial biogenesis (172). In the context of this thesis, Figure 6.4 outlines an expanded framework to include a role for altered mRNA stability to promote mitochondrial biogenesis in obesity.

![Figure 6.4 – Proposed role for mRNA stability during mitochondrial biogenesis in obesity.](image)

Calcium ($\text{Ca}^{2+}$) leak from the sarcoplasmic reticulum (SR) caused by mitochondrial ROS emission activates $\text{Ca}^{2+}$/calmodulin-dependent protein kinase II (CaMKII) and induces mitochondrial biogenesis in obesity. This model can be extended to include a role for mRNA stability (red hatched lines). $\text{Ca}^{2+}$ ions can enhance mitochondrial mRNA stability by altering the conformation of AU-rich elements (AREs) to favour binding of mRNA stabilizing RBPs such as HuR. In addition, CaMKII may also induce post-translational modifications (i.e. phosphorylation) to RBPs such that the binding affinity of AUF1, an mRNA destabilizing RBP, is reduced. Collectively, these proposed events can increase mRNA stability and promote the synthesis of proteins during mitochondrial biogenesis.

Two possible events in this proposed model links $\text{Ca}^{2+}$ to increased mRNA stability and mitochondrial biogenesis in obesity. First, the leak of $\text{Ca}^{2+}$ ions from the SR elevates its cytoplasmic concentration, and can enable direct interaction with mRNA transcripts (261). Upon interaction with divalent metal ions like $\text{Ca}^{2+}$, the secondary structure of the mRNA 3’-UTR can be folded to prevent binding of destabilizing RBPs
Second, CaMKII can phosphorylate RBPs that destabilize mRNA, thereby lowering its ARE binding affinity (42, 261). This reduces competition with HuR, an RBP that promotes mRNA stability (e.g., HuR) and acts as a signal to initiate translation events (108, 209). Altogether, the model proposed in Figure 6.4 highlights areas of future investigation that can expand our current mechanistic understanding of the posttranscriptional regulation of mitochondrial biogenesis in obesity.

6.4 Essential fatty acids and whole-body lipid handling

6.4.1 Diacylglycerol and IR

DAG accumulation within insulin-responsive tissues is linked with IR (39, 71, 169, 386), though not uniformly observed in the literature (15, 79, 265, 348). Mechanistically, DAGs activate PKCs that serine-phosphorylate IRS1 (Figure 1.3) to inhibit insulin signaling. In chapter 5, reduced DAG content could not account for the protective effect of ALA and LA diets against IR; however, altered FA composition of DAGs may have contributed.

DAGs consist of two FAs bound to glycerol, producing several unique species with distinct biological actions, and highlights the complexity of elucidating an overall role for DAG in IR (26). In chapter 5 the FA composition of DAG was measured, but not FA composition within individual DAG molecules. Paradoxically, the incorporation of ALA, LA and other n-3/n-6 PUFAs into the sn-2 position of DAG can enhance activation of novel PKCs (δ, θ, β, and ε) and may promote IR (244, 250, 330). Although PKC activation was not measured, ALA and LA diets protected against IR, implying that EFA-
enriched DAG were not harmful, possibly indicating a lack of enrichment at the \textit{sn}-2 position.

The ALA and LA diets in this thesis were formulated using plant oils (flaxseed and safflower). Plant oils contain naturally occurring DAG, where the majority is the \textit{sn}-1,3 isomer (7:3 ratio of \textit{sn}-1,3-DAG: \textit{sn}-1,2/2,3-DAG) (272) that is unable to activate PKCs (244, 250, 330). Therefore, in the absence of reduced DAG content, a repopulation of DAG favoring increases in \textit{sn}-1,3-DAG rich in ALA and LA may have protected against DAG-induced PKC activation and IR. Previous work points towards a protective effect of ALA/LA-enriched DAG. In ZDF rats, dietary ALA-DAG (7:3 ratio of \textit{sn}-1,3-DAG: \textit{sn}-1,2-DAG) reduced body weight, hepatic TAG accumulation and increased markers of β-oxidation compared to the ALA-TAG diet (272). Also, mice fed a HFD containing LA-DAG (7:3 ratio of \textit{sn}-1,3-DAG: \textit{sn}-1,2-DAG) had a lower body weight, visceral fat mass, and circulating leptin and insulin compared to the HF LA-TAG group after 5 months (273, 274). In humans, LA-DAG oil (50 g/day of predominantly \textit{sn}-1,3-DAG) consumed for 16 weeks led to reduced body weight, visceral fat area (abdomen), and hepatic fat content based on CT images (278). Collectively, these studies, and others (248, 256, 275) demonstrate that DAG enriched with ALA/LA derived from plant oils can exert anti-obesity effects that may protect against IR. Current mechanistic data attributes these effects to both the FA-composition of DAG and structural differences between DAG and TAG, such that dietary DAG oils enhance hepatic UCP2 expression, and β-oxidation within the liver and small intestine (272, 273, 278, 378). Also, ALA and LA diets may have altered the subcellular compartmentalization of DAG, which was not measured in chapter 5. The harmful effects of PKC activation are confined to cellular
membranes (26, 225), and therefore ALA and LA may have reduced membrane-localized DAG to protect against IR. Overall, a more thorough characterization of DAG species, stereochemistry and subcellular compartmentalization will provide further insight on its role in mediating IR.

### 6.4.2 Ceramide and IR

Although elevated ceramide content has previously been linked with IR (28, 151, 267, 403, 405), other studies including data from this thesis have challenged this notion (36, 151, 360). In chapter 5, ceramide content within muscle, liver and EWAT was not reduced by ALA and LA diets, despite the prevention of IR in obese rats. While particular SFA species such as C18:0 ceramides have also been linked with IR in rodents (151, 183, 388) and humans (27), it is unlikely they were reduced given that all diets had equivalent SFA content. Also, previously reported reductions in muscle C18:0 ceramide did not correlate with improvements in insulin-stimulated glucose uptake in obese Zucker rats (151), questioning the importance of specific ceramide species.

An alternate hypothesis is that ALA and LA diets prevented ceramide-induced IR by maintaining membrane fluidity, which has been linked with insulin receptor function (35, 89). Unlike ceramides synthesized from MUFA precursors, those composed of SFAs can form stable lipid rafts within cell membranes that reduce fluidity (316). It has been proposed that insulin receptor function is reduced as the rigidity of cellular membranes increase (i.e., fewer insulin receptors and/or lower binding affinity of insulin to its receptor) (35, 89); therefore, within cell membranes the formation of lipid rafts containing SFA-rich ceramides may contribute to IR. Although PUFAs are not substrates
for ceramide synthesis, their independent incorporation into cell membranes may help maintain overall fluidity and preserve insulin receptor function. This could represent a mechanism counteracting the effect of ceramides at the level of cell membranes, and would not necessitate reductions in tissue ceramide content, providing a possible explanation for observations in this thesis. This is plausible as the FA composition of cell membranes is largely determined by dietary intake (356). Whether insulin action was protected by the enrichment of cell membranes with PUFAs remains to be shown. Future studies can extend the current data by examining the impact of ALA and LA supplementation on membrane fluidity within diverse insulin-responsive tissues. Ultimately, this would provide insight on the contribution of factors that promote membrane rigidity (e.g., ceramide-containing lipid rafts) or fluidity (e.g., unsaturated FAs) in the development of IR. Furthermore, this would deepen our understanding of tissue-specific EFA metabolism, as the activity of enzymes responsible for converting ALA/LA to longer-chain n-3/n-6 PUFAs are associated with IR (see recent review (384)).

6.4.3 EFA metabolism and IR

In recent years it has been proposed that the risk for T2D is linked to alterations in the activity of the pathway that desaturates and elongates EFAs to their longer-chain counterparts (i.e., EPA, DHA and AA). Specifically, a high activity of the rate-limiting delta-6 desaturase (D6D) has been implicated T2D risk, particularly when considering the metabolism of n-6 PUFAs (see reviews (43, 89, 203, 384)). The combination of high D6D activity and high n-6 PUFA (predominantly LA) content in modern diets creates a
“perfect storm”, favoring greater AA formation and pro-inflammatory stress linked with IR. Given that insulin is known to stimulate D6D activity (43), the high insulin levels observed in IR may further accelerate D6D-mediated formation of AA. Whereas, presumably, a high D6D activity would not be considered harmful with diets rich in n-3 PUFA (predominantly ALA), leading to greater EPA and DHA formation. This double-edged sword concept is largely predicated on clinical data from prospective observational studies examining plasma phospholipids and erythrocyte membranes that collectively showed a positive correlation between D6D and T2D risk (140, 200, 204, 297). Also, the adult subjects used are often overweight and pre-diabetic, in which case it is not surprising to find a high D6D activity positively associated with T2D risk (140, 204). This leads to an important consideration regarding the time course in which IR develops, with respect to potential adverse effects of a high D6D/high dietary n-6 PUFA combination. In this regard, results presented in chapters 4 and 5 may provide some initial insights. Obese Zucker rats began the dietary intervention prior to the development of IR, which is known to develop by 15 weeks of age (436). In agreement with this, results in chapter 4 indicate that both ALA and LA protected against IR by 17 weeks of age. These results indirectly suggest that an LA-rich diet combined with high D6D activity is not sufficient to illicit IR, but does not preclude its role in exacerbating pre-existing IR given the preventative approach of this work.

A limitation of the experimental conditions utilized in chapters 4 and 5 is that it is not possible to determine if 1) the improvements seen with ALA and LA stem from these FAs, or whether their endogenous conversion to EPA/DHA and AA, respectively, mediate their beneficial effects, and 2) whether D6D activity plays a role in the
prevention/development of IR. This limitation can be addressed with the use of a D6D knockout mouse, where the endogenous conversion of ALA and LA is prevented (369). The use of a HFD enriched with LA would allow for assessment of whether LA protects against HFD-induced IR, without the confounding effects of its conversion. Furthermore, a HFD enriched with AA would provide distinct insight on this FA, which is thought to be harmful through its metabolism, giving rise to pro-inflammatory mediators (e.g., leukotrienes and prostaglandins) (50). Importantly, this would clarify the role of D6D activity, which is rate-limiting in the conversion of LA to AA (384). Moreover, with a D6D model, a HFD with ALA could be used to distinguish its effects from a HFD with EPA and DHA. This is important as the acyl chain length and degree of unsaturation may attribute unique biological effects. In support, ALA was shown to act independently on risk factors associated with fatty liver disease compared to EPA/DHA (266). However, EPA but not DHA improved age-related loss in skeletal muscle mitochondrial function, further highlighting distinct biological effects of different n-3 PUFAs (179). Altogether, investigations in this thesis have laid the groundwork for future studies examining the relationship between EFAs and their conversion in the context of obesity-related IR.

6.5 Conclusions

This thesis has improved our understanding of how physical activity and dietary fat composition modulate health and metabolism. Findings presented in this thesis support the following conclusions: 1) exercise alters posttranscriptional mechanisms such that mitochondrial biogenesis ensues in a cellular environment favoring mRNA destabilization, 2) ALA and LA supplementation can protect against IR in obese Zucker
rats, 3) ALA and LA appear to exert their beneficial effects independent of changes in skeletal muscle mitochondrial content and function, as well as changes in DAG and ceramide accumulation, and 4) the FA profile of skeletal muscle, WAT and liver are distinctly remodeled following ALA and LA supplementation. Overall, this thesis has demonstrated the effects of lifestyle factors at the cellular, tissue-specific and whole-body level, and emphasizes the power of lifestyle modifications for maintaining optimal health.


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