

**SKELETAL MUSCLE MITOCHONDRIAL CAPACITY IN INDIVIDUALS
WITH IMPAIRED GLYCEMIC CONTROL**

**SKELETAL MUSCLE MITOCHONDRIAL CAPACITY PLAYS A MINIMAL
ROLE IN MEDIATING INSULIN SIGNALING AND REGULATION IN
INDIVIDUALS WITH IMPAIRED GLYCEMIC CONTROL**

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TITLE: Skeletal muscle mitochondrial capacity plays a minimal role in mediating insulin signaling and regulation in individuals with impaired glycemic control

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ABSTRACT

This thesis examined the biochemical role of skeletal muscle mitochondria and metabolic consequences of mitochondrial adaptations to exercise in individuals with poor glycemic control. Mitochondrial dysfunction and/or ectopic lipid accumulation has been implicated in the pathogenesis of metabolic-related diseases such as obesity and type 2 diabetes (T2D). However, whether mitochondrial dysfunction is the cause of insulin resistance and T2D or is a consequence of this disorder remains controversial. Alternatively, pro-inflammatory stress signals initiated through altered secretion of adipocytokines and oxidative stress may be a unifying mechanism underlying insulin resistance and T2D. Furthermore, the impact of exercise on muscle adaptation in insulin-resistant states is not well defined. At rest and prior to exercise training, no evidence of mitochondrial dysfunction or disproportionate intramyocellular lipid (IMCL) accretion was detected in obese, insulin-resistant skeletal muscle biopsy samples *vs.* healthy, lean age-, and fitness-matched men. In response to exercise training (12 weeks, consisting of 32 sessions of 30-60 min @ 50-70% maximal oxygen uptake [VO_{2peak}]), there was an increase in mitochondrial oxidative phosphorylation (OXPHOS) capacity, mitochondrial content, and IMCL deposition with sub-cellular specificity. Exercise training also reduced both skeletal muscle and systemic oxidative damage, already elevated in the obese. The improved adipocytokine profile associated with obesity after training also coincided with improvements in glycemic regulation. Patients with genetic mitochondrial mutations, resulting in skeletal muscle mitochondrial dysfunction have an increase prevalence of dysglycemia/T2D. However, when evaluated against age- and activity-

matched normoglycemic myopathy controls, no differences in mitochondrial electron transport chain protein subunits, mitochondrial or IMCL density, or level of whole-body insulin resistance was detected. In fact, dysglycemic mitochondrial myopathy patients demonstrated *higher* skeletal muscle OXPHOS capacity and Akt activation, a key step in insulin-stimulated glucose transport activity as compared with normoglycemic mitochondrial myopathy patients. Interestingly, a significant impairment in β -cell function (defective insulin secretion), in the dysglycemic patients was observed coincident with elevated glucose levels during the oral glucose tolerance test (OGTT). These findings indicate that insulin resistance does not cause skeletal muscle mitochondrial dysfunction/IMCL accumulation or *vice versa* and provides evidence against a direct link between mitochondrial dysfunction and the development of insulin resistance/T2D. Perhaps, oxidative stress/inflammation and pancreatic β -cell erosion mediate the observed obesity-induced insulin resistance and mitochondrial myopathy-associated T2D, respectively? Twelve weeks of moderate endurance exercise is an effective strategy to improve mitochondrial capacity, oxidative damage, inflammation, and glycemic regulation in insulin-resistant, obese individuals, but an improvement in muscle insulin sensitivity did not appear to be required.

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
8-OHdG	8-hydroxy-2-deoxyguanosine
β -HAD	β -hydroxyacyl-CoA dehydrogenase
Akt	serine/threonine protein kinase Akt
ANOVA	analysis of variance
AUC	area under the curve
BMI	body mass index
COX	cytochrome <i>c</i> oxidase
COX-II	cytochrome <i>c</i> oxidase, subunit II
COX-IV	cytochrome <i>c</i> oxidase, subunit IV
CPT-I	carnitine palmitoyltransferase I
CRP	C-reactive protein
CS	citrate synthase
DAG	diacylglycerol
DI	disposition index
EM	electron microscopy
ETC	electron transport chain
FA	fatty acid
FAT/CD36	fatty acid translocase
FFA	free-fatty acid
FFM	fat-free mass

GLUT	glucose transporter
GTT	glucose tolerance test
HbA _{1c}	hemoglobin A _{1c}
HDL	high-density lipoprotein
HOMA-IR	homeostasis model assessment index of insulin resistance
IDF	international diabetes federation
IFG	impaired fasting glucose
IGI	insulinogenic index
IGT	impaired glucose tolerance
IL-6	interleukin-6
IMCL	intramyocellular lipid
IMF	Intermyofibrillar
IR	insulin receptor
IRS	insulin receptor substrate(s)
ISI	insulin sensitivity (Matsuda) index
ITT	insulin tolerance test
LDL	low-density lipoprotein
Mfn2	mitofusin-2
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
NRF	nuclear respiratory factor
OGTT	oral glucose tolerance test

OXPHOS	oxidative phosphorylation
PDK	phosphoinositide-dependent protein kinase
PGC-1 α	peroxisome proliferator-activated receptor- γ coactivator-1 α
PI3K	phosphoinositol 3-kinase
PIP ₂	phosphatidylinositol 4,5 diphosphate
PIP ₃	phosphatidylinositol 3,4,5-triphosphate
PKB	serine/threonine protein kinase Akt
PolG	mitochondrial DNA mutator mice
ROS	reactive oxygen species
SCHAD	short-chain β -HAD
SEM	standard error of the mean
SOD1	superoxide dismutase 1, cytosolic (Cu/Zn-SOD)
SOD2	superoxide dismutase 2, mitochondrial (Mn-SOD)
SS	subsarcolemmal
T2D	type 2 diabetes mellitus
TEM	transmission EM
TFAM	mitochondrial transcriptional factor A
TNF- α	tumor necrosis factor- α
VO _{2peak}	maximal oxygen consumption
WAT	white adipose tissue
WHO	world health organization
WT	wild-type mice

FORMAT AND ORGANIZATION OF THESIS

This thesis is prepared in the “sandwich” format as outlined in the McMaster University School of Graduate Studies’ Guide for the Preparation of Theses. It includes a general introduction, 3 independent studies prepared in journal article format, and an overall discussion. The candidate is the first author on all the manuscripts. At the time of thesis preparation, Chapters 2, 3 and 4, were under review in peer-reviewed journals.

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CHAPTER 1
INTRODUCTION

1.0 INTRODUCTION

1.1 Obesity Prevalence in Canada

Obesity is the most prevalent nutrition-associated problem in the world today, reaching epidemic proportions in both developed and developing countries (1). It is a key risk factor for a variety of health problems, including diabetes, hypertension, dyslipidemia, heart disease, stroke and cancer (1). In Canada, the prevalence of overweight (i.e., body mass index [BMI] ≥ 25 kg/m²) and obese (i.e., BMI ≥ 30 kg/m²) individuals has increased over recent decades among adults in all areas of the country. According to the most recent estimates from the 2007-2009 Canadian Health Measures Survey, 61% of the adult population are overweight and one in four (24%) is obese (2). The sheer number of people who are overweight and obese underscores a public health challenge.

1.2 Insulin Resistance and Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2D) is rapidly becoming one of the most prevalent health concerns in the developed world. Approximately 2.5 million Canadians have been diagnosed with T2D, with the annual total direct costs of treating the disease approximating \$12 billion (3). Obesity, insulin resistance, and T2D can be viewed as a continuum of metabolic diseases characterized by disordered carbohydrate and lipid metabolism (4-6). The defining features of T2D, which also commonly occurs in obese individuals, are an impaired ability of insulin to stimulate glucose uptake in peripheral tissues (i.e., insulin resistance) and impaired insulin secretion (β -cell dysfunction) (5).

Although genetic factors contribute to the development of insulin resistance and/or T2D, obesity and a lack of physical activity are risk factors, both independently associated with diabetes and diabetes-related co-morbidities (1). Insulin resistance results from defects in insulin signaling pathways involved in initiating glucose transporter 4 (GLUT4) translocation from intracellular pools to the cell membrane (7). Initially, the pancreas can adapt to the increase in peripheral insulin resistance by increasing insulin secretion, but eventually the pancreas becomes overwhelmed and persistent hyperglycemia develops (5). Chronic hyperglycemia is linked to several diabetic complications, including cardiovascular disease, nephropathy, neuropathy, and retinopathy (8). The criteria for the diagnosis of diabetes and intermediate hyperglycemia based on the clinically recommended oral glucose tolerance test (OGTT) procedure is presented in Table 1 (9). Strategies that can improve glycemic regulation are therefore beneficial for improving overall health and reducing the risk of T2D and its associated sequelae.

Table 1: Criteria for the diagnosis of diabetes and/or impaired glucose homeostasis

World Health Organization/International Diabetes Federation	
Diabetes	
Fasting plasma glucose	≥7.0 mM (125 mg/dL)
2-h plasma glucose	OR, ≥11.1 mM (200 mg/dL)
Impaired Glucose Tolerance (IGT)	
Fasting plasma glucose	<7.0 mM (126 mg/dL)
2-h plasma glucose	AND, ≥7.8 and <11.1 mM (≤140 and <200 mg/dl)
Impaired Fasting Glucose (IFG)	
Fasting plasma glucose	≥6.1 and ≤6.9 mM (≥110 and ≤125 mg/dL)
2-h plasma glucose	If measured, <7.8 mM (140 mg/dL)

Adapted from a WHO/IDF consultation report (9). 2-h plasma glucose concentration after ingestion of a 75-g glucose load.

1.2.1 Skeletal Muscle Insulin Signaling

Insulin plays an essential role in metabolic homeostasis by mediating glucose uptake by muscle, liver, and adipose tissue in the postprandial state. The most downstream event in the cascade leading to glucose entry into the cell is the translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane (10). Insulin binding to its receptor on the plasma membrane increases insulin receptor tyrosine kinase activity, resulting in the phosphorylation of insulin receptor substrates (IRS) on tyrosine residues (11). Phosphorylated IRS activates phosphoinositol 3-kinase (PI3K) that catalyzes the generation of phosphatidylinositol 3,4,5-triphosphate (PIP₃) that serves as docking sites for phosphoinositide-dependent protein kinase (PDK) and Akt (11). Akt is the kinase controlling most of the metabolic actions of insulin and is activated by phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ by PDK and mammalian target of rapamycin (mTOR), respectively (10). Defect(s) in any component of the pathway, from the insulin receptor to the translocation of GLUT4 to the plasma membrane, might contribute to insulin resistance (10; 12).

1.3 Endurance Exercise Training

Endurance exercise training leads to numerous physiological adaptations involving multiple organ systems. Increases in skeletal muscle mitochondrial content and proteins that regulate various aspects of cellular metabolism are among the most well characterized adaptations to endurance exercise training (13). These phenotypic adaptations in skeletal muscle allow for more efficient energy production during exercise

and contribute to improved functional performance. Exercise also has numerous health benefits and is recommended for the treatment and prevention of several chronic conditions including obesity, metabolic syndrome, T2D, and cardiovascular disease (6; 14; 15). The skeletal muscle adaptive response to training is thought to be highly specific, depending on the nature of the exercise stimulus (16; 17). For example, traditional endurance exercise training, characterized by prolonged, continuous, low- to moderate-intensity exercise, leads to increased muscle oxidative capacity and improved endurance performance (18; 19). Skeletal muscle is the largest site for postprandial glucose disposal (20), is a crucial regulator of lipid metabolism (21), and is a key determinant of basal metabolic rate (22). As a result, adaptations in skeletal muscle play an important role in exercise-induced improvements in metabolic health. However, little is known regarding the biochemical response of skeletal muscle adaptations to endurance exercise training in the context of insulin resistance and obesity.

1.3.1 Skeletal Muscle Adaptations to Endurance Exercise Training

Endurance training, characterized by repeated sessions of continuous low to moderate-intensity exercise over several weeks, leads to numerous physiological adaptations (18; 23-25). Recently, high-intensity interval training has also been shown to induce skeletal muscle metabolic and performance adaptations that resemble traditional endurance training despite a low total exercise volume (26-28). Among the most prominent is an increase in skeletal muscle mitochondrial content, which is commonly assessed by an increase in the maximal activity or protein content of mitochondrial

enzymes (18; 19; 24). Electron micrograph data indicates that the increase in mitochondrial content is largely the result of increased size (29), which likely reflects the incorporation of new proteins into existing mitochondria and expansion of the mitochondrial reticulum. Some early studies also reported that an increase in mitochondrial number also contributes to the increase in skeletal muscle mitochondrial volume following endurance training (30). Skeletal muscle mitochondria form a dynamic reticular structure that undergoes fusion and fission (31). Therefore, the increase in mitochondrial volume following exercise training may also involve an increase in mitochondrial fusion (32; 33).

Increased skeletal muscle mitochondrial capacity improves metabolic control, with an increase in fat oxidation and a reduction in carbohydrate oxidation during exercise performed at the same absolute intensity after a period of endurance training (18; 25). Enhanced fat oxidation, in turn, is thought to contribute to an increase in endurance capacity through sparing of muscle glycogen and/or reduced lactate (or hydrogen ion) accumulation (18). Endurance training also elevates levels of metabolic transport proteins (e.g., GLUT4, fatty acid translocase [FAT/CD36]) within skeletal muscle, contributing to an enhanced capacity for glucose and lipid uptake (34-38). Improved glucose uptake is indirectly reflected by a training-induced increase in resting muscle glycogen (39; 40), which may also contribute to improved endurance capacity following training.

1.3.2 Endurance Exercise Training Improves Metabolic Health

Exercise training improves metabolic health and reduces the risk for metabolic-

related diseases, including T2D (6; 40; 41). Several prospective randomized controlled studies such as the Finnish Diabetes Prevention Study (42), the Da Qing IGT and Diabetes Study in China (43), the Malmo study in Sweden (44), and the Indian Diabetes Prevention Programme (45) have shown that lifestyle modification involving enhanced physical activity helps to delay or prevent the progression of IGT to diabetes. One of the strongest evidence in direct support of endurance exercise training improving metabolic health and reducing T2D progression (46) and incidence of the metabolic syndrome (47) comes from the Diabetes Prevention Program study conducted in the United States. This randomized, placebo-controlled trial demonstrated that a lifestyle intervention involving modest nutritional changes and 150 min per week of continuous, moderate-intensity exercise (i.e., brisk walking) for approximately three years was superior to pharmacological intervention (metformin) for preventing the development of T2D and reducing the incidence of metabolic syndrome in individuals with impaired glucose control. Likewise, numerous shorter duration intervention studies have reported improvements in markers of metabolic health following several weeks of endurance-type exercise training (48; 49). For example, six months of endurance training involving walking or jogging for 170 min per week resulted in an ~85% increase in insulin sensitivity in overweight or obese individuals (50). Several meta-analyses and systematic reviews have also concluded that endurance-type exercise training improves insulin sensitivity and/or glucose tolerance in obesity and patients with T2D with or without concomitant changes in weight and/or body composition (51-54). Since skeletal muscle is the primary disposal site for ingested glucose (20), adaptations in skeletal muscle are

likely to be involved in improved glycemic regulation following endurance training. However, the independent influence of endurance training on glycemic regulation is unclear, since many of the previously reported improvements in glucose homeostasis with exercise have been obfuscated by concomitant nutrition and/or weight loss interventions (52; 55).

1.4 Molecular Mechanisms of Insulin Resistance

The molecular mechanisms linking obesity to insulin resistance/T2D have not been fully elucidated. However, at least four distinct mechanisms have been proposed: 1) ectopic lipid deposition, particularly in skeletal muscle (56-59); 2) mitochondrial dysfunction, evident by decreased mitochondrial mass and/or function (60-62); 3) increased production of pro-inflammatory adipokines/cytokines (63-66); and 4) increased generation of reactive oxygen species (ROS) (67-69). Improved understanding of the biochemical role of skeletal muscle mitochondria in the context of these mechanisms in obesity and T2D will help advance identification and development of effective treatment options.

1.4.1 The Role of Skeletal Muscle Lipid Accumulation and Mitochondria in the Development of Insulin Resistance

Ectopic (i.e., non-adipose tissue) lipid accumulation, particularly in skeletal muscle (intramyocellular lipid - IMCL), has been hypothesized as a possible mechanism inducing insulin resistance in skeletal muscle. Despite contributing only a small fraction (~1%) to the total muscle fat content (56; 70), several studies have demonstrated

increased IMCL content in obesity and T2D and an inverse relationship between muscle insulin sensitivity and IMCL content (57; 70-79). On the other hand, accumulation of IMCL within skeletal muscle may not be invariably linked to insulin resistance.

Endurance-trained athletes who are markedly insulin-sensitive have higher IMCL levels than lean sedentary subjects, levels similar to those reported in patients with T2D (80; 81). This is also supported by studies demonstrating increased IMCL with exercise training in previously sedentary subjects (80; 82). Furthermore, it is likely that elevated IMCL stores may be only a marker of dysfunctional muscle fatty acid (FA) metabolism and that accumulation of more reactive lipids, such as diacylglycerols (DAG) (83), ceramides (84), or long-chain fatty acyl-CoA's (85) are actually responsible for the insulin resistance. Indeed, there are now plausible mechanistic links between the development of insulin resistance and accumulation of DAG and ceramide in muscle (85). Intramyocellular DAG levels are elevated in a number of models of insulin resistance (83; 86), and ceramide content is increased in muscle from obese insulin resistant humans (84). DAG can activate several isoforms of protein kinase C (PKC), which can impair insulin signal transduction to glucose transport via serine phosphorylation of IRS-1 (87). In addition, ceramides can cause insulin resistance by preventing insulin-stimulated Akt serine phosphorylation and activation (88; 89). In addition to lipid accumulation, the obese/insulin-resistant phenotype is characterized by an impaired capacity for FA oxidation, which is associated with a reduction in the activity of muscle carnitine palmitoyltransferase I (CPT I), the rate-limiting step in the mitochondrial oxidation of FA's (90). Thus it is likely that an impaired ability to

transport and oxidize FA's in skeletal muscle mitochondria of these individuals exacerbates lipid accumulation. Therefore, interventions that increase FA oxidation may exert an insulin-sensitizing effect on skeletal muscle, in part, by reducing the accumulation of cytosolic lipids (91). Endurance training, for example, enhances fat oxidation (18; 92) and improves insulin sensitivity (93). Alternatively, augmenting IMCL synthesis in skeletal muscle, thereby decreasing DAG and ceramide levels may protect against high-fat diet-induced insulin resistance and may offer a new approach to prevent and treat insulin resistance and T2D (94). Thus it is of interest to determine whether the improvement in muscle FA oxidation following endurance training in obese individuals is associated with a reduction in specific IMCL pools, such as DAG and ceramide, that have a direct link with the development of insulin resistance. These observations appear to contradict the association between IMCL and insulin resistance; however, the explanation may lie in the exercise-enhanced oxidative phosphorylation (OXPHOS) capacity and mitochondrial abundance (95-97).

Alternatively, the intracellular distribution of mitochondria and IMCL, and their relative proximity to each other likely mitigates the negative sequelae of augmented intramuscular lipids in exercise-trained muscle (29). This paradox points to physical activity as a major determinant of increased net muscle lipid uptake and utilization.

Increased skeletal muscle mitochondrial content is hypothesized to play a role in mediating exercise-induced improvements in metabolic health and insulin sensitivity (6; 41; 61). This is supported by findings that individuals with obesity, insulin resistance and T2D have reduced markers of skeletal muscle mitochondrial content when compared to

insulin sensitive controls (98-105); and reduced expression in a cluster of nuclear genes responsible for oxidative metabolism (100; 106-108). Other groups have reported impaired *in vivo* mitochondrial function in subjects with insulin resistance (109; 110) and T2D (111). These findings have led to the theory that reduced mitochondrial content and/or oxidative capacity in skeletal muscle contributes to insulin resistance and the development of T2D (60-62; 110; 112) (Table 2); however they do not necessarily establish a directional link between a primary impairment of mitochondrial function leading to IMCL accumulation resulting in impaired glucose uptake.

Reduced mitochondrial content or function is thought to lead to an accumulation of IMCL and intracellular lipid metabolites, such as fatty-acyl CoA derivatives (113), DAG (83; 87) or ceramides (89; 114), which impair insulin signaling by activation of signaling pathways that increase inhibitory serine phosphorylation of the IRS (60-62; 83; 87; 115; 116), resulting in reduced Akt activation (104) and impaired glucose transport (115; 117; 118). If this theory is valid, endurance exercise training may improve insulin sensitivity by increasing mitochondrial content and improving the ability of skeletal muscle to completely oxidize lipid substrates (119). Recently, the hypothesis that impaired skeletal muscle oxidative capacity causes insulin resistance has come under scrutiny lately (120-129) (Table 3). For example, high-fat diets (130; 131) and genetic manipulations (131; 132) induce insulin resistance while concomitantly *increasing* mitochondrial content and fatty acid oxidation (130-132) (Table 4). Notwithstanding, exercise training-induced increases in muscle mitochondrial capacity are still likely to contribute to improved metabolic health.

Table 2: Studies implicating mitochondrial dysfunction in the development of insulin resistance/T2D

Participants	Mitochondrial Function	Physical Activity	References
Healthy, pre-menopausal, lean/obese women	Enzyme activity: CS*, COX, β -HAD CT: thigh	Sedentary: < 2x/wk	Simoneau <i>et al.</i> , 1995.
Lean nondiabetic/Obese nondiabetic/Obese T2D	Enzyme activity: CS, COX	PA status not reported	Simoneau <i>et al.</i> , 1997.
Lean nondiabetic/Obese nondiabetic	Enzyme activity: CS, COX*, β -HAD	Sedentary: similar VO _{2max} across groups	Simoneau <i>et al.</i> , 1999.
Lean nondiabetic/Obese nondiabetic/Obese T2D	Enzyme activity: NADH:O ₂ oxidoreductase*, CS* EM: mitochondria*	PA status not reported, Lean nondiabetic ~10 yr younger	Kelley <i>et al.</i> , 2002.
Lean nondiabetic young/Lean nondiabetic elderly	¹ H-MRS: IMCL* ¹³ C-MRS: TCA flux* ³¹ P-MRS: ATP synthesis*	Sedentary: PA index questionnaire matched across groups	Petersen <i>et al.</i> , 2003.
Lean nondiabetic/Lean insulin-resistant offspring of T2D	¹ H-MRS: IMCL* ³¹ P-MRS: ATP synthesis*	Sedentary: PA index questionnaire matched across groups	Petersen <i>et al.</i> , 2004.
Lean nondiabetic/Obese nondiabetic/Obese T2D	Enzyme activity: succinate:O ₂ oxidoreductase in SS/IMF mitochondria * DNA: mtDNA*; EM: mitochondria*	Sedentary: Lean more PA, VO _{2max} not quantified, Lean ~10 yr younger	Ritov <i>et al.</i> , 2005.
Lean nondiabetic/Lean insulin-resistant offspring of T2D	Protein: COXI*, PGC-1 α , PGC-1 β , TFAM mRNA: PGC-1 α , PGC-1 β , TFAM, NRF-1, NRF-2 DNA: mtDNA ¹ H-MRS: IMCL*; EM: mitochondria*	Sedentary: PA index questionnaire matched across groups	Morino <i>et al.</i> , 2005.
Obese insulin-sensitive/Obese insulin-resistant men	Enzyme activity: CS*, COX, β -HAD Protein: CI*, CII, CIII*, CIV, CV mRNA: PGC-1 α *, NRF-1, TFAM, COX1*, CYTB ¹ H-MRS: IMCL	Sedentary: < 1 h/wk, VO _{2max} matched across groups	Heilbronn <i>et al.</i> , 2007.
Overweight nondiabetic/Overweight T2D	¹ H-MRS: IMCL ³¹ P-MRS: PCr recovery half-time	Sedentary: VO _{2max} matched across groups	Schrauwen-Hinderling <i>et al.</i> , 2007.
Lean nondiabetic/Obese nondiabetic/Obese T2D	Enzyme activity: CS*, β -HAD, NADH oxidase* Protein: cardiolipin; DNA: mtDNA	Sedentary: < 20 min/wk, VO _{2max} similar across groups	Ritov <i>et al.</i> , 2010.

* Significant difference between groups, $P \leq 0.05$.

Table 3: Human studies challenging the theory implicating mitochondrial dysfunction in the development of insulin resistance/T2D

Participants	Assessment of Mitochondrial Function	Physical Activity	References
Overweight nondiabetic/Overweight T2D	Protein: PGC-1 α , COX1, CS Bioluminescent: MAPR TLC: IMCL DNA: mtDNA	Sedentary: VO _{2peak} matched across groups, habitual PA levels similar across groups	Asmann <i>et al.</i> , 2006
Overweight nondiabetic/Obese T2D men	Enzyme activity: CS, CI DNA: mtDNA* Respirometry: O ₂ flux	Sedentary: Routine daily PA (walking, gardening, etc.), VO _{2peak} not assessed	Boushel <i>et al.</i> , 2007
Normal birth weight-lean nondiabetic/Low birth weight-lean nondiabetic men	³¹ P-MRS: ATP turnover rate, PCr recovery mRNA: COX7A1, NDUFB6, PGC-1 α	VO _{2max} matched across groups	Brons <i>et al.</i> , 2008
Obese nondiabetic/Obese pre-diabetic/Obese T2D	¹ H-MRS: IMCL* ³¹ P-MRS: PCr recovery	Low PA levels: Habitual PA questionnaire similar across groups, VO _{2peak} lower in Obese T2D	De Feyter <i>et al.</i> , 2008
Lean nondiabetic Northern Europeans/Lean nondiabetic Asian Indians/Lean T2D Asian Indians	Enzyme Activity: CS* Protein: PGC-1 α , PGC-1 β Bioluminescent: MAPR* mRNA: PGC-1 α , TFAM, NRF-1, GLUT4 DNA: mtDNA* IMCL*	PA levels not reported, matched for PA levels across groups	Nair <i>et al.</i> , 2008
Obese nondiabetic/Obese IGT/Obese T2D	Muscle mRNA transcriptome	VO _{2max} matched across groups	Gallagher <i>et al.</i> , 2010
Lean active/Sedentary overweight FH-/FH+/Obese nondiabetic/Obese T2D	³¹ P-MRS: ATP _{max} synthesis rate*	Sedentary: PA questionnaire/accelerometer, VO _{2max} different across groups	Bajpeyi <i>et al.</i> , 2011

* Significant difference between groups, $P \leq 0.05$.

Table 4: Murine studies challenging the theory implicating mitochondrial dysfunction in the development of insulin resistance/T2D

Model	Assessment of Mitochondrial Function	References
Muscle-specific <i>Tfam</i> knockout mice (Type II fibers)	Enzyme activity: AMPK Protein: GLUT1, GLUT4, AMPK, p-AMPK, ACC, p-ACC mRNA: GLUT1, GLUT4	Wredenberg <i>et al.</i> , 2006
High-fat fed rats	Protein: MCAD*, LCAD*, VLCAD*, CS*, CI*, CII*, Cyt C*, COXI*, COXIV*, mRNA: CPT1, PPAR α *, PPAR δ , PGC-1 α DNA: mtDNA*	Garcia-Roves <i>et al.</i> , 2007
High-fat fed C57BL/6J mice	Enzyme activity: CS*, MCAD*, β -HAD*, CPT1* Protein: PGC-1 α *, CI*, CII*, CIII*, CIV*, CV*	Turner <i>et al.</i> , 2007
Obese diabetic <i>fa/fa</i> Zucker rats	Enzyme activity: CS, COX, VLCAD, SDH ¹ H-MRS: IMCL* ³¹ P-MRS: PCr recovery*	De Feyter <i>et al.</i> , 2008
High-fat fed male Wistar rats	Protein: CS*, COXI*, COXIV*, PGC-1 α *, PPAR δ * DNA: mtDNA*	Hancock <i>et al.</i> , 2008
Lean/Obese diabetic <i>fa/fa</i> Zucker female rats	Enzyme activity: CPT1, CS*, COX, β -HAD* Protein: FABP _{PM} *, FAT/CD36*, DGAT, FATP1*, COXIV* DNA: mtDNA* TLC: IMCL*	Holloway <i>et al.</i> , 2009
Iron-deficient low/high-fat/Normal iron low/high-fat male Wistar rats	Enzyme activity: LCAD*, NADH-dehydrogenase* Protein: CS, COXI*, COXIV*, LCAD*, GLUT4, AMPK, p-AMPK*, PGC-1 α *, succinate-oxidoreductase*, Cyt c* Chloroform/methanol: IMCL*	Han <i>et al.</i> , 2011

* Significant difference between groups, $P \leq 0.05$.

Endurance training-induced increases in skeletal muscle GLUT4 protein content are also believed to play a role in improved glycemic control (133). Numerous studies in humans have demonstrated increased GLUT4 protein content following both short- (~1 wk) and medium-term (4-10 wk) endurance training (134-136). In rodents, the training-induced increase in total GLUT4 protein is proportional to the increase in sarcolemmal GLUT4 translocation in response to a given insulin concentration (37), providing evidence that elevated total muscle GLUT4 content after training may help improve glycemic regulation. Therefore, endurance exercise training may improve insulin action and glycemic control, in part, by improving the capacity for skeletal muscle glucose transport through higher GLUT4 content.

1.4.2 The Role of Inflammation in Insulin Resistance

There are established correlative and causative links between chronic inflammation and insulin resistance in obesity and T2D (137-141). White adipose tissue (WAT) is a major endocrine and secretory organ, which releases a wide range of protein signals and factors termed adipokines. A number of adipokines, including leptin, adiponectin, C-reactive protein (142), tumor necrosis factor- α (143-145), and interleukin-6 (142), are linked to inflammation and the inflammatory response (64-66; 146). Obesity is characterized by a state of chronic low-grade inflammation, with raised circulating levels of inflammatory markers, as well the expression and release of inflammation-related adipokines generally rises as adipose tissue expands (137; 138; 142). The major exception to this pattern of increased production is the adipocyte hormone adiponectin,

the expression and circulating levels of which decline in obesity (147). Given the anti-inflammatory action of the hormone (148) and its role in modulating insulin sensitivity, obesity-induced reductions in adiponectin exacerbates the degree to which adipose tissue is in a state of ‘inflammation’ in the obese (149; 150). The elevated production of inflammation-related adipokines is increasingly considered to be important in the development of diseases linked to obesity, particularly insulin resistance/T2D and the metabolic syndrome (139-141). WAT is involved in extensive cross-talk with other organs and multiple metabolic systems through the various adipokines. Thus, in obesity and T2D, hyperglycemia could lead to insulin resistance in part by an inflammatory response as reflected by release of cytokines into the circulation. Strategies that can improve the adipokine profile are therefore beneficial for improving overall health and reducing the risk of T2D and its associated sequelae.

1.4.3 The Role of Oxidative Stress in Insulin Resistance

Cellular oxidative stress, characterized by the excessive intracellular accumulation of ROS has been implicated in the etiology of insulin resistance-related conditions, obesity and T2D (67-69; 151-153). ROS are oxygen-containing molecules (e.g., hydrogen peroxide [H₂O₂], superoxide radical [\bullet O₂⁻], hydroxyl radical [\bullet OH], peroxy radical [\bullet RO₂⁻], and hydroperoxyl radical [\bullet HO₂⁻]) (69) that either may or may not have unpaired electrons but are highly reactive in tissues. Low basal concentrations of ROS are important for normal cellular redox status, tissue function and intracellular signaling processes (154). The majority of cellular ROS is produced by mitochondria

during ATP synthesis. Free radicals in the form of superoxides are generated by electrons leaking from the electron transport chain (ETC), mainly through complexes I and III (155) and are released into the mitochondrial matrix and/or into the intermembrane space. Cells however, possess inherent antioxidant enzymes to detoxify ROS (156). These include conversion of superoxide to H_2O_2 by mitochondria-localized manganese superoxide dismutase (SOD2) or cytosol-localized copper-zinc SOD1. The antioxidant enzymes catalase and glutathione peroxidase detoxify H_2O_2 by converting them to water and molecular oxygen (157). Yet, if ROS production exceeds the antioxidant capacity to dissipate these reactive species, damage to cellular components (e.g., lipids, DNA and proteins) may ensue and compromise cell function (154). Decreased antioxidant capacity, increased production of ROS, and elevated oxidation products of lipids, DNA, and proteins have been reported in plasma, urine, and various tissues, suggestive of systemic and organ specific oxidative stress in obesity (68; 158-163) and T2D (69; 164). Free radical overproduction is considered one of many mechanisms in the generation of insulin resistance in obesity and T2D. Hence, lifestyle changes that mitigate against ROS production or augment intracellular antioxidant response may be the best preventative and therapeutic approach to oppose the increasing epidemic of obesity and its related sequelae. In line with this, we have shown that an acute bout of endurance exercise coordinately induced an expression program involved in oxidant stress management in healthy men (165) and also resistance exercise training was effective in enhancing the skeletal muscle cellular antioxidant capacity in older adults (166). However, the effects of endurance exercise training on oxidative stress in insulin-resistant obese individuals are

less clear.

1.5 Skeletal Muscle Mitochondria and Insulin Resistance: The Current Challenge

It has been purported that reduced mitochondrial content and/or mitochondrial dysfunction leading to IMCL accumulation is etiologically linked to insulin resistance and T2D. Contrary to this theory is the notion that hyperglycemia and insulin resistance *per se* can induce alterations in mitochondrial function (127; 129; 152; 167). Hence, the underlying mechanisms of mitochondrial dysfunction, whether it manifests as a primary or secondary defect in the pathogenesis of insulin resistance and T2D, or merely coincides with these phenomena is very complex and presently unclear. Furthermore, emerging data have questioned the universality of the causal link between skeletal muscle mitochondrial function and insulin resistance and T2D. Given that the health impact of obesity and related co-morbidities is tremendous, it is necessary to develop therapeutic modalities to improve the metabolic health of those living with obesity. In this regard, exercise training can increase skeletal muscle mitochondrial content and improve insulin sensitivity but the molecular mechanisms that mediate metabolic remodeling of this tissue are not completely understood. Furthermore, the molecular mechanisms of metabolic adaptation in response to exercise are only beginning to be unraveled and there is a paucity of human data in this regard.

1.6 Scope and Nature of this Work

Reduced mitochondrial function, content and size have been associated with conditions of aging, insulin resistance and T2D. However, the cross-sectional nature of most associations limits the determination of cause and effect relationships. Furthermore, whether this subtle dysfunction is due to increased age, age-associated co-morbidities, or merely a result of impaired mitochondrial “fitness” (i.e., low habitual physical activity) is also unclear. This thesis examined the role of human skeletal muscle mitochondria in disorders of impaired glycemic regulation in individuals well-matched for age and physical fitness/activity, as well as the biochemical mechanisms of adaptation in human skeletal muscle in response to endurance exercise training.

The primary purpose of Study 1 (Chapter 2) was to obtain a comprehensive molecular and morphological picture of changes in skeletal muscle mitochondria in the pathogenesis of insulin resistance in sedentary obese men absent of any co-morbidities and age- and physical fitness-matched healthy lean counterparts. At present, the effect of endurance exercise without dietary restrictions and without targeted weight loss on mitochondrial function, IMCL content, and glucose homeostasis is unclear. In addition, it is not known if the response of insulin-resistant obese men to endurance exercise training differs from that of healthy lean control subjects matched for age and fitness level. Hence, the secondary objective was to examine the effects of moderate- but progressive-intensity endurance training on the exercise-responsive mitochondrial proteins involved in mitochondrial biogenesis, glucose homeostasis, sub-cellular distribution of IMCL and mitochondrial ultrastructure. Of note, the subjects in this cohort were moderately obese

but healthy, and did not have other confounding factors that are typically associated with morbid obesity and T2D; and were equivalently active in order to discern if changes in skeletal muscle are inherently related to glucose homeostasis and not inactivity. A major strength of this study was that body composition (body weight, BMI, and percent body fat) after 12 weeks of training was maintained at baseline levels in both lean and obese men. The above approach helped to eliminate the potential impact of weight loss on insulin sensitivity and muscle mitochondria. Thus, changes arising in outcome variables would be attributed solely to the effects of exercise training and not obfuscated by body composition. Endurance exercise was the model utilized since it is the most traditional exercise protocol employed to induce mitochondrial adaptation in skeletal muscle.

Given that obesity is associated with a state of low-grade systemic inflammation (137; 138; 142) and increased oxidative stress (67; 68), it is possible that a similar mechanism, unifying these two phenomena are involved in explaining insulin resistance in obesity. However, the effects of endurance exercise training modulating these outcomes in obesity and insulin resistance are poorly understood. Study 2 (Chapter 3) subsequently examined the role of inflammation and both systemic and skeletal muscle oxidative damage in the pathogenesis of insulin resistance in obesity; and in response to endurance exercise training.

While most of this research was conducted in obese but otherwise healthy individuals, the final study examined the biochemical role of mitochondria on glucose metabolism in patients clinically diagnosed with primary mitochondrial myopathy in order to determine a directional link between intrinsic mitochondrial dysfunction and

T2D. Individuals harboring mitochondrial genetic defects have *bona fide* mitochondrial dysfunction associated with impaired respiratory chain activity, increased IMCL deposition and reduced ATP production (168).

The primary purpose of Study 3 (Chapter 4) was to determine if mitochondrial dysfunction in skeletal muscle precedes the development of T2D in patients clinically diagnosed with a genetic mitochondrial disease. Based on the results of Study 3 in this thesis, as well as previous research indicating impaired insulin secretion as a primary factor in the etiology of T2D in mitochondrial disease populations (169; 170), a secondary purpose of Study 3 was to examine glucose regulation. In order to assess impaired glycemic control, an OGTT was employed and several models were utilized to evaluate the extent of impaired insulin secretion.

Finally, Chapter 5 combines the results and conclusions from the previous chapters and places them in a broader perspective. The applicability of endurance exercise training as a therapeutic strategy to improve metabolic health in obesity and disorders of glucose regulation will be discussed and future research recommendations will be suggested.

1.7 References

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CHAPTER 2

**ENDURANCE EXERCISE RE-DISTRIBUTES INTRAMYOCYELLULAR LIPIDS
BUT ARE NOT IMPLICATED IN THE ETIOLOGY OF INSULIN RESISTANCE
IN PREVIOUSLY SEDENTARY OBESE MEN**

(Submitted in Public Library of Science ONE, 2011)

Endurance Exercise Re-distributes Intramyocellular Lipids but are Not Implicated in the Etiology of Insulin Resistance in Previously Sedentary Obese Men

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Running head: Re-distribution of Skeletal Muscle Lipid Droplets

Nonstandard abbreviations used: COX, cytochrome *c* oxidase; CS, citrate synthase; DAG, diacylglycerol; ETC, electron transport chain; FFM, fat-free mass; HOMA-IR, homeostasis model assessment index of insulin resistance; IMCL, intramyocellular lipid; IMF, intermyofibrillar; Mfn2, mitofusin-2; mtDNA, mitochondrial DNA; OGTT, oral glucose tolerance test; OXPHOS, oxidative phosphorylation; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α ; SCHAD, short-chain β -hydroxyacyl-CoA dehydrogenase; SS, subsarcolemmal; T2D, type 2 diabetes mellitus; TEM, transmission electron microscopy; VO_{2peak}, maximal oxygen consumption

Conflict of interest: The authors have declared that no conflict of interest exists.

Keywords: mitochondrial function, intramyocellular lipid, endurance exercise, obesity, insulin resistance

Abstract

Reduced skeletal muscle mitochondrial oxidative capacity, promoting accumulation of intramyocellular lipid (IMCL), has been implicated in the etiology of insulin resistance in obesity. However, whether impaired mitochondrial function plays a causal role in insulin resistance remains equivocal. Additionally, the impact of physical activity without weight loss on sub-cellular localization of lipids and mitochondria, and insulin resistance has not been examined. We examined markers of mitochondrial function (protein abundance and maximal enzyme activities of electron transport chain subunits), IMCL/mitochondria content in both subsarcolemmal (SS) and intermyofibrillar (IMF) regions by transmission electron microscopy, and intracellular lipid metabolites in *vastus lateralis* biopsies prior to and following twelve weeks of endurance exercise. Sedentary obese men showed no evidence of mitochondrial dysfunction, disproportionate IMCL content in either sub-cellular region, diacylglycerol or ceramide accretion despite marked insulin resistance *vs.* healthy age- and fitness-matched lean controls. Endurance exercise increased mitochondrial oxidative phosphorylation capacity and content, while only mediating moderate improvements in insulin resistance. Exercise promoted re-distribution of IMCL content - decreasing in the SS region and increasing in the IMF region while concomitantly aggregating closer to mitochondria, but no association with insulin resistance was found. Our findings suggest that insulin resistance in obesity develops independent of skeletal muscle mitochondrial dysfunction and is not associated with IMCL content regardless of sub-cellular distribution. In fact, endurance exercise enhanced skeletal muscle mitochondrial capacity and differentially regulated sub-cellular

IMCL pools with only moderate improvements in insulin resistance, further suggesting the lack of a direct association between skeletal muscle mitochondrial function and insulin sensitivity.

Introduction

Obesity is a major risk factor for the development of insulin resistance and many chronic diseases, such as type 2 diabetes mellitus (T2D) [1]. Although the primary cause of insulin resistance in obesity remains elusive, defects in skeletal muscle mitochondrial function [2,3,4,5,6,7,8] including aberrant mitochondrial morphology [2,9] and reduced expression of genes responsible for oxidative metabolism [10,11] leading to accumulation of intramyocellular lipid (IMCL) have been proposed as likely mechanisms mediating the disease process. As such, several studies have shown an inverse association between skeletal muscle IMCL content and whole-body insulin sensitivity in obese individuals [12,13] and T2D [14,15,16]. Similarly, intracellular fatty acid intermediates, such as diacylglycerol (DAG) and/or ceramides, have been shown to interfere with insulin signaling by activating inhibitory serine phosphorylation of insulin receptor substrates [17,18,19,20], resulting in reduced Akt activation [9] and glucose transport [17]. These findings have led to the hypothesis that reduced mitochondrial content and/or oxidative capacity in skeletal muscle contributes to insulin resistance and the development of T2D [8,21,22].

However, in these earlier studies [13,23,24], the subpopulation of subsarcolemmal (SS) or intermyofibrillar (IMF) IMCL was not specifically examined morphologically, which may be more important than IMCL accumulation *per se* for the development of insulin resistance/T2D. The role of IMCL in these sub-cellular regions in obesity and T2D is only beginning to be unraveled and there is a paucity of human data in this regard. The idea for examining IMCL and mitochondria in sub-cellular regions is supported by

findings from Ritov *et al.* who demonstrated that SS mitochondrial electron transport chain (ETC) activity was reduced in obese individuals with or without T2D vs. lean controls [3]. A more recent report indicated that IMF mitochondria content were reduced in subjects with T2D but SS mitochondrial content was similar compared with insulin-sensitive subjects [25]. It is important to note that in the aforementioned studies [3,25], physical fitness was not strictly controlled for. Furthermore, direct assessment of both IMCL and mitochondria content in terms of morphology (i.e., size, number, density), sub-cellular distribution and the relationship to mitochondria, using the transmission electron microscopy (TEM) technique to assess if IMCL/mitochondria distribution in specific subpopulations is related to insulin sensitivity to our knowledge has not been conducted.

Recently, the universality of the hypothesis implicating mitochondrial dysfunction to insulin resistance/T2D has been questioned [26,27,28,29,30,31,32,33,34]. For example, high-fat diet [35,36] and genetic manipulations [36,37] induce insulin resistance while concomitantly increasing mitochondrial content and fatty acid oxidation [35,36,37]. Asian Indians with T2D, in comparison with healthy Northern European Americans matched for age, sex, and BMI, have higher skeletal muscle oxidative phosphorylation (OXPHOS) capacity [30]. Furthermore, accumulation of IMCL within skeletal muscle may not be invariably linked to insulin resistance. Endurance-trained athletes also exhibit increased IMCL content, coupled with an increased abundance of mitochondria and heightened insulin sensitivity [15,24,38]. This paradox points to physical activity as a major determinant of increased net muscle lipid uptake in exercise-trained muscle.

Hence, the nature of mitochondrial dysfunction and the causal relationship between mitochondrial dysfunction and the development of insulin resistance/T2D remains elusive. Furthermore, the impact of physical activity on the compartmentalization of IMCL droplets and mitochondria is not known. The current study aimed to clarify this association by examining skeletal muscle of obese men and their age- and fitness-matched lean counterparts; and the influence of moderate-intensity endurance exercise training on skeletal muscle adaptations.

Results

Lean and Obese Baseline Characterization

Subject characteristics. Lean and obese men were group matched for age and aerobic capacity. A comprehensive description of the participants is provided in Tables 1-2. BW, BMI, waist circumference, fat mass, fat-free mass (FFM), and body fat percentage were markedly higher in obese men *vs.* lean men (Table 1). Fasting serum triglyceride and FFA levels were significantly elevated, whereas fasting serum HDL cholesterol concentrations were significantly lower in obese men *vs.* lean men (Table 2).

Oral glucose tolerance test and insulin resistance. 2-h plasma glucose levels as well as fasting and 2-h plasma insulin levels were significantly higher in obese men *vs.* lean men (Table 2). Plasma concentrations of glucose (AUC_{glucose} ; Figure 1A) and insulin (AUC_{insulin} ; Figure 1B) during the oral glucose tolerance test (OGTT) were significantly higher in obese men *vs.* lean men. Assessment of insulin resistance with the homeostasis model assessment index of insulin resistance (HOMA-IR) showed 68% greater insulin resistance in obese men *vs.* lean men (3.31 ± 0.47 *vs.* 1.97 ± 0.26 , $P \leq 0.02$; Figure 1C).

Mitochondrial function. The transcriptional co-activator peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) is widely regarded as the master regulator of mitochondrial biogenesis [39,40]. We found no difference in whole muscle protein content of PGC-1 α between lean and obese men (Figure 2A). We further analyzed the expression of several key nuclear- and mitochondrial DNA (mtDNA) encoded proteins

that are involved in mitochondrial energy metabolism. The protein content of citrate synthase (CS; marker of mitochondrial abundance), cytochrome *c* oxidase - subunit II (COXII; mtDNA-encoded) and COX - subunit IV (COXIV; nuclear DNA encoded), as well as the maximal activities of CS, COX (marker of mitochondrial oxidative capacity), and short-chain β -hydroxyacyl-CoA dehydrogenase (SCHAD; marker of mitochondrial β -oxidation) were similar in both lean and obese men (Figure 2, A and B). Mitofusin-2 (Mfn-2; mitochondrial fusion protein) protein content was also similar in both groups (Figure 2A). We measured mtDNA copy number (an index of mitochondrial content) in skeletal muscle using quantitative PCR and observed no between-group differences (Figure 2C).

Intramyocellular lipid and mitochondrial content: IMCL and mitochondria are heterogeneously distributed in myofibers. Two sub-cellular fractions of IMCL and mitochondria were characterized based on their location in both the subsarcolemmal (SS) and intermyofibrillar (IMF) regions. Electron micrographs illustrating these subpopulations are shown in Figure 3A-D. A summary of both IMCL and mitochondrial morphology and sub-cellular distribution is provided in Table 3. The *vastus lateralis* IMCL and mitochondrial content in both the SS and IMF sub-cellular regions was similar in lean and obese men (Figure 3, E-J). To examine the potential factor that may be responsible for the difference in insulin sensitivity observed between sedentary lean and obese men, we undertook a novel analysis that characterizes the physical relationship between IMCL and mitochondria. There was no difference in the proportion of IMCL

juxtaposed with mitochondria (i.e., the proportion of IMCL in contact with mitochondria) in both the SS (lean: $41.2 \pm 8.5\%$ vs. obese: $39.7 \pm 4.6\%$) and IMF (lean: $40.9 \pm 15.2\%$ vs. obese: $37.1 \pm 4.6\%$) regions between the two groups (Figure 4).

Insulin signaling, glucose uptake and lipid metabolites. Basal Akt phosphorylation at Ser⁴⁷³ residue, a key step in insulin-stimulated glucose transport activity, and GLUT4 protein content were not different between the two groups (Figure 5A). We also examined intracellular fatty acid intermediates (DAG and ceramides) and did not observe any significant differences in these variables between the two groups (Figure 5B).

Correlations. HOMA-IR was not correlated with mitochondrial function or IMCL/mitochondrial content; however, it was positively correlated with BMI ($r = 0.367$; $P \leq 0.03$), waist circumference ($r = 0.435$; $P \leq 0.01$), and body fat percentage ($r = 0.533$; $P \leq 0.01$).

Effect of Endurance Exercise Training

Subject characteristic. Twelve weeks of endurance exercise training increased aerobic capacity by 18% in lean men and by 15% in obese men ($P \leq 0.001$; Table 1). Waist circumference was reduced by 4% in lean men and by 3% in obese ($P \leq 0.001$); whereas, BW, BMI, fat mass, FFM, and body fat percentage were unchanged by training and remained markedly higher in the obese men ($P \leq 0.02$; Table 1). Endurance training increased FFA concentration ($P \leq 0.03$); whereas, total cholesterol, triglyceride, HDL

cholesterol and LDL cholesterol concentrations remained unchanged (Table 2).

Oral glucose tolerance test and insulin resistance. Endurance training induced a significant reduction (19%) in the 2-hr plasma glucose concentration (from 5.98 ± 0.46 to 4.87 ± 0.32 , $P \leq 0.01$), and a 35% reduction in the 2-h plasma insulin concentration (from 49.68 ± 10.62 to 32.40 ± 3.78 , $P = 0.07$). Endurance training had no effect on the plasma glucose concentration during the OGTT (AUC_{glucose}) (Figure 1A). Endurance training had no effect on the plasma insulin concentration during the OGTT (AUC_{insulin}), which remained significantly higher post-training in the obese group (Figure 1B). Endurance training tended to decrease HOMA-IR by 17% (2.17 ± 0.19 vs. 2.60 ± 0.30 , post- vs. pre-training, respectively, $P = 0.10$; Figure 1C).

Mitochondrial function. Endurance training did not alter whole muscle PGC-1 α protein content (Figure 2A). Endurance training increased the protein contents of CS ($P \leq 0.04$), COXII ($P \leq 0.0001$), COXIV ($P \leq 0.001$) and Mfn2 ($P \leq 0.01$) (Figure 2A) as well as the maximal activities of CS ($P \leq 0.001$), COX ($P \leq 0.03$), and SCHAD ($P \leq 0.01$) (Figure 2B). The effect of endurance training on muscle content of mtDNA was assessed for 8 of the 18 participants, those for whom sufficient pre- and post-training sample were available. Endurance training trended towards an increase in mtDNA copy number by 145% (2782 ± 1125 vs. 1136 ± 389 , post- vs. pre-training, $P = 0.10$; Figure 2C).

Intramyocellular lipid and mitochondrial content. A summary of IMCL and mitochondrial morphology and sub-cellular distribution following endurance training is provided in Table 3. Lean and obese men had similar IMCL content in both the SS and IMF regions following endurance training (Figure 3). Endurance training decreased IMCL size in the SS region (-35%, $P \leq 0.01$), but tended to increase IMCL size in the IMF region (+40%, $P = 0.09$) (Figure 3E). Endurance training decreased the number of lipids in the SS region (-20%, $P \leq 0.001$), but increased the number of lipids in the IMF region (+40%, $P \leq 0.05$) (Figure 3F). Endurance training decreased IMCL density in the SS region (-47%, $P \leq 0.001$; Figure 3G), while it increased IMF IMCL density (+93%, $P \leq 0.02$; Figure 3G).

Lean and obese men had similar mitochondrial content in both the SS and IMF regions following endurance training (Figure 3). Endurance training increased mitochondria size in the SS region ($P \leq 0.0001$) and IMF region ($P \leq 0.001$) by a similar magnitude (40%) in both groups (Figure 3H). The total number of mitochondria in either the SS or IMF sub-cellular regions were unaffected by training (Figure 3I). Endurance training increased mitochondrial density in the SS region (+37%, $P \leq 0.01$), and in the IMF region (+58%, $P \leq 0.01$; Figure 3J). Endurance training increased the proportion of IMCL droplets juxtaposed with mitochondria by 13% in the SS region ($P = 0.08$), and by 36% in the IMF region ($P \leq 0.02$; Figure 4).

Insulin signaling, glucose uptake and lipid metabolites. Endurance training increased the phosphorylation of Akt^{Ser473} protein (from 0.65 ± 0.08 to 0.90 ± 0.12 , $P \leq 0.03$) and

resting whole muscle GLUT4 protein (from 1.52 ± 0.21 to 3.26 ± 0.67 , $P \leq 0.01$; Figure 5A). Endurance training had no effect on either DAG or ceramide content (Figure 5B).

Discussion

The primary finding of the current study was that protein content of mitochondrial biogenesis regulator PGC-1 α and subunits of ETC complexes, maximal activity of mitochondrial OXPHOS and β -oxidation enzymes, and mtDNA copy number were not lower in skeletal muscle of obese men compared with healthy lean controls, despite the obese being insulin resistant. Additionally, we demonstrated that obese men did not have higher skeletal muscle IMCL, DAG or ceramide content or lower mitochondrial density vs. lean controls. Collectively, the current study has shown that the relationship between mitochondrial dysfunction and the development of obesity is not a universal finding. In contrast, the very robust effect of moderate-intensity endurance exercise training stimulating mitochondrial capacity is axiomatic but not universally related to obesity or insulin sensitivity. Furthermore, exercise training mediated a differential response on the localization of IMCL subpopulations. Importantly, body composition was maintained at baseline levels in both groups after 12 weeks of endurance exercise training. This approach helped to eliminate the potential confounding impact of weight loss and/or dietary manipulation on insulin sensitivity and muscle mitochondrial function. Thus, changes arising in outcome variables would be attributed solely to the effects of endurance exercise training and not obfuscated by body composition. The results herein lead us to draw two conclusions: 1) insulin resistance in obesity is not immutably associated with mitochondrial dysfunction or increased accumulation of IMCL/lipid intermediates in skeletal muscle when age, fitness and body compositional changes are controlled, and 2) moderate-intensity endurance exercise training evokes favorable

skeletal muscle mitochondrial adaptations independent of changes in whole-body insulin resistance.

Our findings are in agreement with a growing body of evidence that indicates that insulin resistance is not strongly associated with mitochondrial dysfunction after 12 weeks of endurance exercise training [27,28,30,32,33]. Discordance with previous studies supporting the etiological basis of mitochondrial dysfunction in insulin resistance may be partially explained by differences in study design (e.g., heterogeneity in physical fitness, co-morbidities associated with adiposity, age) and/or methodological differences in assessing mitochondrial function. In contrast to other studies [10,11], where a large difference in demographics existed between the groups, participants in the current study were appropriately age- and fitness-matched sedentary, nondiabetic, lean and obese men. Considering that individuals with obesity, insulin resistance and T2D are generally physically inactive, the impairments in oxidative metabolism might simply be attributed to their sedentary lifestyle and thus the previous associations between muscle mitochondrial function and insulin resistance may be confounded [32,41,42]. This conclusion is supported by a more recent genome-wide RNA expression analyses that found that the skeletal muscle coding transcriptome in T2D was indistinguishable from that of control subjects when subjects were well-matched [29]. Other groups have also demonstrated an absence of significant differences in mRNA and/or protein content of PGC-1 α in subjects with insulin resistance/T2D [30,33]. Furthermore, progressive oxidative dysfunction in skeletal muscle of knockout mice did not result in insulin resistance/T2D [43] and rodents fed a high-fat diet developed muscle insulin resistance

concomitant with an increase in muscle mitochondria and oxidative capacity [36,44].

Together, these findings provide supportive evidence against the theory that insulin resistance or T2D is mediated by a deficiency/dysfunction of muscle mitochondria, and may even result in increased oxidative capacity as a possible adaptive mechanism.

A key feature of the theory linking insulin resistance to mitochondrial dysfunction is the intramyocellular accumulation of lipids, which impair insulin signaling [13,22,23]. This is challenged by the increase in IMCL levels in endurance-trained athletes coupled with an increased abundance of mitochondria and heightened insulin sensitivity [15,24,38]. However, in these earlier studies, the subpopulation of subsarcolemmal or intermyofibrillar IMCL was not specifically examined morphologically. Using TEM to assess IMCL morphology in both sub-cellular regions, we found that these characteristics were similar between lean and insulin-resistant obese men. This contradicts earlier studies showing increased lipid deposition within skeletal muscle [8,45,46] but is supported by others investigating relatives of subjects with T2D [47], overweight [48] and insulin-resistant subjects [49,50,51]. Similarly, examination of the mitochondrial subpopulation morphology did not reveal smaller, less abundant or depleted mitochondrial density in insulin-resistant obese men *vs.* controls, which has been previously reported [2,8,9,12,24,25,45].

More active lipid metabolites (DAG and ceramides) have been implicated in impaired insulin signaling [52], but we also observed no differences in the level of these lipid intermediates between the two groups, supporting recent human [20,53] and rodent [54] data. Phosphorylation of Akt^{Ser473}, a key downstream step for the activation of

glucose transport, and GLUT4 protein contents were assessed in fasted muscle biopsies. No differences in these variables were observed between lean and insulin-resistant obese men. Our observation is consistent with the lack of differences in insulin-activated Akt isoforms in muscle of obese nondiabetic and obese diabetic subjects [55]. Although previous investigations have reported reduced Akt activity [56,57], it is important to note that these studies were conducted in severely obese subjects [57,58] and it is not clear whether physical activity and/or physical fitness were strictly controlled for; consequently, their findings may not be representative of the majority of obese people in society with less severe levels of obesity. The absence of insulin signaling impairments, at least in the markers we assessed, coincide with the lack of increased deposition of IMCL and lipid intermediates in the skeletal muscle of insulin-resistant obese men, which in turn support the biochemical assessments of normal mitochondrial function/ β -oxidation and mitochondrial morphology. Together, these results suggest that when confounding variables such as age and physical fitness are eliminated, insulin resistance is not mediated by skeletal muscle mitochondrial or glucose transport impairments. This is an important consideration because we have previously shown that mitochondrial function is not just influenced by age *per se*, but is strongly modulated by the physical activity status of the participants [38,59].

Insulin-resistant obese men experienced robust increases in mitochondrial content and oxidative function to the same extent as healthy, lean controls, chronically compounded over 12 weeks of endurance exercise, consistent with prior results in overweight/obese and T2D subjects [4,50,60,61,62]. Such increases in mitochondrial

content/function were seen in the setting of changes in aerobic fitness and in the absence of changes in body composition. This observation indicates that physical activity is a chief factor controlling mitochondrial capacity, calling into question whether it is sedentary behavior that is confounding the theory of reduced mitochondrial capacity in obesity, insulin resistance, and T2D.

PGC-1 α (measured at the whole muscle level), which coordinates mitochondrial gene transcription [63,64,65] and the adaptive response to exercise training [66,67], was unaltered following endurance training. This unexpected observation may not be entirely indicative of PGC-1 α activation; rather PGC-1 α activity may be primarily determined by its nuclear localization post-training [40,68]. Greater nuclear PGC-1 α would presumably be more conducive for promoting or maintaining an increase in mitochondrial biogenesis via increased co-activation of transcription factors linked to mitochondrial gene expression. Accordingly, we found increases in several classical markers of mitochondrial biogenesis [69], including the maximal activity and protein contents of CS and COX.

The effect of endurance exercise training on IMCL content in skeletal muscle is equivocal. While endurance exercise has previously been shown to lower IMCL content in T2D [70,71,72], we and others have shown that in nondiabetic untrained subjects, endurance training often elevates IMCL content [38,73,74], presumably as an adaptation to maximize surface area for lipolysis during exercise. The current study is, to our knowledge, the first using TEM to address the role of IMCL in sub-cellular regions in nondiabetic, insulin-resistant obese men with moderate-intensity endurance exercise

training. Here we describe the novel observation that IMCL content is differentially regulated sub-cellularly in myofibers in response to endurance training. In the current study, endurance training mediated a significant decrease in IMCL size, number and density in both groups in the SS region, whereas the opposite effect was observed in the IMF region, whereby IMCL size, number and density increased. Confirming our previous observations [38,75], we have demonstrated that the proportion of IMCL droplets juxtaposed with mitochondria increased in both groups post-training in both sub-cellular regions, potentially contributing to more efficient substrate oxidation. Indeed, we observed a concomitant increase in β -oxidation with endurance training.

In the current study, no clear effect of endurance training on whole-body insulin resistance was observed. It is important to note that this observation was made in the absence of body composition changes (i.e., weight loss); and a ~20% increase in aerobic capacity. Our observation is in accordance with our previous study in which three months of moderate-intensity cycling did not improve insulin resistance in lean and obese women [76]. Of note, women in the latter study did not lose body weight or alter body composition after exercise training. These results are consistent with those reporting only modest or no improvements in insulin sensitivity with exercise training in middle-aged or older men and women [61] or when the effects of weight loss [77] and the last exercise session [78] are accounted for.

In conclusion, this study does not support the notion of impaired skeletal muscle mitochondrial function or IMCL accumulation *per se* being the underlying key defect of metabolism that explains insulin resistance in obese men. Previous associations may in

part be due to a lack of controlling factors, such as age and physical fitness. Additionally, this study illustrates that moderate-intensity endurance exercise training, in the absence of weight loss, evokes favorable mitochondrial adaptations in both lean and insulin-resistant obese men to a similar extent and differentially regulates intracellular subpopulations of lipid substrates. Hence, the metabolic adaptations promoted by moderate-intensity exercise may also have important global health implications for individuals living with obesity-related sequelae. Clearly, future studies are warranted to understand molecular deficits that lead to insulin resistance. It will be of great interest to decipher if insulin resistance-mediated hormonal dysfunction in obesity modulates physical fitness and promote secondary pathology, such as skeletal muscle mitochondrial dysfunction.

Materials and Methods

Subjects. Men were recruited through local advertisements and underwent a telephone and an in-person interview to assess eligibility. The Research Ethics Board of McMaster University approved the experimental protocol (REB project #: 05-053), and subjects provided written informed consent prior to participation in accordance with the guidelines of the Declaration of Helsinki. Inclusion criteria included age from 20-55 yrs and BMI of 18.5-24.9 kg/m² for lean and ≥ 30.0 kg/m² for obese individuals with a self-reported stable BW during the previous 6 mo. Individuals who had evidence of type 2 diabetes, hypertension ($> 140/90$ mmHg), and/or an abnormal exercise stress test, smoked, had orthopedic contraindications to physical activity, or used lipid-lowering, glucose-lowering, antihypertensive, antidepressant or weight-loss medications, or consumed more than two alcoholic beverages per day were excluded. All participants were classified as living a typical Westernized sedentary lifestyle, participating only in routine activities of daily living (walking, gardening, etc.) and not engaged in regular structured or individualized aerobic or strength training programs or athletics. Twenty-four men enrolled in the study, and experimental groups were matched for age and training status (VO_{2peak}/kg FFM/min) when corrected for FFM (Table 1). Six men (3 in the lean group, 3 in the obese group) did not complete the study because of inability/unwillingness to comply with protocol or due to personal or work-related conflicts.

Protocol. All subjects underwent a 12-wk endurance exercise training protocol on a stationary cycle ergometer (Monarck, Cardio Care 827 E), as previously described [76].

Briefly, the protocol commenced with two 30-min biking sessions at 50% $\text{VO}_{2\text{peak}}$ per wk in the first week and increased to three 60-min biking session at 70% $\text{VO}_{2\text{peak}}$ per wk by the final week of training. Training was monitored to ensure that subjects were cycling at the appropriate heart rate.

Metabolic assessments. Prior to and following the intervention (48 h after the last training session), all participants underwent evaluation of insulin resistance, body composition, physical fitness and had a muscle biopsy. Participants refrained from exercise for 48 h preceding the metabolic assessments. After an overnight fast, the glycemic response to a 75-g oral glucose load (300 mL) was determined. Blood samples were collected before and 30, 60, 90 and 120 min during the OGTT. For estimation of whole-body insulin resistance from data obtained during the OGTT, the HOMA-IR [79] was calculated according to the following equation: $\text{HOMA-IR} = I_0 \times G_0 / 22.5$; where I_0 is the fasting insulin concentration (in $\mu\text{U/mL}$), G_0 is the fasting glucose concentration (in mM). The HOMA-IR has been validated against the euglycemic-hyperinsulinemic clamp [79,80]. Fat mass, FFM, and body fat percentage were assessed by dual energy X-ray absorptiometry (GE Lunar, Prodigy, Madison, WI). A symptom-limited maximal oxygen consumption test ($\text{VO}_{2\text{peak}}$) was determined on an electronically braked cycle ergometer and a computerized open-circuit gas collection system (Moxus Modulator VO_2 system with O_2 analyzer S-3A/I and CO_2 analyzer CD-3A, AEI Technologies Inc., Pittsburgh, PA). Subjects cycled (Excalibur Sport, Lode, Groningen, Netherlands) at 50 W for 1 min, thereafter increasing in increments of 25 W/min. $\text{VO}_{2\text{peak}}$ was established when O_2 consumption values reached a plateau or was the highest value during the incremental

ergometer protocol, pedal revolutions could not be maintained over 60 rpm despite vigorous encouragement, and the respiratory exchange ratio was more than 1.12. Subjects were monitored using a 12-lead ECG to rule out any cardiovascular abnormalities.

Blood sample analysis. Blood samples were taken from the antecubital vein after an overnight fast, collected in heparinized vials, placed on ice, centrifuged at 1750 g for 10 min, and stored at -80°C until subsequent analysis. Serum FFA concentration was determined using a commercially available ELISA kit (NEFA kit, Wako Diagnostics, Richmond, VA). Plasma glucose concentration was determined using an automated glucose analyzer (2300 STAT plus, YSI, UK). Plasma insulin concentration was determined using a commercially available ELISA kit (INS kit, BioSource, Belgium, EU).

Muscle biopsies. Samples of *vastus lateralis* were obtained after an overnight fast, as previously described [38]. Biopsies were taken from the same leg prior to and following the intervention with 3-5 cm between the incision sites. Approximately 150 mg muscle tissue was obtained each time and immediately dissected of any adipose and connective tissue. A portion was saved for TEM analysis and the remainder immediately flash frozen in liquid nitrogen. Samples were stored at -80°C for subsequent biochemical and molecular analysis.

Transmission electron microscopy. TEM was used to determine IMCL and mitochondrial characteristics, as previously described [38]. Samples were viewed at 6,500x using a JEOL 1200EX transmission electron microscope. Sixteen micrographs were acquired from 8 randomly sampled longitudinal sections of muscle fibers (2

micrographs/fiber) from each individual muscle - one micrograph acquired near the cell surface representing the SS region and the other acquired of parallel bundles of myofibrils representing the IMF region. Lipid droplets and mitochondrial fragments were circled and converted to actual size using a calibration grid. For each set of 16 images, mean IMCL or mitochondrial size (μm^2), total number of IMCL droplets or mitochondria per square micrometer of tissue ($\#/\mu\text{m}^2$), percentage IMCL or mitochondrial area density (i.e., the fraction of cell area occupied by IMCL or mitochondria), and the percentage of IMCL in contact with mitochondria were calculated in the IMF and SS compartments by digital imaging software (Image Pro Plus, ver. 4.0; Media Cybernetics, Silver Springs, MD), as previously described [38]. The reference for SS space quantification was the cytoplasmic space between the sarcolemma and the first layer of myofibrils.

Homogenization and Immunoblotting. Total protein was extracted from frozen biopsy samples, as previously described [38]. The Lowry assay was used to quantify the total protein content [81]. Proteins were resolved on either 7.5, 10 or 12.5% SDS-PAGE gels, transferred onto Hybond[®] ECL nitrocellulose membranes (Amersham), and immunoblotted using the following commercially available primary antibodies: anti-COX subunit II (cytochrome *c* oxidase - subunit II, MS405) and anti-COX subunit IV (cytochrome *c* oxidase - subunit IV, MS408) were purchased from MitoSciences; anti-GLUT4 from Chemicon (ab1346); anti-phospho-Akt (Ser⁴⁷³, 4060) and anti-PGC-1 α (peroxisome proliferator-activated receptor- γ coactivator-1 α , 4187) from Cell Signaling Technology; and anti-Mfn2 (mitofusin-2; M6444) from Sigma-Aldrich. The anti-CS (citrate synthase) antibody was a generous gift by Dr. Brian Robinson (The Hospital for

Sick Children, Toronto, ON). Anti- β -actin (BD Biosciences) was used as a loading control. Membranes were then incubated with the appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence detection reagent (Amersham). Relative intensities of the protein bands were digitally quantified using ImageJ Version 1.37 statistical analysis software.

Diacylglycerol and ceramide. Muscle DAG and ceramide content was determined as previously described [82]. Briefly, lipids were extracted from freeze-dried muscle, and DAG kinase and [^{32}P]ATP were added to samples preincubated with cardiolipin/oxyglucoside and allowed to react for 2 h. The reaction was stopped and samples were spotted onto thin-layer chromatography plates and developed. Bands representing ^{32}P -labelled phosphatidic acid and ceramide-1-phosphate were dried, scraped from the plate and counted using a liquid scintillation analyzer (Tri-Carb, 2500TR).

Enzyme activity. Muscle lysate CS (EC 2.3.3.1), complex IV (COX, EC 1.9.3.1), and β -oxidation (SCHAD) activity was determined, as previously described [38]. All samples were analyzed in duplicate on a UV spectrophotometer (Cary 300 Bio UV-Visible spectrophotometer, Varian, Palo Alto, CA) and expressed as $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$.

Total DNA isolation. Total DNA was isolated from ~15 mg of skeletal muscle using the Qiagen total DNA isolation kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. DNA samples were treated with RNase (Fermentas, Mississauga, ON) to remove RNA contamination. DNA concentration and quality was

assessed using Nanodrop 2000 (Thermo Scientific, Wilmington, DE).

Mitochondrial DNA content. mtDNA copy number, relative to the diploid chromosomal DNA content was quantitatively analyzed in skeletal muscle using ABI 7300 real-time PCR (Applied Biosystems, CA), as previously described [83]. Primers were designed around ND1 (forward primer, L3485–3504; reverse primer, H3532–3553) and ND4 (forward primer, L12087–12109; reverse primer, H12140–12170) regions of the mitochondrial genome. Nuclear β -globin gene was used as a housekeeping gene.

Statistical analysis. When analyzing differences between lean and obese individuals, statistical analyses were completed using unpaired Student's *t*-tests for independent samples (Statistica, Version 5.0, Statsoft, Tulsa, OK) with adiposity (lean, obese) being the experimental condition. A two-way repeated measures ANOVA (Statistica, Version 5.0, Statsoft, Tulsa, OK) with adiposity (lean, obese) and training (pre, post) being the experimental conditions was completed when analyzing the effect of the endurance exercise program. When statistical significance was achieved, a Tukey's HSD post-hoc test was used to identify individual differences. Correlation analyses were performed using GraphPad Prism (Version 4, GraphPad Software, San Diego, CA). Statistical significance was established at $P \leq 0.05$. Data are presented as means \pm SEM.

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I.A.S., M.J.H., and M.A.T. conceived and designed the study; I.A.S., M.J.H., A.W.G., and J.S. were involved in conducting and coordinating the study; A.W.G. and N.J.M. contributed to the electron microscopy analyses; J.P.L. provided technical assistance in Western blot; G.R.S. contributed to the diacylglycerol and ceramide analyses; I.A.S. and A.S. researched and analyzed data; I.A.S. wrote the article; I.A.S., A.S., M.J.H., J.P.L., S.R., and M.A.T. reviewed and edited the article. All authors read and approved the final article.

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Table 1
Participant characteristics

	Lean		Obese	
	Pre-training	Post-training	Pre-training	Post-training
<i>n</i>	9		9	
Age (yr)	38 ± 3		39 ± 3	
Height (cm)	179 ± 3		180 ± 3	
Body weight (kg)	75.5 ± 3.4	75.2 ± 3.2	108.4 ± 6.1 ^A	107.1 ± 6.5 ^B
BMI (kg.m ⁻²)	23.6 ± 0.5	23.5 ± 0.5	33.6 ± 1.6 ^A	33.1 ± 1.7 ^B
Waist circumference (cm)	86.2 ± 1.4	82.5 ± 1.7 ^C	111.8 ± 3.7 ^A	108.6 ± 4.2 ^{B,C}
Fat mass (kg)	14.9 ± 1.9	14.4 ± 2.1	35.7 ± 3.3 ^A	35.2 ± 3.5 ^B
Fat-free mass (kg)	57.3 ± 2.6	57.9 ± 2.5	68.8 ± 2.7 ^A	68.2 ± 2.9 ^B
Body fat (%)	20.5 ± 2.3	19.7 ± 2.5	33.7 ± 1.4 ^A	33.5 ± 1.5 ^B
Aerobic capacity (mL O ₂ .kg ⁻¹ fat-free mass.min ⁻¹)	46.9 ± 2.1	55.5 ± 2.4 ^C	44.6 ± 2.1	51.1 ± 2.0 ^C

Data are presented as means ± SEM.

^A Obese group data significantly different from lean group data, $P \leq 0.01$

^B Obese group data significantly different from lean group data, $P \leq 0.02$

^C Post-training significantly different from pre-training (main effect), $P \leq 0.001$

Table 2
Metabolic characteristics

	Lean		Obese	
	Pre-training	Post-training	Pre-training	Post-training
Total cholesterol (mM)	4.75 ± 0.36	4.57 ± 0.29	5.26 ± 0.36	5.31 ± 0.35
Triglyceride (mM)	0.92 ± 0.14	0.78 ± 0.12	1.54 ± 0.24 ^A	1.49 ± 0.23 ^B
HDL cholesterol (mM)	1.49 ± 0.07	1.42 ± 0.08	1.20 ± 0.07 ^A	1.22 ± 0.07
LDL cholesterol (mM)	2.84 ± 0.29	2.79 ± 0.26	3.35 ± 0.30	3.41 ± 0.30
FFA (mM)	0.31 ± 0.04	0.53 ± 0.9 ^C	0.53 ± 0.06 ^A	0.61 ± 0.04 ^C
FPG (mM)	5.39 ± 0.14	5.48 ± 0.12	5.68 ± 0.20	5.70 ± 0.24
FPI (μU.mL ⁻¹)	8.07 ± 0.87	7.60 ± 0.89	12.11 ± 1.42 ^A	10.15 ± 1.09
2-h PG (mM)	4.88 ± 0.24	4.37 ± 0.27 ^C	7.09 ± 0.74 ^A	5.43 ± 0.55 ^C
2-h PI (μU.mL ⁻¹)	25.06 ± 6.55	26.46 ± 4.86	74.29 ± 16.89 ^A	39.08 ± 5.21

Data are presented as means ± SEM.

^A Obese group data significantly different from lean group data, $P \leq 0.04$

^B Obese group data significantly different from lean group data, $P \leq 0.01$

^C Post-training significantly different from pre-training (main effect), $P \leq 0.03$

2-h PG, 2-h plasma glucose; 2-h PI, 2-h plasma insulin; FFA, free fatty acid; FPG, fasting plasma glucose; FPI, fasting plasma insulin.

Table 3
Intramyocellular lipid and mitochondrial morphology

	Lean		Obese		Adiposity Effect	Training Effect
	Pre-training	Post-training	Pre-training	Post-training		
Intramyocellular lipid						
IMCL size (μm^2)						
Subsarcolemmal	0.21 \pm 0.02	0.14 \pm 0.02	0.25 \pm 0.04	0.17 \pm 0.01	NS	$P \leq 0.01$
Intermyofibrillar	0.15 \pm 0.02	0.19 \pm 0.04	0.15 \pm 0.02	0.23 \pm 0.03	NS	$P = 0.08$
No. of IMCL ($\# \cdot \mu\text{m}^{-2}$)						
Subsarcolemmal	0.39 \pm 0.02	0.31 \pm 0.04	0.42 \pm 0.03	0.32 \pm 0.04	NS	$P \leq 0.001$
Intermyofibrillar	0.05 \pm 0.01	0.07 \pm 0.01	0.05 \pm 0.01	0.07 \pm 0.01	NS	$P \leq 0.05$
IMCL density (%)						
Subsarcolemmal	7.8 \pm 1.1	4.3 \pm 0.8	10.3 \pm 1.5	5.2 \pm 0.7	NS	$P \leq 0.001$
Intermyofibrillar	0.7 \pm 0.1	1.3 \pm 0.3	0.8 \pm 0.2	1.6 \pm 0.3	NS	$P \leq 0.02$
Mitochondria						
Mitochondria size (μm^2)						
Subsarcolemmal	0.11 \pm 0.01	0.14 \pm 0.01	0.09 \pm 0.01	0.13 \pm 0.01	NS	$P \leq 0.0001$
Intermyofibrillar	0.09 \pm 0.01	0.13 \pm 0.01	0.09 \pm 0.01	0.13 \pm 0.01	NS	$P \leq 0.001$

No. of mitochondria (#.µm ⁻²)						
Subsarcolemmal	1.43 ± 0.16	1.53 ± 0.14	1.31 ± 0.19	1.31 ± 0.12	NS	NS
Intermyofibrillar	0.40 ± 0.04	0.45 ± 0.04	0.37 ± 0.05	0.45 ± 0.06	NS	NS
Mitochondrial density (%)						
Subsarcolemmal	14.5 ± 1.5	20.1 ± 1.5	11.9 ± 1.6	15.9 ± 1.4	NS	<i>P</i> ≤ 0.01
Intermyofibrillar	3.8 ± 0.5	5.8 ± 0.5	3.2 ± 0.4	5.3 ± 0.5	NS	<i>P</i> ≤ 0.01
IMCL Juxtaposed Mitochondria (%)						
Subsarcolemmal	41.2 ± 2.8	48.0 ± 3.3	39.7 ± 4.6	43.4 ± 3.2	NS	<i>P</i> = 0.07
Intermyofibrillar	40.9 ± 5.1	53.8 ± 5.0	37.1 ± 4.6	52.1 ± 6.4	NS	<i>P</i> ≤ 0.02

Data are presented as means ± SEM. IMCL, intramyocellular lipid; NS, not significant.

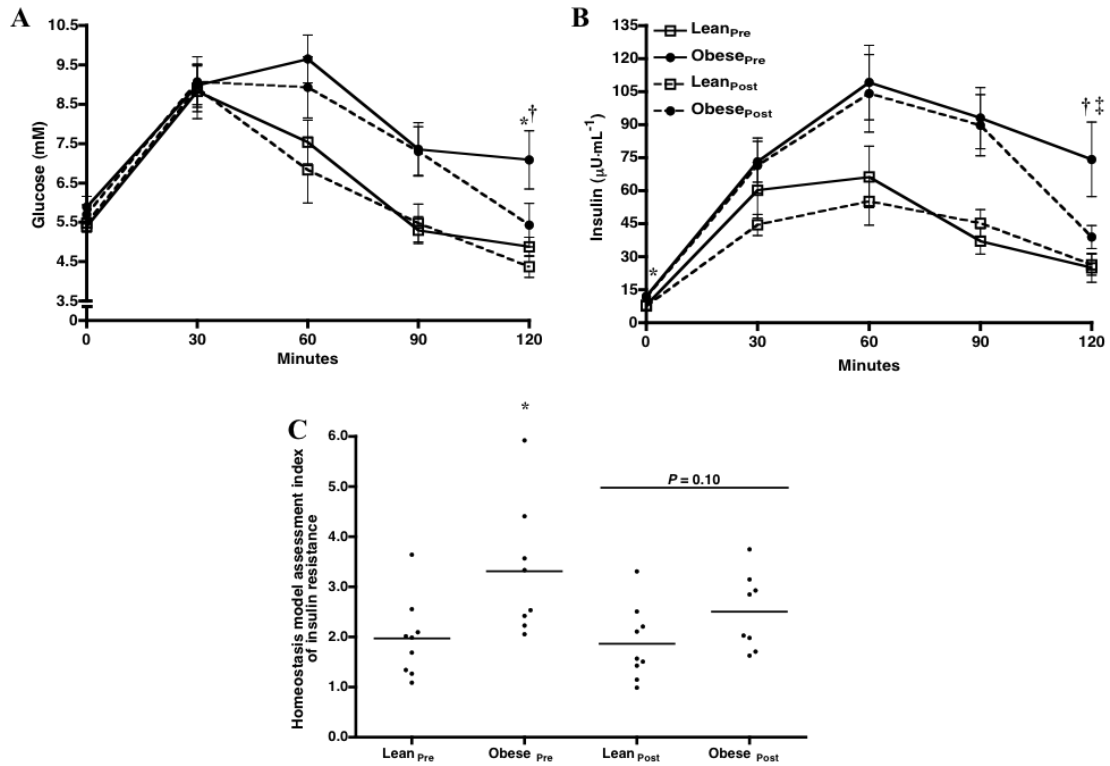


Figure 1. Results of the oral glucose tolerance test. Mean plasma concentrations of glucose (**A**) and insulin (**B**) during a 75-g oral glucose tolerance test, and (**C**) the homeostasis model assessment index of insulin resistance (HOMA-IR) in lean ($n = 9$) and obese ($n = 9$) men prior to and following 12-wk endurance training. (**A**) $P = 0.04$ and $P = 0.28$ for the comparison of the areas under the curve for glucose (AUC_{glucose}) of lean and obese men, pre- and post-training, respectively. 2-hr plasma glucose concentration: * $P \leq 0.01$ lean vs. obese pre-training; † $P \leq 0.01$ pre- vs. post-training (main effect). (**B**) $P = 0.02$ and $P = 0.02$ for the comparison of the areas under the curve for insulin (AUC_{insulin}) of lean and obese men, pre- and post-training, respectively. Fasting plasma insulin concentration: * $P \leq 0.03$ lean vs. obese pre-training; 2-hr plasma insulin concentration: † $P \leq 0.02$ lean vs. obese pre-training; ‡ $P = 0.07$ pre- vs. post-training (main effect). (**C**) HOMA-IR was 68% higher in the obese vs. lean men pre-training and decreased by 17% post-training. * $P \leq 0.02$ lean vs. obese pre-training; $P = 0.10$ pre- vs. post-training (main effect).

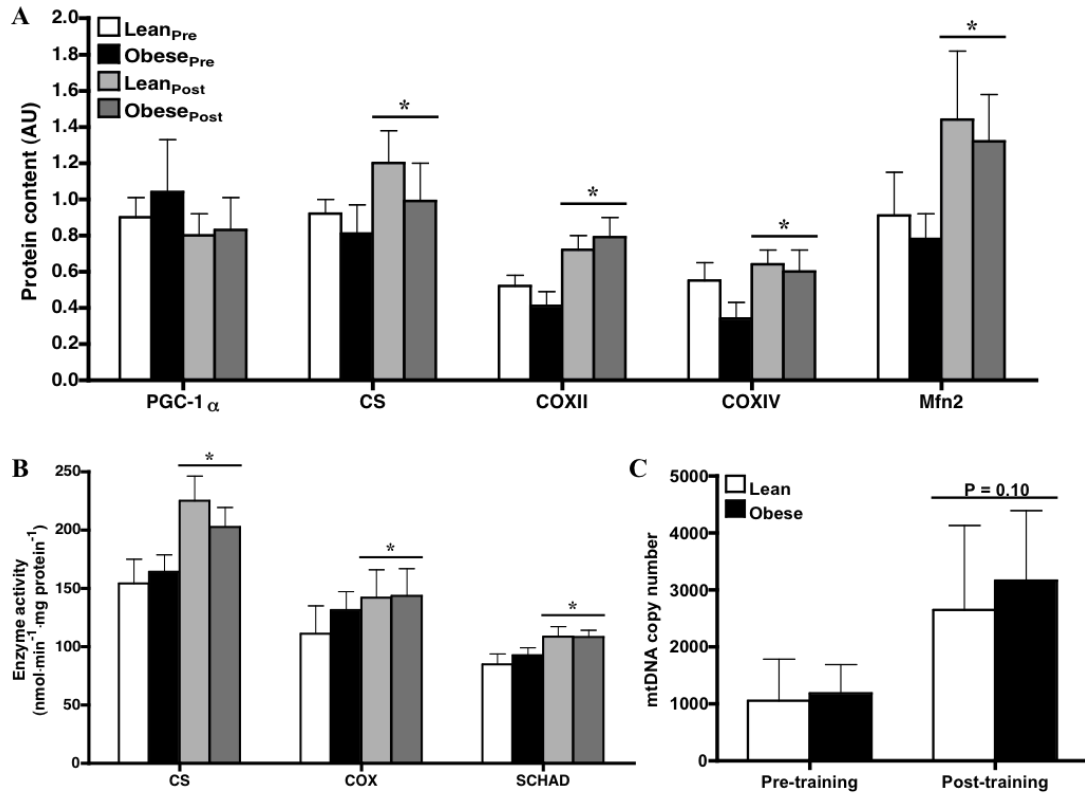


Figure 2. Markers of mitochondrial function. (A) Mitochondrial protein content assessed by Western blot and (B) mitochondrial maximal enzyme activity in skeletal muscle of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12-wk endurance training. (A) PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α ; CS, citrate synthase; COX, cytochrome *c* oxidase - subunits II and IV; Mfn2, mitofusin-2. Results were normalized to β -actin protein content. * $P \leq 0.04$ pre- vs. post-training (main effect). (B) CS, citrate synthase; COX, cytochrome *c* oxidase; SCHAD, short-chain β -hydroxyacyl-CoA dehydrogenase. * $P \leq 0.03$ pre- vs. post-training (Main effect). (C) Mitochondrial DNA (mtDNA) copy number determined by real-time quantitative PCR using a TaqMan probe against NADH dehydrogenase 4 (ND4) and β -globin. mtDNA copy number was calculated as the ratio of ND4 to β -globin in skeletal muscle of lean ($n = 3$) and obese ($n = 5$) men prior to and following 12-wk endurance training. * $P = 0.10$ pre- vs. post-training (main effect).

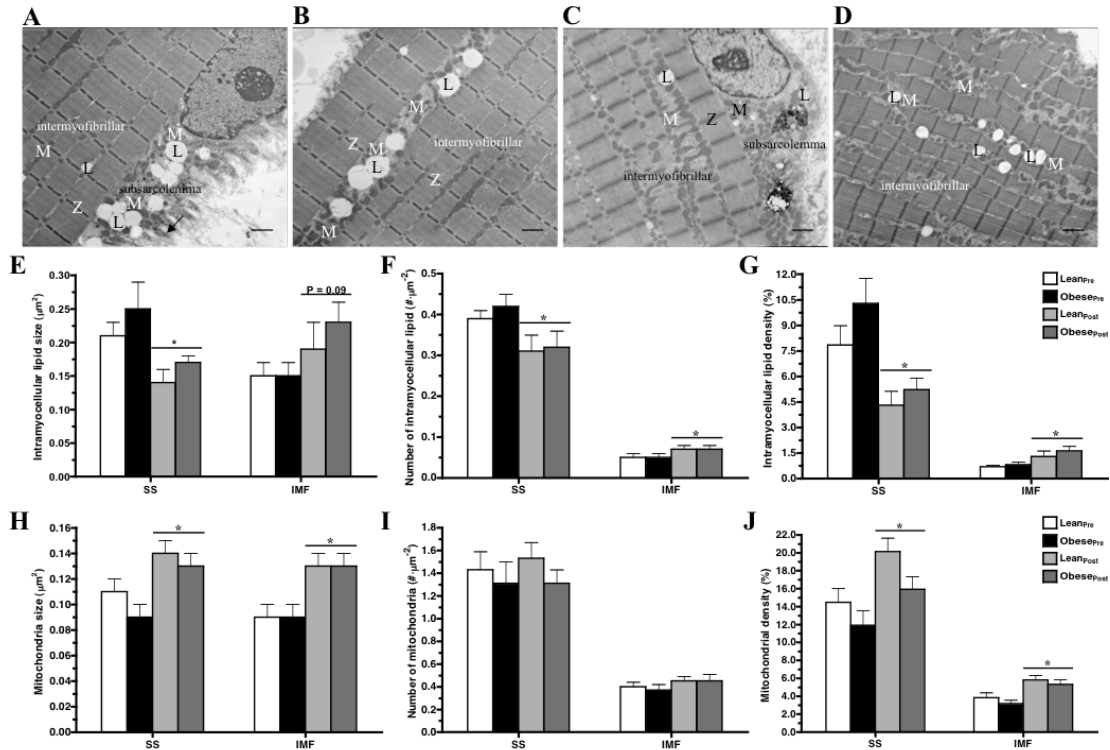


Figure 3. Transmission electron microscopy assessment of intramyocellular lipid and mitochondrial content. Micrographs of a skeletal muscle cell illustrating subsarcolemmal (A) and intermyofibrillar (B) intramyocellular lipid (IMCL) and mitochondria prior to (A,B) and following 12-wk endurance training (C,D). Subsarcolemmal (SS) IMCL and mitochondria are located between the sarcolemma and the most superficial myofibrils. The intermyofibrillar (IMF) IMCL and mitochondria are located between parallel bundles of myofibrils. The micrographs (X6,500 magnification, scale bar: 1 μm) were obtained from a biopsy of the *vastus lateralis* muscle from an obese participant. L, intramyocellular lipid droplet; M, mitochondria, Z, Z-line. IMCL size (E), number (F), and density (G) in SS and IMF regions of skeletal muscle of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12-wk endurance training. * $P \leq 0.05$ pre- vs. post-training (main effect). Mitochondria size (H), number (I), and density (J) in SS and IMF regions of skeletal muscle of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12-wk endurance training. * $P \leq 0.01$ pre- vs. post-training (main effect).

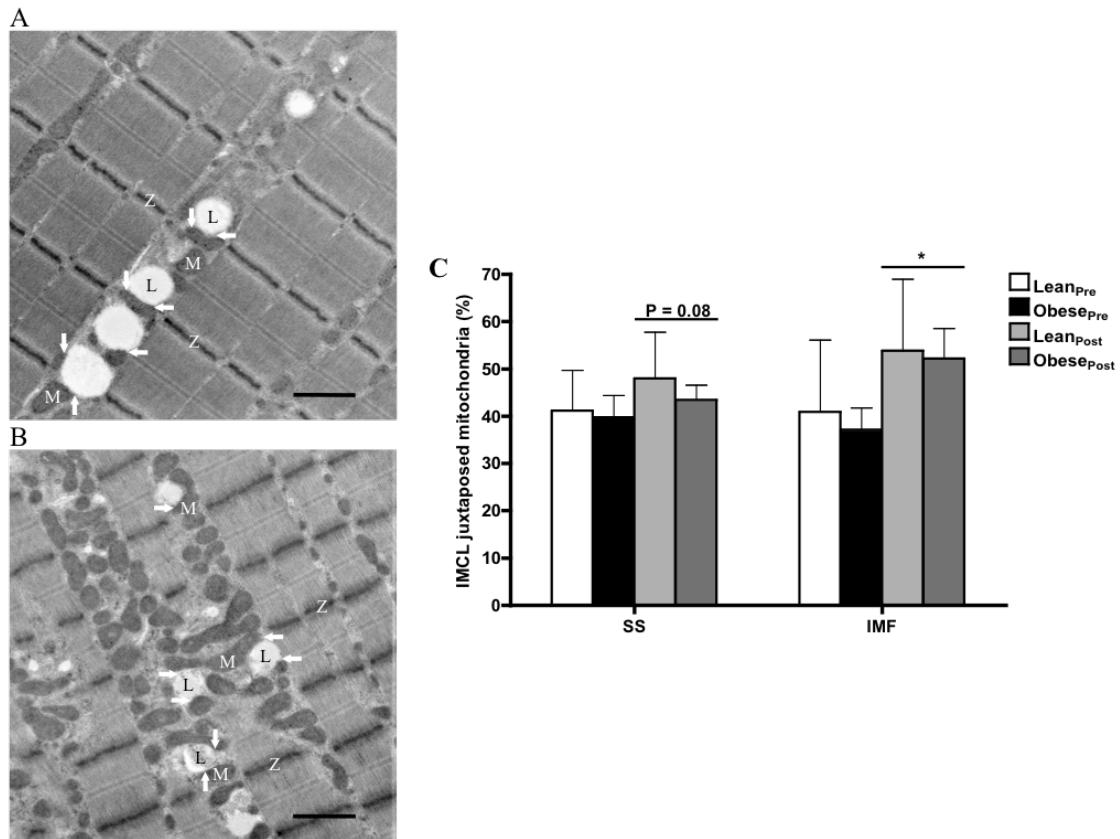


Figure 4. Transmission electron microscopy assessment of intramyocellular lipid and mitochondrial proximity. Representative electron micrographs of a skeletal muscle cell illustrating subsarcolemmal (**A**) and intermyofibrillar (**B**) intramyocellular lipid (IMCL) juxtapsed with mitochondria prior to (**A,B**) and following 12-wk endurance training (**C,D**). The micrographs (X6,500 magnification, scale bar: 1 μ m) were obtained from a biopsy of the *vastus lateralis* muscle from an obese participant. Graph represents the proportion of IMCL juxtapsed with mitochondrial (i.e., the proportion of IMCL in contact with mitochondria) in subsarcolemmal and intermyofibrillar regions of skeletal muscle of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12-wk endurance training. * $P \leq 0.02$ pre- vs. post-training (main effect).

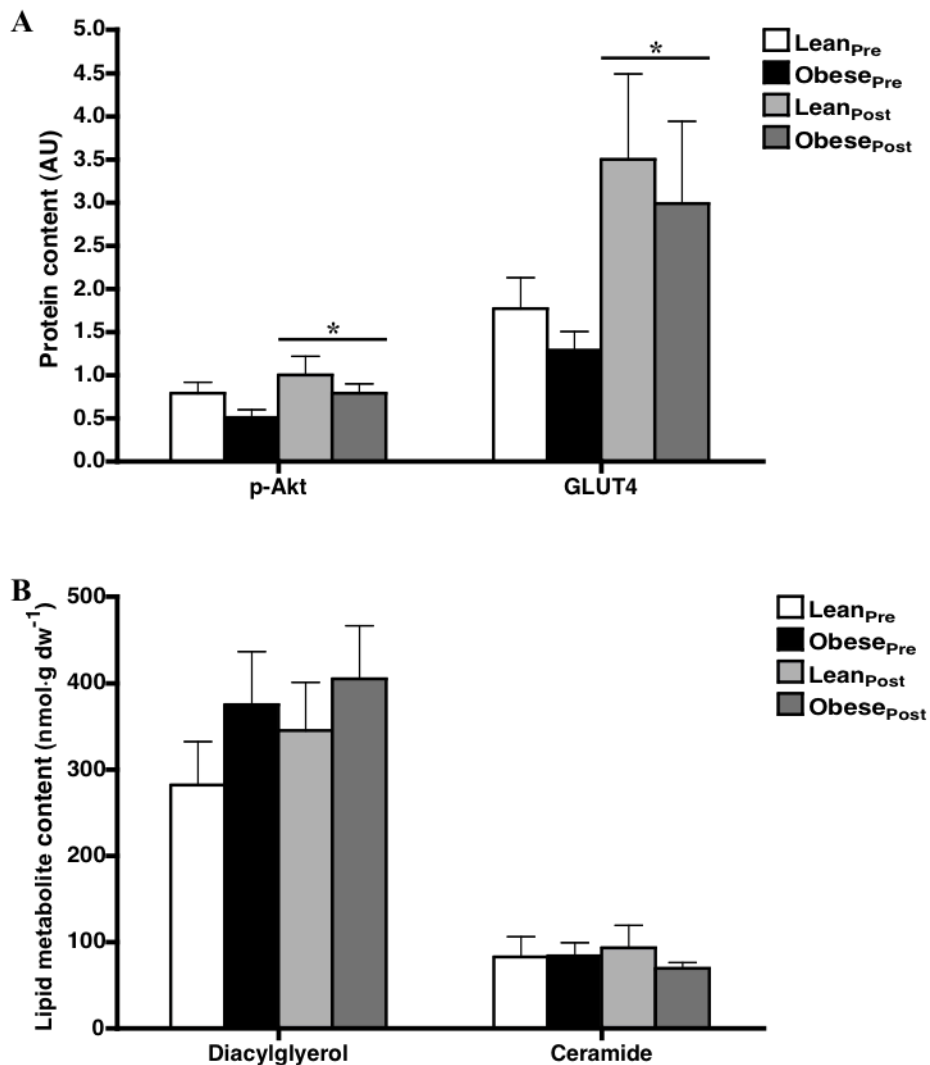


Figure 5. Insulin signaling and lipid metabolite data. (A) Akt phosphorylation at Ser473 residue and GLUT4 protein content assessed by Western blot and (B) diacylglycerol and ceramide lipid content in skeletal muscle of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12-wk endurance training. (A) p-Akt, Akt phosphorylation at Ser473; GLUT4, glucose transporter 4. Results were normalized to β -actin protein content. * $P \leq 0.03$ pre- vs. post-training (main effect).

CHAPTER 3

**ENDURANCE EXERCISE, INDEPENDENT OF WEIGHT LOSS, IMPROVES
BOTH SKELETAL MUSCLE AND SYSTEMIC OXIDATIVE STRESS IN
PREVIOUSLY SEDENTARY INSULIN-RESISTANT OBESE MEN**

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Endurance exercise, independent of weight loss, improves both skeletal muscle and systemic oxidative stress in previously sedentary insulin-resistant obese men

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Abstract

Obesity is associated with a state of low-grade systemic inflammation leading to peripheral insulin resistance. Increased oxidative stress is proposed to link adiposity and chronic inflammation through secretion of adipocytokines. The effects of chronic endurance exercise modulating these outcomes in insulin-resistant obese adult men are unclear. We investigated the influence of moderate endurance cycling training for 3 months on oxidative damage (4-hydroxy-2-nonenal, 4-HNE; protein carbonyls) and antioxidant enzymes (superoxide dismutase, SOD; catalase) in skeletal muscle; urine (8-hydroxy-2-deoxyguanosine, 8-OHdG; 8-isoprostane); and plasma adipocytokines (C-reactive protein, CRP; interleukin-6, IL-6; leptin, adiponectin), in age- and fitness-matched sedentary obese and lean men ($n = 9/\text{group}$). Insulin-resistant obese subjects showed higher 4-HNE, protein carbonyls, Cu/ZnSOD, CRP, IL-6, and leptin, with lower adiponectin, compared to lean controls ($P \leq 0.03$ for all). Training reduced 4-HNE, protein carbonyls, 8-isoprostane, and leptin levels ($P \leq 0.05$ for all); and had a strong tendency to decrease IL-6 and insulin resistance; and increased MnSOD levels ($P \leq 0.01$). In conclusion, regular endurance exercise reduced both skeletal muscle and systemic oxidative damage while improving insulin resistance and adipocytokine profile associated with obesity, independent of weight loss.

Keywords

Obesity, inflammation, reactive oxygen species, oxidative stress, antioxidant enzymes, endurance exercise, skeletal muscle

Introduction

Obesity is an independent risk factor for type 2 diabetes mellitus (T2D), cardiovascular disease, non-alcoholic steatohepatitis, stroke, and cancer [1]. Obesity is associated with chronic low-grade systemic inflammation [2]. Adipocytes are a major source of pro-inflammatory cytokines and are implicated in the onset and progression of obesity, insulin resistance and cardiovascular disease [3]. Increased oxidative stress has been suggested to link adiposity and chronic inflammation [4] through secretion of these adipocytokines [2]. The pro-inflammatory adipocytokines, leptin, interleukin-6 (IL-6), and C-reactive protein (CRP), are higher, whereas adiponectin, the main anti-inflammatory and insulin-sensitizing compound, is lower, with obesity [5]. Adipocytokines are known to modulate various aspects of skeletal muscle metabolism, and therefore any alteration in cytokine secretome during obesity may negatively modulate insulin sensitivity. The mechanisms underlying insulin resistance in obesity remain unclear, although pro-inflammatory stress signals initiated through altered secretion of adipocytokines and oxidative stress are thought to be involved.

Paradoxically, although moderate exercise induces an acute oxidant pulse, it is well established that exercise training results in an increase in skeletal muscle oxidative capacity [6, 7], antioxidant enzymes [8], and insulin sensitivity [9]. These findings are consistent with the *hormesis* hypothesis that adaptations induced by acute exposures to exercise-induced oxidant stress lead to long-term cellular metabolic and redox maintenance. This exercise training adaptation occurs through activation of signaling pathways that lead to increased synthesis of enzymatic and non-enzymatic antioxidants

[10, 11] and decreased production of reactive oxygen species (ROS) during exercise in both human and animal models [12].

In overweight/obese individuals, the effects of endurance training on oxidative stress and inflammation are less clear. In lean and obese men, endurance training without weight loss decreases circulating IL-6 and leptin concentrations with no effect on CRP concentrations [13]. However, other studies have not found an independent effect of exercise training on levels of inflammatory markers and indicate that weight loss might be required to normalize the levels of inflammatory markers [14, 15]. We have previously reported an improvement in cellular skeletal muscle redox status without alteration in systemic inflammation in obese women subjected to aerobic training that led to higher aerobic capacity, independent of weight loss [16]. There are few studies directly examining the association between oxidative stress, inflammation and chronic exercise training in adult obese men [17, 18].

We suspect that peripheral insulin resistance stems from the combination of factors prevalent in obesity, including adipocytokine imbalance, oxidative stress, and systemic low-grade inflammation. We hypothesize that endurance exercise training modulates these factors that contribute to exercise training-mediated improvement in insulin resistance, in part, by augmenting physiological antioxidant mechanisms. In the current study, we examined the relationship between adiposity, adipocytokines, and oxidative stress levels in a cohort of non-diabetic lean and insulin-resistant obese men, group-matched for age and physical fitness. We also investigated the therapeutic effects of endurance training on markers of oxidative damage, inflammation and adipocytokine

levels, independent of changes in body composition.

Materials and Methods

Subjects. Men were recruited through local advertisements and underwent a telephone and an in-person interview to check their medical history in order to assess eligibility for participation. The Research Ethics Board of McMaster University approved the experimental protocol (REB project #: 05-053), and subjects provided written informed consent for participation in the study. The study was conducted in accordance with the guidelines of the Declaration of Helsinki. Inclusion criteria included age from 20-55 years and body mass index (BMI) of ≥ 30.0 kg/m² for obese and 18.5-24.9 kg/m² for lean individuals with a self-reported stable body weight during the previous 6 mo. Exclusion criteria included evidence of diabetes, hypertension ($\geq 140/90$ mmHg), and/or an abnormal exercise stress test. Individuals who smoked, exercised more than 3.5 h/wk at a level more vigorous than walking for the preceding 6 mo, had orthopedic contraindications to physical activity, or used lipid-lowering, glucose-lowering, antihypertensive, antidepressant or weight-loss medications were also excluded. Diet-related exclusion criteria included more than two alcoholic beverages per day. Twenty-four men enrolled in the study, and experimental groups were matched for age and training status (VO_{2peak}/kg FFM/min) when corrected for fat-free mass (FFM) (Table 1). Six men (3 in the lean group, 3 in the obese group) did not complete the study because of inability/unwillingness to comply with protocol or due to personal or work-related conflicts.

Protocol. All subjects underwent a 12-wk endurance training protocol on a stationary cycle ergometer (Monarck, Cardio Care 827 E), as previously described by our

group [19]. Briefly, the protocol commenced with two 30-min biking sessions at 50% VO_{2peak} per week in the first week and increased to three 60-min biking session at 70% VO_{2peak} per week by the final week of training. Training was monitored to ensure that subjects were cycling at the appropriate heart rate.

Metabolic assessments. Prior to and following the intervention, all participants underwent evaluation of insulin resistance, body composition, physical fitness and had a muscle biopsy. Participants were asked not to exercise for 48 h preceding the metabolic assessments. After an overnight fast, the glycemic response to a 75-g oral glucose load (300 mL) was determined. Blood samples were collected before and 30, 60, 90 and 120 min during the oral glucose tolerance test (OGTT). For assessment of insulin resistance, the homeostasis model assessment index of insulin resistance (HOMA-IR) was determined according to the equation: $HOMA-IR = I_0 \times G_0 / 22.5$; where I_0 is the fasting insulin concentration (in $\mu U/mL$), and G_0 is the fasting glucose concentration (in mM) [20]. Fat mass, FFM, and body fat percentage were assessed by dual energy X-ray absorptiometry (GE Lunar, Prodigy, Madison, WI). A symptom-limited maximal oxygen consumption test (VO_{2peak}) was determined on an electronically braked cycle ergometer and a computerized open-circuit gas collection system (Moxus Modulator VO_2 system with O_2 analyzer S-3A/I and CO_2 analyzer CD-3A, AEI Technologies Inc., Pittsburgh, PA). Subjects cycled (Excalibur Sport, Lode, Groningen, Netherlands) at 50 W for 1 min, thereafter increasing in increments of 25 W/min. VO_{2peak} was established when O_2 consumption values reached a plateau or was the highest value during the incremental ergometer protocol, pedal revolutions could not be maintained over 60 rpm despite

vigorous encouragement, and the respiratory exchange ratio was more than 1.12. Subjects were monitored using a 12-lead ECG to rule out any cardiovascular abnormalities.

Blood sample analysis. Blood samples were taken from the antecubital vein after an overnight fast and collected in heparinized or untreated vials, placed on ice, centrifuged at 1750 g for 10 min, and stored at -80°C until subsequent analysis. Serum free-fatty acid (FFA) and adipokines were determined using commercially available ELISA kits: FFA (HR Series NEFA-HR(2), Wako Diagnostics, Richmond, VA, USA); adiponectin (Human Adiponectin Kit DRP300), leptin (Human Leptin Kit DLP00), and interleukin-6 (Human IL-6 Kit D6050) were purchased from Quantikine (R&D Systems, Minneapolis, MN, USA); and C-reactive protein (Human CRP ELISA kit, Alpha Diagnostics International, Cedarlane Labs, Canada). Plasma glucose concentration was determined using an automated glucose analyzer (2300 STAT plus, YSI, UK). Plasma insulin concentration was determined using a commercially available ELISA kit (INS-EASIA, Kit KAP1251, BioSource, Belgium, EU).

Urine sample analysis. Subjects were asked to collect their urine over a 24-h period. Urine samples were stored at -80°C until subsequent biochemical determination. Urine samples were analyzed for markers of oxidative stress using commercially available ELISA kits: 8-isoprostane (Kit 516351, Cayman Chemical, Ann Arbor, MI, USA) and 8-hydroxy-2-deoxyguanosine (8-OHdG; New 8-OHdG Check, Kit KOG-200S/E, JaICA, Shizuoka, Japan).

Muscle biopsies. Samples of *vastus lateralis* were obtained by percutaneous suction-modified Bergström needle biopsy, as previously described by our group [21].

Biopsies were taken from the same leg prior to and following the intervention with 3-5 cm between the incision sites. Approximately 120 mg muscle tissue were obtained each time and immediately dissected of any adipose and connective tissue. Muscle tissue was immediately stored in liquid nitrogen until transferred to -80°C to be stored for subsequent biochemical and molecular analysis.

Homogenization. Total protein was extracted from frozen skeletal muscle biopsy samples as previously described in detail by our group [22]. Briefly, ~ 30 mg of skeletal muscle were homogenized [50 mM TRIS-HCl, 150 mM NaCl, 1% Triton X-100, and 1% sodium deoxycholate, pH 7.4 supplemented with a Complete Mini, EDTA-free protease inhibitor cocktail tablet and a PhosSTOP, phosphatase inhibitor cocktail tablet (Roche Applied Science, Mannheim, Germany) per 10 mL of buffer] on ice in a 2 mL Wheaton glass homogenizer (Fisher Scientific, Ottawa, ON, Canada) using a 1:25 dilution of muscle to buffer. Lysates were centrifuged for 15 min at 600 g at 4°C , and the supernatant was removed, flash frozen, and stored at -80°C until subsequent analysis. The Lowry assay was used to quantify the total protein content of samples as previously described [23].

Immunoblotting. Proteins were resolved on 7.5, 10 or 12.5% SDS-PAGE gels depending on the molecular weight of the protein of interest. The gels were transferred onto Hybond[®] ECL nitrocellulose membranes (Amersham, Piscataway, NJ, USA), and immunoblotted using the following commercially available primary antibodies: anti-4-hydroxy-2-nonenal (4-HNE; ab48506-50), anti-Cu/ZnSOD (ab16831-100), anti-MnSOD (ab13534-50), and anti-catalase (ab16731) were purchased from Abcam Inc. (Cambridge,

MA, USA). Anti-actin (612657, BD Biosciences, Mississauga, ON, Canada) was used as a loading control and to normalize the expression of proteins of interest. Membranes were then incubated with the appropriate anti-mouse or anti-rabbit (depending on the primary antibody source) horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence detection reagent (Amersham, Piscataway, NJ, USA). Relative intensities of the protein bands were digitally quantified (ImageJ, Version 1.37, NIH, Bethesda, MD, USA).

Protein carbonyl. Total protein carbonyl content in muscle lysates was determined using a commercially available kit (Oxyblot Protein Oxidation Detection Kit S7150, Chemicon International, Inc., Temecula, CA, USA). Briefly, 10 μ L of 12% SDS and 20 μ L of DNPH were added to 10 μ L of muscle homogenate, and incubated for 15 min at room temperature. Seventeen microliters of neutralizing solution were added to the samples followed by the addition of 6 μ L β -mercaptoethanol (1:1.7 dilution with ddH₂O). Proteins were resolved on 12.5% SDS-PAGE and transferred onto a Hybond[®] ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA). Relative intensities of the protein bands were digitally quantified (ImageJ, Version 1.37, NIH, Bethesda, MD, USA).

Statistical analysis. When analyzing differences between lean and obese individuals, statistical analyses were completed using unpaired Student's *t*-tests for independent samples (Statistica, Version 5.0, Statsoft, Tulsa, OK, USA) with adiposity (lean, obese) being the experimental condition. A two-way repeated measures ANOVA (Statistica, Version 5.0, Statsoft, Tulsa, OK, USA) with adiposity (lean, obese) and

training (pre, post) being the experimental conditions was completed when analyzing the effect of the endurance exercise program. When statistical significance was achieved, a Tukey's HSD post-hoc test was used to identify individual differences. Correlation analyses were performed using GraphPad Prism (Version 4, GraphPad Software, San Diego, CA, USA). We used a one-tailed test with urinary markers of oxidative damage (8-OHdG and 8-isoprostane), skeletal muscle protein carbonyls and 4-HNE content, as well as plasma adipokines (leptin, adiponectin) and markers of inflammation (CRP, IL-6) because we *a priori* hypothesized that obese individuals would have higher concentrations of these markers (8-OHdG, 8-isoprostane, protein carbonyl, 4-HNE, CRP, IL-6, and leptin), but lower adiponectin levels compared with the lean group; and would be influenced by exercise, based upon earlier work by our group [16]. For all other analyses, a two-tailed test was employed. Statistical significance was established at $P \leq 0.05$. Data are presented as means \pm SEM.

Results

Subject characteristic. Prior to training, obese men had significantly higher body weight, BMI, waist circumference, fat mass, FFM, and body fat percentage vs. lean men (Table 1). Prior to training, obese men had significantly higher serum triglyceride and FFA concentrations, but lower serum HDL cholesterol concentrations vs. lean men (Table 2). Endurance training significantly increased aerobic capacity by 18% in lean men and by 15% in obese men ($P \leq 0.001$) (Table 1). Endurance training reduced waist circumference by 4% in lean men and by 3% in obese men ($P \leq 0.001$), whereas it had no effect on body weight, BMI, fat mass, FFM, and body fat percentage which remained markedly higher in the obese group ($P \leq 0.02$) (Table 1). Endurance training increased FFA concentrations ($P \leq 0.03$); whereas, total cholesterol, triglyceride, HDL and LDL cholesterol concentrations remained unchanged (Table 2).

Oral glucose tolerance test and insulin resistance. Prior to training, obese men had significantly higher plasma insulin area under the curve (AUC_{insulin} , Fig. 1A) and higher plasma AUC_{glucose} (Fig. 1B) vs. lean men after the oral glucose challenge. Prior to training, fasting and 2-h plasma insulin levels as well as 2-h plasma glucose levels were significantly higher in the obese men (Table 2). Endurance training reduced both the 2-h plasma glucose ($P \leq 0.01$) and insulin ($P = 0.07$) concentrations (Table 2). However, post-training plasma AUC_{insulin} concentrations remained significantly higher in the obese vs. lean men (Fig. 1A). There was no difference in the plasma AUC_{glucose} concentrations between the obese vs. lean men post-training (Fig. 1B). Prior to training, HOMA-IR was

68% higher in the obese vs. lean men (3.31 ± 0.47 vs. 1.97 ± 0.26 ; $P \leq 0.02$) (Fig. 1C). Endurance training trended to decrease HOMA-IR by 17% (2.17 ± 0.19 vs. 2.60 ± 0.30 , post- vs. pre-training, respectively; $P = 0.10$). Notably, post-training HOMA-IR values were not different between the two groups (2.51 ± 0.27 vs. 1.87 ± 0.25 , obese vs. lean, respectively) (Fig. 1C).

Inflammation and adipokines. Prior to training, serum adiponectin concentration was significantly lower (6198 ± 507 ng/mL vs. 8505 ± 473 ng/mL, respectively; $P \leq 0.01$), whereas CRP (2611 ± 353 ng/mL vs. 312 ± 84 ng/mL, respectively; $P \leq 0.0001$), IL-6 (0.97 ± 0.20 pg/mL vs. 0.52 ± 0.08 pg/mL, respectively; $P \leq 0.03$) and leptin (12966 ± 1787 pg/mL vs. 3583 ± 543 pg/mL, respectively; $P \leq 0.0001$) concentrations were markedly higher in the obese vs. lean men (Fig. 2). Endurance training significantly reduced serum leptin concentrations (5524 ± 919 pg/mL vs. 8274 ± 1454 pg/mL, post- and pre-training, respectively; $P \leq 0.01$), with a greater reduction observed in the obese men (-34%) compared with the lean men (-22%); however, post-training serum leptin remained significantly elevated in the obese vs. lean men ($P \leq 0.001$) (Fig. 2B).

Endurance training trended to reduce serum IL-6 (-21%, 0.58 ± 0.07 pg/mL vs. 0.73 ± 0.11 pg/mL, post- vs. pre-training, respectively; $P = 0.07$) (Fig. 2C); however, post-training serum IL-6 remained significantly elevated in the obese vs. lean men ($P \leq 0.02$). Serum adiponectin and CRP concentrations were unaltered by endurance training (Figs. 2A and D).

Urinary markers of oxidative damage. Prior to training, obese men trended to have higher urinary 8-OHdG (a marker of DNA damage) and 8-isoprostane (a marker of lipid peroxidation) levels vs. lean men (both $P = 0.09$) (Fig. 3). Endurance training trended to reduce urinary 8-OHdG levels by 15% in obese men only ($P = 0.07$) (Fig. 3A). Endurance training reduced urinary 8-isoprostane levels by 26% ($P \leq 0.02$). Examining the groups independently, the post-training reduction in 8-isoprostane levels was clearly due to the reduction evident only in obese men (-36%; $P \leq 0.02$). Post-training, urinary 8-OHdG and 8-isoprostane levels were not different between obese vs. lean men (Fig. 3).

Skeletal muscle oxidative damage and antioxidant capacity. Prior to training, obese men had higher protein carbonyl (a marker of protein oxidation) and 4-HNE (a marker of lipid peroxidation) levels by 63% ($P \leq 0.02$) and 37% ($P \leq 0.03$), respectively, vs. lean men (Fig. 4). Endurance training significantly reduced protein carbonyl levels by 21% ($P \leq 0.05$) (Fig. 4A). Examining the groups independently, endurance training significantly decreased protein carbonyl levels by 33% in obese men only ($P \leq 0.05$) (Fig. 4A), thus approaching the levels observed in the lean group. Notably, protein carbonyl levels were not different between the two groups post-training. Endurance training decreased 4-HNE levels by 10% ($P \leq 0.04$); however, they remained higher in the obese vs. lean men post-training ($P \leq 0.04$) (Fig. 4B).

Prior to training, only the cytoplasmic antioxidant enzyme Cu/ZnSOD was higher in the obese vs. lean men ($P \leq 0.01$) (Fig. 5A). Endurance training trended to increase Cu/ZnSOD by 27% ($P = 0.10$), with a greater increase observed in the obese men vs. the

lean men (+37% vs. +12%, obese vs. lean, respectively; $P = 0.08$). Endurance training increased the mitochondrial antioxidant enzyme MnSOD by 47% ($P \leq 0.01$), with a greater increase observed in the obese men vs. the lean men (+89% vs. +13%, obese vs. lean, respectively; $P = 0.01$) (Fig. 5B). Neither obesity nor endurance training altered the protein content of catalase (Fig. 5C).

Correlations. A correlation summary between markers of oxidative damage and inflammation vs. anthropometric indices of adiposity and insulin resistance is given in Table 3. Briefly, markers of inflammation in blood (CRP, IL-6) correlated positively with anthropometric measures (waist circumference, BMI, body fat percentage); however, only CRP was significantly correlated with HOMA-IR. Serum leptin correlated positively with anthropometric indices and HOMA-IR, whereas serum adiponectin was inversely correlated with these variables. Both urinary 8-OHdG and 8-isoprostane correlated positively with anthropometric indices and HOMA-IR. Skeletal muscle markers of lipid peroxidation (4-HNE) and protein oxidative damage (protein carbonyls), as well as antioxidant capacity (Cu/ZnSOD), correlated positively with anthropometric indices of adiposity.

Discussion

In this study, we assessed the association between adiposity, inflammation, and oxidative damage after moderate aerobic exercise training in non-diabetic lean and insulin-resistant obese men. We observed that exercise training is effective in substantially improving biochemical, oxidative stress, inflammation, and antioxidant statuses in sedentary lean and obese participants over a period of three months, as well as improving the metabolic health of obese individuals, independent of weight loss. We confirmed herein that obese, insulin-resistant men had elevated skeletal muscle and urinary oxidative stress, and dysregulated adipocytokine profile compared with age- and fitness-matched lean counterparts. Importantly, we showed that moderate intensity cycling training significantly improved the adipocytokine profile with a reduction in leptin and pro-inflammatory IL-6 levels. Furthermore, the effects of exercise training on markers of oxidative stress (8-OHdG, 8-isoprostane, protein carbonyls) were reduced only in obese men. In line with the *hormesis* hypothesis, exercise training led to higher protein content of MnSOD and Cu/ZnSOD. Lastly, we showed that exercise training significantly lowered the 2-hour blood glucose and insulin levels during an oral glucose challenge. These results corroborate our previous findings that exercise training significantly decreased oxidative stress markers of lipid peroxidation and DNA damage in lean and obese women subjected to 12 weeks of exercise training [16]. To our knowledge, we are the first to demonstrate improvement of skeletal muscle and systemic markers of oxidative damage, as well as skeletal muscle antioxidant capacity, in obese men following exercise training in the absence of changes in body weight, BMI or body

fat percentage.

Obesity is strongly associated with higher oxidative stress [5]. One possible obesity-related contributing factor to insulin resistance is oxidative stress-induced lipid peroxidation and the modification of proteins by reactive aldehydes such as 4-HNE, presumably due to an inadequacy of antioxidant defenses in tissues [24]. Recently, Finkel and Holbrook [25] stated that the best strategy to enhance endogenous antioxidant levels may actually be oxidative stress itself, based on the classical physiological concept of *hormesis*. Exercise at high intensity causes a pulsatile increase in oxidative stress due to the generation of ROS that exceed the defense capacity in skeletal muscle [26, 27]. However, it has been consistently observed that individuals undergoing exercise training have high levels of antioxidant enzymes and certain non-enzymatic antioxidants in muscle and demonstrate lower basal levels of oxidative stress, as well as greater resistance to exercise-induced oxidative stress [8]. These adaptations result from cumulative effects of repeated exercise bouts on gene expression of antioxidant enzymes.

Chronically elevated levels of ROS are an important trigger for insulin resistance [28] and subsequent pathophysiology [29, 30]. Thus, the potential clinical significance of lower ROS levels in skeletal muscle through regular physical activity could be its amelioration of skeletal muscle insulin resistance. In the present study, we showed that obese men have elevated skeletal muscle 4-HNE levels compared with healthy lean subjects and that 4-HNE levels correlate well with anthropometric indices of adiposity, namely waist circumference, BMI, and body fat percentage. These findings align with the accumulating evidence suggesting that 4-HNE may play an important role in the

pathogenic cellular changes that cause insulin resistance and other abnormalities in obesity, and that 4-HNE may also mediate disease processes promoted by obesity [31]. Notably, we and others have found that levels of 4-HNE are increased in the blood and/or muscle tissue of obese *vs.* lean subjects [16]. Regular moderate exercise is widely prescribed as an effective preventative measure for obesity and associated co-morbidities [16]. Obese subjects [24] and a genetic animal model of extreme over-eating [32] have lower circulating by-products of lipid peroxidation [24], and improved muscle insulin sensitivity [24], when subjected to exercise training. This suggests that elevated circulating levels of lipid peroxidation by-products can be reduced by exercise intervention. Indeed, 4-HNE levels were lower, along with an improvement in insulin resistance, in obese subjects post-training. Similarly, high protein carbonyl levels are observed in insulin resistance and are correlated with the sequelae of diabetes [33]. We showed elevated skeletal muscle protein carbonyl levels in obese men compared with normal weight controls and a strong positive correlation between protein carbonyls and several clinical indices of adiposity - factors that predict cardiovascular disease and/or T2D [34]. Furthermore, skeletal muscle protein carbonyl levels in the obese group decreased with training approaching levels observed in controls; findings consistent with previous reports [35].

Intracellular enzymes such as SOD and catalase act as primary line of defense to cope with the deleterious effects of reactive oxygen species [36], thereby contributing to an overall decrease in oxidative damage. Lower SOD and catalase activity are associated with insulin resistance [37] suggesting that reduced capacities of antioxidant enzymes

lead to increased oxidative stress in diabetes [38] and obesity [39]. Interestingly, we found an increase in protein content of Cu/ZnSOD in obese men prior to exercise, contrary to other studies [39]. This increase in Cu/ZnSOD protein content likely reflects a compensatory up-regulation of antioxidant enzymes in response to chronically elevated ROS production, as reflected in the higher 4-HNE and protein carbonyl levels in obese men in the current study. We found a significant increase in MnSOD protein expression concomitant with reductions in skeletal muscle 4-HNE, protein carbonyls and urinary 8-OHdG and 8-isoprostane content in the obese men after exercise training. Our observation of an increase in MnSOD protein content in response to endurance exercise training is consistent with previous findings [10, 11], and points to an adaptive response to exercise training, reflecting reduced free radical production and increased enzyme biosynthesis [40]. Collectively, the reductions in urine and muscle of obese and lean men in response to exercise training in (i) lipid peroxidation, (ii) protein oxidation, and (iii) DNA damage, as well as (iv) elevated muscle antioxidant capacity, suggest that exercise training conditioning leads to a greater ability of muscle to readily and rapidly detoxify ROS. This is compatible with both protection against ROS-induced damage and prevention of the formation of additional ROS that may act to mediate further unnecessary adaptive responses [41], thus explaining why trained individuals display less cell damage than untrained subjects [42].

Adipocytes produce a variety of biologically active molecules [43], collectively known as adipocytokines, including CRP, IL-6, leptin, and adiponectin [44]. Dysregulated adipocytokine production contributes to the pathogenesis of obesity-

associated cardio-metabolic risk and/or T2D [34]. Abdominal and visceral adiposity correlate with systemic biomarkers of oxidative stress in both men and women [5], and increased oxidative stress in obesity may in part be the underlying cause of adipocytokine dysregulation and the development of insulin resistance [5]. Here we have demonstrated that in non-diabetic obese and lean men, clinical indices of adiposity closely correlated with systemic biomarkers of oxidative DNA and lipid damage, 8-OHdG and 8-isoprostane, respectively. This is in agreement with recent studies suggesting that systemic oxidative stress correlates with BMI and waist circumference [5]. In addition, we demonstrated that plasma adipocytokine levels in obese men were dysregulated compared with healthy lean controls and that oxidative stress, already augmented in the obese, correlated positively with CRP and leptin, and inversely with adiponectin (data not shown). These results are consistent with a recent study that demonstrated that increased ROS in obesity caused dysregulation of adipocytokine production and that treatment with antioxidants *in vivo* mitigates this dysregulation resulting in improvement of glucose intolerance [5]. Indeed, plasma adiponectin levels were markedly lower and correlated inversely with both oxidative stress (data not shown) and insulin resistance; whereas, leptin, IL-6 and CRP concentrations were elevated in the obese group, but only leptin and CRP levels correlated positively with both oxidative stress (data not shown) and insulin resistance. Several studies have found an effect of obesity on plasma adiponectin, leptin, IL-6 and CRP [44], findings that have been corroborated by the current study. Furthermore, exercise training mitigated some of the adipocytokine dysregulation, namely lowering leptin and IL-6, concomitant with significant reductions in systemic

biomarkers of oxidative stress and glucose/insulin in obese men only. Collectively, these findings suggest that targeting higher oxidative stress in obesity through regular physical activity ameliorates adipocytokine dysregulation leading to improved glucose control. Although we found that exercise training reduced oxidative stress in obese and lean men independent of weight loss, there was no effect of training on adiponectin and CRP in either group. This finding is consistent with numerous studies showing that weight loss is needed to increase adiponectin and to normalize certain inflammatory markers [15].

In the present study, we evaluated multiple markers of oxidative damage and inflammation to ascertain a broader perspective regarding redox balance and fitness level while simultaneously controlling for age, physical fitness and body composition. When lean and insulin-resistant obese subjects were maintained on a moderate but progressive cycling exercise program for three months, their levels of systemic and skeletal muscle oxidative stress decreased and their circulating marker of inflammation had a strong decreasing trend. Multiple glucose homeostatic health indicators improved concomitant with a strong decreasing trend in insulin resistance, demonstrating that lipid, protein, and DNA damage (with their potential detrimental effects on insulin resistance) following exercise training. Thus, chronic endurance exercise may be a useful exercise modality to reduce some of these risk factors associated in the pathogenesis of insulin resistance in obesity.

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I.A.S., M.J.H., and M.A.T. conceived and designed the study; I.A.S. and M.J.H. were involved in conducting and coordinating the study; I.A.S. and A.S. researched and analyzed data; I.A.S. wrote the article; I.A.S., A.S., M.J.H., S.R., and M.A.T. reviewed and edited the article. All authors read and approved the final article.

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List of Abbreviations

4-HNE - 4-hydroxy-2-nonenal

8-OHdG - 8-hydroxy-2-deoxyguanosine

AUC – area under the curve

BMI - body mass index

CRP - C-reactive protein

FFA - free-fatty acid

FFM - fat-free mass

HOMA-IR - homeostasis model assessment index of insulin resistance

IL-6 - interleukin-6

OGTT - oral glucose tolerance test

ROS - reactive oxygen species

SOD - superoxide dismutase

T2D - type 2 diabetes mellitus

VO_{2peak} - maximal oxygen consumption

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Table 1: Subject characteristics

	Lean		Obese	
	Pre-training	Post-training	Pre-training	Post-training
<i>n</i>	9		9	
Age (yr)	38 ± 3		39 ± 3	
Height (cm)	179 ± 3		180 ± 3	
Body weight (kg)	75.5 ± 3.4	75.2 ± 3.2	108.4 ± 6.1 ^a	107.1 ± 6.5 ^b
Body mass index (kg.m ⁻²)	23.6 ± 0.5	23.5 ± 0.5	33.6 ± 1.6 ^a	33.1 ± 1.7 ^b
Waist circumference (cm)	86.2 ± 1.4	82.5 ± 1.7 ^c	111.8 ± 3.7 ^a	108.6 ± 4.2 ^{b,c}
Fat mass (kg)	14.9 ± 1.9	14.4 ± 2.1	35.7 ± 3.3 ^a	35.2 ± 3.5 ^b
Fat-free mass (kg)	57.3 ± 2.6	57.9 ± 2.5	68.8 ± 2.7 ^a	68.2 ± 2.9 ^b
Body fat (%)	20.5 ± 2.3	19.7 ± 2.5	33.7 ± 1.4 ^a	33.5 ± 1.5 ^b
Aerobic capacity (mL O ₂ .kg ⁻¹ fat-free mass.min ⁻¹)	46.9 ± 2.1	55.5 ± 2.4 ^c	44.6 ± 2.1	51.1 ± 2.0 ^c

Data are presented as means ± SEM.

^a Obese group data significantly different from lean group data, $P \leq 0.01$

^b Obese group data significantly different from lean group data, $P \leq 0.02$

^c Post-training significantly different from pre-training (main effect), $P \leq 0.001$

Table 2: Metabolic characteristics

	Lean		Obese	
	Pre-training	Post-training	Pre-training	Post-training
<i>n</i>	9		9	
Total cholesterol (mM)	4.75 ± 0.36	4.57 ± 0.29	5.26 ± 0.36	5.31 ± 0.35
Triglyceride (mM)	0.92 ± 0.14	0.78 ± 0.12	1.54 ± 0.24 ^a	1.49 ± 0.23 ^b
HDL cholesterol (mM)	1.49 ± 0.07	1.42 ± 0.08	1.20 ± 0.07 ^a	1.22 ± 0.07
LDL cholesterol (mM)	2.84 ± 0.29	2.79 ± 0.26	3.35 ± 0.30	3.41 ± 0.30
Free-fatty acid (mM)	0.31 ± 0.04	0.53 ± 0.9 ^c	0.53 ± 0.06 ^a	0.61 ± 0.04 ^c
Fasting plasma glucose (mM)	5.39 ± 0.14	5.48 ± 0.12	5.68 ± 0.20	5.70 ± 0.24
Fasting plasma insulin (μU.mL ⁻¹)	8.07 ± 0.87	7.60 ± 0.89	12.11 ± 1.42 ^a	10.15 ± 1.09
2-h plasma glucose (mM)	4.88 ± 0.24	4.37 ± 0.27 ^c	7.09 ± 0.74 ^a	5.43 ± 0.55 ^c
2-h plasma insulin (μU.mL ⁻¹)	25.06 ± 6.55	26.46 ± 4.86	74.29 ± 16.89 ^a	39.08 ± 5.21

Data are presented as means ± SEM.

^a Obese group data significantly different from lean group data, $P \leq 0.04$

^b Obese group data significantly different from lean group data, $P \leq 0.01$

^c Post-training significantly different from pre-training (main effect), $P \leq 0.05$

Table 3: Univariate correlations between anthropometric indices of adiposity, insulin resistance, and selected variables

Variable	WC	BMI	BF	HOMA-IR
Blood				
C-reactive protein	0.88	0.90	0.80	0.53
Interleukin-6	0.58	0.63	0.54	NS
Leptin	0.89	0.87	0.78	0.57
Adiponectin	-0.41	-0.44	-0.54	-0.36
Urine				
8-hydroxy-2-deoxyguanosine	0.39	0.35	0.47	0.63
8-isoprostane	0.36	0.37	NS	0.60
Skeletal muscle				
4-hydroxy-2-nonenal	0.61	0.61	0.42	NS
Protein carbonyls	0.38	0.40	0.47	NS
Cu/Zn-superoxide dismutase	0.48	0.43	0.39	NS
Mn-superoxide dismutase	NS	NS	NS	NS
Catalase	NS	NS	NS	NS

Pearson correlations (r) for significant ($P \leq 0.05$) relationships. BF, body fat percentage; BMI, body mass index; HOMA-IR, homeostasis model assessment index of insulin resistance; NS, not significant; WC, waist circumference.

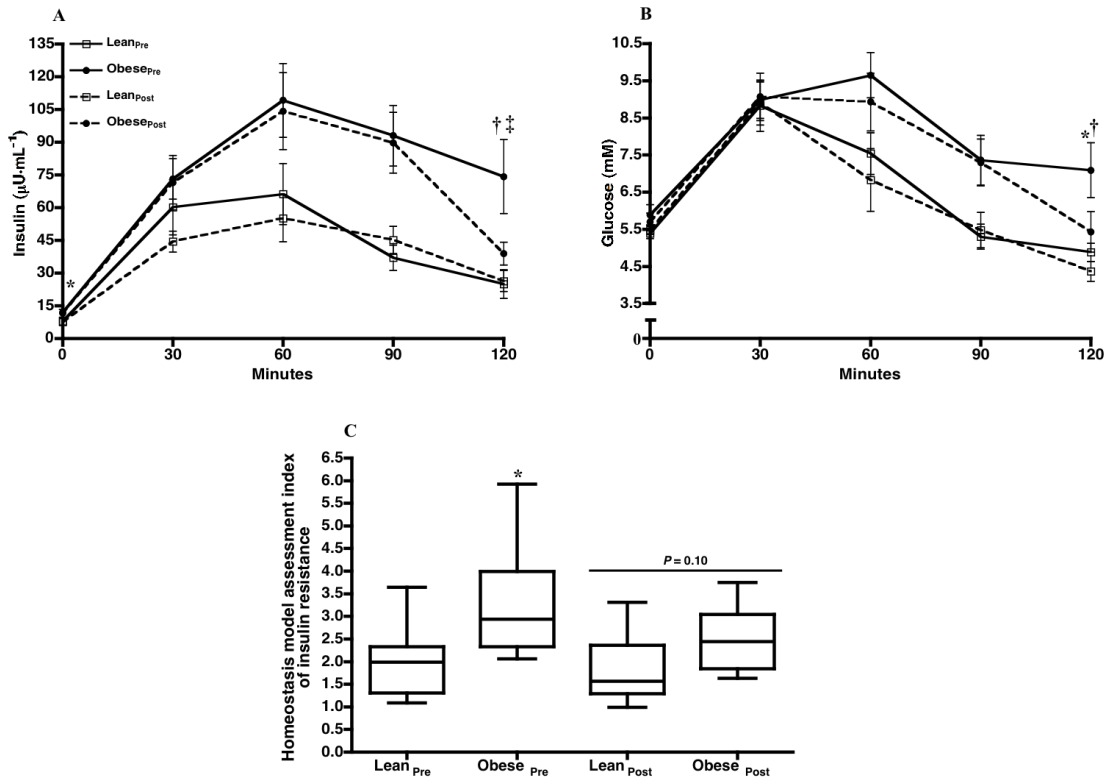


Figure 1: Results of the oral glucose tolerance test. Mean plasma concentrations of insulin (**A**) and glucose (**B**) during a 75-g oral glucose tolerance test (OGTT), and (**C**) box and whisker plot, as medians and inter-quartile range, of the homeostatic model assessment index of insulin resistance (HOMA-IR) in lean ($n = 9$) and obese ($n = 9$) men prior to and following 12 wk of endurance training. (**A**) Obese men had significantly higher OGTT insulin area under the curve (AUC_{insulin}) vs. lean men pre-training ($P = 0.02$) and post-training ($P = 0.02$). *Pre-training fasting plasma insulin concentration, obese vs. lean $P \leq 0.03$; †Pre-training 2-hr plasma insulin concentration, obese vs. lean $P \leq 0.02$; ‡Post- vs. pre-training 2-hr plasma insulin concentration $P = 0.07$. (**B**) Obese men had higher OGTT AUC_{glucose} vs. lean men pre-training ($P = 0.04$). *Pre-training 2-hr plasma glucose concentration, obese vs. lean $P \leq 0.01$; †Post- vs. pre-training 2-hr plasma glucose concentration $P \leq 0.01$. (**C**) Pre-training, obese men had 68% higher HOMA-IR vs. lean men. Endurance training reduced HOMA-IR by 5% in the lean men and by 24% in the obese men ($P = 0.10$ vs. pre-training). *Pre-training, obese vs. lean $P \leq 0.02$. Data are presented as means \pm SEM.

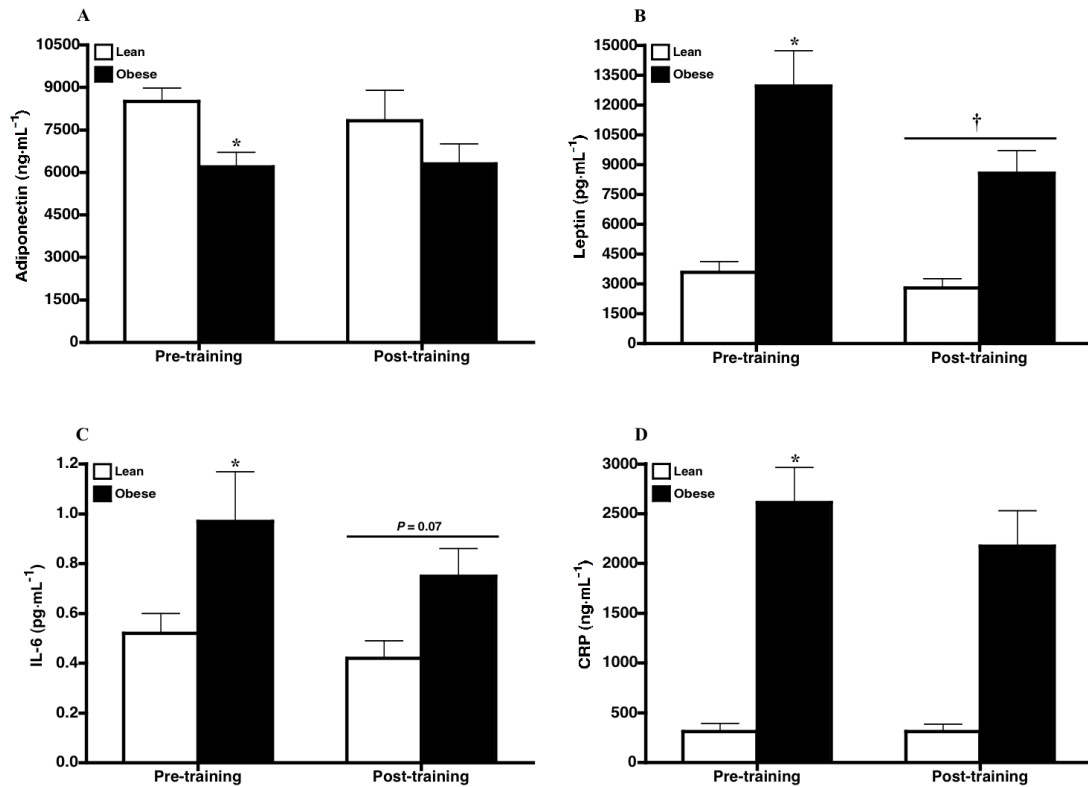


Figure 2: Blood markers of inflammation and adipokines. **(A)** Adiponectin, **(B)** Leptin, **(C)** Interleukin-6 (IL-6), and **(D)** C-reactive protein (CRP) in lean ($n = 9$) and obese ($n = 9$) men prior to and following 12 wk of endurance training. **(A)** *Pre-training, obese vs. lean $P \leq 0.01$. **(B)** *Pre-training, obese vs. lean $P \leq 0.0001$; †Post- vs. pre-training, $P \leq 0.01$. **(C)** *Pre-training, obese vs. lean $P \leq 0.03$; Post- vs. pre-training, $P = 0.07$. **(D)** *Pre-training, obese vs. lean $P \leq 0.0001$. Data are presented as means \pm SEM.

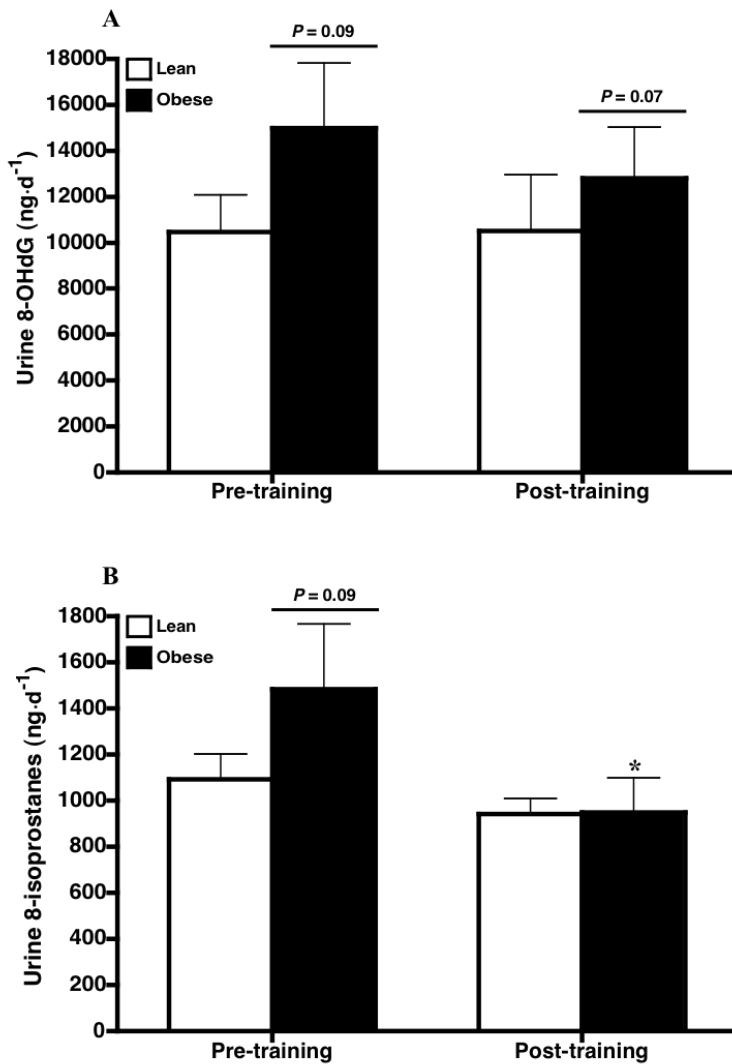


Figure 3: Urinary markers of oxidative damage. **(A)** 8-hydroxy-2-deoxyguanosine (8-OHdG), marker of DNA damage, and **(B)** 8-isoprostane, marker of lipid peroxidation, in 24-h urine samples of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12 wk of endurance training. **(A)** Pre-training, obese vs. lean $P = 0.09$; *Obese men, post- vs. pre-training $P = 0.07$. **(B)** Pre-training, obese vs. lean $P = 0.09$; *Obese men, post- vs. pre-training $P \leq 0.02$. Data are presented as means \pm SEM.

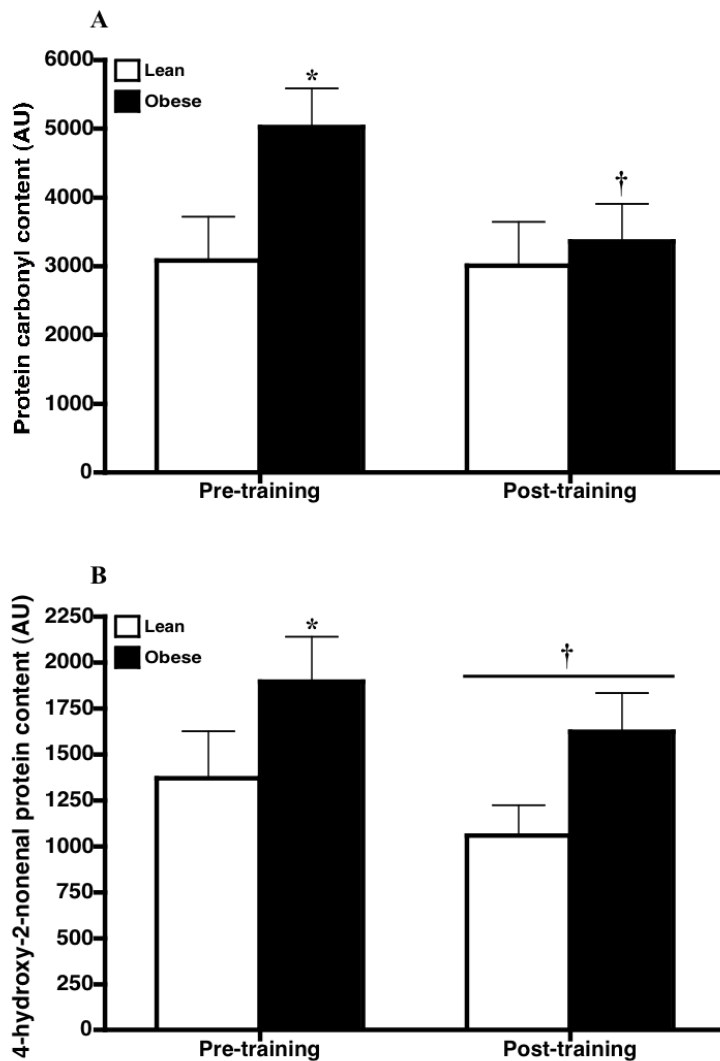


Figure 4: Skeletal muscle markers of oxidative damage. **(A)** Protein carbonyl, marker of protein oxidation, and **(B)** 4-hydroxy-2-nonenal (4-HNE), marker of lipid peroxidation, protein content assessed by Western blot in the *vastus lateralis* of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12 wk of endurance training. **(A)** *Pre-training, obese vs. lean $P \leq 0.02$; †Obese men, post- vs. pre-training $P \leq 0.05$. **(B)** *Pre-training, obese vs. lean $P \leq 0.03$; †Post- vs. pre-training $P \leq 0.04$. Data are presented as means \pm SEM.

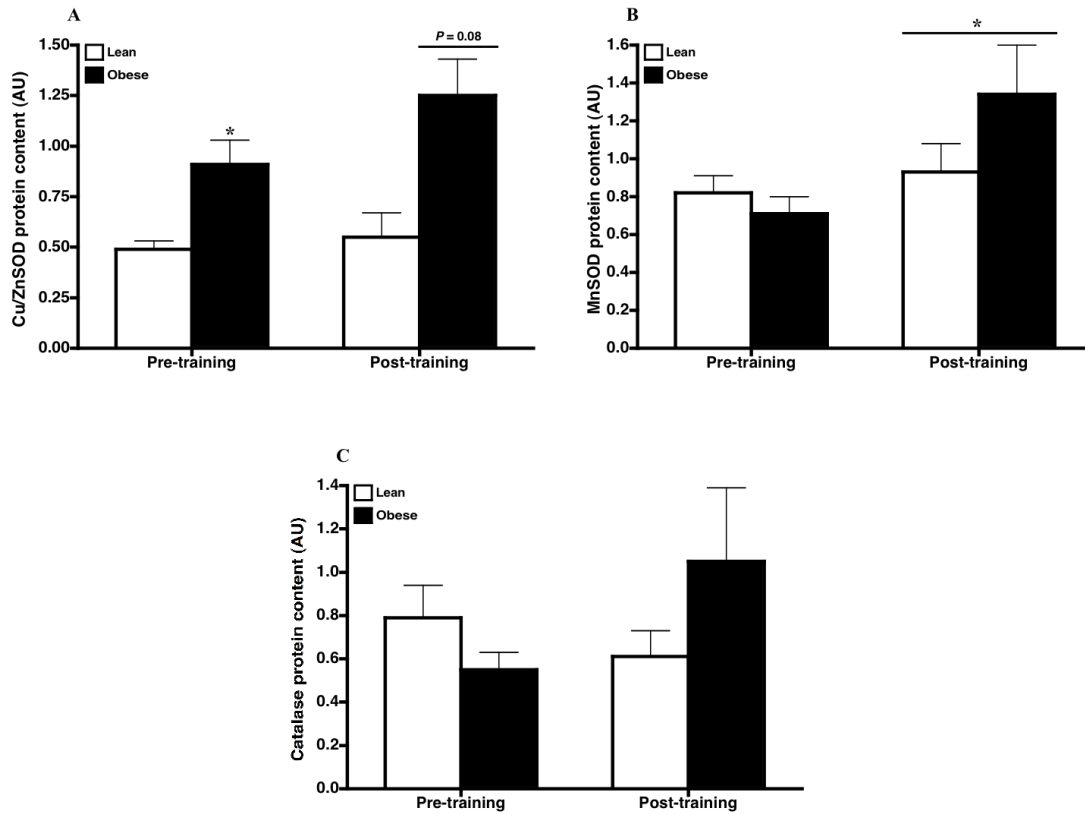


Figure 5: Skeletal muscle markers of antioxidant capacity. **(A)** Copper/Zinc superoxide dismutase (Cu/ZnSOD), **(B)** manganese superoxide dismutase (MnSOD), and **(C)** catalase, protein content assessed by Western blot in the *vastus lateralis* of lean ($n = 9$) and obese ($n = 9$) men prior to and following 12 wk of endurance training. **(A)** *Pre-training, obese vs. lean $P \leq 0.01$; Obese men, post- vs. pre-training $P = 0.08$. **(B)** *Post- vs. pre-training $P \leq 0.01$. Data are presented as means \pm SEM.

CHAPTER 4

**SKELETAL MUSCLE MITOCHONDRIAL FUNCTION DOES NOT
INFLUENCE THE PATHOGENESIS OF DYSGLYCEMIA IN PATIENTS WITH
PRIMARY MITOCHONDRIAL MYOPATHY**

(Submitted in Diabetes, 2011)

Skeletal Muscle Mitochondrial Dysfunction Does Not Influence the Pathogenesis of Dysglycemia in Patients with Primary Mitochondrial Myopathy

Running Title: Mitochondrial Myopathy-associated Dysglycemia

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Abstract

Mitochondrial dysfunction and intramyocellular lipid (IMCL) accretion have been linked to the development of type 2 diabetes. However, the etiological association between these phenomenon are not clear. We examined whether patients harboring genetic mitochondrial mutations leading to muscle mitochondrial dysfunction and IMCL accumulation, was associated with type 2 diabetes. Thirteen patients with mitochondrial disease completed an oral glucose tolerance test (OGTT) and *vastus lateralis* biopsy. Mitochondrial oxidative phosphorylation (OXPHOS) capacity, insulin signaling, OGTT-based measures of insulin resistance/ β -cell function, and electron microscopy-based IMCL/mitochondrial content were assessed. Six patients had an abnormal OGTT (i.e., 2-h glucose ≥ 7.8 mM) while the remaining seven patients had normal glucose responses. OXPHOS enzyme activity and phospho-Akt^{Ser473} were greater and β -cell function was lower (all $P \leq 0.01$) in the dysglycemic vs. normoglycemic patients, yet insulin resistance was similar. Mitochondrial electron transport chain subunit abundance and IMCL/mitochondrial content were similar between groups. Despite mitochondrial dysfunction, dysglycemic patients had higher muscle OXPHOS activity concomitant with augmented distal insulin signaling, but similar IMCL/mitochondrial content vs. normoglycemic patients, indicating a paradoxical dissociation between mitochondrial dysfunction and type 2 diabetes. Consequently, impaired glucose control cannot be attributed to muscle mitochondrial dysfunction, but rather is a function of impaired insulin secretion.

Type 2 diabetes, characterized by persistent hyperglycemia, has achieved an epidemic status worldwide. Although the etiological mechanisms of type 2 diabetes remain elusive, peripheral insulin resistance and reduced insulin secretion are cardinal features that lead to impaired metabolic regulation (1). Recently, impaired skeletal muscle mitochondrial oxidative capacity (2-7), aberrant mitochondrial ultra-structure (2; 8) and reduced expression of genes responsible for oxidative metabolism (9; 10) have been implicated in the development of insulin resistance and type 2 diabetes. This association between muscle mitochondrial dysfunction and insulin resistance in some (2-7), but not all conditions (11-18), has led to controversy regarding the hypothesis that muscle mitochondrial dysfunction contributes to insulin resistance and the development of type 2 diabetes.

Reduced mitochondrial content or function is thought to lead to an accumulation of intramyocellular lipid (IMCL) in skeletal muscle, which impairs insulin signaling by activation of signaling pathways that increase inhibitory serine phosphorylation of the insulin receptor substrates (1; 19). However, impaired oxidative capacity is not the only mechanism for an increase in IMCL, since endurance-trained athletes also have enhanced IMCL storage, coupled with an increased abundance of muscle mitochondria and heightened insulin sensitivity (20; 21). Nair *et al.* recently reported that Asian Indians, in comparison with demographically matched Northern European Americans were severely insulin resistant, in spite of higher muscle mitochondrial ATP production and mitochondrial DNA (mtDNA) copy number (15). Furthermore, a high-fat diet caused insulin resistance while enhancing mitochondrial biogenesis in rats (22). Together, these

results question the association between muscle mitochondrial function, IMCL content and insulin resistance, arguing against the universality of the hypothesis that muscle mitochondrial dysfunction is causal to the development of type 2 diabetes.

Skeletal muscle mitochondrial dysfunction is well documented in patients with primary mitochondrial genetic defects (23; 24). The etiology of the disease can be traced to mutations in the nuclear and mitochondrial genomes that alters activity of the respiratory chain, coincident with IMCL accumulation, resulting in reduced ATP production (24). Furthermore, individuals harboring mitochondrial genetic mutations have an increased prevalence of type 2 diabetes (25). As such, the purported link between mitochondrial dysfunction and the development of type 2 diabetes appears to be valid.

In the current investigation we utilized both patients with primary genetic mitochondrial myopathy and a murine model of mitochondrial dysfunction in order to clarify the etiological association between mitochondrial dysfunction and insulin resistance/type 2 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. Mitochondrial myopathy patients were recruited through a subject recruitment letter from the Neuromuscular and Neurometabolic Disease Clinic, McMaster University Medical Center. All other participants were recruited through local advertisements and underwent a telephone and an in-person interview to assess eligibility. The Research Ethics Board of McMaster University approved the experimental protocol (REB project #: 05-310), and subjects provided written informed consent prior to participation in accordance with the guidelines of the Declaration of Helsinki. Inclusion criteria included age from 30-60 years, BMI of ≤ 30.0 kg/m² with a self-reported stable body weight during the previous 6 months, and normal fasting blood glucose and hemoglobin A_{1c} [HbA_{1c}] measurements in the year preceding the study. Individuals who had a history of type 2 diabetes, hypertension (> 140/90 mmHg), smoked, or used lipid-lowering, glucose-lowering, antihypertensive, antidepressant or weight-loss medications, or consumed more than two alcoholic beverages per day were excluded. All participants were classified as living a sedentary lifestyle, participating only in routine activities of daily living (walking, gardening, etc.) and not engaged in regular structured or individualized aerobic or strength training programs or athletics. Thirteen patients ($n = 9$ women, $n = 4$ men) with mitochondrial myopathy and fifteen healthy, lean controls ($n = 7$ women, $n = 8$ men) volunteered for this study. All mitochondrial myopathy patients met established criteria for the diagnosis of mitochondrial disease (24).

Genetic analysis. Nine myopathy patients had Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), two with electron transport chain complex

I deficiency, one with a 12S rRNA defect (C739T mutation), and one patient with Leber's Hereditary Optic Neuropathy (A14459G mutation). Of the patients with MELAS, five had the A3243G mutation, three with the C3271T mutation, and one with the A3260G mutation.

Metabolic assessments. All participants refrained from strenuous physical activity 48 h preceding the metabolic assessments. After an overnight fast, the glycemic response to a 75-g oral glucose load (300 mL) was determined. Blood samples were collected before and 30, 60, 90 and 120 min during the oral glucose tolerance test (OGTT). Fat mass, fat-free mass, and body fat percentage were assessed by dual energy X-ray absorptiometry (GE Lunar, Prodigy, Madison, WI). Habitual physical activity was assessed using the Baecke questionnaire (26).

Blood sample analysis. Blood samples were taken from the antecubital vein after an overnight fast, collected in heparinized or untreated vials, placed on ice, centrifuged at 1750 g for 10 min, and stored at -80°C until subsequent analysis. Serum free-fatty acid (FFA) was determined using a commercially available ELISA kit (NEFA kit, Wako Diagnostics, Richmond, VA). Plasma glucose concentration was determined using an automated glucose analyzer (2300 STAT plus, YSI). Plasma insulin concentration was determined using a commercially available ELISA kit (INS kit, BioSource, Belgium). HbA_{1c} was determined using an automated Hemoglobin Testing System (VARIANT™ II TURBO, version 3.2, Bio-Rad Laboratories Inc., Hercules, CA).

Classification of glucose tolerance. Using the 2006 World Health Organization (WHO) definition and diagnosis of diabetes mellitus and intermediate hyperglycemia (27),

participants were categorized as normoglycemic (fasting plasma glucose [FPG] < 6.1 mM and 2-h plasma glucose < 7.8 mM) or dysglycemic (either impaired fasting glucose [IFG]: FPG 6.1-6.9 mM and/or impaired glucose tolerance [IGT]: 2-h plasma glucose \geq 7.8 and < 11.1 mM; or diabetes: FPG \geq 7.0 mM and/or 2-h plasma glucose \geq 11.1 mM). Seven mitochondrial myopathy patients were classified as normoglycemic and six as dysglycemic ($n = 1$ IGT; $n = 5$ diabetes). All control subjects were classified as normoglycemic.

Calculations. For estimation of insulin sensitivity from data obtained during the OGTT, the homeostasis model assessment index of insulin resistance (HOMA-IR) (28) and the insulin sensitivity (Matsuda) index (ISI) (29) were calculated according to the following equations: $\text{HOMA-IR} = I_0 \times G_0 / 22.5$; where I_0 is the fasting insulin concentration (in $\mu\text{U/mL}$) and G_0 is the fasting glucose concentration (in mM), and $\text{ISI} = 10,000 / \sqrt{(G_0 \times I_0) \times (\text{mean } G_{\text{OGTT}} \times \text{mean } I_{\text{OGTT}})}$; where G_0 is the fasting glucose concentration (in mg/dL), I_0 is the fasting insulin concentration (in $\mu\text{U/mL}$), and G_{OGTT} and I_{OGTT} are the glucose and insulin concentrations, respectively, during the OGTT. The HOMA-IR and Matsuda index have been validated against the euglycemic-hyperinsulinemic clamp (28-30). Three variables were used for estimation of β -cell function from data obtained during the OGTT. First, the ratio of the total area under the insulin curve to the total area under the glucose curve ($\text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}}$) (31). Second, the insulinogenic index (IGI): $\text{IGI} = (\Delta_{\text{insulin}30-0 \text{ min}} / \Delta_{\text{glucose}30-0 \text{ min}})$ (32). Third, the disposition index (DI): $\text{DI} = \text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}} \times \text{Matsuda index}$ (31).

Muscle biopsies. Samples of *vastus lateralis* were obtained after an overnight fast, as

previously described (20). Muscle tissue was immediately stored in liquid nitrogen until transferred to -80°C to be stored for subsequent biochemical and molecular analysis.

Electron microscopy. Electron microscopy (EM) was used to determine mitochondrial and IMCL characteristics, as described previously (20). Samples were viewed at 7,500x using a JEOL 1200EX transmission electron microscope. Two representative images per longitudinal section of muscle fiber were obtained for a total of eight images per sample. A board-certified trained pathologist blinded to the samples scored mitochondrial pathology. Mitochondrial fragments and lipid droplets were circled and converted to actual size using a calibration grid. Quantification of mitochondrial and IMCL number and area was performed using the Media Cybernetics Image Pro Plus 4.0 computerized image analysis system, as described previously (20).

Homogenization and immunoblotting. Total protein was extracted from frozen biopsy samples, as previously described (20). The Lowry assay was used to quantify the total protein content (33). Proteins were resolved on either 7.5, 10 or 12.5% SDS-PAGE gels, transferred onto Hybond[®] ECL nitrocellulose membranes (Amersham) and immunoblotted using the following commercially available primary antibodies: anti-COX subunit II (cytochrome *c* oxidase - subunit II, MS405), anti-COX subunit IV (cytochrome *c* oxidase - subunit IV, MS408), and MitoProfile[®] Total OXPHOS Cocktail (MS603) were purchased from MitoSciences; anti-GLUT-4 from Chemicon (ab1346); and anti-phospho-Akt from Cell Signaling Technology (Ser⁴⁷³, 4060). The anti-CS (citrate synthase) antibody was a generous gift by Dr. Brian Robinson (The Hospital for Sick Children, Toronto, ON). Anti- β -actin (BD Biosciences) was used as a loading control.

Membranes were then incubated with the appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with an enhanced chemiluminescence detection reagent (Amersham). Relative intensities of the protein bands were digitally quantified using ImageJ Version 1.37 statistical analysis software.

Enzyme activity. Muscle lysate CS, complex IV (COX), and β -oxidation, short-chain β -hydroxyacyl-CoA dehydrogenase (β -HAD) activity was determined, as previously described (20). All samples were analyzed in duplicate on a UV spectrophotometer (Varian Cary 300 Bio UV-Visible spectrophotometer, Palo Alto, CA) and expressed as $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$.

Mouse model of mitochondrial myopathy. We generated the homozygous knock-in mtDNA mutator mouse model of mitochondrial myopathy (PolG; *PolgA*^{D257A/D257A}; $n = 14$ -16; ♀ = ♂; 8 months of age) and littermate wild-type controls (WT; *PolgA*^{+/+}; $n = 14$ -16; ♀ = ♂; 8 months of age) from heterozygous mice (*PolgA*^{+D257A}; a kind gift by Dr. Tomas A. Prolla, University of Wisconsin-Madison, USA). The details of breeding, genotyping, phenotypic characterization, and tissue harvesting from WT and PolG mice were recently described (34). At 30 weeks of age, the mice were subjected to glucose- and insulin-tolerance tests (GTT and ITT) after an overnight fast, as previously described (35; 36). For the GTT, glucose was collected at baseline and 30, 60 and 120 min after mice were given $2 \text{ g}\cdot\text{kg}^{-1}$ glucose (Sigma-Aldrich, St. Louis, MO) by oral gavage (WT, $n = 10$; PolG, $n = 14$). For the ITT ($n = 6$ per group), glucose was collected at baseline and 20, 40 and 60 min after mice were given $1 \text{ IU}\cdot\text{kg}^{-1}$ insulin (Novolin[®]ge Toronto, human biosynthetic insulin, Novo Nordisk, Mississauga, ON) in saline by subcutaneous

injection. Glucose concentration was measured by One Touch Ultra Glucometer (Johnson & Johnson, Milpitas, CA) in tail blood samples collected by repeated puncture.

Statistical analysis. All statistical analyses were performed using Statistica 5.0 software (Statsoft, Tulsa, OK). Statistical significance was determined by 2-tailed Student's *t*-test.

Statistical significance was established at $P \leq 0.05$. Data are presented as mean \pm SEM.

RESULTS

Subject characteristics. Patients with primary mitochondrial myopathy were slightly older, weighed less, had lower fat-free mass, and were less physically active *vs.* healthy lean controls (Table 1). FFA and fasting plasma lactate levels were higher in myopathy patients *vs.* controls (Table 1). No other variables, including BMI, body fat percentage, fat mass, triglyceride, total-, HDL-, and LDL-cholesterol were significantly different between myopathy patients and controls (Table 1).

Skeletal muscle mitochondrial function. We examined the protein expression of several key mitochondrial- and nuclear-encoded subunits that are involved in energy metabolism in the mitochondria. The protein content of CS, core protein 2 subunit of complex-III (CIII), COXII, COXIV, as well as the maximal activity of the COX:CS ratio, were significantly lower in myopathy patients *vs.* controls ($P \leq 0.05$) (Fig. 1A and B). No differences in the protein expression of complex-II (CII) and -V (CV) subunits were found between myopathy patients and controls (Fig. 1A).

Mitochondrial morphology and intramyocellular lipid. Mitochondrial myopathy patients had substantial accumulation of swollen, pleomorphic, oversized mitochondria along with paracrystalline inclusions, vacuolization, and disrupted membranes (Fig. 1C and D). IMCL content was significantly higher in myopathy patients *vs.* controls ($P \leq 0.02$) (Fig. 1E-G).

Oral glucose tolerance test and insulin resistance. Fasting plasma glucose levels did not differ between myopathy patients vs. controls but the 2-h plasma glucose concentration in response to an OGTT was significantly higher in the patient group (Table 1). Myopathy patients also showed impaired clearance of oral glucose loads (AUC_{glucose}) vs. controls ($P \leq 0.01$) (Fig. 2A). No between-group differences were seen for fasting and 2-h plasma insulin levels (Table 1) or plasma insulin levels after the oral glucose challenge (AUC_{insulin}) (Fig. 2B). Assessment of insulin sensitivity revealed similar HOMA-IR (Fig. 2C) and Matsuda index values (Table 1) amongst the two groups. β -cell function measurements were significantly lower in patients vs. controls (Table 1). Examining the data from the myopathy patients closely, we found six patients (46%) had abnormal OGTT results. This subgroup of patients (referred to as ‘dysglycemic’ hereafter) displayed markedly higher 2-h plasma glucose levels vs. patients with normal glucose tolerance results (referred to as ‘normoglycemic’ hereafter) (12.42 ± 0.70 vs. 5.79 ± 0.42 ; $P \leq 0.001$) (Table 2).

Characterization of Patients with Mitochondrial Myopathy with Normal and Impaired Glucose Regulation

Patient characteristics. Dysglycemic and normoglycemic mitochondrial myopathy patients were similar with respect to age, fat-free mass, and physical activity levels. Dysglycemic patients, however, weighed less, had lower body fat percentage and fat mass. No other variables, including triglyceride, total-, HDL-, and LDL-cholesterol, or FFA levels were different between the two groups (Table 2).

Insulin resistance and β -cell function. No differences were found in the fasting plasma glucose, HbA_{1c}, or fasting and 2-hr plasma insulin levels between dysglycemic and normoglycemic patients (Table 2). The AUC_{glucose} was significantly greater ($P \leq 0.001$), while the AUC_{insulin} was significantly lower ($P \leq 0.02$) in the dysglycemic vs. normoglycemic patients (Fig. 3A and B). HOMA-IR (Fig. 3C) and the Matsuda index (Table 2) were similar between the two groups. All β -cell function measurements; AUC_{insulin}/AUC_{glucose}, IGI, and the DI, were significantly lower ($P \leq 0.01$) in the dysglycemic vs. normoglycemic patients (Table 2).

Insulin signaling and glucose transport. In skeletal muscle, basal phosphorylation of Akt^{Ser473}, a key convergent step in insulin-stimulated glucose transport activity was significantly higher in the dysglycemic vs. normoglycemic patients ($P \leq 0.01$) (Fig. 4); yet whole muscle GLUT-4 protein content was not different between the two groups (Fig. 4).

Skeletal muscle mitochondrial function. Protein expression of CS and CII, CIII, COXII, COXIV, and CV subunits were not different between dysglycemic vs. normoglycemic patients (Fig. 5A); however, the COX:CS maximal enzyme activity ratio was significantly higher in the dysglycemic patients ($P \leq 0.01$) (Fig. 5B). Short-chain β -HAD:CS maximal enzyme activity ratio was not different between the two groups (Fig. 5B).

Mitochondrial morphology and intramyocellular lipid. There was no difference in mitochondrial density or any of the IMCL variables between dysglycemic *vs.* normoglycemic patients; however, dysglycemic patients had more abnormal mitochondria (Supplementary Figure 1).

PolG Mutator Mouse Model of Mitochondrial Myopathy

We have recently shown that the mtDNA mutator mouse (PolG) model of mitochondrial dysfunction, harboring proofreading deficient mitochondrial polymerase gamma, shows severe mitochondrial metabolic aberrations in skeletal muscle (34). Hence, we have utilized this as a murine model of mitochondrial disease to further assess if muscle mitochondrial dysfunction is associated with insulin resistance and type 2 diabetes. To assess the ability of PolG mice to dispose of glucose, a GTT was measured after an overnight fast. There was no difference on the total glucose response (AUC_{glucose}) to the oral glucose load between PolG *vs.* WT mice (Fig. 6A). No difference was found in the fasting and 2-hr glucose levels between the two groups. To determine whether PolG mice develop insulin resistance, we performed a 60-min ITT to assess changes in blood glucose in response to an insulin injection. We found no difference in the 30-min blood glucose level (an indicator of insulin resistance) after insulin injection between PolG *vs.* WT mice (Fig. 6B).

DISCUSSION

The observed lower expression of genes encoding mitochondrial proteins in muscle from individuals with type 2 diabetes and their first-degree relatives suggests that impaired mitochondrial function is involved in the development of insulin resistance and/or type 2 diabetes (1; 10). On the other hand, hyperglycemia is able to down-regulate expression of ETC-encoding genes (37), making it difficult to distinguish between cause and consequence of the observed effects. Patients with primary mtDNA mutations have impaired skeletal muscle OXPHOS activity and reduced protein expression of key ETC subunits (24). Additionally, these patients exhibit mitochondrial ultra-structure pathology as well as higher IMCL content (23) and often develop diabetes (25). Hence, individuals with primary mitochondrial disease represent an “experiment of nature” to evaluate whether pathologically intrinsic mitochondrial dysfunction in skeletal muscle is associated with insulin resistance and/or type 2 diabetes. The current study demonstrated that patients with primary mitochondrial disease and impaired glucose homeostasis had higher skeletal muscle mitochondrial OXPHOS capacity than their normoglycemic counterparts. Furthermore, the dysglycemic myopathy patients did not exhibit whole-body insulin resistance, arguing against a direct involvement of a mitochondrial dysfunction in the development of muscle insulin resistance and/or type 2 diabetes.

The OGTT showed that 46% of the mitochondrial myopathy patients met the WHO diagnostic criteria for IGT/diabetes (i.e., dysglycemia), despite the fact that fasting blood glucose concentration and HbA_{1c} were normal. An examination of the data from this subset of mitochondrial myopathy patients with age- and physical activity-balanced

normoglycemic myopathy patients, suggested that the hypothesis of deranged skeletal muscle mitochondria directly leading to type 2 diabetes should be reexamined. Despite improper glucose regulation, dysglycemic patients had higher skeletal muscle OXPHOS capacity vs. normoglycemic patients. Furthermore, protein expression of mitochondrial ETC subunits and mitochondrial density were similar between patients. Similarly, mtDNA mutator mice, harboring extensive skeletal muscle mitochondrial dysfunction and aberrant mitochondrial morphology (34), exhibited no evidence of impaired glucose regulation in response to glucose or insulin loads, thus recapitulating the phenotype characteristic of normoglycemic myopathy patients in the current study. This is further evident by the report that mice heterozygous for the Akita diabetogenic mutation (Akita) crossed with the PolG mouse (PolG-Akita), demonstrated improved glycemic regulation (38). Together, these findings extend the growing evidence against a direct causal link between skeletal muscle mitochondrial dysfunction and type 2 diabetes (13; 15-18; 22; 39). Taken together, this provides supportive evidence against the hypothesis that type 2 diabetes is mediated by a deficiency or dysfunction of muscle mitochondria, and a primary mitochondrial defect may even result in increased oxidative capacity as a possible adaptive mechanism.

Previous studies have shown an inverse association between skeletal muscle IMCL content and whole-body insulin sensitivity (40; 41); however, the proposed relationship between IMCL accumulation and skeletal muscle insulin resistance is ambiguous, as it is strongly influenced by training status and/or habitual physical activity (20; 21). In the current study, EM was utilized to assess skeletal muscle IMCL content

which allows for determination of IMCL size, sub-cellular localization and juxtaposition with mitochondria (20), yet we found no differences between dysglycemic and normoglycemic patient groups with primary mitochondrial disease. More recently, Turner *et al.* demonstrated that fatty acid oxidative capacity in rodent models of lipid-induced insulin resistance was up-regulated (39). As such, the authors concluded that high lipid availability does not lead to IMCL accumulation and insulin resistance by decreasing muscle mitochondrial fatty acid oxidative capacity. Similarly, we found no differences in the ability of dysglycemic patients to oxidize fatty acids, coincident with a lack of disproportionate IMCL accrual in the skeletal muscle of these patients compared with normoglycemic patients. Considering that type 2 diabetes patients are generally physically inactive, the impairments in oxidative metabolism in type 2 diabetes patients might simply be attributed to their sedentary lifestyle. In this regard, it is important to note that in most studies physical activity/fitness has not been (strictly) controlled for. For studies in which physical activity was taken into account, the results suggest that the abnormalities in oxidative metabolism in type 2 diabetes patients can at least partly be attributed to physical inactivity (42; 43). Recent data from respiration measurements on permeabilized muscle fibers show that when O₂ flux is being normalized for mtDNA content or CS activity, no differences in mitochondrial respiration rate are observed between type 2 diabetes patients and healthy controls (17). These results imply that type 2 diabetes patients have normal intrinsic mitochondrial function, but an impaired oxidative capacity due to reduced mitochondrial content, most likely secondary to lower habitual physical activity levels (17; 43). Indeed, we also observed preservation of

skeletal muscle ETC subunit protein expression between dysglycemic and normoglycemic myopathy patients; however, contrary to Boushel *et al.* (17), we found higher mitochondrial OXPHOS capacity and similar mitochondrial density between patient groups. A major strength of the current study is that we examined mitochondrial function in sedentary dysglycemic and normoglycemic patients. The above approach helped to eliminate the potential impact of confounding variables (e.g., age and physical activity) on muscle mitochondria in these patients. Furthermore, exercise intolerance is a common hallmark of patients with primary mitochondrial myopathies (23), as such; physical activity levels between groups were similar, thus ruling out the potential influence of physical activity on our findings.

The current study also aimed to determine whether abnormalities of insulin sensitivity or secretion are present in our dysglycemic patients. We found that basal Akt phosphorylation, a key step for the activation of glucose transport in muscle, was higher in the dysglycemic patients with no difference in GLUT-4 content *vs.* normoglycemic patients. The higher basal Akt activation in the dysglycemic patients is perhaps evidence of a compensatory (possibly genetic, epigenetic, or adaptive) mechanism to augment glucose uptake and non-oxidative glucose metabolism because it requires less mitochondrial involvement (oxidizing glucose *vs.* fatty acids) in efforts to mitigate basal hyperglycemia. Interestingly, no differences in the estimates of insulin sensitivity, fasting blood glucose, or HbA_{1c} levels were observed between the two groups. In general, the response of the signaling protein and peripheral tissues is not consistent with reduced insulin sensitivity in the dysglycemic patients in comparison with normoglycemic

patients. Moreover, the blunted 30-min increase in insulin from baseline concomitant with elevated glucose levels at all time points during the OGTT, and a reduction in β -cell function, demonstrates defective insulin secretion, rather than insulin resistance, contributing to impaired glucose regulation in the dysglycemic patients. Our results are in accordance with studies reporting normal insulin sensitivity (44), but impaired glucose-stimulated insulin secretion (44-47), which strongly depends on intact mitochondrial metabolism (45). Some authors suggest that both reduced insulin secretion and insulin resistance are instrumental in the development of type 2 diabetes (48; 49) amongst mitochondrial myopathy patients. Discordance among the etiology of type 2 diabetes (insulin secretion vs. resistance, or both) in mitochondrial myopathy patients might relate to differing levels of mutant mtDNA in the tissues relevant to glucose tolerance, that is muscle for insulin sensitivity and pancreas for insulin secretion. It is therefore conceivable that once the mutational burden becomes overwhelming on the pancreas, either the glucose sensing function or the insulin production capacity of the pancreatic islet cells becomes affected by the change in mitochondrial function as seen in the subgroup of myopathy patients in the current study.

In the current investigation, OGTT-derived indices were utilized to estimate insulin resistance (HOMA-IR), insulin sensitivity (Matsuda index) and insulin secretion ($AUC_{\text{insulin/glucose}}$, IGI), which have been shown to correlate well with estimates from the more time-consuming, costly and labor-intensive euglycemic-hyperinsulinemic clamp (28-30). Furthermore, the disposition index, obtained from the intravenous glucose tolerance test (IVGTT), provides an integrated measure of β -cell compensation, whereby

insulin secretion is appropriately assessed in relation to prevailing insulin sensitivity. Recently, an OGTT-based measure of β -cell compensatory capacity has been shown to correlate well with the IVGTT (31) and hence was utilized in the current investigation.

In summary, dysglycemic patients with primary mitochondrial myopathy had higher skeletal muscle OXPHOS capacity but similar IMCL/mitochondrial content as normoglycemic patients, demonstrating that muscle mitochondrial dysfunction and IMCL accretion *per se* does not cause diabetes. The present data also suggest that mitochondrial myopathy-associated diabetes initially results from impaired β -cell function, rather than poor insulin sensitivity; however, we cannot discount a role for eventual peripheral (muscle) insulin resistance later on in the progression of the disorder. Finally, we propose that pancreatic-related mitochondrial impairment may result in aberrant number of functioning β -cells or from the inability of these cells to optimally sense glucose, thereby contributing to the pathogenesis of type 2 diabetes in humans with mitochondrial myopathy.

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I.A.S., H.P.D., A.S., S.R., M.P., and M.A.T. conceived and designed the study; I.A.S., H.P.D., and A.S. were involved in conducting and coordinating the study; A.S., J.E.B., and A.C.H. conducted and contributed to the PolG study; I.A.S., H.P.D., and A.S. researched and analyzed data; I.A.S. wrote the article; I.A.S., A.S., S.R., A.C.H., K.M.M. and M.A.T. reviewed and edited the article. All authors read and approved the final article.

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TABLE 1

Clinical and metabolic characteristics of healthy lean control subjects and patients with primary mitochondrial myopathy

	Controls (n = 15)	Myopathy Patients (n = 13)
Age (years)	39 ± 2	45 ± 3*
Body composition		
Weight (kg)	68.9 ± 2.0	60.1 ± 3.9*
BMI (kg.m ⁻²)	23.6 ± 0.5	21.8 ± 1.6
Body fat (%)	26.5 ± 2.8	30.1 ± 3.4
Fat-free mass (kg)	51.1 ± 2.6	40.8 ± 1.5†
Fat mass (kg)	17.9 ± 1.7	19.5 ± 3.1
Fitness		
Physical activity index	7.19 ± 0.30	5.82 ± 0.31†
Lipids		
Triglyceride (mM)	0.89 ± 0.10	1.31 ± 0.30
Total cholesterol (mM)	4.46 ± 0.25	4.58 ± 0.28
HDL (mM)	1.62 ± 0.08	1.41 ± 0.09
LDL (mM)	2.42 ± 0.20	2.75 ± 0.28
Free-fatty acid (mM)	0.45 ± 0.04	0.60 ± 0.06*
Glucose		
Fasting plasma glucose (mM)	5.00 ± 0.07	5.13 ± 0.18
2-h plasma glucose (mM)	5.40 ± 0.33	8.85 ± 1.03†
Insulin		
Fasting plasma insulin (μU.mL ⁻¹)	8.00 ± 0.59	9.19 ± 1.13
2-h plasma insulin (μU.mL ⁻¹)	30.71 ± 7.05	28.96 ± 4.26

Lactate

Fasting plasma lactate (mM)	0.65 ± 0.06	2.44 ± 0.38‡
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Insulin sensitivity / β -cell function

Matsuda index	7.37 ± 0.59	6.23 ± 0.58
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AUC _{insulin} /AUC _{glucose} (pmol.mmol ⁻¹)	140.42 ± 24.00	71.24 ± 17.67*
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Insulinogenic index (pmol.mmol ⁻¹)	87.57 ± 13.56	77.94 ± 14.76
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Disposition index	8.05 ± 1.56	3.72 ± 1.05*
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Data are presented as mean ± SEM. * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$.

TABLE 2

Clinical and metabolic characteristics of patients with primary mitochondrial myopathy with normal and impaired glucose regulation

	Normoglycemic (<i>n</i> = 7)	Dysglycemic (<i>n</i> = 6)
Age (years)	45 ± 3	46 ± 5
Body composition		
Weight (kg)	67.8 ± 3.7	51.1 ± 5.4*
BMI (kg.m ⁻²)	24.4 ± 1.9	18.8 ± 2.1
Body fat (%)	36.6 ± 3.8	22.6 ± 4.1*
Fat-free mass (kg)	42.9 ± 1.4	38.4 ± 2.6
Fat mass (kg)	25.5 ± 3.6	12.5 ± 3.6*
Fitness		
Physical activity index	5.85 ± 0.35	5.79 ± 0.55
Lipids		
Triglyceride (mM)	1.05 ± 0.22	1.61 ± 0.62
Total cholesterol (mM)	4.78 ± 0.20	4.35 ± 0.57
HDL (mM)	1.50 ± 0.12	1.30 ± 0.12
LDL (mM)	2.75 ± 0.21	2.75 ± 0.59
Free-fatty acid (mM)	0.56 ± 0.06	0.66 ± 0.11
Glucose		
Fasting plasma glucose (mM)	4.92 ± 0.12	5.43 ± 0.39
2-h plasma glucose (mM)	5.79 ± 0.42	12.42 ± 0.70‡
HbA _{1c} (%)	5.6 ± 0.1	5.9 ± 0.4
Insulin		
Fasting plasma insulin (μU.mL ⁻¹)	9.58 ± 1.29	8.73 ± 2.06

2-h plasma insulin ($\mu\text{U}\cdot\text{mL}^{-1}$)	32.00 ± 5.08	25.41 ± 7.33
Lactate		
Fasting plasma lactate (mM)	2.11 ± 0.84	2.77 ± 0.60
Insulin sensitivity / β -cell function		
Matsuda index	5.98 ± 0.69	6.57 ± 1.09
$\text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}}$ ($\text{pmol}\cdot\text{mmol}^{-1}$)	118.98 ± 18.16	$15.55 \pm 3.19\ddagger$
Insulinogenic index ($\text{pmol}\cdot\text{mmol}^{-1}$)	119.18 ± 6.09	$28.44 \pm 4.46\ddagger$
Disposition index	5.86 ± 1.27	$0.74 \pm 0.18\ddagger$

Data are presented as mean \pm SEM. * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$.

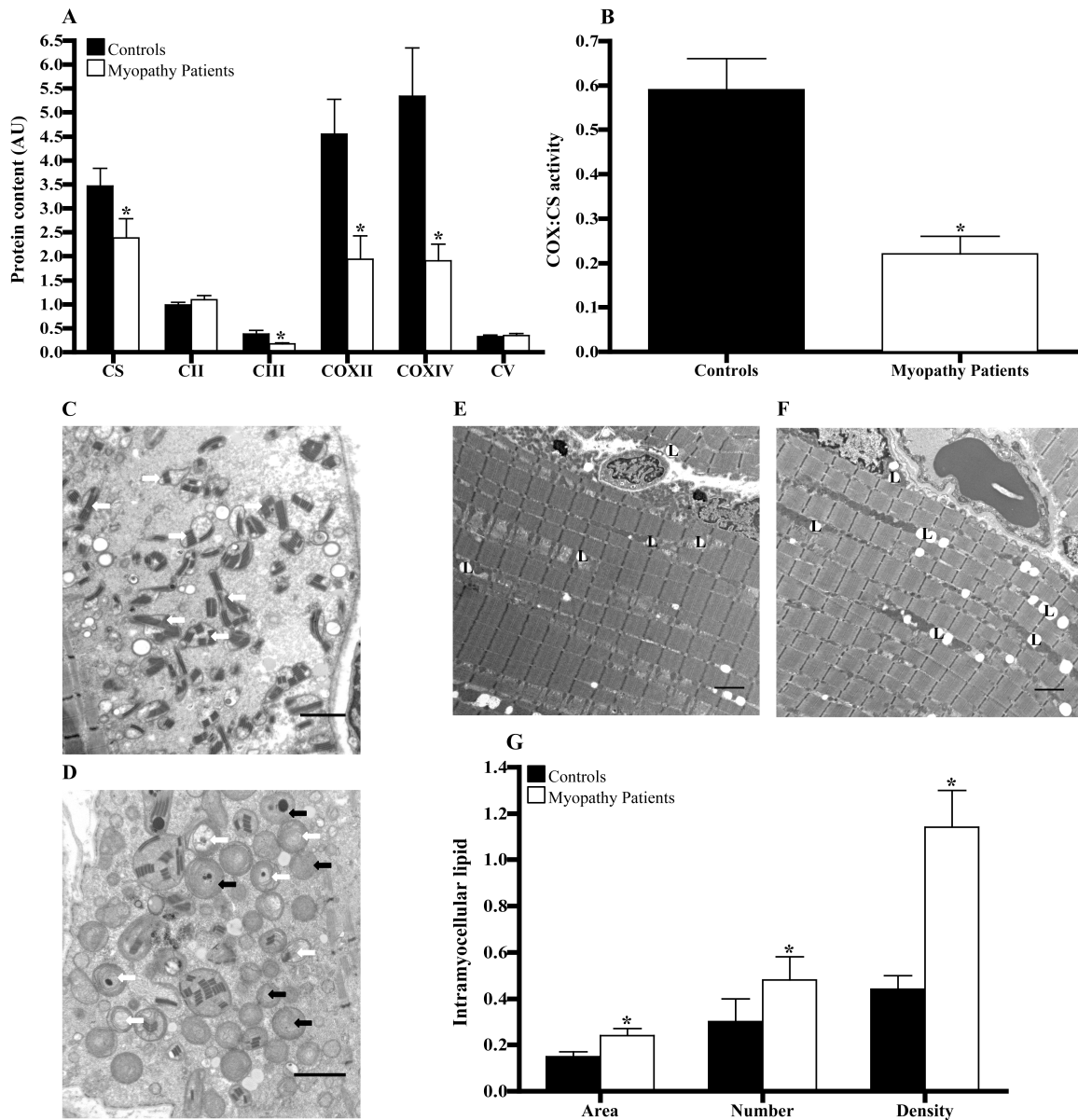


Fig. 1. Skeletal muscle mitochondrial function and intramyocellular lipid content. *A*: Mitochondrial citrate synthase (marker of mitochondrial abundance, CS), and electron transport chain (ETC) subunits – complex II (CII), core protein 2 (subunit of complex III, CIII), cytochrome *c* oxidase (COX) – subunit II (encoded by the mitochondrial genome, COXII) and –IV (encoded by the nuclear genome, COXIV), and complex V (CV) protein expression assessed by Western blot. Results were normalized to β -actin protein expression; and *B*: Mitochondrial maximal enzyme activity of COX (marker of the ETC). Results were normalized to CS maximal enzyme activity, in the *vastus lateralis* of control subjects ($n = 15$) and mitochondrial myopathy patients ($n = 13$). *C-D*: Representative electron micrographs (X7,500 magnification, scale bar: 2 μ m), of *vastus lateralis*

demonstrating mitochondrial morphology, obtained from a mitochondrial myopathy patient. *C*: Arrows indicate paracrystalline inclusions and pleomorphic mitochondria. *D*: Dark arrows indicate swollen mitochondria and white arrows indicate mitochondria with disrupted membranes. *E-F*: Representative electron micrographs (X7,500 magnification, scale bar: 2 μm), of *vastus lateralis* demonstrating intramyocellular lipid (IMCL), obtained from a *E*: control subject, and *F*: mitochondrial myopathy patient. “L” indicates IMCL droplets. Electron micrographs were obtained by transmission electron microscopy. *G*: Graph of intramyocellular lipid content quantified as area (μm^2), number ($\#/\mu\text{m}^2$), and density (%). * Significantly different from control subjects, $P \leq 0.05$. Data are presented as mean \pm SEM.

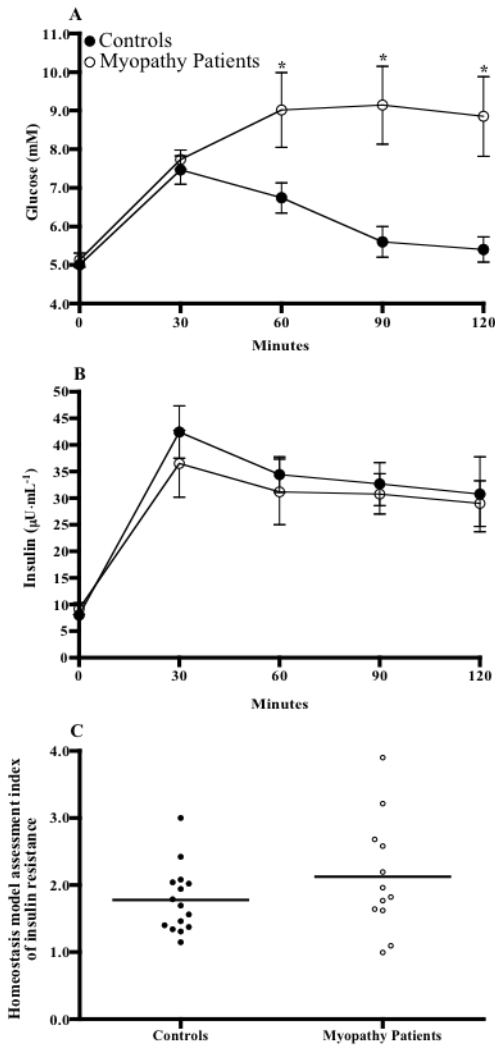


Fig. 2. Oral glucose tolerance test and insulin resistance. Mean plasma concentrations of *A*: glucose (mM) and *B*: insulin ($\mu\text{U}\cdot\text{mL}^{-1}$) during a 75-g oral glucose tolerance test (OGTT), and *C*: the homeostasis model assessment index of insulin resistance (HOMA-IR), in control subjects ($n = 15$) and mitochondrial myopathy patients ($n = 13$). *A*: $P \leq 0.01$ for the comparison of the areas under the curve for glucose concentration of control subjects and mitochondrial myopathy patients. * Significantly different from control subjects, $P \leq 0.02$. Classification of glucose tolerance for dysglycemic patients: ($n = 1$, impaired glucose tolerance [IGT]: 2-h plasma glucose ≥ 7.8 and < 11.1 mM; $n = 5$, diabetes: 2-h plasma glucose ≥ 11.1 mM). Data are presented as mean \pm SEM.

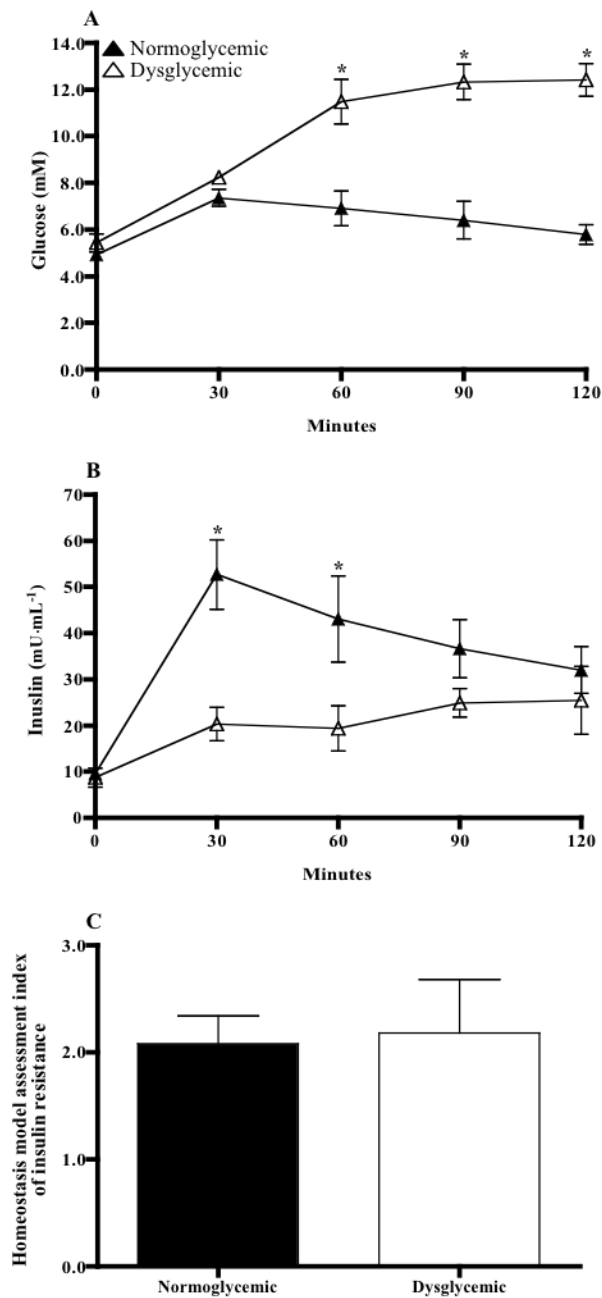


Fig. 3. Oral glucose tolerance test and insulin resistance in mitochondrial myopathy patients. Mean plasma concentrations of *A*: glucose (mM) and *B*: insulin ($\mu\text{U}\cdot\text{mL}^{-1}$) during a 75-g oral glucose tolerance test (OGTT), and *C*: the homeostasis model assessment index of insulin resistance (HOMA-IR), in normoglycemic ($n = 7$) and dysglycemic ($n = 6$) mitochondrial myopathy patients. *A*: $P \leq 0.001$ for the comparison of the areas under the curve for glucose concentration of normoglycemic and dysglycemic mitochondrial myopathy patients. * Significantly different from normoglycemic patients,

$P \leq 0.01$. *B*: $P \leq 0.02$ for the comparison of the areas under the curve for insulin concentration of normoglycemic and dysglycemic mitochondrial myopathy patients. * Significantly different from normoglycemic patients, $P \leq 0.05$. Data are presented as mean \pm SEM.

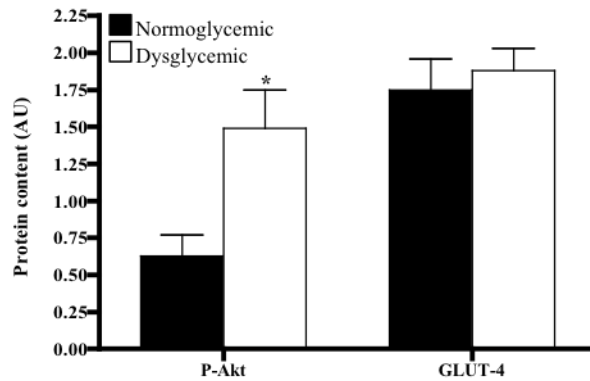


Fig. 4. Distal insulin signaling and glucose transport in mitochondrial myopathy patients. Protein expression of Akt phosphorylation at Ser473 residue (P-Akt) and whole muscle GLUT-4, assessed by Western blot in the *vastus lateralis* of normoglycemic ($n = 7$) and dysglycemic ($n = 6$) mitochondrial myopathy patients. * Significantly different from normoglycemic patients, $P \leq 0.01$. Results were normalized to β -actin protein expression. Data are presented as mean \pm SEM.

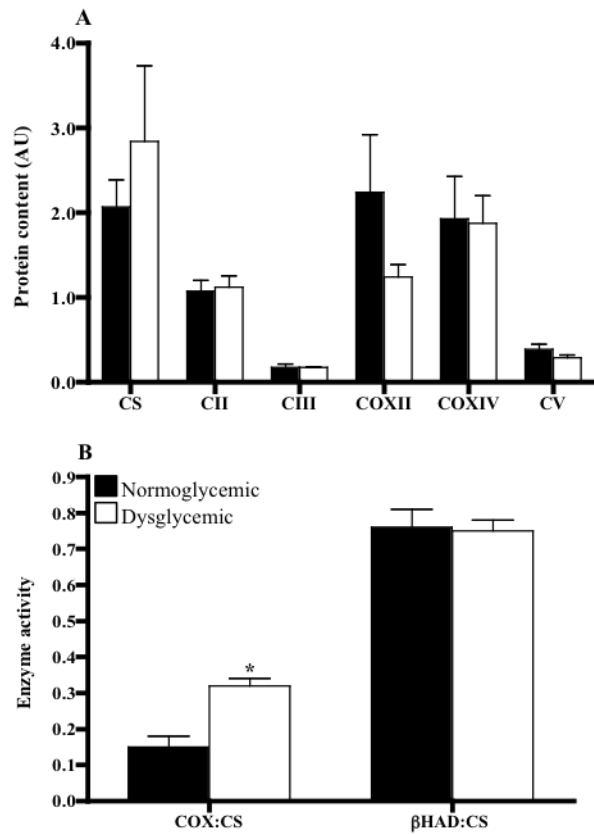


Fig. 5. Skeletal muscle mitochondrial function in mitochondrial myopathy patients. *A*: Mitochondrial citrate synthase (CS), and electron transport chain subunits – complex II (CII), core protein 2 (complex III, CIII), cytochrome *c* oxidase (COX) – subunit II (COXII) and –IV (COXIV), and complex V (CV), protein expression assessed by Western blot. Results were normalized to β -actin protein expression; and *B*: Maximal enzyme activities of mitochondrial COX and β -oxidation - short-chain β -hydroxyacyl-CoA-dyhydrogenase (β -HAD). Results were normalized to CS maximal enzyme activity, in the *vastus lateralis* of normoglycemic ($n = 7$) and dysglycemic ($n = 6$) mitochondrial myopathy patients. * Significantly different from normoglycemic patients, $P \leq 0.01$. Data are presented as mean \pm SEM.

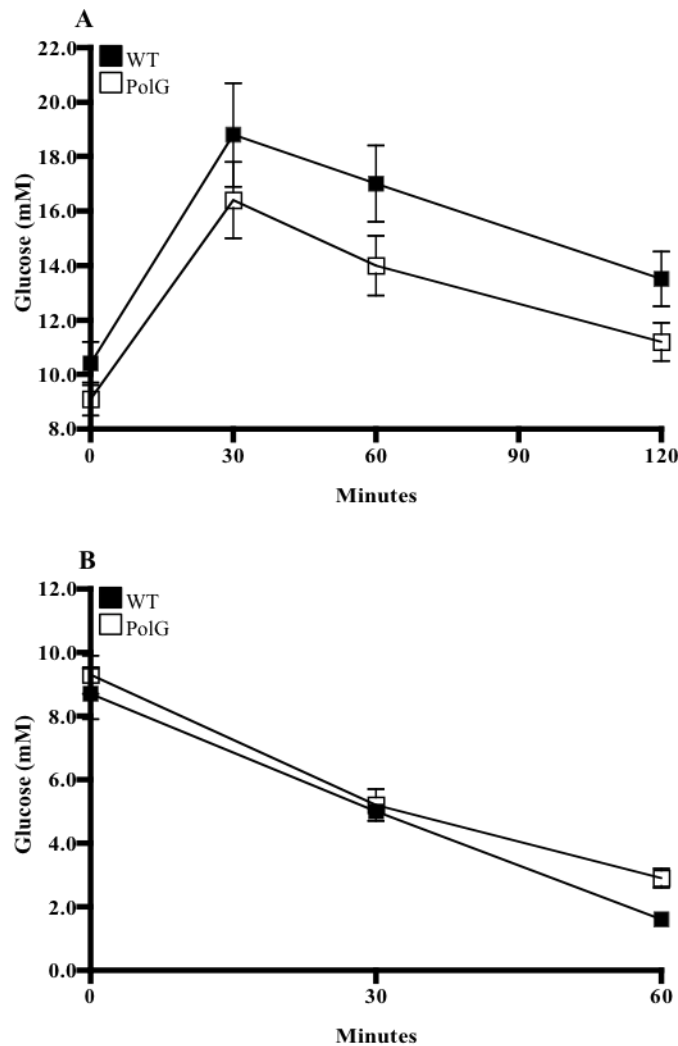
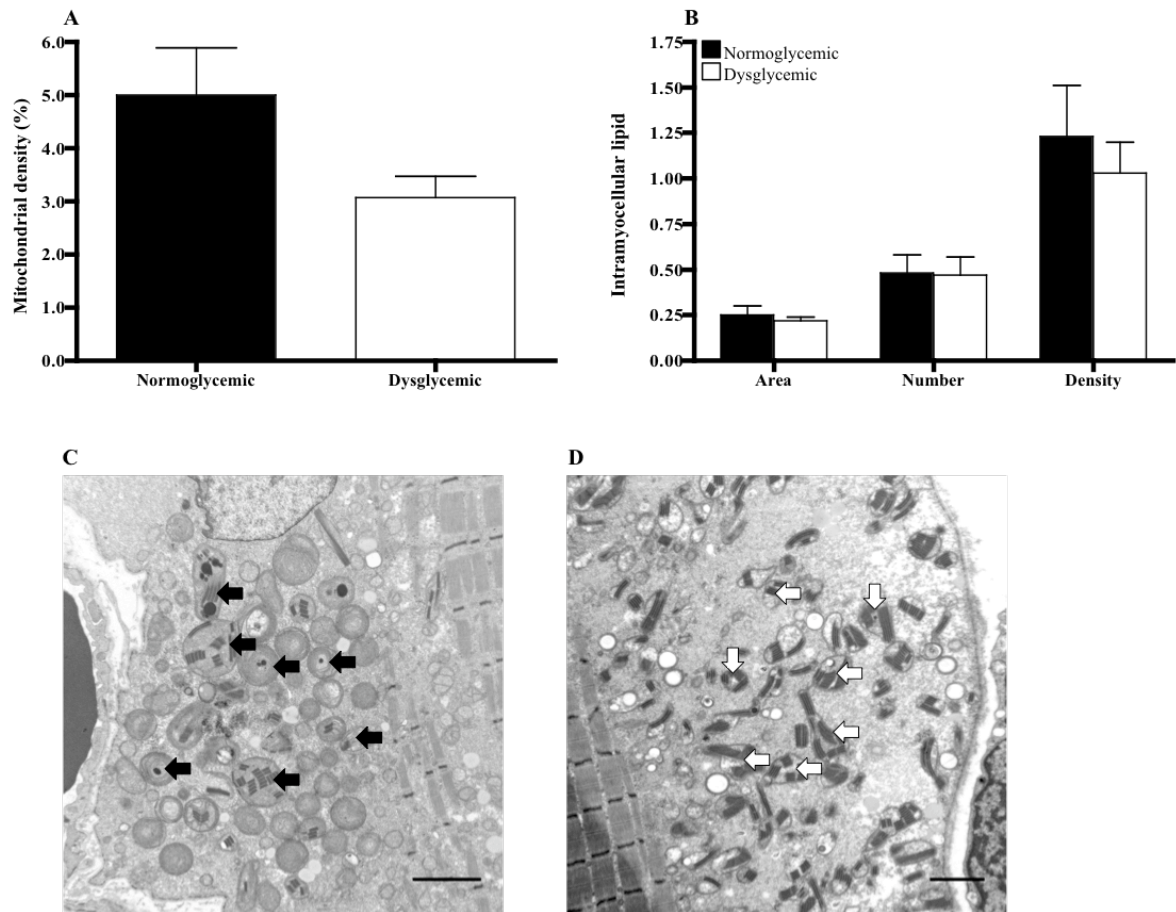


Fig. 6. Glucose and insulin tolerance test in PolG mutator mouse model of mitochondrial myopathy. *A*: Glucose tolerance test (GTT), and *B*: insulin tolerance test (ITT), assessed after an overnight fast in wild-type (WT, $n = 6-10$) and polymerase gamma (PolG, $n = 6-14$) mice. WT and PolG mice were assessed at 30 weeks of age. Data are presented as mean \pm SEM.

SUPPLEMENTARY DATA



Supplementary Figure 1. Mitochondrial morphology and intramyocellular lipid content in mitochondrial myopathy patients. *A*: Mitochondrial density (%), *B*: Intramyocellular lipid content quantified as area (μm^2), number ($\#/\mu\text{m}^2$), and density (%), and *C-D*: Mitochondrial morphology, assessed by transmission electron microscopy in the *vastus lateralis* of normoglycemic ($n = 7$) and dysglycemic ($n = 6$) mitochondrial myopathy patients. *C-D*: Representative electron micrographs (X7,500 magnification, scale bar: 2 μm), of a skeletal muscle cell obtained from a *C*: normoglycemic and *D*: dysglycemic mitochondrial myopathy patient. Arrows indicate mitochondrial abnormalities (i.e., paracrystalline inclusions, disrupted membranes). Data are presented as mean \pm SEM.

CHAPTER 5

DISCUSSION

5.0 DISCUSSION

5.1 Summary

This thesis examined the biochemical adaptations of human skeletal muscle in response to endurance exercise training with a focus on the role of human skeletal muscle mitochondria in disorders of impaired glycemic regulation and the relationship to obesity. The first two studies explored biochemical markers of mitochondrial function (Chapter 2) and oxidative stress/inflammation (Chapter 3) implicated in the development of insulin resistance and in response to medium-term endurance exercise. Mitochondrial content and mitochondrial enzyme capacity was not diminished in insulin-resistant obese men compared with healthy lean controls when appropriately matched for age and physical fitness, although evidence of cellular damage and low-grade systemic inflammation coincident with dysregulated adipokine levels was clearly evident. Endurance exercise was equally effective in increasing aerobic fitness, mitochondrial capacity, insulin signaling and GLUT4 content to a similar extent in both lean and obese men; however, improvements in oxidative stress, inflammation and adipokine profile were skewed towards obese men. Study 3 (Chapter 4) examined the role of impaired skeletal muscle mitochondrial function in the development of type 2 diabetes mellitus (T2D) in patients diagnosed with primary mitochondrial disease. The skeletal muscle of these patients was indistinguishable from that of age- and physical activity-matched normoglycemic patients, in regards to mitochondrial content, mitochondrial oxidative capacity and intramyocellular lipid (IMCL) accumulation. Insight into potential mechanisms was provided based on our observation of diminished pancreatic β -cell secretion rather than

whole-body resistance. Hence, we hypothesized that aberrant mitochondria of the pancreatic islet cells lead to the development of dysglycemia. Collectively, these studies facilitate in elucidating our understanding of mitochondrial function and its role in disease pathology as a whole. The present chapter integrates these findings and highlights their relative contributions to the field. Potential limitations, unanswered questions, and future directions are discussed throughout.

5.2 Mitochondrial Dysfunction in Insulin Resistance and Type 2 Diabetes: Culprit, Victim, or Bystander?

Several studies have demonstrated that individuals with obesity, insulin resistance and T2D have reduced markers of skeletal muscle mitochondrial content (1-9), defective mitochondrial oxidative capacity (10-14) and reduced expression of a cluster of nuclear genes responsible for oxidative metabolism (7; 15-17). These findings have led to the theory that reduced mitochondrial content and/or oxidative capacity in skeletal muscle causes insulin resistance/T2D (18-22). However, whether perturbations in skeletal muscle mitochondria are central to the pathophysiology of insulin resistance/T2D is robustly debated (23-26). The absence of a role of muscle mitochondria contributing to the etiology of impaired glucose homeostasis in Study 1 adds further scrutiny to theory implicating mitochondrial dysfunction in insulin resistance/T2D. Our findings support the idea that muscle mitochondrial dysfunction in obesity is not the leading cause of insulin resistance. However, we do not preclude a subsequent role of mitochondrial dysfunction as a component of a “vicious cycle” exacerbating the consequences of insulin resistance/T2D as the disease progresses. The reasons for the disparity between our

findings and previous studies are not clear, but we believe this may be related to the fact that our insulin-resistant obese men were younger, presented no co-morbidities (i.e., healthy obese), and were appropriately matched demographically (i.e., age and physical fitness) in contrast to other studies. In essence, we have “clamped” other confounding factors and have truly explored the question as to whether obesity *per se* was associated with impaired mitochondrial function and structure. Importantly, we also used a wide range of methodologies and techniques to study mitochondrial biogenesis and density (such as, qPCR, Western blot, enzyme activity, electron microscopy). However, these markers may not necessarily provide the *in vivo* mitochondrial bioenergetic capacity or functionality of coupled muscle mitochondria *in vitro* and thus conclusions are limited. Nevertheless, given that VO_{2peak}/kg FFM was not different between lean and obese men in the current study provided very strong *in vivo* evidence that there were no differences in mitochondrial bioenergetic capacity.

Petersen *et al.* has shown that lean offspring of diabetic patients have reduced insulin sensitivity in parallel with elevated IMCL content (11). Compared with controls, these subjects also show reduced skeletal muscle ATP synthesis in response to insulin stimulation as measured by magnetic resonance spectroscopy (^{31}P -MRS) saturation transfer. These measurements reflect impaired baseline activity of mitochondrial oxidative phosphorylation (OXPHOS) (11; 27). Insulin-resistant offspring of diabetic parents also show a reduction in skeletal muscle mitochondrial content (1). The lean offspring of diabetic parents in these studies may represent a narrowly selected cohort of individuals that do not necessarily reflect the early pathophysiology of T2D in this

heterozygous disease process. In addition, the measurement of basal oxidative phosphorylation flux does not reflect maximal oxidative capacity of skeletal muscle, and whether baseline perturbations are sufficient to initiate the development of skeletal muscle insulin resistance has been questioned (24). Furthermore, the role of a genetic link, whether mitochondrial in origin, epigenetic, or otherwise has not been considered. This is important especially to differentiate between genetic *vs.* lifestyle induced T2D. Strong epidemiological and experimental evidence indicates a link between the intrauterine growth environment and adult diseases, such as obesity, hypertension, T2D, and cardiovascular disease (28; 29). The evidence suggests that gestational or overt diabetes in pregnancy can affect diabetes risk in offspring (30). For example, in longitudinal studies of Pima Indians, offspring of mothers with established disease during pregnancy develop T2D earlier than those born to mothers without diabetes (31-33). Furthermore, obesity and T2D were more frequent among siblings born to the same mother after she developed diabetes (31). Additionally, non-genetic manipulations (nicotine exposure) in female rats during pregnancy has been shown to negatively influence postnatal mitochondrial capacity in the offspring (34). These results clearly demonstrate that the aforementioned observations in the offspring of diabetic patients studies by Shulman's laboratory (1; 11) may very likely be attributed to an epigenetic phenomenon or at least suggestive of it. The possibility exists that the genetic linkage of T2D may have more of mitochondrial etiology than lifestyle-mediated T2D. Lastly, the reduced mitochondrial content and/or function observed in previous studies may merely be a consequence of reduced physical activity of T2D patients (26; 35). Reduced physical

activity and sedentary lifestyle are implicated in the development of obesity and T2D (36). Additionally, our lab and others have shown that even a short-term (two weeks) reduction in physical activity results in ~20-30% reduction in mitochondrial biogenesis and oxidative capacity (37). In the current studies, we carefully selected only sedentary subjects to avoid confounding effects of physical activity between obese subjects and controls (Study 1) and between mitochondrial myopathy patients (Study 3). This is an important consideration because we have previously shown that mitochondrial OXPHOS capacity and size increases with training (38). The results in Chapter 2 further shows that in response to endurance exercise training, mitochondrial biogenesis and oxidative capacity is significantly enhanced in obese individuals concluding that their lack of optimal mitochondrial function is in part due to their sedentary lifestyle (39). On the other hand, mitochondrial dysfunction might be a parallel process that is present with insulin resistance/T2D. Two DNA microarray studies found a coordinated reduction in the expression of genes encoded by peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) in the skeletal muscle of patients with T2D (16; 17). However, in contrast to these studies, we did not observe any difference in the protein expression levels for PGC-1 α in our insulin-resistant obese men. Whether or not mRNA expression of genes involved in mitochondrial bioenergetics play a key role in the insulin sensitivity of our subjects requires further investigation. Furthermore, Morino *et al.* found no changes in PGC-1 α , nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (TFAM) mRNA levels in the insulin-resistant offspring of T2D patients in comparison to their healthy controls but a reduction in mitochondrial density

and *in vivo* function (1). Consistent with this, Gallagher *et al.* found that the muscle mRNA transcriptome is invariant with respect to insulin and glucose homeostasis but provided evidence that insulin resistance in humans may be related to coordinated changes in multiple microRNAs, which act to target relevant signaling pathways (40). Together, these results suggest that reduced mitochondrial biogenesis cannot fully account for the impaired glucose homeostasis and that other unknown or regulatory factors involved in the regulation of mitochondrial biogenesis may be responsible for the reduced skeletal muscle mitochondrial function/content in lean insulin-resistant offspring or T2D. Finally, the role of the obese state in the down-regulated expression of the PGC-1 α and its target genes is an important question that remains to be answered.

Alternatively, it cannot be excluded that a reduced mitochondrial function may be the consequence of insulin resistance/T2D (12; 41; 42). In support of this hypothesis are the findings that high-fat fed mice develop glucose intolerance/insulin resistance prior to the onset of reactive oxygen species (ROS)-induced mitochondrial abnormalities (43; 44). In Study 1, muscle mitochondrial OXPHOS capacity and content was preserved, despite elevated glucose and insulin levels *vs.* healthy lean controls. This finding suggests that mitochondrial function does not result in a functional defect secondary to an impaired response to insulin and corroborates the Brons *et al.* study, in which lean men with low birth weights – an independent risk factor of insulin resistance and T2D, had elevated plasma glucose and insulin concentrations while mitochondrial function *in vivo* was identical to that of controls (45). However, it is difficult to reach any definitive conclusion as to the true sequence of events and whether or not mitochondrial

dysfunction is the causal culprit or the victim of insulin resistance. To explore the former, we utilized patients with primary mitochondrial genetic defects, who manifest many skeletal muscle mitochondrial impairments (46; 47) purported in obese, insulin resistant and patients with T2D (21). Although a subset of mitochondrial myopathy patients (Study 3) were classified as impaired glucose tolerant (IGT) or diabetic, when compared with demographically matched normoglycemic myopathy patients, muscle mitochondrial dysfunction could not account for their dysglycemia. Moreover, Akt activation and mitochondrial OXPHOS capacity was *higher* in the dysglycemic group. Similarly, in Asian Indians, diabetes *per se* was not linked with mitochondrial dysfunction (48). In fact muscle mitochondrial ATP production and mitochondrial DNA (mtDNA) copy number was *higher* in insulin-resistant Asian Indians vs. Northern European American counterparts (48). These data suggest that muscle mitochondrial dysfunction is not the primary causal event in T2D and may even result in increased mitochondrial function or insulin sensitivity/signaling as a possible adaptive response to metabolic defects that initiate in other metabolically active tissues (i.e., pancreas) as may be operational in the dysglycemic mitochondrial myopathy patients (21; 49).

In summary, it is important to notice that a down-regulated OXPHOS gene expression profile and reduced mitochondrial function/content is only reported in subjects that were already insulin resistant or have established T2D and had higher IMCL levels compared to their control groups. Hence it is difficult to ascertain a “cause-and-effect” relationship between mitochondrial deficits and the onset on insulin resistance. Therefore, more research is clearly needed to elucidate the specific role of mitochondrial function in

the etiology of T2D. The variability in mitochondrial content and function in these different studies of subjects with obesity, insulin resistance, and established T2D not only underscores the complexity of the disease progression that may range from initial adaptive to more chronic maladaptive changes, but the skeletal muscle mitochondrial biology in the context of the homeostasis of the other peripheral organs that together coordinate systemic insulin sensitivity. Nonetheless, if mitochondrial function proves to be of relevance in the etiology of insulin resistance/T2D, it provides a fundamental explanation for the well-documented effects of physical activity and exercise training – major enhancers of mitochondrial function and biogenesis – on glucose homeostasis and T2D risk. If anything, the interest in mitochondrial function has taught us once more that the growing prevalence of physical inactivity in developed countries may be more important in the etiology of insulin resistance/T2D than sometimes appreciated.

5.3 Intramyocellular Lipid and Fatty Acid Intermediates

The relationship between IMCL content and skeletal muscle insulin resistance has been reported in both animal (50-53) and human studies (3; 54-57). In most of the earlier studies, biochemical extractions were performed to quantify muscle lipid content but were unable to differentiate between IMCL and extramyocellular lipid (EMCL) deposits. Contamination of biopsy samples with EMCL has been shown to affect the results (58). Since the introduction of ¹H-magnetic resonance spectroscopy (¹H-MRS), a noninvasive method to quantify both IMCL and EMCL content *in vivo* (59), numerous studies have shown IMCL accretion to be associated with obesity (60; 61) and insulin resistance/T2D

(62-64). Direct visualization of IMCL aggregates by using histochemistry has confirmed the presence of greater IMCL content in obese (65) and/or T2D patients (9; 57; 66) compared with healthy, lean controls. However, it is important to note that in order to obtain a *bona fide* assessment of IMCL content in terms of morphology (i.e., size, number), sub-cellular distribution and the relationship to mitochondria, the electron microscopy (EM) technique provides the only true comprehensive insight. The available evidence suggests that in a variety of conditions, there is an association between skeletal muscle lipid content and insulin resistance. However, the reported correlations between IMCL content and insulin resistance (54; 55; 64), disappear with the inclusion of well-trained endurance athletes (66; 67). This is due to the fact that endurance-trained athletes are markedly insulin sensitive (68-70), despite their elevated IMCL content (66; 67) (71), referred to as the “athlete paradox” (66) (38; 72). The disconnect between IMCL levels and insulin sensitivity has more recently been demonstrated by findings from Kiens’s laboratory which show higher whole body insulin sensitivity and insulin-stimulated glucose uptake in skeletal muscle in women despite a higher IMCL concentration *vs.* men (73). The higher insulin sensitivity at the muscular level in women could not be explained by higher protein expression of the IR, Akt, or GLUT4 or a higher ability of insulin to stimulate signaling through Akt (73). Furthermore, in these earlier studies, the subpopulation of subsarcolemmal (SS) or intermyofibrillar (IMF) IMCL was not specifically examined morphologically, which may be more important than IMCL accumulation *per se* for the development of insulin resistance/T2D. The role of IMCL in these sub-cellular regions in obesity and T2D is only beginning to be unraveled and there

is a paucity of human data in this regard. The idea for examining IMCL and mitochondria in sub-cellular regions is supported by findings from Ritov *et al.* who demonstrated that SS mitochondrial ETC activity was reduced in obese individuals with or without T2D *vs.* lean controls (6). Using EM to quantify IMCL content (size, number, and density) in both sub-cellular fractions, we found that these characteristics were similar between insulin-resistant obese men and lean controls (Study 1) and between dysglycemic myopathy patients *vs.* normoglycemic myopathy patients (Study 3). Although these findings contradict earlier studies showing increased lipid deposition within skeletal muscle (11; 63; 74), they are indeed supported by others investigating relatives of subjects with T2D (75), overweight (76) and insulin-resistant subjects (7; 12; 77).

Other groups have reported similar IMCL content between controls and insulin-resistant subjects, but unlike our current results, a concomitant reduction in mitochondrial function was also found (12; 77). This suggests that IMCL levels combined with compromised mitochondrial function may contribute to insulin resistance. Hence, we hypothesized that the capacity to store lipid as IMCL along with appropriate mitochondrial function relies on the juxtaposition of IMCL with mitochondria, and this in turn may influence myocellular insulin sensitivity (38). We therefore undertook a novel analysis that characterizes the physical relationship between IMCL and mitochondria, an observation that exploits the technical advantage of EM. The proportion of IMCL droplets juxtaposed with mitochondria was similar between insulin-resistant obese men *vs.* lean controls (Study 1) in both sub-cellular regions and was not related to insulin resistance. Although this observation was contrary to our hypothesis, we believe this

finding adds further support disassociating the IMCL-insulin resistance paradigm. Rather, IMCL may be a marker of other fatty acid intermediates such as diacylglycerol (DAG), fatty acyl-CoA and ceramides that can directly interfere with muscular insulin signaling (78), but we also observed no differences in the level of lipid intermediates between lean and insulin-resistant obese men, corroborating recent human (79; 80) and rodent (81) data.

It is important to note that explanations beyond mitochondrial dysfunction for IMCL accumulation exist. For example, mitochondrial reactive oxygen species (ROS) emission has been shown to serve as both a gauge of energy balance and a regulator of cellular redox environment, linking intracellular metabolic balance to the control of insulin sensitivity (82). Additionally, studies in obese rats (83; 84) and obese humans (85) have suggested that the rate of fatty acid transport into insulin-resistant muscles is increased. Among the putative fatty acid transporters, only the plasmalemmal content of FAT/CD36 has been shown to be upregulated in obese Zucker rat skeletal muscle (84), and in obese and diabetic human muscle (85). Taken altogether, these observations suggest that the increased plasmalemmal FAT/CD36 accounts for the increased rate of fatty acid transport into insulin-resistant skeletal muscle. In obese Zucker rats, there is an increased capacity for fatty acid oxidation, and yet IMCL are seen to accumulate (86). This suggests that the increased rate of fatty acid transport into insulin-resistant muscle exceeds the capacity for fatty acid oxidation and, therefore, more of these fatty acids that have entered the muscle are esterified (86). This theory also supports, in part, the mechanism proposed by Muoio's group (87): namely, that in insulin-resistant muscle, the

excess lipids available to mitochondria exceed the capacity for their oxidation. Thus, it appears that a key event in raising IMCL concentrations is the increase in fatty acid transport, mediated by the increase in plasmalemmal FAT/CD36, not mitochondrial dysfunction. In this manner, an increased delivery of fatty acids into muscle can overwhelm the increased capacity for their oxidation, which results in the accumulation of IMCL.

In summary, although the accumulation of IMCL coincides with the development of insulin resistance, the relationship is most likely indirect. Several intermediates of fat metabolism have been postulated to interfere with insulin signaling. While the potential for intermediates to accumulate in situations with high fat availability and low fat oxidation (i.e., reduced physical activity) exists, the balance between availability and oxidation may be more crucial.

5.4 Insulin Signal Transduction

Insulin stimulates glucose transport into tissues (e.g., adipose, skeletal muscle) by causing the translocation of the insulin-responsive glucose transporter, GLUT4, from the intracellular location to cell surface membranes. Thus, alterations in the trafficking, budding, fusion, or activity of GLUT4 have been postulated to explain the mechanisms of insulin resistance and T2D (88-90). Alternatively, insulin resistance/T2D could result from alterations in upstream insulin signaling events, resulting in decreased GLUT4 translocation to the plasma membrane. In Studies 1 and 3, GLUT4 protein content was not different between individuals with impaired glucose homeostasis and their

counterparts. Of note, whole muscle GLUT4 content was assessed, which do not necessarily reflect GLUT4 activity or actual membrane-bound protein levels, which may be more reflective of insulin sensitivity status and is of important consideration for future studies. To explore the possibility of impaired insulin signaling upstream of GLUT4, we examined the phosphorylation of Akt^{Ser473}, which has been implicated in the regulation of GLUT4 translocation (91) and its impaired activation is associated with insulin resistance (92) although controversial (93). We found no difference in the basal phosphorylation of Akt^{Ser473} between our insulin-resistant obese men and healthy, lean controls (Study 1); and in our dysglycemic mitochondrial myopathy patients, we found *higher* basal Akt^{Ser473} phosphorylation vs. normoglycemic myopathy patients. Importantly, the activation of Akt requires its phosphorylation at both Ser⁴⁷³ and Thr³⁰⁸ (94), thus the precise role of Akt in regulating glucose transport and metabolism in this thesis remains unclear.

Reduced insulin-stimulated glucose transport activity in patients with T2D and the offspring of type 2 diabetic parents (78; 95; 96) has been attributed to increased serine phosphorylation of IRS, which, in turn, blocks insulin receptor kinase phosphorylation on critical tyrosine sites that are required for PI3K association and activation (78).

Consistent with this hypothesis, recent studies have demonstrated serine phosphorylation of IRS on Ser³⁰², Ser³⁰⁷, Ser⁶¹², and Ser⁶³⁶ in several insulin-resistant rodent models (95; 97; 98), as well as in lean insulin-resistant offspring of parents with T2D (1). Attempts were made to probe for phosphorylated serine residues on IRS via Western blot but these efforts were unsuccessful because of potential antibody-specificity issues in human skeletal muscle in our hands. It is possible that any number of upstream insulin signaling

impairments could account for the dysregulated glucose homeostasis observed in our subjects; however, because whole-body insulin resistance was measured it remains unknown whether increased IRS serine phosphorylation would have delineated the pathogenesis of insulin resistance as multiple tissues (i.e., adipose, liver, skeletal muscle) play a role in maintaining whole-body glucose homeostasis.

5.5 Endurance Exercise Training

5.5.1 Unequivocal Skeletal Muscle Mitochondrial Improvements in Both Lean and Obese Men

Endurance exercise training has long been known to increase skeletal muscle mitochondrial content and improve functional exercise capacity in healthy individuals (99; 100). The purported link between reduced skeletal muscle mitochondrial content and insulin resistance by some (1-5; 10-12), but not all investigators (25; 26; 45; 101-104), suggests that endurance exercise training may be an efficacious treatment/therapeutic modality to increase mitochondrial content and improve the ability of skeletal muscle to oxidize substrates (105). However, the details of exercise-mediated muscle adaptations in insulin resistance and obesity are only beginning to be unraveled. The results from Study 1 suggest that considerable capacity exists in insulin-resistant obese men to augment mitochondrial content/function in response to moderate-intensity endurance exercise training. In particular, the coordinated increase in mitochondrial OXPHOS enzymes and protein content of ETC subunits, in addition to mitochondrial size/density, to the same extent in both lean and insulin-resistant obese men, provided evidence that exercise induced mitochondrial biogenesis irrespective of adiposity. Despite the well-established

increase in muscle mitochondrial content following exercise training, the molecular mechanisms promoting mitochondrial biogenesis in skeletal muscle remain elusive. PGC-1 α , a transcriptional co-activator touted as a therapeutic target in many chronic degenerative diseases, has generated particular interest in recent years as a critical regulator of mitochondrial gene transcription (106; 107). In turn, PGC-1 α has been implicated as a key regulator of the adaptive response to exercise in skeletal muscle (108). Although resting total PGC-1 α protein was unchanged following exercise training in Study 1, this may not be indicative of PGC-1 α activation; rather PGC-1 α activity may be primarily determined by its sub-cellular location (109; 110) such that more PGC-1 α protein is present in the nucleus in response to training. Greater nuclear PGC-1 α would presumably be more conducive for promoting or maintaining an increase in mitochondrial biogenesis via increased co-activation of transcription factors linked to mitochondrial gene expression. Furthermore, PGC-1 α mRNA in skeletal muscle of individuals with obesity has been reported to be lower compared to lean controls (111; 112) and microarray studies have also reported reduced PGC-1 α mRNA expression (16) and coordinated down-regulation of PGC-1 α target genes (17) in individuals with insulin resistance and T2D. Although PGC-1 α expression at the protein level was not different between insulin-resistant obese and healthy lean men prior to endurance training in the current study, examining PGC-1 α mRNA, its downstream targets, and sub-cellular localization could help reveal more insight to the metabolic regulation of skeletal muscle adaptation to exercise. This was not attempted in this thesis, primarily due to tissue limitations. Nevertheless, if reduced levels of skeletal muscle PGC-1 α or its downstream

targets are linked with insulin resistance/T2D (16; 17), strategies that increase the activation or expression of PGC-1 α may be of therapeutic benefit (113-116).

Accordingly, we found increases in several classical markers of mitochondrial biogenesis, including the maximal activity and protein contents of CS and COX. Future studies examining sub-cellular localization of PGC-1 α and molecular targets in response to exercise in obesity, insulin resistance and T2D could provide further insight into the regulation of muscle adaptation to increased contractile activity (113).

5.5.2 Differential Regulation of Intramyocellular Lipids

The effect of endurance exercise training on IMCL content in skeletal muscle is equivocal. While endurance exercise has previously been shown to lower IMCL content in T2D (117-119), we and others have shown that in nondiabetic untrained subjects, endurance training often elevates IMCL content (38; 120-122), presumably as an adaptation to maximize surface area for lipolysis during exercise. To our knowledge, only one other paper has used EM to assess whether differences in the content of SS and IMF lipids present in insulin resistant conditions are differentially regulated with endurance training (123). Study 1 demonstrated differential regulation of sub-cellular IMCL stores in response to moderate-intensity exercise training and represents a novel contribution to the literature. IMCL content in the SS region was decreased whereas IMCL content in the IMF region was increased as well as their juxtaposition relative to mitochondria increased irrespective of sub-cellular regions. The results suggest that studying lipids in their respective SS and IMF cellular milieu may provide greater insight into its role in skeletal

muscle metabolism and association with disease pathology. Measuring the sub-cellular distribution of IMCL droplets, and possibly proteins regulating IMCL dynamics, may be a more fruitful approach to the molecular study of muscle adaptation to exercise. The mechanisms leading to the differentially regulated IMCL content in sub-cellular fractions upon exercise training were beyond the scope of this thesis. The data was primarily interpreted to represent a shift/redistribution in sub-cellular localization of IMCL from the SS region to the IMF region in response to exercise. A recent report suggested that SS fraction of IMCL were elevated in patients with T2D whereas no differences in IMF lipids were found compared to controls (123). The abnormality of SS lipids in T2D suggests that this fraction of lipids could be an important factor contributing to the regulation of skeletal muscle insulin sensitivity in certain diseases (123). We did not confirm this in Study 3, in that dysglycemic myopathy patients had similar total IMCL content compared with their normoglycemic myopathy counterparts. However, contrary to the aforementioned study (123), dysglycemic myopathy patients had elevated IMF, not SS lipids compared to healthy controls (data not shown). As such, the sub-cellular distribution of IMCL may represent the next phase of study in understanding the biology of this lipid pool in the context of muscle physiology and pathology.

5.5.3 Improvements in Markers of Inflammation: Links with Insulin Resistance

The production of adipocyte-derived proteins (such as leptin, adiponectin) and pro-inflammatory cytokines (such as, C-reactive protein [CRP], interleukin-6 [IL-6], tumor necrosis factor- α [TNF- α]) is increased in obesity (124; 125), and raised

circulating levels of several acute-phase proteins and inflammatory cytokines has led to the view that obesity is characterized by a state of chronic low-grade inflammation, and that this links causally to insulin resistance and T2D (126-129). Study 2 was therefore designed to test whether altered adipocyte-derived proteins and pro-inflammatory cytokines are associated with obesity and whether they could explain whole-body insulin resistance. This expectation was confirmed by us, in that insulin-resistant obese men had elevated CRP, IL-6, and leptin levels but reduced adiponectin levels compared with healthy, lean controls and that these adipokines were associated with whole-body insulin resistance. Study 2 also aimed to determine whether markers of systemic inflammation and adipokines could be modulated with moderate-intensity endurance exercise training since a sedentary lifestyle is a strong and independent risk factor for many chronic diseases, including those that are associated with persistent, systemic inflammation (130). Exercise training significantly lowered leptin and IL-6 levels corroborating findings by other researchers (131-133); however, the mechanisms leading to the exercise-mediated reduction in inflammation was not ascertained this study. Of note, it has been demonstrated that plasma concentrations of IL-6 increases during muscular contractions and enhances insulin action (134), but on the other hand, IL-6 has been associated with obesity and reduced insulin sensitivity (135-137). Perhaps an acute increase in circulating levels of IL-6 enhances fat oxidation, improves insulin-stimulated glucose uptake, and has anti-inflammatory effects (134; 138). However, elevated chronic levels of IL-6, as seen in obesity and patients with T2D, play a pathogenetic role in the disease process.

The inflammatory response that emerges in the presence of obesity has been

suggested to be triggered by and to reside predominantly in adipose tissue, although other metabolically critical sites, particularly the liver, pancreas, skeletal muscle might also be involved during the course of the disease (128; 139). For example, inflammatory cytokines are able to induce insulin resistance in muscle cells in culture (140; 141) and *in vivo* by infusion into humans, resulting in decreased glucose uptake and glycogen synthesis (142). In our investigation, despite dysregulated adipokine levels in the insulin-resistant obese men, muscle mitochondrial function and insulin signaling was unaffected, at least in the markers we assessed. We did however note significant inverse correlations between whole-body insulin resistance and adiponectin, which has been beneficially linked with insulin sensitivity and have anti-inflammatory and anti-atherogenic activity (143), and positive correlations with markers of inflammation. These results suggests that whole-body insulin resistance in obesity may be attributable to defects in tissues other than skeletal muscle and that inflammatory changes are likely to be the primary abnormality in these groups of subjects. We believe that inflammatory cell recruitment and changes in cytokine production may be primarily involved in maintaining and/or exacerbating the insulin resistant state associated with obesity. However, this aspect has not yet been thoroughly explored in this thesis. Future studies examining the molecular mechanisms involved in the inflammatory response in skeletal muscle in response to exercise in humans and how it might relate to peripheral insulin resistance and muscle metabolism in obesity could help support the findings in Chapter 3 and provide further insight into the regulation of muscle adaptation to increased contractile activity. Recently, the Nod-like receptor (NLR) family of innate immune cell sensors, such as the

nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (Nlrp3) inflammasome have been implicated in the pathogenesis of obesity-induced inflammation and insulin resistance (144). Calorie restriction and exercise-mediated weight loss in obese individuals with T2D was associated with a reduction in adipose tissue expression of Nlrp3 as well as with decreased inflammation and improved insulin sensitivity (144); however, to-date no one has examined Nlrp3 in response to endurance exercise training without weight loss in skeletal muscle. There are many unanswered questions in this regard and this area is ripe for future research.

5.5.4 Improvements in Markers of Systemic and Muscle Oxidative Stress: Links with Insulin Resistance

Oxidative stress resulting from increased production of reactive oxygen species (ROS) (or their inadequate removal), has been associated with obesity (145; 146) and implicated in the pathogenesis of T2D (147), insulin resistance and associated sequelae (148-151). In addition to the many health benefits conferred by endurance exercise training (see below), an acute bout of exercise can increase antioxidant capacity and decrease the production of free radicals in lean humans and lean and obese animals (152; 153). In the basal state, trained individuals have lower levels of oxidative stress and higher antioxidant enzyme activity (154); however, the effect of endurance exercise on oxidative stress in obese individuals has not been determined. Study 2 was therefore designed to test whether systemic and skeletal muscle oxidative stress was elevated in obesity and whether moderate-intensity endurance exercise training could modulate the balance between oxidative stress and antioxidant capacity. Indeed, insulin-resistant obese

men had elevated markers of systemic oxidative DNA damage (8-hydroxy-2-deoxyguanosine [8-OHdG]) and lipid peroxidation (8-isoprostane) as well as increased muscle protein damage (protein carbonyls) and lipid peroxidation (4-hydroxy-2-nonenal). Furthermore, exercise training was a potent stimulus to reduce markers of oxidative stress (8-OHdG, 8-isoprostane, protein carbonyls) in obese men only while concomitantly increasing the antioxidants, Mn-superoxide dismutase (SOD) and Cu/ZnSOD, despite no change in body weight or composition. Our findings are consistent with studies that have shown elevated oxidative stress in obesity (145; 146; 155), and imply that improvement in fitness levels through regular endurance exercise can attenuate obesity-induced oxidative stress. We have previously reported that obese women participating in regular moderate endurance cycling for 12 weeks decreased systemic, but not skeletal muscle markers of oxidative damage (155). Study 2 expands our previous work by providing insight into the biochemical events that potentially mediate both skeletal muscle and systemic adaptive response to aerobic exercise training in men, with notable differences. This suggests the effects of exercise training on skeletal muscle and systemic oxidative stress and inflammation may be gender specific and regulated by estrogen (156), but further studies are warranted to determine whether alterations in both systemic and skeletal muscle oxidative damage and circulating markers of inflammation with endurance exercise training are *bona fide* gender-specific adaptations. Although the molecular mechanisms mediating stress-induced insulin resistance was not explored, it has been suggested that the accumulation of modified cellular components such as proteins, lipids, or DNA in obesity activate inhibitory signaling cascades (i.e., serine

phosphorylation of IRS) or the “stress-activated protein kinases” of the mitogen-activated protein (MAP) kinase family (157). Further studies are needed to elucidate the exact mechanisms mediating improved oxidant-antioxidant balance and glycemic control with endurance exercise in skeletal muscle of obese, insulin resistance and T2D patients.

5.5.5 Improvements in Metabolic Health

Obesity is a risk factor for a variety of health problems, including T2D, hypertension, dyslipidemia, coronary artery disease, stroke, osteoarthritis and cancer. In addition to affecting personal health, the increased health risks translate into an increased burden on the health care system. The cost of obesity in Canada has been estimated to be \$2 billion a year or 2.4% of total health care expenditures in 1997 (158). Clearly, establishing interventions that can slow, and hopefully reverse, the alarming increase in obesity prevalence in Canada and globally has enormous public health and health care spending implications. To date, the most effective non-pharmacological intervention to improve glycemic regulation and reduce the risk of sequelae in individuals with insulin resistance/T2D is lifestyle modification focused on increased physical activity (i.e., regular exercise training) (159). Study 1 was therefore designed to test whether improvements in properties of skeletal muscle are linked to improved metabolic health. Twelve weeks of moderate-intensity endurance exercise training involving 32 sessions which each session requiring 30-60 minutes time commitment at 50-70% $\text{VO}_{2\text{peak}}$ significantly improved systolic and diastolic blood pressure (data not shown), waist circumference, skeletal muscle resting GLUT4 content, resting muscle glycogen (data not

shown), functional exercise capacity and 2-hr plasma glucose and insulin levels in response to an oral glucose load. Although phosphorylation of Akt^{Ser473} also increased in muscle following training, whole-body insulin resistance (estimated by the homeostasis model assessment index of insulin resistance [HOMA-IR]) only trended to decline. Importantly, these changes occurred in the absence of weight loss. Our findings corroborate findings from other researchers who also report the benefits of exercise as a preventive and therapeutic intervention for chronic disorders even in the absence of weight loss (160). From this thesis, we cannot deduce if the training-induced increase in mitochondrial capacity is responsible for the improved glycemic control. However, we found no correlation between (changes in) insulin resistance and skeletal muscle mitochondrial function, although a parallel improvement of both variables was seen upon exercise training. These results might suggest that insulin resistance and muscle mitochondrial function are two effects of exercise training that are not necessarily causally related and that additional exercise-related factors could be responsible for the training-induced improvement in glycemic control. Alternatively, weight loss alone has been shown to be more pivotal than physical activity for amelioration of insulin resistance in obesity (161). The maintenance of baseline body composition upon twelve weeks of exercise training is a strength of this study as it helps to eliminate the potential impact of weight loss on insulin sensitivity and muscle mitochondria. Thus, changes arising in outcome variables would be attributed solely to the effects of exercise training and not confounded by body composition. Further research is needed to examine the effects of more chronic moderate-intensity endurance exercise in combination with

weight loss by diet on disease markers.

5.6 Concluding Remarks

Whether the central mechanism driving insulin resistance and the development of T2D and associated sequelae include abnormalities in impaired insulin signal transduction, extracellular factors such as adipokines, and intracellular molecules including IMCL, oxidative stress, or other forms of mitochondrial dysfunction remain unknown. What is clear is that each of the aforementioned mechanisms has been linked to the pathogenesis of insulin resistance and T2D and more importantly can be modulated by moderate-intensity endurance exercise. The studies in this thesis characterized the biochemical role of mitochondria in human skeletal muscle in individuals with impaired glucose homeostasis and highlighted the potency of endurance exercise in inducing skeletal muscle adaptations associated with improved metabolic health despite weight loss. Importantly, in subjects with poor glycemic control, skeletal muscle mitochondrial function and IMCL accumulation *per se* were not central mechanisms contributing to their pathology, as evidenced by preservation of their mitochondrial OXPHOS capacity. Collectively, these findings improve our understanding of the basic regulation of skeletal muscle adaptation to exercise that is critical for reducing morbidity and mortality (162; 163).

On a broader scale, these results also have substantial implications for exercise therapy in metabolic disorders and to reduce chronic disease in the general population. Increased physical activity is associated with reduced risk of numerous chronic diseases

(164; 165) and those who most engage in physical activity have the lowest rates of all-cause mortality (166). Despite the well-known widespread benefits of regular exercise, the vast majority of individuals in developed nations do not achieve sufficient physical activity to accrue health benefits (167). Thus, pressure must be placed on health promotion/public health programs to continue emphasizing physical activity as the cornerstone of obesity treatment and prevention because the many important benefits of exercise can, unfortunately, not be measured on a scale.

5.7 References

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