The effects of interleukin-6 and estrogen on lipolysis and glucose homeostasis

by

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Abstract

The Effects of Interleukin-6 and Estrogen on Lipolysis and Glucose Homeostasis

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Cytokines and hormones exert significant control over lipolysis and whole-body glucose homeostasis; two of these include interleukin-6 (IL-6) and 17-β-estradiol, or estrogen (E2). IL-6 is released from skeletal muscle during exercise alongside elevated epinephrine (Epi), however, it is unknown whether they synergistically regulate lipolysis in skeletal muscle. Other studies suggest that E2 also regulates metabolism, given that ovariectomized (OVX) rodents and menopausal women demonstrate visceral adipose accretion, derangements in lipolysis and perturbed glucose homeostasis. Exercise has been postulated as a viable alternative to hormone replacement therapy (HRT) during E2 deficiency since it improves insulin signaling and glucose tolerance, but the precise mechanisms of E2 action on lipid and glucose metabolism are unknown.

In the first study of this thesis, IL-6 independently stimulated subtle increases in glycerol accumulation, in vitro, in EDL muscle from wildtype (WT) mice, but not soleus. Conversely, soleus had elevated basal glycerol accumulation in IL-6 KO mice; and a significant response to Epi only occurred in WT mice. This highlighted a fiber-type specific role of IL-6 in stimulating lipolysis in fast-twitch skeletal muscle. In the second study, the relative efficacies of E2 and exercise were compared in treating glucose intolerance observed in OVX rats. Our most novel finding showed that 4 weeks of treadmill training was equally effective as daily E2 supplementation in improving whole-body glucose homeostasis. In order to explore the role of E2 in adipose tissue metabolism
(study 3), β3 agonist CL 316, 243 was injected in vivo or applied to adipose explants from SHAM, OVX or OVX E2 rats to examine lipolysis. Plasma FFAs were different, and lower in OVX and OVX E2 rats relative to SHAMs, but glycerol, HSL Ser660 and Ser563 phosphorylation were not impaired in vivo, and E2 had no direct effect on lipolysis ex vivo. This suggests that changes in adipose lipolysis consequent to E2 loss are secondary to changes in a circulating factor or adipocyte morphology.

Taken together, the findings in this thesis suggest that intact IL-6 signaling and the presence of E2 are required for functional lipolysis and the presence of E2 is critical for maintaining glucose homeostasis.
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Abbreviations

2-DG \( [^3\text{H}]\)-2-deoxyglucose
AC adenylate cyclase
ACTH adrenocorticotropic hormone
aFABP/ALBP/aP2 adipocyte fatty acid binding protein
Akt/PKB akt/protein kinase B
AMP adenosine monophosphate
AMPK adenosine monophosphate protein kinase
ANOVA analysis of variance
ArKO aromatase knockout
AS160 akt Substrate of 160 kDa
ATGL adipose triglyceride lipase
ATOC adipose tissue organ culture
ATP adenosine triphosphate
AUC area under the curve
\( \beta \)HAD \( \beta \)-3-hydroxyacyl CoA dehydrogenase
BSA bovine serum albumin
\( \text{Ca}^{2+} \) calcium
CaMK \( \text{Ca}^{2+}/\text{calmodulin-dependent protein kinase} \)
cAMP cyclic adenosine monophosphate
CE conjugated estrogen
CGI-58/ABHD5 comparative gene identification-58
cGMP cyclic guanosine monophosphate
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<td>CL</td>
<td>CL 316, 243</td>
</tr>
<tr>
<td>core1</td>
<td>anti-ubiquinol-cytochrome C reductase core protein 1</td>
</tr>
<tr>
<td>COXIV</td>
<td>cytochrome oxidase complex IV</td>
</tr>
<tr>
<td>CPT1</td>
<td>carnitine palmitoyl transferase 1</td>
</tr>
<tr>
<td>CS</td>
<td>citrate synthase</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>E1</td>
<td>estrone</td>
</tr>
<tr>
<td>E2</td>
<td>17-β-estradiol</td>
</tr>
<tr>
<td>E3</td>
<td>estriol</td>
</tr>
<tr>
<td>EDL</td>
<td>extensor digitorum longus</td>
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<td>epinephrine</td>
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<tr>
<td>EPT</td>
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<td>ERK1/2</td>
<td>extracellular regulated kinases 1/2</td>
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<td>ERKO</td>
<td>estrogen receptor knockout</td>
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<tr>
<td>ERβ</td>
<td>estrogen receptor β</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
</tr>
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<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>fatty acid translocase/cluster of differentiation 36</td>
</tr>
<tr>
<td>FATP</td>
<td>fatty acid transport protein</td>
</tr>
<tr>
<td>FDB</td>
<td>flexor digitorum brevis</td>
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<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>G0S2</td>
<td>G0/G1 switch gene 2</td>
</tr>
<tr>
<td>GDR</td>
<td>glucose disposal rate</td>
</tr>
<tr>
<td>GIR</td>
<td>glucose infusion rate</td>
</tr>
<tr>
<td>GLUT2</td>
<td>glucose transporter 2</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter 4</td>
</tr>
<tr>
<td>Glyc&lt;sub&gt;RA&lt;/sub&gt;</td>
<td>glycerol rate of appearance</td>
</tr>
<tr>
<td>GP130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTT</td>
<td>glucose tolerance test</td>
</tr>
<tr>
<td>HGP</td>
<td>hepatic glucose production</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>HSL</td>
<td>hormone sensitive lipase</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IL-6rα</td>
<td>interleukin-6 receptor α</td>
</tr>
<tr>
<td>IMTG</td>
<td>intramuscular triglyceride</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IRS-1</td>
<td>insulin receptor substrate 1</td>
</tr>
<tr>
<td>iWAT</td>
<td>inguinal white adipose tissue</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>MAG</td>
<td>monoacylglycerol</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>MAGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Nepi</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomized</td>
</tr>
<tr>
<td>PAT proteins</td>
<td>perilipin-adipophilin TIP47 family</td>
</tr>
<tr>
<td>PCOS</td>
<td>polycystic ovarian syndrome</td>
</tr>
<tr>
<td>PDK</td>
<td>pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor γ co-activator 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidyl-4, 5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol-3,4,5-triphosphate</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
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<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLIN</td>
<td>perilipin</td>
</tr>
<tr>
<td>PPARα</td>
<td>peroxisome proliferator receptor α</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator receptor γ</td>
</tr>
<tr>
<td>PPT</td>
<td>propyl pyrazole triol</td>
</tr>
<tr>
<td>r.p.</td>
<td>retroperitoneal</td>
</tr>
<tr>
<td>rIL-6</td>
<td>recombinant interleukin-6</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
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<td>SOCS3</td>
<td>suppressor of cytokine signaling 3</td>
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xix
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>VAT</td>
<td>visceral white adipose tissue</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
<tr>
<td>α2AR</td>
<td>α2 adrenergic receptor</td>
</tr>
<tr>
<td>β1,3AR</td>
<td>β1-3 adrenergic receptor</td>
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CHAPTER 1: Literature Review

Regulation of glucose homeostasis, lipid metabolism and substrate utilization by IL-6 and estrogen

1. Glucose homeostasis

Maintenance of plasma glucose, typically between 4-7 mM, is coordinately regulated by a concerted balance of intestinal absorption, uptake by virtually all cell types in central (brain) and peripheral tissues (skeletal muscle, adipose, liver) and hepatic glucose production (HGP; the sum of glycogenolysis and gluconeogenesis) (239). Glucose is a primary energy substrate and is robustly regulated by insulin. At the tissue level, insulin acts to increase glucose uptake in skeletal muscle and adipose tissue, inhibit HGP at the liver and blunt adipocyte lipolysis. Insulin resistance can result in profound dysregulation of these metabolic pathways as a consequence of abnormalities in the insulin signaling cascade. These disruptions include decreases in IR concentration, kinase activity, content or phosphorylation of IRS-1/2, PI3K activity or GLUT4 translocation (reviewed below).

Glucose tolerance is recognized as the ability to clear a bolus of glucose from circulation to peripheral tissues, often measured during oral or intraperitoneal glucose tolerance testing (GTT) in humans or rodents. Consequently, glucose intolerance results in pre-diabetic hyperglycemia and a loss of glucose homeostasis. Glucose intolerance and/or insulin resistance are hallmark features of several metabolic disorders, including: obesity, the Metabolic Syndrome, Type 2 Diabetes, polycystic ovarian syndrome (PCOS),
menopause (or estrogen deficiency), hyperlipidemia and hypertension. A constellation of endocrine hormones, cytokines and signaling molecules are able to individually influence whole-body glucose homeostasis and in recent years, both interleukin-6 (IL-6) and 17-β-estradiol (estrogen, E2), have gained appreciable roles as potent effectors. This thesis will address the abilities of IL-6 and E2 to modulate molecular pathways underlying carbohydrate and lipid metabolism, which in turn can influence whole body glucose tolerance and homeostasis.

1.1 Insulin signaling

Insulin is a post-prandial hormone released from the pancreas that stimulates glucose uptake into insulin-sensitive tissues, such as adipose tissue and liver, with approximately 80% of whole body glucose uptake occurring in skeletal muscle (56). Postprandial extracellular blood glucose enters pancreatic β cells via GLUT2 and is converted to ATP, which causes closure of $K_{\text{ATP}}$ channels, membrane depolarization, opening of voltage-gated $\text{Ca}^{2+}$ channels and exocytosis of insulin granules to the plasma membrane (260).

The molecular pathway of insulin signaling is well characterized and initiated in insulin-sensitive tissues (see Appendix IX). Once circulating insulin peptide binds to the α subunit of the insulin receptor (IR), this results in the auto-phosphorylation of tyrosine residues in the β IR subunits, increasing inherent catalytic kinase activity and subsequent tyrosine phosphorylation of insulin receptor substrate (IRS)-1. After its phosphorylation and activation, IRS-1 interacts and docks with the p-85 subunit of phosphatidylinositol-3 kinase (PI3K), thereby activating the catalytic p-110 subunit, which phosphorylates
phosphatidyl-4, 5-bisphosphate (PIP2) in the cell membrane to produce phosphatidylinositol-3,4,5-triphosphate (PIP3). Increased PIP3 propagates a kinase cascade involving PIP3-dependent kinases 1 and 2 (PDK1/2). PDK1/2 activates protein kinase B (PKB), or Akt, predominantly the Akt2 isoform, which phosphorylates Akt substrate 160kD (AS160). Phosphorylation of AS160 on its phospho-Akt (PAS) motif causes its dissociation from intracellular, endosomal vesicles containing glucose transport protein 4 (GLUT4) to facilitate glucose clearance (272). Regulation of this pathway is of paramount importance for blood glucose maintenance.

1.2 Insulin action in skeletal muscle, adipose and liver

Insulin signaling in skeletal muscle, adipose tissue and liver produces distinct functional outcomes in the context of glucose homeostasis. Stimulation of glucose uptake in skeletal muscle, and to a lesser extent, adipose tissue; inhibition of lipolysis in adipose tissue; and inhibition of hepatic glucose production are important downstream physiological processes dependent on operative insulin signaling.

1.3 Insulin-stimulated glucose uptake into skeletal muscle

In skeletal muscle, glucose transporter proteins GLUT1 and GLUT4 are stored in intracellular, multivesicular endosomes in the basal state (234). GLUT proteins are translocated to the cell membrane as the distal outcome of the insulin cascade, though almost all uptake can be accounted for by GLUT4 isoform recruitment to the plasma membrane (178). Two distinct signaling pathways are able to incite GLUT4 recruitment: exercise/contraction (reviewed in a subsequent section) and insulin (205). Separate
insulin or contraction-responsive GLUT4 “pools” have been identified in skeletal muscle (45, 61, 113), which, in combination, can have additive effects on glucose transport in skeletal muscle (312, 334). Defects in insulin signaling at the level of PI3K and Akt, and even as proximal as IRS-1 phosphorylation, have been documented in insulin resistant subjects (158). Taken together with diminished or fully ablated GLUT4 movement (331), marked reductions in insulin-stimulated glucose uptake by skeletal muscle precede the diagnosis of T2D (306). This highlights the importance of intact insulin signaling and downstream glucose uptake by skeletal muscle in the context of whole-body glucose regulation.

1.4 Suppression of adipose tissue lipolysis by insulin

The excessive release of free fatty acids (FFAs) into circulation has been established as a causal link (22, 145) and correlates strongly with an insulin resistant state in humans and rodents (273). Controlled FFA efflux, or lipolysis, from adipose tissue and proper lipid sequestration into adipose TAG stores are critical processes for attenuating increases in blood FFAs, preventing ectopic fat accumulation, maintaining glucose homeostasis and preserving normal insulin signaling (191). Insulin-mediated suppression of glycerol release from adipose tissue is promoted via PKB/Akt phosphorylation and activation of phosphodiesterase 3B, which degrades cyclic AMP (cAMP), thereby suppressing PKA activation and downstream lipolytic action by hormone sensitive lipase (HSL; regulatory mechanisms presented in detail in a subsequent section) and perilipin in adipocytes (63). Insulin also independently stimulates protein phosphatase-1, which dephosphorylates HSL and decreases lipolytic rate (237), and has been shown to possibly
suppress glycerol efflux in 3T3-L1 adipocytes (150). In addition to its effects on mobilization, insulin also serves to promote re-esterification of FFAs in adipose tissue, thereby decreasing glycerol/FFA efflux and enhancing storage in adipose depots (36). Evidence of impaired antilipolytic action in adipocytes is evident in non-insulin resistant, obese subjects and Zucker rats (138, 294), whereas other studies show no impairment of insulin-mediated lipolytic suppression in basal or catecholamine-treated environments (8, 9, 26). Nonetheless, the ability of insulin to regulate lipolysis and FFA storage in adipose tissue is well documented and critically important for glucose homeostasis.

1.5 Inhibition of hepatic glucose production by insulin

Plasma glucose concentration is a function of insulin-mediated i) glucose disposal by peripheral tissues, ie. skeletal muscle, adipose and liver, and ii) hepatic glucose production (HGP; (55)), which is the summation of glycogen breakdown (glycogenolysis) and gluconeogenesis (161). Hepatic glucose production is therefore a critical pathway in the regulation of glucose homeostasis. Insulin acts directly at the liver to decrease HGP in a dose dependent fashion, whereby ~40-1190 uU/mL blunted glucose release by 68-95% in humans (54). Insulin also indirectly suppresses HGP by limiting gluconeogenic precursor release, ie. glycerol, FFAs, from peripheral tissues (240, 279). Hepatic glucose production can be elevated due to increased diet or adipose-derived FFAs when lipolysis is dysregulated (161, 162, 284) and FFAs can independently induce hepatic insulin resistance by impairing signaling, thereby diminishing the ability of insulin to suppress HGP (20, 172).
2. Hormone and cytokine regulation of glucose metabolism: the roles of estrogen and interleukin-6

Several endocrine hormones and tissue-derived cytokines are able to independently modulate the insulin signaling cascade and downstream effects in insulin-sensitive tissues. Recent evidence highlights interleukin-6 (IL-6) and estrogen as autocrine and paracrine effectors of skeletal muscle, adipose and liver insulin action, which drive glucose homeostasis.

2.1 Estrogen production and molecular signaling

Estrogen is classified as a steroid hormone, interacts with androgen and/or estrogen receptors (ER) to elicit physiological effects and exists in three major forms. In females, estrone (E1) is predominant during menopause; 17-β-estradiol (E2) is the most bioactive form in premenopausal women; and estriol (E3), primarily expressed during pregnancy (278). In females with normal menstrual cycling, E2 is produced in thecal cells of ovaries by aromatase driven conversion of androstenedione to E1 to E2. During ablated ovarian estrogen production, E2 is no longer generated in the ovaries, but is produced in extragonadal sites such as mesenchymal cells of adipose tissue, vascular endothelium, in smooth muscle cells of the aorta and at several locales in the brain (277). Typical concentrations of estrogens and androgens in females during ovulation, post-menopause and following ovariectomy are listed below (278):
Table 1: Sex Hormones, their sources and concentration ranges in human females, OVX females and males

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Ovulatory Females</th>
<th>Post-menopausal Females</th>
<th>Post-bilateral oophorectomy (OVX) Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone (E1)</td>
<td>17-200 pg/mL</td>
<td>7-40 pg/mL</td>
<td></td>
<td>10-60 pg/mL</td>
</tr>
<tr>
<td>Estradiol (E2)</td>
<td>50-350 pg/mL</td>
<td>15 pg/mL</td>
<td>&lt; 10 pg/mL</td>
<td>10-40 pg/mL</td>
</tr>
<tr>
<td>Estriol (E3)</td>
<td>Main form during pregnancy</td>
<td></td>
<td></td>
<td>no role</td>
</tr>
<tr>
<td>Estrone sulfate (E1S)</td>
<td>&gt;1000 pg/mL</td>
<td>350 pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androgens</td>
<td>20 ng/dL</td>
<td>0.8 ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (T)</td>
<td>20 ng/dL</td>
<td>10 ng/dL</td>
<td>~200-700 ng/mL</td>
<td></td>
</tr>
</tbody>
</table>

The classical (genomic) mode of estrogen signaling involves E2 binding to ERα or ERβ receptors in the nucleus, which homo or heterodimerize as an E2-ER-ER complex, and subsequently bind to estrogen response elements (EREs) to act as ligand-dependent transcription factors (TFs) (21). Hormone binding also induces conformational changes that promote recruitment of TF co-activator proteins. Although many of the effects of E2 are mediated by the action of ERs on gene expression, some downstream signal transduction happens on an acute timescale (non-genomic), likely initiated by membrane-bound ER isoforms (21). Non-genomic actions of E2 are characterized by actions of protein kinases, namely tyrosine kinase and mitogen activated protein kinase (MAPK) in MCF-7 cells (194) and PI3K in human endothelial cells (108); thus it is not
surprising that E2 could modulate insulin signaling proteins and glucose metabolism in skeletal muscle, adipose and liver, but these mechanisms are not well elucidated.

2.2 Changes in E2 in humans and implications for insulin sensitivity and glucose homeostasis

There are several physiological and pathological relationships that illustrate the relationship between glucose homeostasis and fluctuations in circulating estrogen, such as the menstrual cycle (66, 69), pregnancy (268), menopause (35, 40) and polycystic ovarian syndrome (PCOS) (58), where hormonal concentrations are variable but correlative with indices of insulin sensitivity or glucose tolerance. The risk for Type 2 Diabetes is influenced by estrogen status, given that premenopausal females have increased insulin sensitivity compared with age-matched males (60, 209), and elevations in fasting blood glucose and insulin occur in postmenopausal women despite matching to age and body mass index versus premenopausal females (173). Postmenopausal women have lower content of GLUT4 protein in skeletal muscle compared with premenopausal women, which is implicated in reductions in insulin sensitivity, but is also a normal consequence of aging (120). Taken together, this suggests that E2 plays an important role in glucose homeostasis, which can become dysfunctional in its absence.

2.3 In vitro estrogen signaling in molecular mechanisms underlying glucose homeostasis

Several pathways have been identified in skeletal muscle, adipose and liver in which E2 modulates signaling cascades that influence glucose homeostasis. In 2006, Nagira et al found that treatment of 3T3-L1 adipocytes with E2 does not enhance insulin-
induced IR phosphorylation, but increases insulin-dependent IRS-1 and Akt phosphorylation, IRS-1/p85 interaction, as well as insulin-stimulated glucose uptake, but only at the lower dose (10^{-8} M vs 10^{-5} M; (203)). Similarly, in rat L6 myotubes E2 induces translocation of GLUT4 to the membrane in a dose-dependent fashion via ERα specific signaling (81). Administration of the ERα selective agonist propyl pyrazole triol (PPT) increases insulin-stimulated glucose uptake in adipocytes and phosphorylates Akt^{Ser473}, AS160 and adenosine monophosphate-activated protein kinase (AMPK) in C2C12 myocytes, implicating a role for ERα-specific glucose uptake (51, 52, 92). Interestingly, Rogers et al found that ex vivo E2 treatment (10nM) results in rapid phosphorylation of Akt, AMPK and TBC1D1/4 insulin signaling proteins in absence of any change in 2-DG transport in rat soleus (258). Furthermore, in vivo treatment of rats with PPT for 3 days augments insulin-stimulated Akt and AS160 phosphorylation and glucose uptake into soleus and extensor digitorum longus (EDL) in rats (92). This highlights the ability of E2 to affect insulin signaling, though dose dependency seems apparent and the exact mechanisms and functional outcomes remain uncertain.

2.4 In vivo effects of estrogen on glucose homeostasis in humans

Acute administration of conjugated estrogen (CE) can modulate insulin action and glucose homeostasis in humans. In postmenopausal women, CE infusion reduces serum insulin levels during a two-stage hyperinsulinemic euglycemic clamp, suggesting higher hepatic insulin clearance in the presence of E2 (227). Reduced plasma insulin concentrations were not mirrored by a proportional decrease in GIR, further suggesting improved insulin action in the presence of E2. This is the sole study examining very acute
effects of E2 on insulin action in humans; however, significant limitations prevented conclusions due to the possibility that other constituents of CE (estrone sulfate, equilin sulfate, 17-βdihydroequilin) may have had an effect on insulin secretion or clearance. Mechanisms regarding HGP and/or glucose disposal were also not examined. To isolate the specific effects of E2, the same authors utilized a 24 hour, transdermal, physiological dose of 17-βE2 alone, and found no effect on insulin area under the curve (AUC) or calculated glucose disposal rate (GDR) during a hyperglycemic clamp (228). However, fasting glucose was significantly reduced after 24 hour E2 treatment and GDR was inversely correlated with number of years post-menopause (228). In accordance with this study, Pereira et al found a beneficial effect of 1 week transdermal E2 on GDR in a hyperinsulinemic-euglycemic clamp in early menopausal women (<6 years postmenopause), but not late (>10 years) (229). Despite unresolved results from studies looking at acute effects of E2 on glucose metabolism, the collective data does not rule out the possibility that short term E2 treatment can affect indices of insulin sensitivity and/or glucose homeostasis in humans. However, the results tend to be variable due to subject age, time spent in menopause, duration and method of hormone delivery, degree of adiposity and circumstance for sustained estrogen loss, which vary drastically across studies.

On a longer-term, chronic scale, there is strong evidence that hormone replacement therapy (HRT) can improve whole-body insulin action, most notably presented in the large-scale Heart and Estrogen/Progestin Replacement Study (HERS) and Women’s Health Initiative Hormone Trial (WHI). In these studies, the incidence of insulin resistance was 35% lower in women assigned to HRT versus the control group
(140), and fasting insulin and glucose were also reduced in women after one year on HRT (186). Additional evidence of HRT improving insulin action has been cited (50, 67, 68, 133, 213); however, some studies have found no improvement in insulin sensitivity after treatment with unopposed E2 or HRT with progesterone included (62, 151) and one study found increased insulin resistance in women taking oral E2 or HRT (269). A major limitation of this area is that chronic HRT has been shown to limit accretion of visceral adipose in postmenopausal women, which is inversely related with indices of glucose intolerance, insulin resistance and T2D. Thus, it is difficult to conclude whether E2 exerts positive effects independent of changes in adiposity, or whether hormonal effects are secondary to changes in body composition induced by HRT. Nonetheless, the evidence is largely positive in terms of the effects of HRT in women and discrepant effects can be accredited to variations in route of delivery (although underlying mechanisms have not been explored), composition of HRT administered, antagonistic effects of progesterone and variability in inherent subject characteristics (ie. age, adiposity, activity level), similar to the limitations in studies examining acute E2 dosing.

Despite evidence for E2 having positive effects on glucose regulation, the large-scale, randomized control WHI was halted prematurely due to elevated risk of breast cancer, thrombosis and coronary heart disease amongst the HRT-treated group and spurred conclusions that in terms of clinical recommendations, HRT should not be prescribed due to its severe health risks (204). However, a significant critique of this study is that subjects were much older than when HRT would normally be initiated. In older age brackets, the physiological/metabolic impairments consequent to E2 loss may have advanced past the “critical window/timing effect” proposed, which suggests that the
restorative ability of hormone re-introduction must occur in the early stages of menopause (264, 305). To address this, the Kronos Early Prevention Study (KEEPS) is currently assessing the use of HRT within 36 months of menopause onset, in women aged 42-58 years, with early indications of HRT acting effectively in protecting subjects from metabolic syndrome (106). In any case, there is a sparse amount of data from non-observational, intervention-based clinical trials regarding the health effects of HRT, which has led to precipitous declines in prescription by physicians. It is of critical importance to propose and research alternative treatment options in order to preserve women’s health and maintain glucose regulation after menopause. One promising substitute may be the use of exercise training, which has proven effective in maintaining insulin sensitivity/glucose tolerance in human females and OVX rats (68, 270).

2.5 *In vitro and rodent models to study role of E2 in mediating glucose homeostasis*

Part of the difficulty in assessing metabolic functions of E2 lies in aging as a related and confounding factor in human subjects. Many physiological processes change alongside decreases in sex hormones due to menopause, and the increased propensity to accumulate visceral adipose mass also complicates the ability to discern the effects of estrogen from changes in fat storage on glucose homeostasis and insulin sensitivity. Since visceral adipose tissue is often cited as the primary risk factor for development of Type 2 Diabetes, change in body composition is a critical factor to consider when investigating the effects of E2 on metabolic function. Rodent models have been useful in providing mechanistic and cellular approaches to understanding how estrogen status impacts glucose regulation.
The ovariectomized (OVX) model is one of the most commonly utilized for studying ablation of female sex steroids on metabolic and physiological function, whereby both ovaries are cauterized and removed to prevent gonadal release of E2, progesterone and follicle-stimulating hormone. Some criticism lies in the abrupt nature of the surgical procedure and rapid hormone loss, which does not mirror what occurs in humans with natural menopause. However, it is important to remember that many premenopausal women undergo OVX/oophorectomy for other clinical reasons. This model also permits the study of ablated E2 function in the absence of aging and provides the ability to prospectively study E2 loss, versus observational human studies, in which timecourse cannot always be analyzed. Two days post-OVX, 70-90% reductions in circulating E2 are commonly observed (94, 280), as some E2 is still produced peripherally in adipose tissue. One subtle distinction between mouse and rat models of OVX are that rats seemingly undergo more pronounced hyperphagia versus mice, and interestingly, pairfeeding does not prove effective in preventing changes in body mass relative to a SHAM surgery operated control (Appendix A).

The OVX mouse and rat models closely mimic postmenopausal changes in humans, given the well-documented body weight gain (116, 256) and visceral adipose accretion (11) that occurs subsequent to bilateral ovary removal. Disrupted E2 signaling in vivo is phenotypically similar in OVX, estrogen receptor alpha (ERα) and aromatase knockout (ArKO) rodents, which are characterized by marked visceral adipose accumulation, elevated fasting insulin, decreased lipid oxidation, fasting hyperinsulinemia, increased lipid species accumulation in muscle (most notably triacylglycerol (TAG) and diacylglycerol (DAG) moieties), adipose tissue inflammation,
elevated HGP and hypersensitivity to developing non-alcoholic fatty liver disease (33, 125, 185, 195, 249). All of these factors converge to regulate whole body glucose homeostasis and insulin sensitivity in vivo and are robustly modulated by estrogen. Regardless of the animal model of impaired E2 function, it is clear that E2 loss produces striking disruptions in metabolic and physiological function. However, the underlying mechanisms remain largely unclear.

Evidence of impaired skeletal muscle glucose uptake in OVX rats is equivocal. Some studies show lower skeletal muscle GLUT4 content (271) and reduced [3H]-2-deoxyglucose (2-DG) uptake into soleus and/or EDL (271), while others find no change in GLUT4 content or glucose transport in OVX rats (37, 90, 180). In spite of these equivocal findings of altered glucose uptake, marked glucose intolerance is evident in OVX rats and mice by 10-15 weeks post-surgery, as determined by an oral GTT (13, 180, 271). Furthermore, several studies document improvements in fasting glucose, fasting insulin and glucose tolerance after acute or chronic E2 supplementation (52).

Interestingly, ERα knockout (ERαKO) mice show significant decreases in GLUT4 content in skeletal muscle, which is not recapitulated in ERβ knockout models. This suggests that the insulin-sensitizing effects of E2 are likely mediated through the ERα receptor isoform, since the effects observed in ERαKO mice are not found in ERβKOs.

In addition to E2, other cytokines and hormones are able to exert distinct effects on insulin signaling proteins that help dictate glucose homeostasis. Of these, interleukin-6 has been shown to affect pathways in human and rodent models in the context of glucose metabolism.
2.6 Interleukin-6 (IL-6) production, molecular signaling and mechanisms underlying glucose homeostasis

Interleukin-6 (IL-6) is a cytokine released from immune cells during an inflammatory response, and has recently been shown to be secreted from adipose tissue (199). IL-6 signal transduction is initiated through binding to either membrane bound IL-6 receptor α (IL-6rα), GP130 receptor complex (96) or the soluble IL-6 receptor (254). Downstream signaling targets include AMPK, STAT3 and SOCS3, the latter of which inhibits IL-6 mediated cellular signaling (49, 146). Obesity and disease states are associated with elevations in circulating IL-6 (149, 266); however, whether or not IL-6 is pro or anti-inflammatory in nature is controversial. Paradoxically, a role of IL-6 in substrate utilization during exercise is becoming increasingly apparent, as IL-6 appears to be released from skeletal muscle during contractile activity and is elevated post-exercise, a period during which insulin action is also improved (289, 323).

In vitro findings are equivocal regarding the effects of IL-6 on glucose transport. One study found an augmented effect on insulin-stimulated 2-DG uptake in adipocytes treated with IL-6 for 5 hours (295), which parallels findings of increased basal and insulin stimulated glucose uptake, GLUT4 translocation and AMPK activation in IL-6 treated L6 myotubes (39). Acute, high dose IL-6 exposure seems to almost unanimously stimulate GLUT4 translocation and glucose transport in skeletal muscle (3, 84, 146, 207); however, some evidence exists demonstrating reduced glucose transport and IRS-1, GLUT4 and PPARγ mRNA in 3T3 adipocytes exposed to IL-6 for 30 minutes to 24 hours (266). In humans, acute recombinant human IL-6 (rhIL-6) infusion at a physiological dose has been shown to increase whole body glucose disposal during a
hyperinsulinemic-euglycemic clamp (39). Conversely, adipose IL-6 content correlates inversely with glucose transport in adipocytes isolated from obese subjects (15). Whole-body IL-6 knockout mice (IL6−/−) become obese in the absence of IL-6 signaling, and intracerebroventricular IL-6 injections increase energy expenditure and partially reverse weight gain (313) However, exercise-induced muscle glucose clearance and phosphorylation of AMPK are not impaired, suggesting that IL-6 may not be essential for glucose uptake during exercise (212).

In addition to having a role in glucose metabolism and homeostasis, IL-6 can also exert its effects via fatty acid oxidation and lipolysis by increasing AMPK, cAMP, and glycerol release from EDL, C2C12 myocytes, and 3T3L1 adipocytes (39, 146, 231). As previously mentioned, increases in circulating FFAs negatively regulate insulin signaling and action; therefore, proper lipolytic signaling is imperative for maintaining whole body glucose homeostasis. This pathway receives separate input from both E2 and IL-6 and their effects on lipolytic enzymes and FFA/glycerol release are described below.

3. Triacylglycerol lipolysis: enzymes, lipid droplet proteins, regional tissue-specific differences and regulation by estrogen and IL-6

White adipose tissue (WAT) represents a considerable energy sink that is readily available during times of energetic stress (167), as FFAs are essential substrates for energy provision. Mobilization of FFAs from adipose and skeletal muscle TAG stores is a finely regulated process and has a large (patho)physiological role in cellular signaling health and disease (261).
3.1 Adipose tissue lipolysis

Circulating FFAs are transported into WAT and skeletal muscle via FAT/CD36, fatty acid binding protein (FABP) and fatty acid transport protein (FATP) transporters on the plasma membrane (27). For peripheral storage, FFAs are esterified into triacylglycerol (TAG) pools and stored inertly in the lipid droplet of adipocytes or as intramuscular TAG (IMTG) in skeletal muscle (319). During times of energy demand, TAGs can be hydrolyzed through a tightly regulated enzymatic process (lipolysis) during which fatty acids are cleaved from the glycerol backbone (243, see Appendix IX). The fate of liberated FFAs can be oxidation in skeletal muscle for high energy phosphate (adenosine triphosphate; ATP) provision, or re-esterification into the adipose TAG pool, the latter of which accounts for 60% of FFAs (19, 64). Liberation of FFAs via lipolysis is complex, but well-characterized, and involves three specific lipases working in sequential order: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MAGL), which coordinately work to cleave FFAs from glycerol. An increasing number of lipid droplet proteins (e.g. perilipins and associated proteins) are being discovered as modulators of lipolysis in adipose tissue (63, 128).

Until 2004, HSL was considered the primary lipase for TAG hydrolysis. However, HSL-null mice display a phenotype of normal body weight but decreased fat mass, and preservation of ~40% of the wildtype lipolytic rate, as well as accumulation of DAG in adipose tissue, but not TAG (99, 316). This suggested that a) HSL is more important for cleaving FFAs from DAG to MAG species, and b) a previously unidentified lipase existed with a preference for hydrolyzing TAGs to DAGs. In 2004, three independent groups discovered ATGL/desnutrin, which is the enzyme responsible for catalyzing
hydrolysis of TAG to DAG (129, 310, 333). ATGL activity is enhanced by physical interaction with co-activator protein comparative gene identification-58 (CGI-58) (166), but reduced by G_{o}/G_{i} switch gene 2 (G0S2)(327). Post-translational phosphorylation of ATGL on the murine Ser^{406} residue, possibly by AMPK, has been shown to increase lipolysis (1). Overexpression of ATGL in rodents results in increased TAG breakdown, whereas ATGL-null mice have increased adipose mass and ectopic fat accretion surrounding the heart.

HSL has wide substrate specificity and can act on TAG, DAG, MAG and cholesterol and retinyl esters, but has been shown in vitro to have 10-fold higher activity in the presence of DAGs versus TAGs (155); thus, HSL is rate limiting only after ATGL has hydrolyzed the first ester bond linking glycerol and FAs. HSL is phosphorylated by PKA on Ser^{563} and Ser^{600} residues, which are considered the major PKA-targeted HSL sites in adipocytes (7). This modification causes its translocation from the cytosol to the lipid droplet to increase TAG hydrolysis. Additional kinases, such as ERK1/2, PKC/MAPK, cGMP-dependent kinase have also been shown to phosphorylate HSL. Interestingly, AMPK phosphorylates the Ser^{565} residue in adipose tissue (318) and prevents subsequent action of PKA on HSL, strongly suggesting AMPK acts as an antilipolytic “brake” on the system. This is discordant with AMPK-mediated phosphorylation of ATGL, and while it is clear that AMPK-mediated HSL Ser^{565} phosphorylation decreases lipolysis, opposing actions in the same pathway require further elucidation. The combined action of ATGL and HSL are responsible for 95% of TAG hydrolysis in adipose tissue (276). Final hydrolysis of MAG is completed by MAGL,
which is not regulated by hormonal control and simply dependent on substrate availability.

Several lipid droplet-associated proteins have recently been discovered in adipose tissue and are involved in lipolysis. These include: perilipin A, perilipin-adipophilin TIP47 family (PAT proteins), adipocyte fatty acid binding proteins (aFABP/ALBP/aP2), caveolin 1 and comparative gene identification 58 alpha/beta hydrolase domain containing protein 5 (CGI-58/ABHD5). Mechanisms of regulation are largely unknown in these proteins. Perilipin A (PLIN1) appears to maintain low basal lipolysis and act as a lipolytic barrier until energetic demand, possibly by blocking neighbouring HSL and ATGL from hydrolyzing TAG pools (196, 329). The presence of PLIN1 is necessary for PKA-induced HSL translocation to the lipid droplet, however, it is still uncertain whether PLIN1 phosphorylation (particularly on Ser517) is obligatory for HSL movement (197). Additional PLIN proteins (1-5) work alongside lipolytic enzymes to regulate basal and stimulated lipolysis.

3.2 Catecholamine regulation of lipolysis

Catecholamines, epinephrine (Epi) and norepinephrine (NEpi) both exert potent control over lipolysis. During fasting and exercise, catecholamines reach the adipose tissue via circulation (Epi) or by direct sympathetic innervation (NEpi) and subsequent lipolytic signaling is initiated by four types of G-protein coupled receptors (GPCR): β₁AR, β₂AR, β₃AR and α₂AR (206). Binding of catecholamines can be either stimulatory or inhibitory on lipolysis depending on which receptor type they bind. β₁AR, β₂AR, β₃AR receptors associate with the stimulatory Gₛ subunit of the GPCR, whereas α₂AR contain
an inhibitory $G_i$ subunit (160). Activation of either GPCR causes interaction with the enzyme adenylate cyclase (AC) activity, which is inhibited by $G_i$ or stimulated by $G_s$. When any of the $\beta$-AR family is bound by catecholamines, AC increases intracellular cyclic adenosine monophosphate (cAMP) by converting ATP to cAMP, which activates cAMP-dependent protein kinase A (PKA) and exerts lipolytic control by phosphorylating numerous downstream proteins and enzymes (165). Epi has been shown to bind more potently to $\beta_2$AR, whereas NEpi has a greater affinity for $\beta_1$AR, but both cause release of FFA and glycerol from adipose tissue (114).

3.3 In vivo effects of estrogen on lipolysis

The loss of sex hormones in women triggers an accelerated increase in central adiposity (301) that is exacerbated in women not taking HRT (82, 131). Increased fat deposition could be a consequence of decreased fat oxidation, greater TAG storage or impaired lipolysis, although this data is limited. Intravenous delivery of 0.25mg conjugated estrogen (CE) prior to a hyperinsulinemic-euglycemic clamp in postmenopausal women reduced basal glycerol rate of appearance ($\text{Glyc}_{\text{RA}}$) in abdominal and femoral SAT as measured by microdialysis, but had no whole-body effect on potentiating the inhibition of $\text{Glyc}_{\text{RA}}$ by insulin (226). In a previous study, Van Pelt et al noted reductions in serum glycerol after acute CE treatment (adjusted for insulin concentrations) and postulated E2-mediated clearance from circulation as a mechanism, although this has never been measured (227). It is unclear why whole-body lipolysis would decrease with E2 in one case, but remain unchanged in similar subjects in the follow up study. Given the pleiotropic effects of E2 on different tissues and variability in
ERα/β receptor expression, it is possible that other fat depots (ie. VAT or muscle) were unaffected by E2, or that day-to-day variability prevented definitive findings. Gormsen et al found similar results after treating postmenopausal women with one 4mg dose of E2. Local epinephrine-stimulated glycerol release was reduced in the presence of E2 at femoral and abdominal SAT locales, as measured by microdialysis interstitial glycerol concentrations (89). Importantly, these studies attempted to measure the in vivo effects of E2 on fat storage and mobilization but did not measure any representative visceral depots, where adipose has been shown to accumulate in women without E2. Therefore, the acute effects of E2 on lipolysis in human subjects have been inconclusive thus far and require further investigation.

Additional cross-sectional studies have shown, interestingly, that NEpi-stimulated β-adrenergic lipolytic sensitivity and lipolytic rate are highest in nonportal (retroperitoneal, subcutaneous abdominal) versus portal fat depots (omentum, mesenteric) in women, which is opposite to men, and that depot specific differences disappear post-menopause (241). Femoral adipocytes also displayed a blunted lipolytic response compared with abdominal adipocytes (242); however, in another study there was no difference in β-adrenergic stimulation in subcutaneous abdominal or gluteal adipocytes between peri- and postmenopausal women (72). Taken together, the protection against central adiposity in premenopausal, estrogen replete women could be due to the increased lipolytic sensitivity in the abdominal area. The likelihood of adipose accretion due to positive energy imbalance is high, however, this hypothesis is unresolved, as there are no longitudinal studies that examine energy intake versus expenditure during the transition to menopause (235).
Transcriptional regulation by chronic E2 supplementation may also underlie mechanisms regulating lipolysis in females. Treatment of postmenopausal women with 50 μg/day ethinyl estradiol for 3 weeks lowered lipolytic response to NEpi in abdominal SAT depot, but not femoral SAT, which may be due to upregulation of α2-adrenergic receptor content in SAT by E2 (225). In contrast, E2 had no effect on anti-lipolytic α2 receptors in intra-abdominal fat, suggesting that plasticity in the SAT depot may confer the typical gynoid fat distribution pattern that is lost postmenopause in women (225). This is the only study to date, in humans, that examined the chronic effects of E2 supplementation and unfortunately, was not designed to assess mechanisms.

In rodent models, loss of E2 function in OVX rats/mice and ERαKO mice produces dysregulation of lipolysis that results in elevated circulating FFAs, which can be reversed with E2 supplementation (52, 53). In conjunction with perturbed lipolysis, there is evidence of reduced oxidative enzymes carnitine palmitoyl transferase (CPT1) and β-3-hydroxyacyl CoA dehydrogenase (βHAD) activities in skeletal muscle of OVX rats, that are subsequently restored with 14 days of E2 pellets (38). Although FFA oxidation was not measured, a reduction in this pathway could also contribute to elevated plasma FFAs. Loss of E2 has been shown to cause elevated basal lipolysis in isolated adipocytes and increase FFA release from adipose tissue, which may be explained by elevated ATGL content and increased interaction with CGI-58, elevated PLIN2 content and decreased PLIN1, which taken together, result in increased basal lipolysis (51, 320). However, a critical point to note is that E2 treatment can reverse the marked hyperphagia that occurs with E2 deletion in rodents, which results in reduced energy intake, and an overall lower degree of adiposity (193, 250). Restoration of normal lipolytic function
may be due to E2-induced reduction in adipocyte size and lower basal lipolysis (200, 317). Thus, in absence of a pairfeeding regime, it is impossible to discern whether effects on lipolysis in OVX versus OVX + E2 rats are due to E2 alone, or secondary to changes in adipose tissue morphology due to differences in energy intake (52). This is important to consider when assessing chronic E2 treatment and effects on lipolysis; in vitro experiments may provide more insight into the direct mechanisms of E2 influence in lipolytic function.

3.4 In vitro effects of estrogen on lipolysis

There are no studies to date that examine the direct effects of E2 on lipolytic rate, enzymes or re-esterification in ex vivo tissue samples from skeletal muscle or adipose tissue. Thus far, E2 has been reintroduced in vivo without complementary in vitro or culture experiments, which limits the ability to comment on any direct action of E2 in lipolysis independent of circulating factors. There is also an absence of data examining the short-term effects of E2 on lipolysis in adipose tissue or skeletal muscle. Goodyear and Greenberg investigated the isolated, acute effects of E2 incubation on soleus and EDL; however, the outcome measures were restricted to insulin signaling protein activation and glucose uptake (258). Since estradiol treatment has been shown to activate AMPK in skeletal muscle (258), this would presumably activate HSL Ser\(^{565}\) in muscle and/or adipose tissue and subsequently blunt lipolysis, but this has yet to be confirmed.

Work from the 1980s examined Epi-treated lipolysis in extracted parametrial fat pads (VAT) from OVX rats either E2 replete or supplemented with low (1.2\(\mu\)g/animal/10 days) or high (120\(\mu\)g/animal/10 days) E2 and noted a potentiation of HSL activity to Epi
in the presence of E2 in conjunction with a decrease in fat cell size (17). Similarly, treatment of OVX rats with 5μg/day/4 days or 20μg/7 days of E2 resulted in 50-70% increase in parametrial adipocyte response to the β-adrenergic agonists isoproterenol, Epi, forskolin and 3-isobutyl-1-methylxanthine (IBMX) (219, 224). The apparent ability of E2 to augment β-adrenergic responsiveness occurred in absence of changes in basal lipolysis after E2 re-introduction alone (17, 219, 224), which conflicts with human studies showing elevations in circulating FFAs in E2-diminished women. More work is required to examine the direct effects of E2 devoid of central action on energy intake or confounding systemic circulating factors, on lipolytic signaling and glycerol/FFA release. In addition to E2, other circulating factors are able to exert control over the lipolytic signaling cascade, including IL-6.

3.5 In vivo and isolated effects of IL-6 on lipolysis

In humans, low and high dose infusion of recombinant IL-6 (rhIL-6) for 3 hours causes a 50% increase in circulating FFAs in the absence of any changes in plasma insulin, epinephrine, glucagon or peripheral insulin resistance (102). These findings are paralleled by experiments in rats, where acute IL-6 infusion causes elevations in plasma FFAs (208). In accordance with stimulatory effects during infusion, systemic IL-6 concentrations increase robustly during exercise (70, 223), in a mode, intensity and duration type manner, and can mediate lipid metabolism, most notably fat oxidation and lipolysis (102). This is likely for fuel provision during times of energy stress, as FFAs can act as an energy substrate. This data highlights the ability of IL-6 to directly stimulate changes in lipolysis. The mechanisms underlying exercise-induced IL-6 production are
unclear, but may be attributable to nitric oxide (290), reactive oxygen species (154), mitogen activated protein kinases (MAPK) c-jun NH$_2$-terminal kinase (JNK) and p38 (78, 179), intracellular Ca$^{2+}$ (142) and/or glycogen content (288). Until recently, this increase in plasma IL-6 was thought to be attributed to an exercise-induced, whole-body inflammatory response. However, there is evidence for up to 100-fold increases in IL-6 mRNA localized within human skeletal muscle post-exercise, suggesting that IL-6 could be secreted from active muscle as opposed to immune cells (143, 291). This provides reasoning for the potential of IL-6 to influence lipolysis within skeletal muscle in an autocrine loop, or by paracrine mechanisms in other tissues (ie. adipose) after it is secreted into systemic circulation.

In vitro experiments have yielded evidence for IL-6 increasing basal and isoproterenol-stimulated glycerol release from human breast adipocytes (220). Similarly, Kelly et al. have shown that treatment with 120 ng/mL IL-6 induces lipolysis in rat EDL muscle (146). IL-6 can activate AMPK in both adipose tissue and skeletal muscle by increasing intracellular cAMP concentrations and the AMP: ATP ratio (39, 85, 146, 147, 267), although the precise mechanism is unknown. Work by Bruce et al also showed that IL-6 treatment in isolated rat soleus increased the oxidation of both exogenously and endogenously (intramuscular triglycerides, IMTG) derived FFAs by 50% in the absence of any alterations in FFA transport (32). Interestingly, IL-6 also reduced insulin’s attenuation of FFA oxidation and limited re-esterification into IMTG (32). This isolated model strongly suggests that IL-6 has independent effects on fat metabolism, independent of circulating hormonal or serum factors (102), and is in accordance with whole-body data from Van Hall demonstrating increased fat oxidation with IL-6 infusion. Data
highlighting the role of IL-6 in lipolysis and lipid utilization remains sparse, but generally indicates the individual ability of IL-6 to induce systemic FFA appearance, and lipolysis in isolated adipose and muscle tissues. It is also important to note that while IL-6 influences FFA mobilization, its effect is marginal compared to catecholamines.

3.6 Region-specific differences in adipose lipolysis

Adipose tissue mass can range anywhere from 5-60% of total body weight in humans, with upwards of 80% residing in subcutaneous (SC; gluteal, femoral, inguinal) stores, and 10-20% as intra-abdominal (VAT; intra- and retroperitoneal, omental, mesenteric) in men versus 5-10% in women (170). Additionally, skeletal muscle contains 10–50 μmol/g tissue of TAG in the form of lipid droplets that provide a fuel source during times of energy demand (101). Evidence continues to suggest that regional localizations of adipose tissue have heterogenous characteristics in terms of lipolytic rate, sensitivity to adrenergic stimulation and dichotomous traits between males and females, likely due to specific steroid hormone exposure, although these mechanisms are unclear (170, 300). Regional differences in adipose insulin sensitivity and suppression of lipolysis represent a critical mechanism of lipolytic regulation. In adipocytes isolated from humans, insulin-induced inhibition of glycerol release and insulin binding are significantly higher in subcutaneous versus visceral (omenta) adipose depots, despite no differences in IR content (24). The reduced antilipolytic effect of insulin in visceral adipose has been attributed to lower IR and IRS-1 phosphorylation and reduced PI3K activity (332).
In humans, basal, isoproterenol, dibutyryl cAMP and forskolin-stimulated glycerol release in adipocytes has been shown to have highest absolute concentrations, but less responsiveness in SC adipocytes or tissue explants (74, 189, 246, 251, 311). However, the sensitivity and responsiveness (fold increase) to isoproterenol or alternative \( \beta \)-adrenergic agonists is highest in VAT tissue, likely due to increased content of \( \beta_1 \)AR and \( \beta_2 \)AR and lower content of inhibitory \( \alpha_2 \)AR (74, 112, 115, 188). In females, \( \alpha_2 \) adrenergic, antilipolytic receptor content and sensitivity are markedly lower in abdominal versus gluteal adipocytes and may explain some degree of sex-based discrepancies in catecholamine-induced lipolysis (311).

A diversity of lipolytic responses can be seen across various depots (236). In rodents, SC adipose is characterized by highest antilipolytic \( \alpha_2 \)AR expression (48, 225) and lowest response to NEpi and insulin versus intra-abdominal depots (48, 300). Similar to humans, isoproterenol, forskolin and dbcAMP-stimulated adipocytes show regional variation with maximum responsiveness from epididymal (VAT) > retroperitoneal (VAT) > subcutaneous tissues in male rats, due to lower \( \beta_3 \)AR content, and reduced HSL activity and mRNA expression in SC fat (299). In female rats SC adipocytes exhibit reductions in stimulated cAMP production coupled with decreased \( \beta_1 \)AR and \( \beta_2 \)AR content relative to representative VAT depots (59, 107, 159). Aside from lipolytic mechanisms, regional differences related to FFA uptake and storage governed by Fatty Acyl Synthase (FAS) and LPL expression may also account for some degree of lipolytic disparity between depots (48), while anatomical differences in innervation and vascularization may also play a role in lipolysis across various adipose depots. Importantly, in 1991, Langin et al compared beta-adrenoreceptor content across 5 species and found that all but human
adipocytes contained a third, previously unidentified \( \beta_3 \)AR which mediate the majority of catecholamine-stimulated lipolysis in rodents (164). An additional \( \beta_3 \)AR agonist, CL 316, 243, has been used in numerous studies to evoke a lipolytic response in rodent adipocytes (41, 98, 265), but predictably, is a poor inducer in human adipose tissue (80). Interestingly, rat adipocytes have weak \( \alpha_2 \)AR response and lower receptor content when compared with other species (160). These are important considerations when comparing lipolytic responses across different species and models of study.

3.7 Skeletal muscle lipolysis

Skeletal muscle stores lipids in the form of intramuscular triglycerides (IMTG), which are found in lipid droplets and can range from 10–50 \( \mu \)mol/g tissue (101). This substrate pool can provide a substantial amount of fuel to muscle via IMTG lipolysis, especially at rest or during low- moderate intensity exercise (259). Importantly, although key enzymes are unchanged, the lipolytic process has notable differences compared with adipose tissue. Several studies have noted increases in muscle TAG hydrolysis during exercise in humans (77, 122), but even in the absence of circulating factors and hormones, contraction can elicit reductions in endogenous IMTG stores in a rate-dependent fashion (64). Lipolytic responsiveness to contractile activity itself is unique to skeletal muscle compared with adipose tissue; IMTGs have been shown to account for 74-80% of lipid provision in isolated contracting soleus (8-40 tetani/minute) (64), with minor contributions from exogenous FFAs, glucose and glycogen.

In terms of hormonal regulation, the \( \beta_2 \)AR mediates catecholamine-stimulated lipolysis in skeletal muscle (100). ATGL and HSL are both expressed, stimulated by
catecholamines in a similar fashion to adipocytes, and correlate with IMTG content and oxidative capacity of the muscle fiber type (5, 136). Interestingly, PLIN 1 is not expressed in skeletal muscle, but it is possible that PLIN 3 and PLIN5 exhibit protein-protein interactions with CGI-58 and work to prevent its interaction with ATGL at rest (182). Epinephrine and muscle contraction can independently induce HSL activity; their effects are additive and thereby suggest their signaling mechanisms are at least partly distinct (163). Analogous to differences in adipose tissue depot lipolysis, fiber type specific characteristics are evident in skeletal muscle, such that physiological concentrations of Epi evoke loss of endogenously pre-labeled [$^{14}$C] palmitate in oxidative soleus, but not glycolytic flexor digitorum brevis (FDB; (230)). This fiber type specificity is perhaps not surprising, as soleus fibers tend to accumulate and oxidize more exogenously labeled palmitate than glycolytic muscles FDB and epitrochelaris (EPT), thereby demonstrating a greater FFA sink and reliability on lipolytic release of FA for high energy phosphate provision (65). In vivo, it has been found that insulin (198) and glucose (25) have little to no effect on glycerol release from skeletal muscle in humans, contrary to their observed abilities to suppress adipose tissue lipolysis. Endogenous skeletal muscle lipolysis may increase after exercise training (127), as some studies have shown a greater depletion of intramuscular fat stores post-exercise in trained subjects (122, 232), in spite of no change in whole-body lipolytic rate (153) and a decreased $\beta_2$ response after endurance training (79).
4. Aerobic exercise and improvements in glucose transport, insulin sensitivity and whole body glucose homeostasis

Exercise has been shown to have both acute and lingering effects on skeletal muscle glucose disposal and whole-body insulin sensitivity. Despite often normal GLUT4 gene expression in insulin resistant or glucose intolerant individuals, insulin resistance can be attributed to dysfunctional GLUT4 translocation or docking at the plasma membrane, or lower overall transporter activity (4, 83). However, even in an insulin resistant state, glucose transport and recruitment of GLUT4 during exercise is normal (148). Exercise and isolated muscle contractions can facilitate GLUT4 translocation and skeletal muscle glucose disposal in complete absence of insulin via pathways that bypass insulin signaling proteins, such as AMPK, Ca$^{2+}$/calmodulin dependent kinase (CaMK), NO and protein kinase C (PKC) and rapid increases in GLUT4 mRNA expression and protein content (134, 157, 252). Separate insulin or exercise-responsive vesicular GLUT4 pools exist and respond to distinct molecular signals (177). This highlights the importance and utility of exercise/muscle contraction as a therapeutic tool in preventing or treating glucose transport amidst disruptions in the insulin signaling pathway. Interestingly, the acute effect of muscle contraction on glucose uptake is followed by continued glucose clearance into skeletal muscle for 16-48 hours post-exercise (23, 244).

Repeated bouts of aerobic exercise have been shown to induce sustained improvements in insulin sensitivity and glucose homeostasis, as evidenced by improvements in oral GTT (OGTT) in humans or IPGTT in rodents after >1 week of either voluntary or regimented exercise training (18, 29, 109, 118, 257). The increase in
insulin action post-exercise could be due to enhancement of the insulin signaling cascade, although results have been inconclusive, likely due to large variability in exercise intensity, duration and/or modality and differences in fiber types and measurement time points. Exercise does not seem to modulate insulin binding to IR (304), and changes in IRS-1 and IRS-2 content have either increased after one day of swim training in rats and returned to normal after 5 days (44), are unaffected after 7 days training in obese subjects (211) or decreased below sedentary levels in habitually trained men (328). In conjunction with these findings, exercise does not seem to independently mediate PI3K activation (87), however, exercise has been shown to increase insulin-stimulated PI3K activity in habitually or seven days trained humans (119, 152). Taken together, elevated post-exercise insulin sensitivity does not seem to be due to changes in insulin signaling proteins. Importantly, GLUT4 mRNA and protein content increase rapidly by 16 hrs. post-exercise, and are augmented two fold further after a second successive day of swimming in rats (244). Increased GLUT4 translocation is also elevated in the hours post-exercise, and would contribute to increased glucose uptake and post-exercise insulin sensitivity (104).

In addition to increasing glucose uptake, exercise promotes fat oxidation in mitochondria of skeletal muscle (171) through activation of peroxisome proliferator receptor α (PPARα) and by upregulating proteins involved in mitochondrial biogenesis (reviewed in (248)), such as peroxisome proliferator-activated receptor γ co-activator 1 (PGC-1α; (10, 325)), which is phosphorylated by AMPK and upregulated by PGC-1α-dependent binding to its promoter region (126). Exercise-induced increases in citrate synthase (CS) and electron transport chain proteins such as cytochrome oxidase complex
4 (COXIV) and ubiquinol-cytochrome C reductase core protein 1 (core1) are notable in skeletal muscle (192, 324) and adipose tissue post-training (297). Among numerous other beneficial adaptations, exercise training has been shown to reduce DAG and ceramide lipid species, which directly interfere with proper insulin signaling (296, 330), in skeletal muscle of obese subjects (31). This suggests exercise may partition lipids away from storage and towards oxidation, thereby promoting greater oxidative capacity, which is positively associated with and predictive of whole-body insulin sensitivity (30).

Interventions that augment oxidative enzyme content and/or mitochondrial biogenesis, such as exercise, can effectively combat chronic disease initiated by insulin resistance or glucose intolerance.

5. Conclusions

In summary, the maintenance of whole-body glucose homeostasis, which is partially governed by insulin signaling and functional lipolysis in adipose tissue and skeletal muscle, is of paramount importance. Factors such as cytokines and hormones, more specifically IL-6 and E2, are able to exert considerable control over glucose disposal and FFA metabolism, as evidenced by disrupted lipolysis and glucose handling in their absence. The precise molecular mechanisms by which IL-6 and E2 affect metabolic function in skeletal muscle and adipose are unclear, but could have large implications either at rest or during situations of increased energy demand. Furthermore, aerobic exercise training has been shown to promote adaptations that favour improvements in glucose homeostasis, and recently it has been suggested that E2 could induce similar signaling mechanisms. This remains inconclusive. The full extent of the effects of IL-6 on skeletal muscle lipolysis, and E2 on glucose homeostasis and adipose
lipolysis are still not understood, but warrant further investigation given their potential to significantly modulate whole-body metabolism and glucose homeostasis.
CHAPTER 2: Thesis Objectives

Although the molecular mechanisms of IL-6 action and lipolysis are each well-characterized in skeletal muscle, an important question remains in whether this cytokine affects intramuscular lipolysis in a fiber type specific pattern. Given that IL-6 has been shown to elicit FFA mobilization in fast-twitch, glycolytic skeletal muscle, this provided rationale for studying and comparing its effects in slow-twitch, oxidative fibers. Another critical question is whether any synergy between IL-6 and known lipolytic stimulator Epi is observed. Since both IL-6 and Epi are released during prolonged muscle contractions, independent and/or coordinate regulation by IL-6 and Epi could hold relevance in the context of fuel utilization during acute exercise. The main hypothesis of this study was that IL-6 and Epi interact to augment lipolysis in both fiber types of skeletal muscle. The first study of this thesis sought to address these objectives and research questions.

In addition to cytokines, there is growing evidence for the ability of hormones, more specifically E2, to exert regulation over lipolytic function in muscle and adipose tissues, glucose uptake in skeletal muscle and whole-body glucose homeostasis. It is inconclusive whether E2 can directly stimulate glucose uptake in muscle, or whether whole body loss of E2 impairs disposal into muscle. In any case, progression through menopause in women is associated with a loss of glucose homeostasis and increased risk for diabetes, but the mechanisms are unknown. Unfortunately for many females, repletion of E2 via HRT places them at risk for deleterious side effects (ie. stroke), but exercise has been postulated as a potential alternative. Therefore, the purpose of the second study in this thesis was to directly compare the efficacies of daily E2 or exercise at reversing glucose intolerance observed in OVX rats and explore the underlying mechanisms in
regards to insulin signaling in adipose tissue, skeletal muscle and liver. It was expected that E2 and exercise would be comparable at restoring glucose homeostasis by means of improving glucose uptake in skeletal muscle of OVX rats.

The role of E2 in adipose tissue lipolysis is not well understood and has yielded conflicting results due to variation in species of study, methodology used and timing, composition, method and dose of E2. The second study of this thesis unexpectedly generated results suggesting that E2 exerts significant influence over insulin signaling in adipose tissue, which produced a set of hypotheses extending towards adipose tissue lipolysis. However, very limited data exists that details casual, mechanistic effects of E2 on adipose lipolytic function. To gain insight into the potentially direct influence of E2 in visceral and subcutaneous adipose depots, explant, ATOC and in vivo methods were used in combination to study genomic versus non-genomic, and whole-body effects of E2 on lipolysis in OVX rats. Impairments in stimulated lipolysis were expected in both adipose depots OVX rats in vivo. Ex vivo, it was hypothesized that lipolytic response would be blunted in OVX rats, but restored with two weeks of E2 supplementation, suggesting a genomic effect of E2. Non-genomic effects of E2 on adipose tissue have never been studied, but based on human studies, an acute, non-genomic effect of E2 was expected.
CHAPTER 3: IL-6 and epinephrine have divergent fiber type effects on intramuscular lipolysis

Abstract

IL-6 is an exercise-regulated myokine that has been suggested to increase lipolysis in fast-twitch skeletal muscle. However, it is not known if a similar effect is present in slow-twitch muscle. Furthermore, epinephrine increases IL-6 secretion from skeletal muscle, suggesting that IL-6 could play a role in mediating the lipolytic effects of catecholamines. The purpose of this study was to determine whether IL-6 stimulates skeletal muscle lipolysis in a fiber type dependent manner and is required for epinephrine-stimulated lipolysis in murine skeletal muscle. Soleus and extensor digitorum longus (EDL) muscles from male C57BL/6J wild-type and IL-6−/− mice were incubated with 1 µM (183 ng/ml) epinephrine or 75 ng/ml recombinant IL-6 (rIL-6) for 60 min. IL-6 treatment increased 5′-AMP-activated protein kinase and signal transducer and activator of transcription 3 phosphorylation and glycerol release in isolated EDL but not soleus muscles from C57BL/6J mice. Conversely, epinephrine increased glycerol release in soleus but not EDL muscles from C57BL/6J mice. Basal lipolysis was elevated in soleus muscle from IL-6−/− mice, and this was associated with increases in adipose triglyceride lipase (ATGL) and its coactivator comparative gene identification-58 (CGI-58). The increase in ATGL content does not appear to be due to a loss of IL-6’s direct effects, because ex vivo treatment with IL-6 failed to alter the expression of ATGL mRNA in soleus muscle. In summary, IL-6 stimulates lipolysis in glycolytic but not oxidative muscle, whereas the opposite fiber type effect is seen with epinephrine. The absence of IL-6 indirectly upregulates lipolysis, and this is associated with increases in ATGL and its coactivator CGI-58.
Introduction

Circulating IL-6 levels have been shown to increase during exercise (71, 76, 212, 217, 315), and this is thought to be the result of increases in the secretion of IL-6 from skeletal muscle (291). This observation has fueled the hypothesis that IL-6 may have direct effects on muscle substrate metabolism, which indeed has been borne out by several studies (32, 84, 102, 146). For example, treatment with recombinant IL-6 (rIL-6) has been shown to increase the phosphorylation of 5′-AMP-activated protein kinase (AMPK), the so-called master regulator of energy metabolism, in both isolated rodent skeletal muscle and in human skeletal muscle in vivo (146). Moreover, IL-6 increases skeletal muscle glucose transport (84), although this effect seems to be present only in fast-twitch skeletal muscles (84, 96).

In addition to exogenous carbohydrates, intramuscular lipids serve as an important fuel source for ATP generation during exercise (318). For instance, skeletal muscle contains 10–50 μmol/g tissue of triacylglycerol (TAG) in the form of lipid droplets (101). TAG hydrolysis is governed by the activities of distinct lipases, adipose triglyceride lipase (ATGL) which preferentially hydrolyzes TAG, and hormone-sensitive lipase (HSL) which preferentially hydrolyses diacylglycerol (DAG) (121). ATGL is less well studied but is known to require the coactivator protein, comparative gene identification-58 (CGI-58), for full activation (166). HSL is better studied and is regulated via several reversible phosphorylation sites (Ser563, Ser659, Ser600, and Ser565) by protein kinase A, extracellular signal-regulated kinase, and AMPK, respectively (255, 318). In addition to its stimulatory effects on glucose transport in muscle, IL-6 has recently been shown to stimulate intramuscular lipolysis in rat extensor digitorum longus
(EDL) muscle, as indicated by an increase in glycerol release (146). However, it is not clear whether this occurs in oxidative muscle fibers as well, i.e., in a fiber type dependent manner.

Epinephrine is recognized as a major regulator of intramuscular lipolysis, and an estimated 50% of free fatty acids oxidized during exercise are derivatives of catecholamine-stimulated intramuscular lipolysis (93, 216). Physiological epinephrine concentrations have been shown to induce endogenous TAG breakdown in oxidative, but not glycolytic, rat muscle (230). Epinephrine infusion also produces robust increases in plasma IL-6 in both rats (57) and humans (283) and has been shown to increase IL-6 secretion from skeletal muscle in vivo (88). Exercise intensity, arterial epinephrine concentration, and skeletal muscle IL-6 secretion have also been closely correlated (111). Taken together, these data raise the question as to whether epinephrine's effects on intramuscular lipolysis are entirely direct, or are in part mediated via stimulation of IL-6 release, which can then stimulate lipolysis through an autocrine/paracrine effect.

Therefore, the primary aims of this study were to determine 1) whether there are fiber type specific differences in the ability of IL-6 to stimulate lipolysis, and 2) whether IL-6 is required for epinephrine-stimulated TAG hydrolysis in skeletal muscle. We hypothesized that IL-6 would stimulate lipolysis in both oxidative and glycolytic skeletal muscle and that it would be required for epinephrine-mediated lipolysis. To address these questions, we utilized ex vivo incubations of soleus and EDL muscles obtained from wild-type (WT; C57BL/6J) or whole body IL-6-deficient (IL-6−/−) mice.
Methods

Materials and reagents

Reagents, molecular weight markers, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON, Canada). Western Lightning Plus enhanced chemiluminescence (ECL) was purchased from Perkin Elmer (NEL105001EA). The following primary antibodies were purchased from Cell Signaling Technology: phospho-HSL (Ser\textsuperscript{563} catalog no. 4139, Ser\textsuperscript{660} catalog no. 4126), total HSL (catalog no. 4107), ATGL (catalog no. 2138), AMPK-α (catalog no. 2532), p-AMPK Thr\textsuperscript{172} (catalog no. 2531), p-STAT3 Tyr\textsuperscript{705} (catalog no. 9138), Signal transducer and activator of transcription 3 (STAT3); (catalog no. 4904), p-AKT (virus oncogene cellular homolog) Thr\textsuperscript{308} (catalog no. 9275), p-AKT Ser\textsuperscript{473} (catalog no. 9271), and glycoprotein130 (GP130; catalog no. 3732). Antibodies against the β\textsubscript{2}-adrenergic receptor (catalog no. sc9042) and the α-subunit of the IL-6 receptor (catalog no. sc-660) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for α-tubulin were purchased from Abcam (catalog no. ab4074) and CGI-58 from Novus Biologicals (catalog no. NB110–41576). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Free glycerol was measured using a commercially available fluorometric kit from BioVision (catalog no. K630–100). SuperScript II reverse transcriptase, random primers, and dNTP were products from Invitrogen (Burlington, ON, Canada). Taqman gene expression assays for mouse β-actin (catalog no. 4352933E) and ATGL (catalog no. 4351372) were from Applied Biosytems (Foster City, CA). Murine rIL-6 was purchased
from Peprotech (catalog no. 216–16). Epinephrine (catalog no. E4642), and all other chemicals were purchased from Sigma.

Animals

All protocols were approved by the University of Guelph Animal Care Committee and followed Canadian Council on Animal Care guidelines. Twelve-week-old male IL-6−/− mice (Jackson Laboratories B6.12952-IL6tmkopl/J) and age-matched C57BL/6J WT mice were housed two per cage, with a 12:12-h light-dark cycle and were fed standard rodent chow ad libitum. There were no differences in body weight between WT and IL-6−/− mice (28.9 ± 0.5 g WT; 27.7 ± 0.9 g IL-6−/−).

In vitro experiments

On the day of the experiment at ~9–10 AM, mice in the fed state were anaesthetized with pentobarbital sodium (5 mg/100 g body wt.). Soleus and EDL muscles were chosen to make fiber type comparisons of epinephrine and IL-6 stimulated lipolysis. Murine soleus muscle is composed of ~58% type I and 42% IIA fibers, while EDL is 49% IIB and 51% IIA (34). Muscles were removed and preincubated for 30 min at 30°C in oxygenated (95% O₂-5% CO₂) Krebs-Henseleit solution containing the following constituents (in mM): 125 NaCl, 5 KCl, 2.5 CaCl₂, 1.25 KH₂PO₄, 1.18 MgSO₄, 24 NaHCO₃, and 5 glucose. BSA (2%, fatty acid free) was added to media for lipolysis, but not for the signaling experiments. Soleus and EDL muscles from IL-6−/− and WT mice were incubated in the absence or presence of 1 μM (~183 ng/ml) epinephrine or 75 ng/ml murine rIL-6 for 60 min to measure lipolysis. Media were immediately frozen at −80°C,
and free glycerol concentration was subsequently measured as an index of ex vivo lipolysis. In a separate set of experiments, soleus and EDL muscles were incubated with 1 μM epinephrine or 75 ng/ml rIL-6 for 60 min to assess signaling. Previous work examining the effect of IL-6 on lipid and glucose metabolism in isolated muscle strips used IL-6 concentrations ranging from approximately 10 to 120 ng/ml (84, 146). Based on these previous studies we chose to use a concentration of 75 ng/ml. This concentration of IL-6 is much higher than circulating levels following aerobic exercise (~25 pg/ml) (315). However, the interstitial concentration of IL-6 surrounding muscle has been reported to increase to the nanogram per milliliter range after moderate physical activity (262). All muscles were blotted, frozen in liquid nitrogen, and stored at −80°C until analyses were performed.

To assess the effects of IL-6 on ATGL and SOCS3 gene expression in soleus, muscles were excised from C57BL/6J mice and incubated in Krebs-Henseleit buffer with or without 75 ng/ml rIL-6 for 6 h. Samples were intermittently gassed to maintain viability. All muscles were blotted, frozen in liquid nitrogen, and stored at −80°C until analyses were performed.

Free glycerol assay

Glycerol concentration in the incubation media was measured using a fluorometric assay on a black 96-well plate. Briefly, a glycerol standard curve was prepared by diluting a 1 mM kit standard to a range of 0.1–1.2 nmol/well. All standards and samples were loaded in triplicate and wells were adjusted to 50 μl total volume with assay buffer; and 50 μl enzyme reaction mix (46 μl assay buffer, 2 μl glycerol probe, 2 μl
glycerol enzyme mix) were added to samples and standards. Reactions were incubated at room temperature for 30 min and were protected from light. Concentrations were determined fluorometrically at excitation/emission wavelengths of 535/590 nm. Glycerol concentration was normalized to tissue weight (nmol/g wet weight).

**Western blotting**

Soleus and EDL muscles were homogenized in a 25:1 volume-to-weight ratios of ice-cold cell lysis buffer supplemented with PMSF and protease inhibitor cocktail (Sigma catalog nos. 78830 and 9599). Samples were homogenized in a FastPrep-24 instrument (MP Biomedicals catalog no. 116004500) for two 30-s intervals and then centrifuged at 1,500 g for 15 min. Protein concentration of the supernatant was determined using the bicinchoninic acid method (282), and equal amounts of protein were separated on 10% gels to assess the protein content of ATGL, p-HSL Ser^{660}, p-HSL Ser^{563}, HSL, p-AMPK Thr^{172}, AMPK, p-STAT3 Tyr^{705}, STAT3, CGI-58, p-AKT Thr^{308}, p-AKT Ser^{473}, total AKT, GP130, β_{2}-adrenergic receptor, and IL-6 receptor. Proteins were transferred to nitrocellulose membranes at a constant 200 mA per tank and subsequently blocked in Tris-buffered saline-0.01% Tween (TBST) supplemented with 5% nonfat dry milk for 1 h at room temperature with gentle shaking. Membranes were incubated at 4°C overnight in primary antibodies diluted 1:1,000 in TBST with 5% BSA. The following day blots were washed with TBST and then incubated in TBST-1% nonfat dry milk supplemented with 1:2,000 horseradish peroxidase conjugated goat ant-rabbit or anti-mouse secondary antibody for 1 h at room temperature. Bands were visualized using ECL and quantified using Alpha Innotech software.
**Glucose tolerance tests**

Intraperitoneal glucose tolerance tests were performed as an assessment of whole body glucose homeostasis. Mice were fasted for 6 h prior to an intraperitoneal injection of glucose (2 g/kg body wt.). Blood glucose levels were determined by tail vein sampling at the indicated intervals using a glucometer. Changes in glucose over time were plotted, and the area under the curve was calculated.

**Real time PCR**

RNA was isolated from soleus tissue using the Qiagen RNeasy kit according to the manufacturer's instructions. cDNA was synthesized by using 1 μg of RNA and SuperScript II reverse transcriptase, random primers, and dNTP. Real time PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems), as described previously (75). Each well (20 μl total volume) contained 1 μl cDNA template, 8 μl RNase free water, 1 μl gene expression assay, and 10 μl Taqman Fast Universal PCR Master Mix. Results for ATGL expression were normalized to the mRNA expression of β-actin. The expression of β-actin did not change with treatment [mean cycle threshold (CT) values: 22.84 ± 0.33 vehicle vs. 23.46 ± 0.77 IL-6 treated]. Relative differences between control and IL-6-treated samples were determined using the $2^{-\Delta\Delta CT}$ method (174). The amplification efficiencies of the gene of interest and the housekeeping gene were equivalent.
Statistical analysis

Comparisons between groups within a given genotype were made using a paired, two-tailed Student's t-test. Differences between genotypes was assessed using an unpaired t-test. Statistical significance was established at $P < 0.05$.

Results

IL-6 increases lipolysis in glycolytic but not oxidative muscle

Previous work has shown that IL-6 stimulates lipolysis in adipocytes (220), rat EDL muscle (146), L6 myotubes (231), primary human myocytes (3), and in humans during whole body infusions (288). However, it is not known whether IL-6 induces lipolysis in oxidative skeletal muscle or if fiber type differences exist. To address this question we treated isolated mouse EDL and soleus muscles with either epinephrine or IL-6 ex vivo. The protein content of the IL-6 receptor, GP130, and the $\beta_2$-adrenergic receptor was not different in EDL and soleus muscles harvested directly from the mouse compared with muscles that had been incubated (data not shown). As shown in Fig. 1, IL-6 failed to stimulate lipolysis in isolated mouse soleus muscle. Similarly, there were no changes in the phosphorylation of STAT3 or AMPK in response to IL-6 (Fig. 1D). A 60-min treatment with 75 ng/ml IL-6 tended to increase glycerol release from isolated mouse EDL muscle by $\sim 33\%$ compared with vehicle controls ($P = 0.07$). In conjunction with these findings, 60 min of IL-6 exposure increased the phosphorylation of STAT3 and AMPK by $\sim 40\%$ and $54\%$, respectively ($P < 0.05$) in EDL muscle (Fig. 1D).
**Figure 1:** IL-6 induces lipolysis in soleus but not extensor digitorum longus (EDL) mouse skeletal muscle. IL-6 (75 ng/ml) treatment for 60 min increased lipolysis in EDL (A) but not soleus (B) isolated muscle. The phosphorylation of STAT3 and 5′-AMP-activated protein kinase (AMPK) was increased in EDL (C) but not soleus muscle (D) following treatment with 75 ng/ml IL-6 for 60 min. Data are means ± SE. Quantified Western blot data are expressed relative to the vehicle control, n = 6 for soleus and n = 10 for EDL muscle (*P < 0.05). Representative Western blots are shown to the right of the quantified data in C and D.
Basal lipolysis is elevated in oxidative skeletal muscle from IL-6−/− mice and not further increased by epinephrine

To assess the interaction between IL-6 and epinephrine in stimulating skeletal muscle lipolysis, we assessed basal and epinephrine-stimulated lipolysis in skeletal muscle from WT and IL-6−/− mice. As some have shown that IL-6−/− mice have impaired glucose homeostasis (187), and given that this could impact lipolysis, we measured glucose tolerance in chow-fed WT and IL-6−/− mice. Glucose tolerance (Fig. 2) and body weights were similar between genotypes. Basal lipolysis was elevated by ~40% (P < 0.05) in soleus muscle from IL-6−/− mice (Fig. 3A). Epinephrine significantly increased glycerol release ~50% in WT soleus muscle (10.7 ± 0.72 vehicle, 16.07 ± 2.51 nM·gram tissue−1·h−1 epinephrine) but had no effect in IL-6−/− mice (Fig. 3B). Thus, despite comparable absolute lipolytic rates with epinephrine stimulation, the fold change from basal to epinephrine-mediated lipolysis was significantly higher in WT mice (Fig. 3C). Basal glycerol release was not different in EDL muscle from WT and IL-6−/− mice (Fig. 3D). In contrast to soleus muscle, epinephrine did not stimulate lipolysis in glycolytic skeletal muscle in either genotype (Fig. 3, E and F).
**Figure 2:** Glucose tolerance is normal in IL-6−/− knockout (KO) mice. Mice were injected intraperitoneally with 2 g/kg body wt. glucose and changes in blood glucose over time determined. Data are means ± SE for 8–10 animals per group. WT, wild type.

*ATGL and CGI-58 protein content are elevated in soleus muscle from IL-6−/− mice*

Protein content of both ATGL and its coactivator CGI-58 was significantly higher in soleus muscle from IL-6−/− compared with WT mice (Fig. 4). There were no genotype
differences in total or phosphorylated HSL Ser^{563} or Ser^{660} residues. Similarly, the phosphorylation of AKT on serine and threonine residues was also comparable.

**Figure 3:** Basal lipolysis is elevated in soleus from IL-6^-/- mice. A: basal lipolysis is elevated in soleus muscle from IL-6^-/- compared with WT mice. B: epinephrine treatment (1 μM, 60 min) does not cause further increases in lipolysis. C: change in (epinephrine minus basal from muscles from the same animal) glycerol release. Basal (D), epinephrine (E), and change in glycerol release (F) in isolated EDL muscle from WT and IL-6^-/- mice. Data are means ± SE for 6–10 muscles per group. *P < 0.05 compared with corresponding group in the same condition.
Figure 4: Basal adipose triglyceride lipase (ATGL) and comparative gene identification-58 (CGI-58) are elevated in soleus muscle from IL-6 \(-/-\) mice. The protein abundance and/or phosphorylation of hormone-sensitive lipase (HSL) and AKT were not different in soleus muscle from WT vs. IL-6 \(-/-\) mice; however, ATGL and CGI-58 were significantly increased. Data are means ± SE for 10 muscles per group and are expressed relative to WT mice. Representative blots are shown to the right of the quantified data. *p < 0.05 compared with WT.

**IL-6 does not directly alter expression of ATGL in isolated soleus muscle**

Since elevated ATGL protein expression was associated with increases in basal lipolysis in IL-6\(^{(-/-)}\) soleus muscle, we questioned whether IL-6 could directly regulate ATGL mRNA in mouse soleus muscle. Treatment of isolated soleus muscle with 75
ng/ml IL-6 for 6 h did not alter the mRNA expression of ATGL (Fig. 5) or the mRNA expression of SOCS3, a marker of IL-6 signaling.

![Graph showing mRNA expression of ATGL and SOCS3](image)

**Figure 5:** IL-6 does not induce changes in ATGL gene expression in isolated soleus muscles. Soleus muscles from C57BL/6J mice were treated with or without 75 ng/ml IL-6 for 6 h and the mRNA expression of suppressor of cytokine signaling 3 (SOCS3) and ATGL determined. Data are means ± SE and quantified relative to β-actin.

**Discussion**

The role of IL-6 as a regulator of skeletal muscle lipolysis has not been extensively examined. Kelly et al. (146) have previously shown that 60 min of treatment with 120 ng/ml IL-6 induces lipolysis in isolated rat glycolytic (EDL) muscle. In our current study, we have extended these findings to demonstrate a distinct fiber type
response. Lipolysis tended to increase in glycolytic EDL, but not in oxidative soleus muscle, during incubation with IL-6, and indices of IL-6 signaling, p-STAT3 and p-AMPK, were also increased after IL-6 treatment in EDL, but not in soleus muscle. IL-6 signal transduction is initiated by the binding of IL-6 to the IL-6 receptor GP130 complex (254), with some evidence to suggest that the soluble IL-6 receptor potentiates the effects of IL-6 (96). The blunted responsiveness to IL-6 of soleus compared with EDL muscle is not explained by differences in the protein content of the IL-6 receptor or GP130. In fact we found that GP130 protein content was similar between muscles, whereas IL-6 receptor α-protein content was actually higher in soleus compared with EDL muscle (data not shown). IL-6 signaling is negatively regulated by the suppressor of cytokine signaling 3 (SOCS3). SOCS3 binds and inhibits janus kinases, critical components of the proximal IL-6 signaling pathway (12). Recent data have shown an association between increases in SOCS3 protein content and attenuated IL-6 signaling in human skeletal muscle, independent of reductions in the content of the IL-6 receptor and GP130 (135). Thus it seems plausible that differences in the content or cellular localization of SOCS3 could explain the marked differences in IL-6 responsiveness between soleus and EDL muscles ex vivo.

As with IL-6, we also found fiber type specific differences in the response to epinephrine. However, in this instance epinephrine stimulated lipolysis in oxidative soleus but not glycolytic EDL muscle. These findings are consistent with previous work from Peters et al. (230) who demonstrated that physiological doses of epinephrine (0.1, 2.5, 10 nM) activated intramuscular triglyceride (IMTG) hydrolysis but not glycogenolysis in isolated rat soleus muscle (230). The lack of a lipolytic response to
epinephrine in EDL muscle is unlikely to be related to a limitation in IMTG content, as we and Kelly et al. (146) have shown that IL-6 can stimulate lipolysis in rat and mouse EDL muscle. It is possible that the absence of an epinephrine effect in EDL could be explained by lower expression of β-adrenergic receptors in glycolytic muscle (130).

It should be acknowledged that we are measuring the net accumulation of glycerol in the incubation medium as an index of lipolysis following treatment with IL-6 or epinephrine. Several groups have shown that glycerol is taken up by skeletal muscle (110, 314); thus the accumulation of glycerol in the incubation buffer in our experiments could be a function of both release and uptake. However, we feel that, in our ex vivo experiments, glycerol uptake was unlikely to be a significant confounding factor. First, given the relatively large volume for the glycerol to dissipate, uptake would likely be minimized. Second, if glycerol uptake was significant and was different in oxidative and glycolytic fibers, this would be difficult to reconcile with our observation of opposite patterns of hormone-activated lipolysis, i.e., glycerol accumulation tended to be increased following IL-6 treatment in glycolytic muscle, but was increased in oxidative muscle with epinephrine. Thus we would attribute change in net glycerol accumulation largely to changes in glycerol efflux.

The fiber type differences observed with both IL-6 and epinephrine-mediated lipolysis may lend insight into the hormonal regulation of IMTG hydrolysis during exercise. During low- to moderate-intensity exercise (<65% \( V_{\text{O}_{2\text{max}}} \)), slow-twitch fibers are predominantly recruited, circulating epinephrine increases, but increases in plasma IL-6 are negligible. As exercise intensity increases, fast-twitch fibers are recruited and robust increases in IL-6 are observed (144, 217). Taken in the context of our ex vivo data,
it seems reasonable to speculate that, during low-intensity exercise, lipolysis in slow-twitch muscle fibers is stimulated primarily by catecholamines, whereas, during higher-intensity exercise, IL-6 increases lipolysis in the recruited fast-twitch muscle fibers.

Although IL-6 alone was not sufficient to stimulate lipolysis in mouse soleus muscle, we could not discount the fact that it could be playing a permissive role in mediating the stimulatory effects of epinephrine. To address this question, we incubated soleus muscle from WT and whole body IL-6−/− mice ex vivo with epinephrine. As expected, epinephrine induced a >50% increase in lipolysis in soleus muscle from WT mice. Surprisingly, basal lipolysis was significantly elevated in soleus muscle from IL-6−/− relative to WT mice, but was not further increased with epinephrine. Enhanced basal lipolysis was associated with increases in the protein content of ATGL and its coactivator CGI-58. ATGL is critical for basal and catecholamine-stimulated lipolysis and requires CGI-58 for full TAG hydrolase activation (166). Adenoviral ATGL overexpression in 3T3-L1 adipocytes caused increases in lipolysis, while siRNA-mediated knockdown decreases lipolysis under basal and isoproterenol-treated conditions (150, 333). Similarly, ATGL-null mice exhibit decreased plasma free fatty acids at rest and are not able to increase lipolysis during exercise (121). Given these findings, our observation of increased basal lipolysis in soleus muscle from IL-6−/− mice may be causally related to elevated ATGL and CGI-58 protein expression. The inability of epinephrine to further stimulate lipolysis in soleus muscle from IL-6−/− mice initially suggests that IL-6 may be required for epinephrine-induced lipolysis. However, as discussed later, we were unable to demonstrate IL-6 signaling in soleus muscle; therefore, the lack of a further effect was most likely due to lipolysis already being elevated.
The increase in ATGL protein content in soleus muscle from IL-6−/− mice does not appear to be due to the loss of a direct effect of IL-6, as incubating soleus muscles with a high dose of IL-6 failed to increase the phosphorylation of AMPK and STAT3 or induce SOCS3 mRNA, markers of IL-6 signaling. As would be expected given these results, IL-6 also did not directly alter ATGL mRNA expression. Although ATGL mRNA expression was not reduced, it could be argued that IL-6 mediates its effects on ATGL through a posttranscriptional event. While plausible, this would be difficult to reconcile with the apparent lack of IL-6 signaling in isolated soleus muscle.

Our understanding of the mechanisms regulating the expression of ATGL in skeletal muscle is limited. In adipose tissue, insulin decreases ATGL expression at the transcriptional level (43, 150, 156). Interestingly, some but not all, have reported that IL-6-deficient mice develop glucose intolerance (97, 187, 313). Thus an increase in ATGL protein content could be secondary to impaired insulin action in muscle. However, in the current study, glucose tolerance was nearly identical in IL-6−/− compared with WT mice, and the phosphorylation of AKT on serine and threonine residues in soleus muscle from IL-6-deficient mice in the fed state was not impaired. Collectively, these results would argue against a role of impaired insulin action and point toward an unidentified mechanism regulating the increased expression of ATGL in soleus muscle from IL-6−/− mice.

In summary, we have provided novel data demonstrating fiber type specific effects of IL-6 and epinephrine on lipolysis in isolated murine skeletal muscle. Although we originally speculated that IL-6 may be required for the full effects of epinephrine to stimulate muscle IMTG lipolysis, the response to the hormones was divergent. While
epinephrine has been shown to preferentially stimulate lipolysis in oxidative muscle, IL-6 stimulates lipolysis in glycolytic but not oxidative muscle. Moreover, the ablation of IL-6 leads to increases in basal lipolysis and a blunting of further lipolytic activation by epinephrine only in oxidative muscle. The increase in basal lipolysis does not appear to be a consequence of the removal of a direct effect of IL-6, as we are unable to demonstrate known IL-6 signaling in the soleus muscle. Thus, through both direct (glycolytic muscle) and unidentified indirect effects (oxidative muscle), IL-6 is able to regulate skeletal muscle lipolysis.

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**Disclosures**

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

**Author Contributions**

Author contributions: T.L.M., Z.W., D.J.D., and D.C.W. conception and design of research; T.L.M., Z.W., S.F.-C., and D.J.D. performed experiments; T.L.M., Z.W., S.F.-C., D.J.D., and D.C.W. analyzed data; T.L.M., S.F.-C., D.J.D., and D.C.W. interpreted results of experiments; T.L.M. and Z.W. prepared figures; T.L.M., D.J.D., and D.C.W.
drafted manuscript; T.L.M., Z.W., D.J.D., and D.C.W. edited and revised manuscript; T.L.M., Z.W., S.F.-C., and D.C.W. approved final version of manuscript.
CHAPTER 4: Exercise training is an effective alternative to estrogen supplementation for improving glucose homeostasis in ovariectomized rats

Abstract

The irreversible loss of estrogen (specifically 17-β-estradiol; E2) compromises whole-body glucose tolerance in women. Hormone Replacement Therapy (HRT) is frequently prescribed to treat estrogen deficiency, but has several deleterious side effects. Exercise has been proposed as an HRT substitute, however, their relative abilities to treat glucose intolerance are unknown. 30 ovariectomized (OVX) and 20 SHAM (control) rats underwent glucose tolerance tests (GTT) 10 weeks post surgery. Area under the curve (AUC) for OVX rats was 60% greater than SHAM controls (p= 0.0005). Rats were then randomly assigned to the following treatment groups: SHAM sedentary (sed) or exercise (ex; 60 min, 5x/wk.), OVX sed, ex or E2 (28 ug/kg b.w/day) for 4 weeks. OVX ex rats experienced a ~45% improvement in AUC relative to OVX sed rats, whereas OVX E2 underwent a partial reduction (17%; p=0.08). Maximal insulin-stimulated glucose uptake in soleus and EDL was not impaired in OVX rats, or augmented with exercise or E2. Akt phosphorylation, did not differ in soleus, EDL or liver of any group. However, OVX ex and OVX E2 experienced greater increases in p-Akt Ser473 in VAT and SC tissues compared with SHAM and OVX sed groups. Mitochondrial markers CS, COXIV and core1 were increased in soleus post-training in OVX ex rats. The content of COXIV was reduced 52% and 61% in SC of OVX sed and E2 rats, compared to SHAM controls, but fully restored in OVX ex rats. In summary, exercise restores glucose tolerance in OVX rats more effectively than E2. This is not reflected by alterations in muscle maximal insulin response, but increased insulin signaling in adipose depots may underlie whole-body improvements.
Introduction

Compared with age-matched males, premenopausal women display protection against insulin resistance (60, 209) attributed to the beneficial effects of estrogens on insulin action, glucose homeostasis and body composition. The decline and eventual loss of circulating estrogen, specifically bioactive 17-β-estradiol (E2) is independently associated with increased risk for developing Metabolic Syndrome (reviewed by 3, 38).

Apart from a classical role in reproduction, estrogens confer a beneficial phenotype by regulating metabolic pathways, including mitochondrial function and biogenesis, fatty acid transport and oxidation, and lipogenesis and lipolysis (52, 73, 125, 214, 258, 285, 307). Rodent models of estrogen deficiency or ablated receptor signaling, such as estrogen receptor α (ERα) and aromatase knockout mice and ovariectomized (OVX) rats/mice, are similarly characterized by visceral adipose accumulation, reduced lipid oxidation and increased ectopic accumulation of diacylglycerols (DAG) and ceramides, hyperinsulinemia, increased hepatic glucose output and elevated inflammatory markers in adipose and liver. All of these perturbations converge to negatively influence whole-body glucose homeostasis (33, 125, 185, 195, 249).

Hormone replacement therapy (HRT) is primarily prescribed to treat symptoms of estrogen deficiency and curtail development of cardiovascular disease, osteoporosis and cognitive impairments (16, 141). The ability of estrogens and HRT to regulate whole-body glucose homeostasis has gained considerable attention in women’s health research (190). Some formulas of HRT have favourable effects on glucose homeostasis (6, 140, 186) while other studies have found the opposite and often attribute deteriorations in insulin sensitivity to the inclusion of progesterone with conjugated estrogens (287).
Regardless, the general efficacy and safety of HRT has been controversial since The Women’s Health Initiative (WHI) halted their large-scale HRT trials due to unacceptable hazard ratios for stroke, thrombosis, coronary heart disease and elevated risk of endometrial, breast and ovarian cancers (263). Despite the adverse health outcomes of HRT, it is undisputed that glucose metabolism is improved by estrogen-alone replacement in humans and rodents (reviewed in (270, 298). In order to circumvent the need for HRT, especially as the prevalence of T2D continues to increase worldwide, it is critical to develop alternative therapies/treatments that restore the positive metabolic effects of E2.

Since aerobic exercise is insulin-sensitizing (322), training may be an effective substitute or adjunct for HRT (286). Evans et al demonstrated comparable beneficial effects of exercise versus conjugated estrogen plus medroxyprogesterone in modulating glucose tolerance and body composition in healthy postmenopausal women (68). As evidenced in rodent studies, twelve weeks of exercise training initiated at the onset of OVX maintained normal skeletal muscle glucose uptake, prevented visceral adipose accretion and improved whole-body glucose tolerance in OVX rats, indicating that exercise training averts metabolic dysfunction (168, 270). However, to our knowledge, studies have not yet compared the abilities of exercise and E2 to treat, rather than prevent, glucose intolerance induced by estrogen deficiency.

The primary aim of the current study was to examine the effects of aerobic exercise training in treating impaired whole-body glucose homeostasis already present in OVX rats, and to compare this to an oral E2 treatment. Since skeletal muscle glucose uptake accounts for the majority of clearance from plasma, this was measured under basal
and insulin-stimulated conditions in soleus and EDL. Insulin signaling and markers of mitochondrial content in soleus, EDL, liver and adipose tissue were assessed to gain an understanding of tissue specific effects of E2 depletion, and subsequent restoration or exercise training in OVX rats.

Methods

Materials and Reagents

Reagents, molecular weight markers and nitrocellulose membrane were purchased from BioRad (Mississauga, ON, Canada). Western Lightning Plus enhanced chemiluminescence (ECL) was purchased from Perkin Elmer (NEL105001EA). The following primary antibodies were purchased from Cell Signaling: phospho-Akt Thr$^{308}$ (cat. # 9275), phospho-Akt Ser$^{473}$ (cat. # 9271), Akt pan (cat. #4691). Antibodies against cytochrome oxidase complex IV (COXIV; cat. #16056), citrate synthase (CS; cat. #96600), anti-ubiquinol-cytochrome C reductase core protein 1 (core1; cat. 110252) were acquired from Abcam. NP40 cell lysis buffer was acquired from Life Technologies and PMSF and protease inhibitor cocktail were obtained from Sigma (cat. #78830 and 9599). Insulin (Humulin, rDNA origin) was purchased from Eli Lilly (Toronto, ON, Canada).

Animals

All procedures were approved by the Animal Care Committee at the University of Guelph and followed CCAC guidelines. Female Sprague Dawley rats were purchased from Charles River Laboratories at 4 months of age. Two days prior to arrival, all rats underwent either bilateral ovariectomy (OVX; 30 animals total) or SHAM surgery (SHAM; 20 animals total) under ketamine-atropine-xylazine anesthesia performed by
Charles River technicians. Two flank incisions were made on the dorsal side, ovaries were identified and either removed by cauterization (OVX) or left intact (SHAM). OVX surgeries were verified during terminal experiments by recording uterine weight.

For the first 10 weeks, animals were grouped in cages of 3-4 as either SHAM or OVX with no further stratifications. Rats were housed in a temperature controlled room with a 12:12 hour standard light-dark cycle. All animals had ad libitum access to food and were fed a phytoestrogen-purified, soy protein free diet (Harlan 2020X, 16% calories from fat). A pre-measured, excess amount of food was weighed and placed in each hopper and the amount leftover per cage was weighed and recorded every 2-3 days. Body mass was recorded weekly throughout the study.

**Glucose tolerance tests**

At week 10, all rats underwent an intraperitoneal glucose tolerance test (ipGTT) to assess whole-body glucose homeostasis. Previous pilot work (unpublished data) determined that OVX rats were markedly glucose intolerant compared to SHAM controls at this time point. In order to allow comparisons between E2 and ex treatment approaches, versus prevention, confirmation of intolerance was required prior to administering either therapy. Rats were fasted for 6 hours and injected with a 2g/kg bolus of glucose. Blood glucose levels were measured from the tail vein at 0, 15, 30, 45, 60, 90 and 120 minutes using a handheld glucometer (FreeStyle Lite). Area under the curve (AUC) for the glucose response was calculated for each rat.

Tail vein blood samples were taken from each rat at 0 and 15 minutes in order to measure serum insulin. Samples were collected in microvette capillary tubes (Sarstedt
Microvette 300 Z), allowed to clot for 30 minutes and then centrifuged for 10 minutes at 10000 x g. The supernatant was removed and stored at -80°C for further analysis. At 15 weeks, GTTs and blood sampling procedures were repeated after a four week intervention period (outlined below).

After the 10 week GTT, SHAM rats were assigned to remain sedentary (SHAM sed) or undergo exercise treatment (SHAM ex). OVX animals were also allocated to sedentary (OVX sed) and exercise (OVX ex) groups, with an additional OVX group receiving oral 17-β-E2 treatment (OVX E2). All treatments were for 4 weeks. Rats were distributed amongst the various treatment groups such that each group had a similar average glucose tolerance, as determined by the mean AUC. This was done to prevent inherent differences in baseline glucose tolerance from skewing comparisons between groups after the 4 week treatments.

*Estrogen and exercise training protocols*

Training protocol: Two days prior to the commencement of training, animals received two acclimatory training sessions (<5 min, 10 m/min, 0% incline). For the following 4 weeks, training took place on 5 consecutive days each week. Animals started at 10 m/min at a 0% incline for 20 min and the speed and incline were increased to 15 m/min at a 10% for 45 min by the fifth training session. Speed and duration were then increased to 16 m/min, with 5-10 minute bursts of 20 m/min., totaling 60 min of total running time by the tenth training session. This was maintained for the remainder of the study. Since OVX rats had a visibly reduced running capacity, SHAM ex rats were
matched daily to the training regime of the OVX group. This was done to ensure training
effect comparisons were equal between SHAM and OVX groups.

Estrogen protocol: Rats assigned to E2 treatment received a daily dose of 17- β-
estradiol delivered via chocolate hazelnut spread (Nutella), as described by Ingberg et al (123). A concentrated stock solution of 17-B-E2 was made weekly by dissolving
powdered estradiol in sesame oil to yield a concentration of 28 ug 17-B-E2/5 uL sesame
oil/1g nutella per kg body mass each day. All rats consumed the dose of estradiol in less
than a minute. Rats not receiving estradiol received a similar dose of nutella with sesame
oil as a control.

Terminal surgeries and muscle glucose uptake experiments

Rats were allowed a 48 hour recovery period following their last training session
prior to surgical procedures in order to avoid the residual effects of the last bout of
exercise. Animals were fasted overnight prior to terminal experiments and anaesthetized
with an intraperitoneal injection of pentobarbital sodium (6 mg per 100 g body mass)
prior to all surgical procedures.

For glucose uptake, two strips from the soleus and EDL muscles of each animal
were carefully dissected and assigned to one of the following conditions: 1) basal soleus,
2) insulin-stimulated soleus (10 mU/mL), 3) basal EDL or 4) insulin-stimulated EDL (10
mU/mL). Glucose transport was calculated as accumulation of intracellular 3-O-
[^3H]methyl-D-glucose as previously reported (201).
In vivo insulin signaling

A third muscle strip from the soleus and EDL of one leg, as well as samples from perigonadal visceral adipose (VAT) and inguinal adipose (SC) from one side of the body, were removed and snap frozen as the basal condition for subsequent in vivo insulin signaling analysis. Animals were then injected with a 7.5U/kg dose of insulin. Fifteen minutes later, samples from the contralateral leg (soleus and EDL), VAT and SC, as well as the liver were dissected, snap frozen, and stored as the insulin-stimulated condition for western blotting (Akt phosphorylation).

Insulin ELISA

Serum samples from the 10 and 15 week GTTs were analyzed for insulin concentration using a commercially available kit (Millipore EZMRI-13K) according to manufacturers’ instructions. Accuracy and intra-assay comparisons were validated using two quality control standards (run in triplicate) provided with each kit. Serum samples were assayed in duplicates.

Western Blotting

Equal amounts (20 μg) of basal and insulin-stimulated samples were separated by electrophoresis on 10% gels to assess content of phospho- Akt Ser^{473}, phospho-Akt Thr^{308} and total Akt (Akt pan) in each tissue, following protocols previously published (201, 292). Basal samples were separated on 10 or 15% gels to assess content of mitochondrial proteins in response to training (COXIV, CORE1, CS), GLUT4 and ponceau staining.
was used as a loading control. Bands were visualized using ECL and quantified using densitometry on Alpha Innovate Software.

Statistics

All data is expressed as mean ± SE. For measures comparing mean values between SHAM sed, SHAM ex, OVX sed, OVX E2 and OVX ex basal, in the absence of any insulin or time points as factors, a one-way ANOVA was used to assess group differences. For measures involving insulin treatment or multiple time points, a two way ANOVA was used. Tukey’s post hoc test was used in both ANOVA tests if significant differences were detected. In cases where data failed the Shapiro-Wilk test, a log(10) transformation was applied to normalize data. In all tables and figures, letters are used to denote statistical significance, such that means sharing a letter are not significantly different. All alpha values were set to $\alpha = 0.05$. 
Results

*Ovariectomy induces hyperphagia and increased body mass*

Throughout the duration of the study, OVX rats had significantly higher body mass relative to SHAM controls, regardless of sedentary, E2 or exercise treatment (Table 2). Food intake was markedly increased in the OVX groups, but tapered over time (Table 2) and was not significantly different from SHAMs by week 5. In pilot work, we tested the ability of pair-feeding OVX rats to SHAM controls to prevent changes in body mass induced by hyperphagia; however, this did not prevent greater weight gain post-OVX. Given this finding and the fact we did not want to restrict food intake in the OVX trained group, all groups were fed ad libitum.

*Glucose tolerance is impaired in OVX rats at 10 weeks, is partially restored after 4 weeks of E2 treatment, and fully restored by exercise training*

During the 10 week GTT, AUC was significantly increased in OVX rats relative to SHAM controls. Glucose tolerance remained consistent in OVX sed rats at 15 weeks (416 ± 32, 10 wks vs. 446 ± 39, 15 wks; Figure 6B), whereas OVX E2 and OVX ex rats demonstrated 21 and 41% improvement (i.e. reduction) in their AUCs, respectively. Importantly, exercise restored GTT AUC to a greater extent than E2 compared to the OVX sed group.
Table 2: Body mass, food intake and terminal uterine weight measurements.

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<td>SHAM sed</td>
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<td>62.6 ± 2.1a</td>
<td>66.1 ± 1.7a</td>
<td>51.1 ± 1.9a</td>
<td>42.0 ± 1.3a</td>
<td>75.2 ± 2.9</td>
<td>69.1 ± 1.4</td>
<td>43.4 ± 1.4</td>
<td>58.5 ± 0.9</td>
<td>49.8 ± 0.5</td>
<td>53.0 ± 4.8b</td>
<td>37.0 ± 3.9b</td>
<td>42.2 ± 2.3a</td>
<td>44.8 ± 2.0a</td>
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<td>SHAM ex</td>
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<td>76.7 ± 3.3b</td>
<td>52.8 ± 1.4b</td>
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<td>44.9 ± 1.8</td>
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<td>0.89 ± 0.09a</td>
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<td>0.95 ± 0.09a</td>
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Data are presented as mean ± standard error, n= 20 and 30 for SHAM and OVX rats from weeks 1-10, and n=10 for all groups from week 11-14. Groups which share a letter within
each parameter within each week are not statistically different, as assessed by unpaired t-test or one-way ANOVA. Statistical significance accepted at p < 0.05.

Figure 6: Calculated area under the curve (AUC) during A) the 10 week GTT and B) 15 week GTT. Plasma insulin concentrations at 0 and 15 minutes during the 15 week GTT are shown in panel C. Data are presented as mean ± standard error, n= 20 and 30 for SHAM and OVX rats in panel a), respectively, and n=10 for all other groups. Groups which share a letter are not statistically different. Statistical significance accepted at p <
0.05. In panel C: plasma insulin concentrations at the 15 minute time point are trending towards statistical significance vs. basal values, p= 0.06 as assessed by two-way ANOVA.

**Figure 7:** Basal and maximally insulin-stimulated glucose uptake in soleus and EDL (A, D); protein content of insulin signaling markers before (basal) and after (+insulin) 7.5 U/kg b.w injection in soleus p-Akt Ser 473 and p-Akt Thr308 (B, C) and EDL p-Akt Ser473 and p-Akt Thr308 (E, F). Data are expressed as mean ± standard error, n=10 per group. Groups which share a letter are not significantly different. In panel A: bars with an asterisk denote a significant insulin effect relative to the basal control within each group.
Statistical significance is accepted at p < 0.05. In panel D: p = 0.07 for the effect of insulin. In panel B, within +insulin group differences: OVX ex vs. SHAM sed p = 0.06, OVX ex vs. SHAM ex p = 0.08, OVX E2 vs. SHAM sed p = 0.07, OVX ex vs. SHAM ex p = 0.09; panel C: OVX ex vs. OVX E2 p = 0.09, SHAM ex vs. SHAM sed p = 0.09 as assessed by two-way ANOVA.

Plasma insulin response is not impaired in OVX rats during GTT

In order to assess whether reduced peak plasma insulin concentrations may have contributed to the whole-body glucose intolerance observed in OVX sed rats, or whether a greater initial plasma insulin response in OVX E2 and ex groups could explain the improved glucose tolerance, blood was sampled at 0 and 15 minutes during the GTT (Figure 6C). OVX sed rats had significantly elevated plasma insulin at 15 min (6.6 ± 0.6 ng/mL) relative to E2 or exercise treated OVX groups (3.1 ± 0.2, 3.9 ± 0.8 pg/mL).

Ex vivo glucose uptake in soleus and EDL is unchanged by OVX, E2 or exercise training

Given that whole-body glucose disposal is primarily mediated by skeletal muscle (56), we measured maximal insulin-stimulated 3-O-[3H]methyl-D-glucose uptake into soleus and EDL. There were no significant differences between SHAM or OVX phenotype or sedentary, E2 or exercise treatment in uptake in either muscle (Figures 7A and D). As expected, there was a significant effect of insulin in all groups.
Figure 8: Protein content of insulin signaling markers before (basal) and after (+insulin), 7.5 U/kg b.w injection of insulin in liver p-Akt Ser473 and Thr308 (A, B) and total Akt (C). Data are expressed as mean ± standard error, n = 10 per group. There were no statistical differences in content between groups on either Akt reside. Statistical significance is accepted at P < 0.05.
In vivo insulin signaling (pAkt) is improved in VAT and SC adipose tissue with E2 and exercise treatment in OVX rats

There were no differences in phosphorylation of either Akt Ser473 or Thr308 residues amongst the groups in soleus (Figures 7B and 2C), or in EDL (Figures 7E and F), which mirrored our glucose uptake data. p-Akt Thr308 was lower in the SHAM ex group of both tissues. Total Akt was not different in basal or insulin-stimulated conditions in either soleus or EDL.

Changes in hepatic phosphorylation of p-Akt Ser473 (Figure 8A), p-Akt Thr308 (Figure 8B) and total Akt (Figure 8C) followed the same pattern, such that SHAM ex was reduced relative to all other groups (only statistically significant in total Akt). Therefore, the phosphorylation of Akt, at least under insulin-stimulated conditions, appears unaffected by OVX, E2 or ex.

In VAT (figures 9A and B), insulin-stimulated pAkt Ser473 was highest in OVX ex rats, and significantly greater than SHAM sed, SHAM ex and OVX sed groups. Group differences were less robust with the Thr308 residue, but still reflected the same pattern, as OVX ex insulin-stimulated p-AktThr308 showed trends for differences from SHAM sed and OVX sed groups (p=0.08 and 0.053, respectively; Figure 9B). Basal and insulin-stimulated p-Akt Ser and Thr were not reduced in VAT from OVX sed rats relative to SHAM sed controls, per se, but the relative fold increase was less than E2 or ex groups.

In SC tissue (Figures 9C and D), OVX E2 and OVX ex rats showed significantly greater insulin-stimulated p-Akt Ser473 relative to SHAM and OVX sed groups (Figure 9C). This finding was mirrored by p-AktThr308 (Figure 9D), where OVX ex rats had significantly higher levels than SHAM sed or ex rats. Since there were statistical
differences in basal p-Akt Thr308 in SC adipose, the fold-change between basal and insulin-stimulated phosphorylation in each group was also calculated to determine relative insulin induction of the Akt signaling cascade. OVX ex rats displayed the greatest increase (7.6 ± 2.5 fold), which was significantly greater than SHAM sed and ex rats, but not OVX sed or OVX E2 groups. The OVX E2 group exhibited a 4.2 ± 0.8 fold change, whereas SHAM sed, SHAM ex and OVX sed only increased AktThr308 phosphorylation 0.8 ± 0.3, 0.8 ± 0.4 and 1.7 ± 0.5 fold. Total Akt expression was not different under basal or insulin-stimulated conditions in VAT or SC tissues.

Total GLUT4 content is not changed by OVX, E2 or exercise

Total tissue content of GLUT4 protein (quantified at the 45kDa band) was not different in soleus, EDL, VAT and SC adipose tissue in SHAM vs OVX or after sed, E2 or exercise treatment, as shown in Figures 10A-D.
**Figure 9:** Protein Content of insulin signaling markers before (basal) and after (+ insulin) 7.5 U/kg b.w injection of insulin in VAT p-Akt Ser473 and Thr308 (A, B) and SC adipose (C, D). Data are expressed as mean ± standard error, n=10 per group. There were no statistical differences in content between groups on either Akt residue. Statistical significance is accepted at p < 0.05.
Markers of mitochondrial content are significantly increased by exercise training in soleus and SC tissues

Citrate synthase, COXIV and core1 content were measured to assess changes in markers of mitochondrial content after exercise or E2 treatment. Citrate synthase and COXIV were significantly increased in soleus after exercise training in OVX rats (Figure 11A), and core1 expression also approached significance (p=0.06). Mitochondrial markers were unchanged by OVX, E2 or ex in EDL, liver and VAT as shown in panels B, C and D respectively. In SC adipose tissue, both E2 and ex treatment increased citrate synthase in OVX rats to a greater extent than SHAM or OVX sed groups (Figure 5E). COXIV expression was significantly lower in SHAM ex, OVX sed and OVX E2 groups, but robustly improved by exercise training in OVX rats. Changes in core1 approached significance in SC adipose tissue (p=0.08), but overall content was not different between groups.
**Figure 10:** GLUT4 protein content in soleus, EDL, VAT and SC tissues (A-D). Data are expressed as mean ± standard error, n = 10 per group. Groups were not significantly different, as assessed by one-way ANOVA. Statistical significance is accepted at P < 0.05.
Figure 11: Protein content of mitochondrial markers citrate synthase, COXIV, and core1 in soleus, EDL, liver, VAT and SC tissues (A-E). Data are expressed as mean ± standard error, n = 10 per group. Within each target protein in each tissue, groups which share a letter are not significantly different. Statistical significance is accepted at P < 0.05. In panel A: between-group differences in core1 P = 0.06, in panel B: between-group differences in COXIV P = 0.06, in panel E: between-group differences in core1 P = 0.08 as assessed by one-way ANOVA.

Discussion

As women transition through perimenopause to irreversible estrogen deficiency, increased fat mass, particularly in the visceral depot, decreased lean body mass and
impaired whole-body glucose metabolism are commonly experienced (298). Menopause induces a 60% increase in risk for developing Metabolic Syndrome independent of age, BMI and physical activity (218) and is often combatted with various HRT regimes. The E2 dosage administered, as well as the inclusion of other hormones, namely progesterone, can elicit drastically different effects in vivo. Physiological levels of E2 can positively mediate glucose homeostasis, whereas high or low concentrations appear to be more detrimental (202, 203). The dose administered in the present study was 28 μg/kg b.w/day, which has been shown in previous work to produce circulating E2 concentrations within a normal physiological range (278). Instead of implanting silastic capsules, which often cause an early E2 spike and subsequent taper in release (123), we adapted methods utilized by Isaksson et al to elicit a physiological dosing regime (124). Here we chose to administer E2 in a sesame oil vehicle delivered within nutella, in order to precisely manipulate the dose received per day and avoid large day-to-day variability. The palatability of nutella also ensured that the complete dose was ingested quickly. This methodology was effective in evoking a decline in hyperphagia, a common endpoint of E2 action.

Previous pilot work (unpublished data) and the current study demonstrate that body mass increases within one week post-OVX in rats. This persists with sedentary treatment and develops into profound glucose intolerance by 10 weeks, which is partially reversed with four weeks of E2 administration. Others have demonstrated the abilities of E2 treatment and exercise, independently and synergistically, to effectively attenuate metabolic dysfunction that occurs in estrogen deficient women and OVX rodents, prior to the onset of disrupted glucose homeostasis. Preventative effects include lowered fasting
plasma glucose, improved insulin response during a GTT and reduced fat mass in humans (68) and rodents (270) after 8 to 12 weeks of exercise training or E2 administration, as well as improved insulin-stimulated skeletal muscle glucose uptake in OVX rats (270). The most novel finding in our current study is the ability of exercise to effectively reverse OVX-induced glucose intolerance from a treatment, as opposed to a prevention approach, whereas E2 was only partially restorative. After showing marked whole-body improvements with exercise and E2 treatments, we sought to investigate skeletal muscle function and cell signaling changes to gain an appreciation for how both treatments modulate glucose homeostasis.

Given that skeletal muscle accounts for ~85% of glucose disposal (56), we anticipated that muscle glucose uptake might explain the differences observed in the whole-body GTT responses. For this purpose, basal and insulin-stimulated uptake of 3-O-[³H]methyl-D-glucose uptake into isolated soleus and EDL was measured. Previously reported effects of OVX on ex vivo muscle glucose uptake have been equivocal. Saengsirisuwan et al reported 29% and 43% reductions in maximal insulin-stimulated 2-deoxyglucose (2-DG) uptake in soleus and EDL of OVX rats, respectively (270). Impairments were prevented with E2 and exercise treatment independently, but not synergistically. Conversely, Gorres et al found no impairment in skeletal muscle 2-DG uptake in OVX rats under similar experimental conditions, despite rats being sedentary or further challenged with a high fat diet (90). Here we show no impairment in basal or maximal insulin stimulated glucose uptake in soleus or EDL in the OVX sed group, and no augmentation after E2 or exercise treatment in either SHAM or OVX rats. Insulin-stimulated phosphorylation of Akt Thr308 and Ser473 residues mirrored the functional
glucose uptake data in both muscles, such that there were no discernable effects of OVX, E2 or exercise treatment. Unfortunately, tissue yield was inadequate for measuring plasma membrane GLUT4 content, but total tissue homogenate GLUT4 remained unchanged in soleus and EDL. It is possible that while maximal insulin-stimulated glucose uptake and insulin signaling were unaffected, changes in sensitivity elicited by a half maximal dose may have occurred and gone undetected.

The discrepant finding of whole-body glucose intolerance in the absence of impaired, albeit maximal, insulin signaling and glucose uptake in soleus and EDL in OVX sed rats prompted us to measure markers of insulin signaling in other insulin-sensitive tissues. Interestingly, the increases in pAkt Ser473 and Thr308 content from basal to insulin-stimulated condition in VAT were highest in OVX ex rats versus SHAM and OVX sed/E2 groups. This was even more pronounced in the SC adipose depot, where four weeks of E2 or exercise treatment significantly increased insulin induction of Akt phosphorylation on the Ser473 site. Similarly, the fold-increase in SC Akt Thr308 phosphorylation was significantly blunted in the OVX sed group, but recovered in E2 and exercise groups. Taken together, this would suggest that exercise training and E2 administration induced adipose tissue-specific effects that may have led to improvements in glucose tolerance. It is possible that exercise and E2-driven increases in Akt phosphorylation could increase VAT and SC adipose tissue glucose uptake leading to improvements in whole-body glucose tolerance. It is not known whether E2 or exercise can cause increases in glucose uptake in adipose tissue of an estrogen deficient rodent. However, this could be important since OVX rats accumulate up to 70% more SC fat than SHAM controls, and there would be a greater absolute amount of tissue able to clear
glucose during a GTT challenge (335). Orava et al recently demonstrated that glucose uptake into SC adipose tissue is similar to skeletal muscle in healthy subjects, on a per tissue mass basis, which suggests that SC adipose compartments may represent an important site for whole-body glucose homeostasis (215). Limitations to the current study are that neither body fat content, nor glucose uptake or membrane-localized GLUT4 expression were measured in isolated fat depots to assess this possibility.

Perhaps the most encouraging information for justifying exercise as an alternative to synthetic E2 administration is the degree of metabolic benefit incurred from relatively short-term, medium intensity exercise. The treadmill training protocol was reduced in duration and intensity from previous studies used by our group (253, 281) as the OVX females had visible limitations in exercise capacity. Nonetheless, this modified, less intense, training program still induced robust improvements in whole-body glucose tolerance in the OVX rats comparable to those seen with E2 administration. It is important to note that SHAM ex rats were exercise-matched to OVX ex rats, such that they completed the same duration and intensity (speed, incline). The SHAM ex groups would presumably have been exercising at a lower relative workload, and thus did not demonstrate the same degree of increased mitochondrial content as the OVX rats subjected to a relatively more intense exercise regime.

In OVX ex rats, markers of mitochondrial content were increased in soleus, but not EDL, likely indicating a lack of fast-twitch recruitment. In another study, by 8 weeks post-OVX rats demonstrated lower ATP synthesis, blunted citrate synthase activity, lower use of palmitoyl-carnitine and glycerol phosphate substrates and decreased PGC-1a content, attributed to lower mitochondrial content in soleus and white gastrocnemius, all
of which were reversible by E2 treatment (42). Reductions in mitochondrial markers were minimal at 10 weeks post-OVX in the present study, and are consistent with previous findings of normal electron transport chain (ETC) protein content in OVX mouse skeletal muscle (125). However, subtle perturbations did occur in adipose tissue COXIV and core1 content and interestingly, VAT and SC depots were most responsive to E2 and exercise-mediated increases in CS, COXIV and core1. It is uncertain whether these are direct effects of E2 on VAT and SC tissue depots, or if changes in mitochondrial protein content are secondary to whole-body effects of E2 repletion, or whether increases in mitochondrial content are responsible for improvements in whole-body glucose tolerance.

Conclusions

In conclusion, aerobic exercise training approximates the effects of E2 administration in reversing whole-body glucose intolerance observed 10 weeks post-OVX in female rats. The contributing impairments and subsequent improvements are not due to changes in maximal insulin-stimulated glucose uptake in soleus or EDL muscle, or in the capacity of insulin to induce Akt phosphorylation in muscle or liver. Rather, the E2 and exercise induced effects appear to be reflected in an adipose-specific manner, such that VAT and SC demonstrated the most robust increases in pAkt Thr308 and Ser473 content. Taken together, this study is the first to show that exercise is an effective therapy for restoring glucose tolerance in estrogen deficient rats when compared with synthetic E2 administration, and suggests that exercise may provide an alternative option to HRT for estrogen deficient women.
**Author contributions:** TLM and DJD planned experiments; TLM, KLR and DJD performed terminal surgeries and tissue analyses; MJH, DTC, SD and TLM trained and maintained animals and assisted with tolerance tests and tissue sampling; TLM and DJD prepared the manuscript with editing from MJH, DTC, KLR. Thank you to David C. Wright for editing and insight during manuscript preparation.
CHAPTER 5: Estrogen does not directly stimulate adipose lipolysis ex vivo, or increase circulating FFA levels in OVX rats in vivo

Submitted to Adipocyte, August 2016
Abstract

Estrogen (E2) is a steroid hormone that can act via genomic and non-genomic signaling pathways to regulate fat metabolism. Loss of E2 is associated with a robust increase in visceral adiposity; however, the mechanisms by which E2 modulates adipose mass, depot distribution and lipolysis are poorly understood. We sought to measure basal and β₃-stimulated FFA and glycerol release in vivo and ex vivo in E2 deficient or supplemented rats, or with direct, acute E2 ex vivo exposure. For two weeks, ovariectomized (OVX) and OVX rats treated with an oral dose of 28 μg/kg b.w of E2 per day (OVX E2) were pairfed to SHAM controls and fed Harlan 2020X phytoestrogen-free purified diet (n=12 per group). After 2 wks, half of the animals in each group received an in vivo saline injection or 1 mg/kg CL 316, 243. Plasma FFA concentrations, but not glycerol, were lower in OVX and OVX E2 rats compared with SHAM controls (p=0.02) and a significant CL response was present in all groups (p<0.001). HSL Ser660 and Ser563 activation were unaffected by OVX or OVX E2 in retroperitoneal (r.p.) or inguinal (iWAT) adipose depots in vivo. Similarly, ex vivo FFA, glycerol and HSL phosphorylation were comparable to SHAMs in r.p. and iWAT explants from OVX and OVX E2 rats. To assess whether E2 can directly impart effects on lipolysis, r.p. and iWAT tissue was treated with E2, CL or E2 + CL for 2, 4 or 8 hours using adipose tissue organ culture (ATOC). While CL elicited robust lipolysis (p<0.001), E2 only had a minor effect on attenuating r.p. FFAs at 8 hrs. (p=0.10). Adipocyte size was modestly (~40%) increased in OVX rats, but did not reach significance. Taken together, this data suggests that changes in circulating FFAs in OVX rats is not due to non-genomic E2 action, but suggest chronic, genomic E2 exposure is critical for maintaining adipose tissue lipolysis.
Introduction

Estrogen is a steroid hormone capable of exerting genomic, transcription-based and rapid, non-genomic signaling within the cytoplasm (21). Despite the well-characterized binding of E2 to its nuclear receptor (estrogen receptor, ERα or β (73)), and a noted ability of estrogen to influence food intake, energy expenditure and physical activity (309), the pathways by which bioactive 17-β-estradiol (E2) regulates adipose tissue metabolism are largely unknown. Estrogen loss, particularly during menopause in humans, has been shown to cause a shift from subcutaneous to visceral fat storage and derangements in lipolytic responsiveness (241, 303). There is some evidence in humans that estrogen is antilipolytic, through its potentiation of insulin's suppression of lipolysis (227), and inhibition of catecholamine-stimulated lipolysis in subcutaneous adipose tissue (89). Exactly how E2 is able to modulate adipose mass, fat distribution and lipolysis is not clear.

Insulin suppresses lipolysis in vitro (24) and when infused in vivo (36). Free fatty acid (FFA) mobilization can be stimulated by catecholamines epinephrine (Epi) and norepinephrine (NE) (160). Binding of Epi or NE triggers activation of β1,3 adrenergic receptors, followed by adenylate cyclase (AC) activation, increased intracellular concentrations of cyclic adenosine monophosphate (cAMP) and elevated protein kinase A (117), which activates a sequential cascade of enzymes and lipid droplet proteins to reduce TAG to FFAs and glycerol. Adipose triglyceride lipase (ATGL) cleaves triacylglycerol (TAG) to diacylglycerol (DAG, (333)); hormone sensitive lipase (HSL) is phosphorylated at Ser 563, 659 and 660 residues to act as a DAG hydrolase (318) and monoacylglycerol lipase catalyzes the last step. Reduced AC activity and cAMP
production have been observed in visceral adipose tissue from ovariectomized (OVX) animals (159). It is less clear whether lipolysis itself and/or enzymes essential to this process are altered in the prolonged absence of E2. In OVX mice, impaired lipolytic responsiveness to acute exercise has been attributed to reduced translocation of ATGL, blunted phosphorylation of HSL Ser660 and reduced PLIN1, however, lipolysis was not directly measured (320).

Our understanding of the mechanisms by which E2 regulates lipolysis in vivo are largely derived from the OVX rodent model, given the complexities of aging, mode of E2 delivery, dose, duration and methodological limitations in humans. As measured by microdialysis, OVX rats had 25 and 50% reductions in basal and β2-stimulated glycerol release, respectively, in subcutaneous tissue versus intact rats, and lipolytic responsiveness to isoproterenol was restored after 7 days of E2 treatment (53). Whole-body increases in circulating FFA concentrations have also been observed in OVX mice treated with E2 for 60 days versus untreated OVX (52). These findings suggest that chronic, genomic mechanisms of E2 may increase adipose tissue lipolysis. In visceral adipocytes isolated from OVX animals, basal and catecholamine-stimulated lipolysis are lower (52, 53, 159, 219) compared to controls. In vivo replacement with E2 for 4-30 days potentiates visceral lipolytic responsiveness to catecholamines in vitro. Only one study has explored the relationship between estrogen status and subcutaneous responsiveness to isoproterenol (159), in which femoral adipocytes from OVX rats were comparable to SHAM rats.

Although these studies provide insight into the effects of E2 loss on adipose tissue metabolism, the majority fail to control for hyperphagia by pairfeeding, thereby making it
difficult to conclude whether the chronic effects of E2 on lipolysis are direct or exert effects secondary to reductions in energy intake (2) or changes in fat cell size (52). While one study in postmenopausal women demonstrated an ability of acute conjugated estrogen (CE) infusion to reduce glycerol rate of appearance from subcutaneous adipose tissue (226), infusion of rats with the E2 analogue moxestrol had no effect on lipolysis (53). E2 has been shown to induce rapid, non-genomic Akt and AMPK signaling in muscle (51, 258); however, it is uncertain whether E2 activates non-genomic signaling in adipose tissue.

The aim of this study was to compare lipolytic responsiveness of OVX versus SHAM control rats to the β₃ agonist CL 316, 243 (CL). This compound has been used in numerous other studies to stimulate lipolysis in white adipose tissue (41, 80). Lipolysis was quantified, using both FFA and glycerol concentrations with and without 14 days of E2 administration, both in vivo and ex vivo using adipose tissue explants. We coupled these experiments with acute, ex vivo treatment of cultured adipose tissue with E2, CL or a combination of both. Given the paucity of experiments investigating the relationship between subcutaneous adipose lipolysis and E2, both inguinal (subcutaneous) and retroperitoneal (visceral) depots were examined to better appreciate FFA mobilization during E2 deficiency and repletion.
Methods

Animal Care

All procedures were approved by the University of Guelph Animal Care Committee and followed CCAC guidelines. Female Sprague Dawley rats were purchased from Charles River Laboratories at 4 months of age, ~250 g. Two days prior to arrival, all rats underwent either bilateral ovariectomy (OVX; 24 animals total) or SHAM surgery (SHAM; 12 animals total) under ketamine–atropine–xylazine anesthesia by Charles River technicians. Two flank incisions were made on the dorsal side, and ovaries were identified and either removed by cauterization (OVX) or left intact (SHAM). Proper technical surgery was verified during terminal experiments by recording uterine weight, which was significantly atrophied in OVX and OVX E2 rats versus SHAM controls (0.67 ± 0.07 g SHAM, 0.12 ± 0.007 g OVX and 0.12 ± 0.01 g OVX E2).

SHAM and OVX animals were placed in cages with four animals per/cage in a temperature (28°C) controlled room on a 12:12 hour standard light-dark cycle. All rats consumed a phytoestrogen-purified, soy protein free diet (Harlan 2020X) with 16% of calories from fat. Since OVX rats become markedly hyperphagic post-surgery, a pairing feeding regime was utilized. An excess amount of food was placed in the SHAM cage hoppers, weighed each morning, and OVX cages subsequently received the average amount of pellets consumed by the SHAM rats on the previous day. All rats had body mass recorded weekly.

For the 2-8 hour lipolysis time course pilot experiment, female Sprague Dawley rats were received from Charles River at ~250-300g and were acclimated in similar housing conditions for one week prior to final experiments. These rats were allowed ad
libitum access to Harlan 2020X diet and were not fasted prior to tissue harvesting.

Estrogen dosing protocol

Half of the OVX rats (n=12) were assigned to oral E2 treatment on a daily basis. Rats were administered a dose of 17-β-estradiol (E2) delivered via chocolate hazelnut spread (Nutella), as described by Ingberg et al. (Ingberg et al. 2012). A concentrated stock solution was made weekly by dissolving powdered E2 in sesame oil, which was further diluted and mixed to yield a dose of 28 μg E2/5 μL sesame oil/1 g nutella per kg body mass each day. All rats consumed the dose of E2 in less than a minute. SHAM and OVX rats not receiving E2 received a similar dose of nutella with sesame oil as a control.

Terminal surgeries and in vivo lipolysis assessment

Animals were not fasted overnight prior to terminal experiments in order to avoid increases in lipolysis preceding experiments with adipose explants or adipose tissue organ culture (ATOC). Rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg per 100 g body mass). Half of each SHAM, OVX or OVX E2 group (n=6) received a body mass adjusted saline injection and had ~200mg from each depot partitioned towards explant experiments (detailed below). The other half (n=6) of each group of animals received a 1 mg/kg dose of the β3 agonist CL 316,243 (CL) to assess in vivo lipolytic function. After 30 min post-saline or CL injection, inguinal adipose tissue (iWAT) was removed from the outer wall of the abdominal cavity as a representative subcutaneous depot; retroperitoneal (r.p.) adipose tissue was harvested from behind the kidneys as a representative visceral depot. Total fat pads were carefully
removed from each animal and weighed. iWAT and r.p. samples were snap frozen for subsequent analysis and stored at -80°C. A terminal blood sample was obtained via cardiac puncture, set aside for 1 hr., and centrifuged at 10000 x g for 15 min to collect serum, which was then stored at -80°C for subsequent analyses.

**Adipose Tissue Explants**

To assess lipolysis in vitro in SHAM, OVX and OVX E2 rats, adipose tissue explants were prepared immediately after terminal surgeries. Adipose tissue (iWAT and r.p.) were harvested from each saline-injected rat and ~150 mgs placed into petri dishes with 3 mL of M199 supplemented with 1% antibiotic/antimycotic, 2.5 nM dexamethasone and 2% BSA. In each dish, tissue was cut into ~50 mg fragments and left to equilibrate for 2 hrs. in a cell incubator, humidified and maintained at 37 °C and 95% CO₂/5% O₂. Following 2 hrs., cultures were treated with sterile H₂O as the control condition or 1 μM CL 316,243 for another 2 hrs. At the end of the experiment, 200 μL of media was collected, adipose tissue was rinsed in ice-cold PBS, strained, snap frozen in liquid nitrogen and stored at -80°C for further analyses. Tissue mass was recorded in order to normalize FFA and glycerol accumulation per gram of adipose tissue.

**Adipose Tissue Organ Culture**

A separate set of experiments was completed using female Sprague Dawley rats to assess the direct and combined effects of E2 and CL on adipose tissue lipolysis. Adipose tissue organ culture (ATOC) is a well-characterized method used to analyze isolated changes in adipose tissue metabolism with a longer washout period, uncoupled
from systemic factors and crosstalk with other tissues. Prior to terminal surgeries, rats were not fasted. Each animal was anaesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg per 100 g body mass), iWAT and r.p. depots were excised.

Four separate dishes per iWAT or r.p. depot were prepared with ~200 mg sections of adipose tissue. Tissue in each dish was minced into 5-10 mg fragments and placed into 4.5 mL M199 supplemented with 1% antibiotic/antimycotic, 50 μU insulin and 2.5 nM dexamethasone. Samples were left to equilibrate for 24 hrs. at 37°C, at which point the media was removed and changed to M199 supplemented with the same doses of antibiotic and dexamethasone, 2.5% bovine serum albumin (BSA) and no insulin. For the 2-8 hour time course experiment, one dish per depot was treated with each of the following conditions: sterile H₂O, 1 μM CL 316,243, 10 nM 17-β-estradiol, or 1 μM CL 316,243 plus 10 nM 17-β-estradiol. At 2, 4 and 8 hrs., 200μL samples of media were drawn and snap frozen for later analysis. After 8 hrs., tissue was rinsed with ice-cold phosphate buffered saline (PBS), strained, and adipose tissue minces were collected and frozen in liquid nitrogen and stored at -80°C for later analysis.

**Western blotting**

Clamp-frozen iWAT and r.p. adipose samples were homogenized in a 2 : 1 volume-to-weight ratio of NP40 cell lysis buffer supplemented with Protease Inhibitor Cocktail (PIC) and phenylmethylsulfonyl fluoride (PMSF) using the Fast Prep-24 Homogenizer. Lysed samples were centrifuged for 15 min at 2500 g at 4°C to separate the protein supernatant and remaining fat cake. The protein concentration of the supernatant was determined using the BCA method and the CV for this assay is accepted
at <5%. Equal amounts of protein (20μg) were loaded and separated on 10% acrylamide gels and wet transferred onto nitrocellulose membrane for 1 hr. at 200 mA. Membranes were blocked in Tris buffered saline–0.01% tween (TBST) supplemented with 5% non-fat dry milk at room temperature for 1 hr. with gentle shaking, rinsed with TBST and further incubated in TBST plus 5% BSA-diluted primary antibodies (1:1000) overnight at 4°C with gentle shaking. After 24 hrs., membranes were briefly washed in TBST and then incubated in TBST–1% non-fat dry milk supplemented with HRP conjugated secondary antibody (donkey anti-rabbit) for 60 minutes at room temperature. Bands were visualized using ECL and captured using the Alpha Innotech Imaging system. Each membrane was stained with ponceau and imaged to control for equal loading, although protein targets of interest were not normalized to ponceau density, since this did not change across groups or with CL treatment.

**Free fatty acid and glycerol concentrations**

Free fatty acid (FFA) in serum from saline and CL-injected rats, and from all ATOC/explant media samples, was measured using a commercially available kit (Wako Diagnostics, HR Series NEFA(HR)2 and detected on a 96 well plate. Glycerol was measured in serum and ATOC samples from SHAM, OVX and OVX E2 control or CL-treated samples using a commercially available kit (Sigma, MAK117) and 96 well plate according to manufacturers instructions.

**Insulin ELISA**

Serum samples from saline-injected SHAM, OVX and OVX E2 rats were
analyzed for insulin concentration using a commercially available kit (Millipore EZMRI-13K) according to manufacturers’ instructions. Accuracy and intra-assay comparisons were validated using two quality control standards (run in triplicate) provided with each kit. Serum samples were assayed in duplicate.

**Histological Analysis**

Five micrometer sections were mounted on 1.2-mm Superfrost slides, stained with modified Harris hematoxylin and eosin stock with phloxine (Fisher Scientific), and imaged (Olympus FSX 100 light microscope, Olympus, Tokyo, Japan). Two images per depot from each animal (~250-300 total cells) were captured and used to determine cross-sectional area (ImageJ software, National Institute of Mental Health, Bethesda, MD).

An estimation of cell number was calculated as described previously (31, 32). Briefly, the diameter of the cell (determined from cross-sectional area) was used and cells were assumed to be spherical; thus the adipocyte volume could be calculated. The density of the lipid in the cell (0.915g/mL) was then used to calculate the mass of a single cell (181). Finally, the mass of the fat pad was divided by the mass of a single cell to estimate cell number.

**Statistics**

All data are expressed as mean ± SEM. For comparing caloric intake and body mass, a two-way repeated measures ANOVA was used to assess group differences at each week and the interaction between Group x Time was analyzed. For measures involving CL 316,243 treatment in explants from SHAM, OVX and OVX E2 groups, a two-way ANOVA was used and the interaction between Group x Treatment (CL) was
analyzed. A two-way repeated measures ANOVA was utilized to assess differences in the 2, 4 and 8 hr. ATOC time course experiment. For adipocyte size measured by frequency distribution histograms, one way ANOVAs were run at each bin size, per depot. Tukey’s post hoc test was used in both ANOVA tests if significant differences were detected. In cases where data failed the Shapiro-Wilk test, a log(10) transformation was applied to normalize data. All alpha values were set to $\alpha = 0.05$ and significant differences are indicated using symbols visible in the figure legends.
Results

Pairfeeding does not prevent a greater body mass increase in OVX rats, but daily estradiol slows the rate of gain in OVX E2 treated rats

Table 3: Fat pad mass and serum insulin in SHAM, OVX and OVX E2 rats

<table>
<thead>
<tr>
<th>Fat pad mass (g)</th>
<th>Retroperitoneal</th>
<th>Inguinal</th>
</tr>
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<tbody>
<tr>
<td>SHAM</td>
<td>3.62 ± 0.31</td>
<td>2.67 ± 0.15</td>
</tr>
<tr>
<td>OVX</td>
<td>3.48 ± 0.25</td>
<td>3.67 ± 0.25*</td>
</tr>
<tr>
<td>OVX E2</td>
<td>3.41 ± 0.22</td>
<td>3.14 ± 0.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Insulin (pg/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>5.39 ± 0.27</td>
</tr>
<tr>
<td>OVX</td>
<td>4.68 ± 1.45</td>
</tr>
<tr>
<td>OVX E2</td>
<td>4.43 ± 1.06</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM; n=12 for each group. Statistical significance is accepted at p < 0.05; * denotes significant difference compared with SHAM rats.

Daily caloric intake was similar between groups, indicating successful pairfeeding (Figure 12A). Despite utilizing a daily pairfeeding regime to control for caloric intake in hyperphagic OVX rats, body mass was significantly increased in OVX and OVX E2 rats at week one; by week two OVX rats were still significantly heavier, but E2 treatment attenuated mass and OVX E2 were no longer different from SHAM controls (Figure 12B). Notably, both OVX and OVX E2 groups has significantly more mass accumulation during weeks 1-2, and rate of growth decreased in all groups between weeks 2 and 3 (inset Figure 12B). Interestingly, total r.p. fat pad mass did not differ between groups, but OVX and OVX E2 rats had significantly more iWAT (3.66 ± 0.25 and 3.14 ± 0.15 versus
2.64 ± 0.15 g, Table 3). This relationship held true even after correcting for body mass (0.010 ± 0.001 g/kg SHAM, 0.012 ± 0.001 OVX and 0.010 ± 0.0004 OVX E2). Uterine mass was significantly less in OVX and OVX E2 groups compared to SHAM controls, indicating atrophy and successful ovary removal in OVX and OVX E2 rats versus SHAM controls (0.67 ± 0.07g SHAM, 0.12 ± 0.007g OVX and 0.12 ± 0.01g OVX E2).

**Figure 12:** Food intake (A), body mass (B) and (inset) rate of increase in body mass in SHAM, OVX and OVX E2 rats. Data are presented as mean ± SEM. Statistical significance is accepted at p < 0.05; † denotes significant difference compared to SHAM control within weekly time point, ‡ denotes significant difference compared to OVX within weekly time point.
In vivo, basal and CL-stimulated plasma FFAs are reduced in OVX and OVX E2 rats, but the response to CL 316, 243 is normal.

In order to assess in vivo lipolytic response, rats were injected with a body mass adjusted dose of CL 316, 243. Basal and CL-mediated plasma FFA concentrations were reduced in OVX and OVX E2 rats versus the SHAM group, as indicated by a statistically significant group effect (Figure 13A). This was unique to FFAs, as there were no group or interaction effects evident in glycerol concentrations from the same rats (Figure 13B). The lipolytic responsiveness to CL was similar across groups and a significant CL effect was present regardless of ovarian phenotype (Figure 13B).

**Figure 13:** Plasma FFA (A) and glycerol (B) concentrations in control or CL 316, 243 treated rats. Data are presented as mean ± SEM; n=6 for control and CL groups in SHAM, OVX and OVX E2 groups. Statistical significance is accepted at p < 0.05; * denotes significant Treatment (CL) effect compared to own control (ie. SHAM CL vs. SHAM con); † denotes significant group effect compared to SHAM control.
Activation of key lipolytic enzymes p-HSL Ser660 and p-HSL Ser563 were measured in vivo by western blotting of r.p. (Figures 14A-B) and iWAT (Figures 14 C-D) depots to address mechanisms underlying reduced FFAs. There were no notable differences in basal or CL-treated phospho-HSL in r.p. or iWAT depots, as there were no group or interaction effects. Total HSL content was not different between groups (data not shown). Similar to FFAs and glycerol, there was a significant CL effect on HSL phosphorylation and activation in both depots, but no group effect between SHAM, OVX or OVX E2 rats.

**Figure 14:** Lipolytic signaling protein content in r.p. and iWAT depots (in vivo) from control or CL 316,243 treated rats. In the r.p. depot A) p-HSL Ser660 and B) p-HSL Ser563; and in the iWAT depot C) p-HSL Ser660 and D) p-HSL Ser563. Data are presented as mean ± SEM; n=6 for control and CL groups in SHAM, OVX and OVX E2.
Statistical significance is accepted at p < 0.05; * denotes significant treatment effect compared to own control.

Ex vivo lipolytic response is normal in OVX rats

To address whether depot-specific lipolytic alterations exist in OVX rats, adipose tissue explant experiments were completed in r.p. and iWAT of SHAM, OVX and OVX E2 groups to compare with in vivo findings. While differences in r.p. FFA concentrations (Figure 15A) and glycerol (Figure 15B) did not reach statistical significance, group effects were p=0.06 and 0.08, respectively, and the CL effect was significant in SHAM, OVX and OVX E2 rats. This was mirrored by robust increases in activation of p-HSL Ser660 and Ser563 in the CL-treated explants (Figures 15C-D). There were no significant changes in total HSL content between groups (data not shown).

Similar to the r.p. depot, the FFA and glycerol concentrations in iWAT explants were not different between SHAM, OVX, or OVX E2 rats, as indicated by insignificant group and interaction effects (Figures 16A and B). The observed trends in lipolysis were mirrored by the activation of p-HSL Ser563 and Ser660 residues in iWAT, which did not statistically differ between groups (Figures 16C and D), but a CL effect was statistically present in functional and western blotting measurements. Interestingly, the interaction effect (p=0.07) and group effect (p=0.11) in p-HSL Ser660 from iWAT tissue almost reached significance, presumably due to the elevated content in CL-treated OVX E2 rats. This trend was similar in the Ser563 residue, in which OVX E2 rats experienced a higher, but not statistically significant increase in activation. Total HSL was not significantly different across SHAM, OVX or OVX E2 groups (data not shown).
Estrogen does not independently increase lipolysis in adipose tissue, nor does it act in synergy with beta adrenergic stimulation

Since changes in whole-body, circulating FFA concentrations were observed in OVX rats, we conducted ATOC experiments to assess whether underlying mechanisms are directly due to addition of E2. Treatment with E2 (10 nM) did not have a significant effect on inducing lipolysis at 2, 4 and 8 hrs. in either r.p. or iWAT depots (Figures 16A and B). As expected, CL induced marked increases in FFA; however, E2 did not potentiate the lipolytic effect of CL, and although not statistically significant, the addition of E2 to CL in r.p. tissue fragments (Figure 17A) tended to attenuate FFA accumulation at 8 hrs. versus CL alone (p=0.10). Thus, differences in lipolytic function observed in Figure 2 do not appear to be directly due to E2.
Figure 15: FFA (A) and glycerol (B) concentrations in explant media from r.p. depots in control or CL 316, 243 treated rats. Panels C) and D) show protein content of p-HSL Ser600 p-HSL Ser563 in the r.p. depot. Data are presented as mean ± SEM; n=6 for control and CL groups in SHAM, OVX and OVX E2 groups. Statistical significance is accepted at p < 0.05; * denotes significant treatment effect compared to own control.
Figure 16: FFA (A) and glycerol (B) concentrations in adipose tissue explant media from iWAT depots in control or CL 316,243 treated rats. Panels C) and D) show protein content of p-HSL Ser600 p-HSL Ser563 in the iWAT depot. Data are presented as mean ± SEM; n=6 for control and CL groups in SHAM, OVX and OVX E2 groups. Statistical significance is accepted at p < 0.05; * denotes significant treatment effect compared to own control.
Figure 17: Time course of FFA release in control or CL 316,243 treated rats. Adipose tissue from r.p. (A) and iWAT (B) depots were treated with CL (1 uM), E2 (10 nM), or CL + E2 (1 uM + 10 nM) for 2, 4 and 8 hrs. Data are presented as mean ± SEM; n=6 for control and CL groups in SHAM, OVX and OVX E2 groups. Statistical significance is accepted at p < 0.05; † denotes significant difference compared to control at specific time point.

Circulating insulin is not different in OVX rats versus E2-treated OVX or SHAM controls

In order to determine whether the blunted FFA concentrations observed in vivo in OVX rats can be attributed to a systemic factor suppressing lipolysis, we measured circulating insulin concentrations in saline- treated SHAM, OVX and OVX E2 rats. There were no differences in insulin concentrations between groups (5.40 ± 0.27, 4.68 ± 1.45 and 4.43 ± 1.06 ng/mL respectively; Table 3).
Figure 18: Adipocyte cross sectional area and relative frequency % histograms in r.p. and iWAT tissue. (A) Subcutaneous (iWAT) cells tend to be larger in OVX rats (p=0.12), with no change in r.p. B) Representative images of H&E stains in both depots C) Histogram of retroperitoneal white adipose tissue (r.p.) cross sectional area and (D) inguinal (iWAT). Data are presented as mean ± SEM; n=5 per adipose depot per group.

Adipocyte cross-sectional area of iWAT from OVX rats is slightly elevated

Since iWAT fat pad mass was greater in OVX rats vs the SHAM or OVX E2 groups, we measured hematoxylin and eosin (H & E) stained slides to assess whether adipocyte hypertrophy could explain, or be associated with differences in adrenergic responsiveness to CL in the OVX and OVX E2 rats in vivo. Although not statistically significant, cross-sectional area was 40% and 26% increased in iWAT from OVX rats.
compared with SHAM and OVX E2 rats, respectively (Figures 18A and B). Given the tendency towards increased size, we next measured the frequency distribution of cross-sectional areas. While OVX rats tended to have a greater fraction of adipocytes over 6000 um² in both tissue types, these changes were not significantly different between groups (Figures 18C and D). From histological analyses of cell area, a gross index of cell size was also calculated to assess whether adipocyte hyperplasia is affected by E2 deficiency or re-introduction. Similar to cross-sectional area, mean adipocyte number was elevated 38% in OVX iWAT relative to SHAMs, but this did not reach statistical significance, and neither did cell number in r.p. adipose (data not shown).

Discussion

Estrogen loss is associated with increased adiposity in women (176, 303); this may be due to changes in adipogenesis, lipolysis or FFA oxidation. While E2 or HRT administration can prevent adipose accumulation in rodents (95, 219, 238, 293) and preserve gynoid fat distribution in women (245), the effects of E2 on in vivo and ex vivo basal and catecholamine-stimulated lipolysis in adipose tissue have been largely unexplored. We sought to address whether E2 can affect lipolysis in vivo and ex vivo within subcutaneous (iWAT) and visceral (r.p.) depots. The principal findings of this study were that: 1) in vivo FFA, but not glycerol concentrations, differed in OVX and OVX E2 rats compared with SHAM controls two weeks post-surgery; 2) lipolytic responsiveness and HSL activation with CL 316, 243, a β₃ adrenergic agonist, were not reduced in OVX rats in vivo; 3) ex vivo, lipolysis and activation of p-HSL Ser563 and Ser660 were not impaired in r.p. or iWAT explants from OVX rats, or augmented with oral E2 treatment; 4) ex vivo, E2 does not independently increase lipolysis in r.p. or
iWAT tissue, but had a subtle tendency to attenuate FFA accumulation in r.p. adipose after 8 hr. CL exposure; and 5) cross-sectional cell area and number were not significantly increased in iWAT adipose tissue from OVX rats, but tended to be slightly larger unless E2 treatment was administered.

The in vivo effects of E2 on lipolysis are sparse and yield conflicting results. In humans, Van Pelt et al found that acute conjugated estrogen (CE) infusion reduced basal glycerol release from subcutaneous adipose depots in postmenopausal women by 8-16% (226). The same group has found either reductions in plasma glycerol (227), or no observable effect with CE infusion (226). In rats, Darimont et al. found a 50% decrease in basal lipolysis in parametrial adipose tissue 8 days post-OVX, as measured by in situ microdialysis (53). In contrast to humans, reduced basal lipolysis was increased after 7 days of subcutaneous E2 injection. In our OVX and OVX E2 rats, in vivo plasma FFA, but not glycerol concentrations were reduced two weeks post-surgery versus SHAM controls. Daily E2 supplementation had no influence in restoring FFA concentrations to resemble SHAM rats. Reduced FFAs could indicate an increase in insulin-mediated suppression of lipolysis or hyperinsulinemia; however, several studies are in accordance with ours and show similar circulating insulin in human subjects with and without CE infusion and between intact and OVX rodents (52, 86, 226, 320).

Several factors could account for in vivo discrepancies between studies: subcutaneous versus parametrial fat pads in methodology, CE versus E2 administration, or species-specificity in β-adrenergic receptor distribution amongst female humans and rodents (164). Additionally, all studies previous to ours quantified glycerol accumulation or rate of appearance as the sole functional lipolysis marker; it therefore possible that E2
loss or repletion affect glycerol and FFA efflux, or futile cycling (321), which is not necessarily reflected by reporting only one measurement. Measuring plasma FFAs in circulation imposes limitations to our interpretations, as FFA uptake by peripheral tissues and oxidation by muscle and liver were not measured and would presumably affect systemic concentrations. It is also possible that altered glyceroneogenesis is a functional outcome of E2 loss, which we could not measure, but would affect glycerol concentrations in vivo.

Whether E2 status affects catecholamine response in vivo has been sparsely examined. In vivo treatment of female and male rats with E2 has been reported to increase lipolytic responsiveness to catecholamines in isolated adipocytes (17, 302). In humans, a single dose of E2 attenuated catecholamine-stimulated lipolysis in femoral subcutaneous adipose (89). We sought to extend observations of resting lipolysis by measuring in vivo lipolytic response to the \( \beta_3 \) agonist CL 316, 243 in subcutaneous and visceral depots. In our hands, the CL-induced effect was significant in eliciting whole-body lipolysis in SHAM, OVX and OVX E2 rats. Unlike Darimont et al. (53), who found increased responsiveness of subcutaneous adipose to isoproterenol infusion after 7 days of E2 injection, we did not observe an augmentation of plasma FFA or glycerol in OVX E2 rats after 2 weeks of treatment.

To gain insight into lipolytic mechanisms in OVX rats, we sampled r.p. and iWAT tissues from saline and CL-injected groups to assess HSL signaling in visceral and subcutaneous depots, respectively. In vivo, CL-stimulated HSL phosphorylation in r.p. adipose tissue at Ser660 and Ser563 were not different in SHAM, OVX, or OVE E2 groups. Trends were similar in iWAT. This would suggest that an upstream part of the
lipolytic cascade, a circulating factor, or adipose tissue morphology could be mediating alterations in lipolytic function in vivo. Others have shown reductions in isoproterenol and ACTH-stimulated adenylate cyclase (AC) activity and cAMP concentrations in parametrial, but not subcutaneous adipocytes isolated from OVX rats (159). A low dose of ethinyl E2 for 10 days or high dose for 3 days have both been observed to increase HSL activity in rats with intact ovaries (17). However, our study and others suggest that E2 may not directly regulate HSL, since total HSL, p-HSL Ser660 and 563 protein content in adipose and HSL mRNA expression in adipocytes did not change with OVX or OVX E2 (52). This data provides little insight into the effects of E2 on HSL activation or content, since these particular in vivo experiments were not designed to examine direct effects imparted by E2.

In vitro, visceral adipocytes from OVX rodents have shown elevated basal FFAs and reduced catecholamine responsiveness (159). In vivo E2 treatment for 4-30 days augmented in vitro response to β2 agonists forskolin, IBMX, epinephrine and isoproterenol in OVX E2 rats versus OVX and produced an overall improvement in lipolytic response (103, 219, 222, 243). Each of these studies examined adipocytes isolated by collagenase, devoid of additional cell types in adipose or cell-to-cell communication. Using explants, there were trends for in vitro r.p. FFA and glycerol release (group effects p=0.06 and 0.08, respectively) to be different amongst SHAM, OVX and OVX E2 rats, which was unexplained by similar trends in signaling in this depot. In iWAT, the CL effect was significant in each group, but there were no overall differences in FFA or glycerol accumulation. A dose of 28 μg/kg/day E2 in vivo did not elicit a significantly augmented response to CL in either depot. However, the interaction
between SHAM, OVX or OVX E2 and CL was nearly significant in iWAT p-HSL Ser563 and HSL660, which was unexpected. Despite well-documented evidence of estrogen deficient women accumulating visceral adipose tissue, the changes we (180) and others (89, 225) have observed in subcutaneous tissue suggest it may be more responsive to E2. Pedersen et al. found that women taking HRT had significantly elevated α2A antilipolytic receptor mRNA relative to the control group, and this was specific to subcutaneous fat (225). Healthy subcutaneous explants treated with 100 nM E2 for 24 hrs. also showed elevated α2A mRNA and receptor binding, coupled with an attenuated response of isolated adipocytes to epinephrine (225).

When considering results garnered from human and rodent models in terms of E2 action on lipolysis, human studies have almost exclusively used acute, non-genomic approaches (ie. 90-120 min. dose/infusion of E2 or CE) and assessed subcutaneous tissue, whereas rodent work has primarily utilized chronic, genomic methods (ie. 4-30 days, low to high dose E2) and assessed parametrial, visceral adipose. It is therefore not surprising that conclusions have been difficult to reconcile. To gain more insight and further examine possible mechanisms behind direct, non-genomic effects of E2 on lipolysis, we treated isolated iWAT and r.p. adipose fragments from female rats with E2 or E2 + CL for 2, 4 and 8 hrs. ex vivo and measured FFA release using ATOC. At the E2 dose administered (10 nM), there was no direct effect on stimulating lipolysis with or without CL. A useful next step would be to infuse rodents with E2 or CE and measure glycerol and FFA efflux from tissue, or plasma concentrations, in order to better compare model-specific findings in the context of E2 and lipolysis.
As another potential mechanism underlying changes in lipolysis between SHAM, OVX and OVX E2 rats, gross indices of adipocyte morphology were assessed. Fat pad mass was significantly greater in the iWAT depot of OVX rats versus SHAM controls as previously reported (309). Accordingly, OVX mean cell size and number were increased ~40% each in iWAT, although this did not reach statistical significance, whereas OVX E2 rats did not display different adipose depot or cell size compared with SHAMs. It has been suggested that adipose expansion during nutrient excess is depot specific, such that visceral accumulation is characterized by hypertrophy and hyperplasia drives subcutaneous growth in males (137). Our data suggest the possibility that subcutaneous adipocytes from OVX rats are modestly larger and more numerous than SHAM controls. Adipose tissue has demonstrated β adrenergic resistance to catecholamines (28, 132, 247) and reduced β1,2,3 receptor content and signaling (46), in vitro and in vivo, in obese rodents and humans, and has been postulated as a critical mechanism that contributes to fat accumulation. By two weeks post-OVX, it is possible that the development of iWAT hypertrophy results in defects in β adrenergic stimulation, further promoting adipose accretion and persistent disruptions in lipolysis. Other studies have shown increases in total visceral and subcutaneous adipose mass in rodent models of E2 deficiency at time points past the 2 weeks used in the current study (308, 309). Lipolytic function has not been examined in later stage E2 loss in rodents, or menopause in women, but hypertrophy is evident and may provide mechanistic insight into the etiology of adipose accumulation and impaired mobilization.

In conclusion, basal and β3-stimulated FFAs in OVX and OVX E2 rats were different than SHAM controls in vivo, in absence of any change in circulating insulin or
glycerol concentrations. Ex vivo, basal and CL-stimulated lipolysis and HSL activation were not impaired in subcutaneous or visceral adipose from OVX rats. Neither in vivo or ex vivo indices of lipolysis were altered by physiological doses of E2 for two weeks, but the interaction between SHAM, OVX, OVX E2 and produced a weak, but statistically insignificant interaction to regulate HSL Ser563 in iWAT explants. Acute E2 had no bearing on FFA or glycerol accumulation, or CL-induced lipolysis in either adipose depot in vitro, but had a subtle trend towards to attenuating CL-stimulated FFA accumulation. Lastly, subtle increases in fat pad weight and cell size specific to the iWAT depot occurred in vivo. Thus, changes in circulating FFA do not seem to be due to any rapid, non-genomic effects of acute E2 signaling, but rather reflect the importance of chronic estrogenic exposure for proper in vivo and subcutaneous adipose tissue lipolysis in rats.
CHAPTER 6: Integrative Discussion

6.1 IL-6 and intramuscular lipolysis

The first study in this thesis examined the ability of IL-6 to influence lipolysis in skeletal muscle, and more specifically whether IL-6 is required for eliciting IMTG breakdown, or plays a permissive role in the lipolytic effects of the catecholamine epinephrine (Epi). Incubation of isolated muscles with 75 ng/mL recombinant IL-6 produced a modest, non significant increase in lipolysis in fast-twitch, glycolytic EDL and had no detectable effect in slow-twitch, oxidative soleus. Similarly, IL-6 treatment increased p-AMPK and STAT3 in EDL but not soleus, indicating that indices of IL-6 signaling were increased in fast but not slow twitch fibers. In wildtype mice, Epi stimulated lipolysis in soleus, but this effect was ablated in global IL-6 knockout mice. Soleus from IL-6 KO mice had elevated basal lipolysis and increased ATGL and CGI-58 protein expression; however, this did not appear to be a consequence of IL-6 deletion directly, as incubation with IL-6 did not alter ATGL mRNA in soleus. Our data suggest that subtle increases in lipolysis occur with IL-6 incubation in fast-twitch fibers and conversely, Epi stimulates lipolysis in slow-twitch soleus. This is in agreement with previous reports of IL-6 stimulated lipolysis in rat EDL (146), and selective activation of slow-twitch soleus with exposure to Epi in vitro (230). In summary, IL-6 stimulates a modest increase in lipolysis in EDL in vitro, but not soleus. The presence of IL-6 appears to be required for the lipolytic effects of Epi in soleus, but does not elicit an independent effect in slow-twitch fibers.
Future directions and limitations

Since the publication of this data, others have shown that after 6 hrs. fasting in mice, plasma IL-6 is significantly elevated together with plasma FFAs and HSL Ser660 phosphorylation in epididymal WAT (326). Interestingly, IL-6 mRNA was increased in skeletal muscle, but not WAT, and the elevation in FFAs was abolished in IL-6 KO mice or in wildtype mice injected with an IL-6 neutralizing antibody. This would imply that the presence of IL-6 may be necessary, or permissive, for lipolysis in WAT even if the cytokine is derived from muscle. Deletion of IL-6 during development in the KO model may have produced metabolic adaptations secondary to ablation of IL-6, but it is interesting that with IL-6 neutralization in adult mice, plasma FFAs are reduced. When coupled with the findings in this thesis, the notion of muscle-derived IL-6 as a necessary lipolytic fuel sensor in times of energy demand, such as fasting and muscle contraction, becomes more evident. One study has since challenged the notion of IL-6 as a sensor in the context of carbohydrate metabolism, as O’Neill et al found that glucose uptake, carbohydrate utilization and AMPK activation were unchanged in IL-6 KO mice during acute exercise versus wildtype mice. In our hands, intact IL-6 signaling appears to be required for Epi-stimulated ex vivo lipolysis in soleus; however, it is unclear whether this effect is present in vivo with exercise. O’Neill et al concluded that while IL-6 appears important for skeletal muscle metabolism at rest, its effect may be overridden by stronger stimuli during exercise, such as sympathetic innervation or stress factors. Nonetheless, skeletal muscle derived IL-6 appears to regulate WAT lipolysis and skeletal muscle FFA mobilization, but the exact role of IL-6 in fat metabolism is not entirely clear.
Other lipid droplet proteins that regulate intramuscular lipolysis have been characterized more extensively since the IL-6 publication in this thesis. While we showed indirect effects of IL-6 deletion on ATGL and CGI-58 protein content, it is possible that IL-6 could change the activity of these proteins or modulate additional lipid droplet proteins, such as PLIN 1-5, to influence lipolytic rate. IL-6 has been shown to directly activate AMPK (84, 146) and IL-6 infusion increased whole-body lipolysis and fat oxidation in humans (102); however, its influence on other aspects of fat metabolism in muscle has been relatively unexplored. It is tempting to speculate that IL-6 modulates FA transporters to some degree, given its ability to increase FATP4 mRNA in primary muscle cells (3). Whether muscle-derived IL-6 can regulate FFA transporter (ie. CD36, FATP, FABPpm) content, localization, membrane docking or activity and/or FFA re-esterification into the TAG pool has not been explored using intact muscle. Had we been able to measure FFA release into the media, this would have allowed a calculation of FFA: glycerol to approximate re-esterification; unfortunately, the concentration was below detectable levels for commercially available kits, as an insignificant amount of FFAs are released from skeletal muscle lipolysis.

6.2 Estrogen, glucose uptake and glucose homeostasis

In the second study of this thesis, the aim was to determine at what point OVX rats become glucose intolerant, and compare the abilities of 4 weeks of aerobic exercise training with a daily, physiological administration of E2 to subsequently restore glucose tolerance. Marked glucose intolerance occurred by 10 weeks; at 14 weeks the OVX ex group had significantly improved glucose tolerance compared with OVX sedentary rats, whereas E2 treatment partially (statistically insignificant) restored glucose tolerance.
Increased plasma insulin was not responsible for improved glucose clearance in the OVX ex group. Surprisingly, basal and insulin-stimulated glucose uptake and Akt Ser473 and Thr308 phosphorylation in skeletal muscle were not impaired in OVXs, nor increased in the OVX ex group. Although we did not measure glucose uptake into adipose tissue depots, insulin injections during terminal surgeries allowed global quantification of insulin signaling (Akt phosphorylation) in numerous peripheral tissues (soleus, EDL, liver, iWAT, VAT). There were no differences in soleus or EDL insulin signaling in regards to exercise, E2 treatment, or OVX. However, p-Akt was increased in subcutaneous AT from OVX E2 and OVX ex rats, suggesting the possibility that insulin action was improved specifically in this tissue in both treatment groups. Overall, the most impactful finding in this study is that exercise appears to be more effective than E2 at treating perturbed glucose tolerance secondary to E2 deficiency. This warrants investigation in humans, as findings would be significant for the status of women’s health.

*Future directions and limitations*

While other studies have acutely exercised OVX mice with a single bout to assess lipolytic signaling (320), or used chronic repeated bouts to prevent OVX-elicited metabolic dysfunction (168, 233, 271), their exercise tolerance is visibly limited compared with ovary intact rats, yet this hasn’t been directly measured. Underlying mechanisms for reduced exercise tolerance in OVX rats could be lower sporadic activity (309), excess adipose accretion and biomechanical difficulties (95) or possibly sympathetic dysfunction (169). Whether a similar impairment occurs in postmenopausal women has not been confirmed. Furthermore, it is possible that treadmill running would
not be most effective for improvements in glucose homeostasis in terms of practicality and adherence in women. Others have used team sport or group activity as a means to introduce exercise training, and significant improvements in vascular parameters were reported (210). Our current findings indicate that even with a relatively modest stimulus in treadmill training compared with a standard protocol in males or intact female SHAMs, there were significant improvements in glucose tolerance after 4 weeks. One logical, next step approach would be to assess the efficacy of different exercise doses or types at mitigating glucose intolerance during E2 deficiency; for example, whether high intensity interval training or a more prolonged, lower intensity series of bouts prove most beneficial.

Human studies often recruit pre versus postmenopausal women, in which case subjects are often age-matched, but aging could still confound the effects of E2, given that changes in metabolism, hormone secretions and body composition occur independently with increasing age (14) and could affect glucose homeostasis. Despite some criticisms of the OVX model being inappropriate due to the abrupt cessation of ovarian hormones, one strength of this thesis is that any observable effect in the E2 group is due to the physiological effects of the hormone in absence of age-related influences. Alternative models of estrogen loss include ERα, ERβ or aromatase knockouts that progress through all developmental stages in absence of E2 receptor binding, with likely compensatory changes in other metabolic pathways (47, 139, 249). It is also important to note that it is not only E2 that is lost with ovarian removal, so hormones such as progesterone are often included with E2 when re-introduced to rats or humans (66, 105). It is becoming increasingly evident that E2 has a generally positive effect on glucose
homeostasis and related parameters, such as mitochondrial function (42), whereas inclusion of progesterone may be deleterious (175). However, it is critical to remember E2 loss alone does not drive the metabolic consequences of OVX, since other ovarian factors, such as progesterone, could play a role in metabolic regulation. More work is required to assess the underlying mechanisms of action of ovarian secreted hormones, and how these factors interact to regulate glucose homeostasis.

Another future area of pursuit would involve exploring dose-dependent and timing effects of estrogen and exercise. A particularly interesting viewpoint is the “timing hypothesis”, which suggests that E2 exerts positive effects up to a certain point beyond menopause, after which these effects stop being beneficial and may actually cause harm (307). Subsequent studies should focus on a time course of E2 deletion and whether an optimal age for E2 utilization exists in combatting metabolic disease, particularly in humans; specifically whether E2 and exercise could be used as adjunct or successive therapies depending on age. Stratification of subjects in observational studies is of critical importance for dissecting the mechanisms of E2 timing and action, and may dovetail with research examining changes in ER content across peripheral tissues with menopause, E2 repletion or exercise. Perhaps E2 and exercise can be used in succession as staged, step-wise treatment options in postmenopausal women, since E2 and HRT combined do not appear to elicit synergistically positive effects on glucose metabolism, but each approach appears to be beneficial for glucose tolerance and homeostasis. Accordingly, one major limitation of the second study in this thesis was the lack of plasma E2 measurements. Several kits are available for measuring estradiol, but the cross-reactivity with testosterone, androgens, estrone, estriol, and other estrogen metabolites prevent a reliable
17-β-E2 measurement. A previous study with the same dose and mixture of estradiol, sesame oil and nutella produced circulating 17-β-E2 within a physiological range in rats (123, 124). Since the precise plasma concentrations were not quantified, this restricted us from ensuring OVX rats were E2 deficient, or that the particular dose we utilized elicited elevated E2 in the OVX E2 group. To circumvent this limitation, confirmed uterine atrophy is commonly used as a proxy for E2 depletion; furthermore, the dose administered produced functional outcomes in this study, as observed by significant improvements in glucose homeostasis. Development of an accurate ELISA or similar kit to reliably measure 17-β-E2 would be valuable for future studies.

There is increasing evidence that estrogen is critical for glucose homeostasis in not only females, but males as well, suggesting we are in the initial stages of understanding E2 metabolism and its physiological impact. For example, one case study has shown that a unique aromatase gene mutation in men produces extremely deleterious effects (183). This 29 year old man presented with insulin resistance, T2D and liver steatohepatitis that were each improved with 17-β-E2, but worsened with testosterone treatment. Coupled with evidence of insulin resistance in male ERα knockout mice, this suggests a vital role for estrogen signaling completely devoid of its necessity for fertility and reproduction in women. One hypothesis worth pursuing is that a functional estrogen:testosterone ratio is imperative for maintenance of glucose homeostasis, or that loss of adequate estradiol precedes insulin resistance in men and women. While many relationships show an association between estrogen status and carbohydrate metabolism (35, 213), other comorbidities and confounding factors are often present, such as aging or non-alcoholic fatty liver disease (33, 183), which independently contribute to insulin
resistance. More mechanistic exploration is required to assess the direct role of estrogen in insulin sensitivity: Is it required, or permissive of insulin release at the pancreas? Does its deletion act on specific tissues to predispose men and women to insulin resistance? Does manipulating the biochemistry of sex steroid hormone production allow preservation of glucose homeostasis, such that estrogen is maintained in a greater ratio to testosterone?

The potential for overlap between estrogen and exercise signaling pathways is intriguing and may provide insight into why exercise could be an effective alternative to HRT or synthetic E2 administration, aside from its well-documented adaptations that benefit glucose homeostasis. One viable area of research may lie in the Estrogen Related Receptor (ERR) family, which is upregulated by PGC-1α after exercise, regulates mitochondrial biogenesis (274), and may also restore classical ERα/ERβ mediated signaling (275). It is tempting to speculate that exercise training could, therefore, rescue estrogen signaling in deficient women and mitigate the need for pharmacological E2 regimes. Since our understanding of the targets downstream of ERα/ERβ is still quite limited, this system may require further analyses before integration with ERR is possible. However, this would be a logical extension of estrogen and exercise in the context of glucose metabolism. Some have also suggested that the relative tissue distribution or ratio of ERα:ERβ could change with metabolic perturbations, ie. high fat diet, to alter aspects of glucose homeostasis (91). It is then conceivable that the robust biochemical adaptations induced by exercise training could produce changes in E2 receptor distribution in skeletal muscle, adipose and liver.
6.3 Estrogen and adipose tissue lipolysis

The purpose of the third study in this thesis was to determine if adipose tissue lipolysis becomes dysfunctional during estrogen deficiency, in both visceral and subcutaneous depots, using in vivo and in vitro methods. Basal and CL 316,243-stimulated lipolysis were different, and tended to be lower in OVX and OVX E2 rats versus SHAM controls, which would imply some level of derangement in lipolysis. However, in r.p. and iWAT depots harvested after in vivo injection with CL, HSL Ser 660 and Ser 563 phosphorylation did not appear to be compromised. In vitro, there were no significant differences between SHAM, OVX or OVX E2 groups in FFA or glycerol release in adipose explants, or HSL phosphorylation in r.p. adipose. Basal and CL-stimulated lipolysis were not different in iWAT from SHAM, OVX or OVX E2 rats, despite the noted near-significant interaction effect of E2 and CL in augmenting HSL Ser563 phosphorylation in the E2-treated group. To ascertain if these effects, although subtle, were due to E2 directly or an indirect secondary mechanism, r.p. and iWAT fragments were cultured using ATOC and treated with E2, CL or E2+ CL for 2, 4 and 8 hours, and we found no ability of E2 to stimulate lipolysis or act in synergy with CL at the concentrations used. The next step was to assess whether a secondary factor could be driving changes in lipolysis in the in vivo and in vitro models. Circulating insulin levels were not different in SHAM, OVX or OVX E2 rats in vivo, but iWAT adipose mass was significantly greater than SHAM rats, which was attenuated with 2 weeks of oral E2 treatment. Accordingly, although not statistically significant, cell size was 40% larger in adipocytes from OVX rats in the iWAT depot and r.p. adipocytes were unaffected by
OVX or E2 supplementation. Overall, E2 appears to be necessary in maintaining in vivo, whole-body adipose lipolysis, although mechanisms do not appear to be direct.

**Future directions and limitations**

One critical consideration in this field is that all OVX studies, with the exception of one (243), have been performed in rats with intact adrenal glands. An insightful approach in future studies would be to utilize a dual ovariectomized-adrenalectomized rodent model, since adrenals produce estrogenic precursors that can be converted by aromatase to bioactive estrogen. Furthermore, and importantly, adipose tissue expresses aromatase and ERα and ERβ receptors (184) that bind 17-β-E2 with high affinity (224). It is possible that paracrine estrogen is actively regulating adipose tissue, yet this has not been examined. Whether this signaling pathway is of increased importance with greater fat pad mass and estrogen deficient obesity is a distinct possibility. The relationship between estrogen status and adiposity is becoming more clear, such that its loss leads to increased fat mass in women, but the relationship with aromatase expression and the contribution of adipose tissue to estrogen production warrants investigation.

All of the studies examining adipose tissue lipolytic function and signaling utilized E2 fed or injected rats and subsequently isolated adipocytes by collagenase treatment, which destroys complex cellular interactions during extraction (52, 159, 219, 221, 224, 225). This methodology may explain the in vivo to ex vivo differences in basal lipolysis, as intact adipose tissue maintains autocrine communication between adipocytes and neighbouring cells. Importantly, in all studies except one to date (3), rodents were administered estradiol in the absence of pair-feeding, rendering it impossible to comment
on whether lipolytic impairments or subsequent improvements in OVX rodents were due to direct effects of E2 on adipose tissue, secondary to hormone-induced changes in central appetite regulation and hyperphagia (1), or unique to isolated adipocytes. One major advantage of this study was the utilization of adipose tissue explants and ATOC, which both preserve non-adipocyte to adipocyte cell communication and more closely represent an intact adipose tissue environment. Regarding adipose tissue explants, an acute, 2 hr. washout period prior to treating with CL in the SHAM, OVX and OVX E2 rats was purposely chosen to allow recovery from surgery and presumably allow the unique sex steroid environments to be maintained during experiments. Since we could not accurately measure E2 in plasma or ATOC media, it is impossible to comment on the inherent presence of E2 during explants. Regardless, this was the preferred method, since differences in SHAM to OVX characteristics would be more likely washed out after a 24 hr. versus 2 hr. recovery period. Conversely, with ATOC we used a longer, 24 hr. washout period to measure the direct effects of E2 and better rid adipose tissue of any genomic, chronic effects of E2 signaling/metabolism compared with a shorter washout. This is an important distinction from explants and could explain why findings have been discrepant regarding the effects of E2 on lipolysis. Pairing in vivo and in vitro approaches within the same rats allowed a unique perspective and dissection of genomic and non-genomic mechanisms of E2 action. There is little to no information available that details cytoplasmic signaling cascades elicited by E2 binding to its membrane receptor in muscle, adipose and liver, and this would be a critical advancement in our understanding in estrogen metabolism. Others have identified an ability of E2 and its metabolites to activate AMPK and Akt (51, 52, 258), but this requires validation and investigation into
which kinases are involved in the underlying mechanisms, and what type of effect is produced functionally.

Several factors are worth considering when evaluating estrogenic effects in females, as there have been relatively few studies and therefore, limited attempts at repeatability. Many authors have cited differences in estrogen composition- ethinyl estradiol, 17-β-estradiol, conjugated estrogens, estrogen plus progesterone; route of delivery- oral ingestion, transdermal, various injection sites; duration of supplementation- acute (1-4 hours), or more chronic timescales (1-4 weeks), or upwards of 4 weeks; dosage; time point of estrogen implementation- immediately after E2 loss, or upwards of 8 weeks after depletion. Different permutations of these factors mean that this research area is quite inconclusive in terms of metabolic effects of E2 in fat metabolism, listing no effect of estrogen, augmented lipolytic response, or attenuated response in the presence of estrogen. To further complicate this, some studies have aging as an additional factor to consider. As biomedical, metabolism and human health research progresses to utilizing females more frequently, our understanding of metabolic regulation in females will continue to become clearer. In the meantime, sparse data and few studies, when compared with our knowledge of males, incites a massive limitation in our understanding of estrogen and its function.
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Appendix I: Pairfeeding does not attenuate weight gain in OVX rats compared with ad libitum OVX rats

Figure 19: Body mass in SHAM, OVX pairfed and OVX ad libitum fed rats. Body mass in SHAM, OVX pairfed and OVX ad libitum fed rats. Data are presented as mean ± SEM. Statistical significance is accepted at p < 0.05; * denotes significant difference compared to SHAM control within weekly time point, † denotes significant difference compared to OVX pairfed within weekly time point.
Appendix II: Composition of Harlan 2020X Diet

## 2020X

Teklad Global Soy Protein-Free Extruded Rodent Diet

Product Description: 2020X is a fixed formula, non-sterilizable extruded diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. 2020X does not contain alfalfa or soybean meal, thus minimizing the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces cholesteryl, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. Also available certified (20200X) and irradiated (2020IX). For autoclavable diet, refer to 20200AS (sterilizable).

### Macronutrients

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>19.1</td>
</tr>
<tr>
<td>Fat (acid hydrolysis)</td>
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</tr>
<tr>
<td>Carbohydrate (available)</td>
<td>47.0</td>
</tr>
<tr>
<td>Crude Fiber</td>
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<tr>
<td>Neutral Detergent Fiber</td>
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</tr>
<tr>
<td>Ash</td>
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<tr>
<td>Energy Density</td>
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</tr>
<tr>
<td>Calories from Protein</td>
<td>24</td>
</tr>
<tr>
<td>Calories from Fat</td>
<td>16</td>
</tr>
<tr>
<td>Calories from Carbohydrate</td>
<td>60</td>
</tr>
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</table>

### Minerals

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
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</tr>
<tr>
<td>Phosphorus</td>
<td>0.7</td>
</tr>
<tr>
<td>Non-Phytate Phosphorus</td>
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</tr>
<tr>
<td>Sodium</td>
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</tr>
<tr>
<td>Potassium</td>
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</tr>
<tr>
<td>Chloride</td>
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</tr>
<tr>
<td>Magnesium</td>
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</tr>
<tr>
<td>Zinc</td>
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</tr>
<tr>
<td>Manganese</td>
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</tr>
<tr>
<td>Copper</td>
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</tr>
<tr>
<td>Sulfate</td>
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</tr>
<tr>
<td>Iron</td>
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</tr>
<tr>
<td>Selenium</td>
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### Amino Acids

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<th>Value (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic</td>
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</tr>
<tr>
<td>Glutamic</td>
<td>3.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.2</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.7</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.6</td>
</tr>
<tr>
<td>Proline</td>
<td>1.8</td>
</tr>
<tr>
<td>Serine</td>
<td>0.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.3</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Valine</td>
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<tr>
<td>Phenylalanine</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Arginine</td>
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### Vitamins

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<thead>
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<tr>
<td>Vitamin D</td>
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</tr>
<tr>
<td>Vitamin E</td>
<td>110</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin B6</td>
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</tr>
<tr>
<td>Vitamin B2</td>
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<tr>
<td>Niacin</td>
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<tr>
<td>Riboflavin</td>
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<tr>
<td>Pantothenic Acid</td>
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<tr>
<td>Vitamin B6 (pyridoxal)</td>
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<tr>
<td>Thiamine</td>
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<tr>
<td>Folate</td>
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<tr>
<td>Choline</td>
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### Fatty Acids

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<tr>
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</tr>
</thead>
<tbody>
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<td>C16:0 Palmitic</td>
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</tr>
<tr>
<td>C18:0 Stearic</td>
<td>0.1</td>
</tr>
<tr>
<td>C18:1 n-9 Oleic</td>
<td>1.1</td>
</tr>
<tr>
<td>C18:2 n-6 Linoleic</td>
<td>3.6</td>
</tr>
<tr>
<td>C18:3 n-3 Linolenic</td>
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</tr>
<tr>
<td>Total Saturated</td>
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</tr>
<tr>
<td>Total Monounsaturated</td>
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<tr>
<td>Total Polyunsaturated</td>
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### Other

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>–</td>
</tr>
</tbody>
</table>

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* Other extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to either extract, the fat values for acid hydrolysis will be approximately 1% point higher.

* Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

* Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicelluloses, and lignin. Crude fiber methodology underestimates total fiber.

* Energy density is a calculated estimate of metabolizable energy based on the Atwater factors assigning 4 kcal to protein, 9 kcal to fat, and 4 kcal to available carbohydrates.

* Indicates added amount but does not account for contribution from other ingredients.

* 1 IU vitamin A = 0.3 μg retinol

* 1 IU vitamin D = 25 μg cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.
Appendix III: Tissue homogenization protocol

Homogenization buffer
NP40 Cell Lysis Buffer (Thermo Fisher Scientific):

- 50 mM Tris, pH 7.4
- 250 mM NaCl
- 5 mM EDTA
- 50 mM NaF
- 1 mM Na3VO4
- 0.02% NaN3

Supplemented with:
- 1 mM Protease Inhibitor Cocktail (PIC; Sigma)
- 1 mM PMSF (Sigma)

Cell Lysis buffer, PIC and PMSF are stored at -20°C and thawed on ice prior to use day-of.

Protocol
- Combine Cell Lysis buffer, PIC and PMSF and store on ice
- Prepare labeled lysing matrix D homogenization tubes
- Quickly add 10x tissue weight in Lysis Buffer volume to muscle (ie. ~25 mg in 250 µL buffer), 3x volume to VAT or iWAT frozen tissue, 2x volume to explant or ATOC samples, or 30x volume to snap frozen liver in separate homogenization tubes (MP Biomedicals)
- Homogenize using FastPrep sample preparation system (MP Biomedicals)
- Transfer homogenate to a 1.5 mL eppendorf tube
- Spin eppendorf tube at 1500 x g for 15 min. at 4°C
- Aliquot the supernatant into 1.5 mL eppendorf tube **for adipose samples, avoid transferring the fat cake portion that is evident superficially
- Store tubes at -80°C
Appendix IV: BCA protein assay protocol

Used to determine protein concentration in muscle, adipose or liver to prepare for western blotting

- Dilute homogenate (v/v) in distilled H₂O
  - 1:10 muscle, 1:5 adipose, 1:20 liver
  - Maintain homogenate tubes on ice while running the BCA assay
- Prepare BSA standards by serially diluting 2 mg/mL BSA to 1, 0.5, 0.25, 0.125, 0.65 and 0.32 mg/mL
- Add 10 µL of sample or standard to a 96 well plate in triplicate
- Prepare BCA reagent by adding 200 µL 4% (w/v) CuSO₄ with 10 mL bicinehoncininic acid (Sigma)
- Add 200 µL solution to each well containing sample or standard and mix gently
- Incubate, covered, at 37°C for 30 min (in oven or incubator)
- Using spectrophotometer microplate reader (Softmax software):
  - Select the BCA Assay protocol
  - Reading type: absorbance, endpoint
  - Wavelength: 562 nm
  - Setup template to include samples and standards, including the dilution factor utilized
  - Accept adjusted concentrations if the CV per triplicate is <5%; standard curve should read at r² = 0.99-1.00
- Discard diluted samples and move forward to western sample preparation protocol
Appendix V: Western Blotting

Reagents:
1. Laemmeli Buffer 4.0x
   - 50 mL 0.5 M Tris-HCL (pH 6.8)
   - 8.2 g SDS
   - 40 mL glycerol
   - 500 µL 1% Bromophenol Blue
   - 31 mg DTT to 500 µL of 4.0x Laemmeli buffer

   • Bring volume to 100 mL with ddH2O and pH to 6.8.

2. 10 X Running Buffer
   - 30 g Tris Base
   - 144 g Glycine
   - 10 g SDS

   • Bring to 1 L with ddH2O, dilute 10X to 1X for day-of use

3. 1 X Transfer Buffer
   - 80 mL 10 X Transfer Buffer
   - 160 mL Methanol
   - 560 mL ddH2O

4. 10 X Tris Buffered Saline (TBS)
   - 24.2 g Tris Base
   - 80 g NaCl

   • Adjust pH to 7.6 with HCl
   • 1 X TBS: 100 mL TBS, 900 mL ddH2O, 500 µL Tween 20

Protocol:

Gel Electrophoresis assembly:
• Clean glass plates, rinse one long and one short plate per planned gel well with ddH2O. Wipe plates carefully with KimWipes allow to dry.
• Assemble the glass plates and place into casting frame keeping the short plate facing towards the front. Ensure both plate bottoms are flush on a level surface and lock the green pressure cams to secure plates in place. Secure the casting frame in the casting stand.

Cast Gel:
• Prepare running gel to be cast prior to the stacking fraction. For this thesis, 10% acrylamide gels were used for every protein target, with the exception of 15% for COXIV.
• ** add APS and Temed very quickly at the end, swirl gently to initiate polymerization and pour between the two plates.
• Add ddH₂O to the top of the running gel before it sets in order to eliminate air bubbles and maintain a straight polymerization line.
• After the running gel polymerizes, drain ddH₂O.
• Pour the stacking solution between glass plates until the top of the short plate is reached. Insert the comb (10 or 15 wells, depending on sample number) and allow gel to polymerize.

Gel Electrophoresis:
Assembly of electrophoresis module and sample loading:
• Remove casting frame from stand, place gel cassettes into green electrode assembly.
• Slide the gel cassettes into the green clamping frame with electrodes. Press down on the electrode assembly while closing the two cam levers of the frame, make sure gels are aligned horizontally and are placed as far down as possible in the clamp. Place whole assembly into the mini-tank.
• Be sure the short plate faces inward toward the middle white barrier. Use tall electrodes if only running 1-2 gels; add the short assembly if running 3-4 in one tank. If only using one gel, be sure to use a buffer dam.
• Fill between the gels with 1X running buffer. Gently tip the mini-tank to allow running buffer to escape the gel space and rinse the wells created previously by the green comb.
• Load one well of 7 µL molecular weight marker on the leftmost well of each gel. Load samples slowly into each subsequent well (~5-40 µg protein, depending on the target(s)) from left to right. Record order of sample loading.
• Fill the tank to the 2 or 4 gel line with 1X running buffer.
• Connect the apparatus to the power pack. Run at 100 V until sample exits the stacking gel, then increase to 120-140V until sample reaches the bottom of the gel.
• Remove the gels to a tray for preparing transfer units.

Transfer Blotting
• Prior to use, each nitrocellulose membrane must be cut to the dimensions of the gel and immersed in running buffer for for 5-10 minutes.
• Soak the membrane, 4x filter paper per cassette, and 2x fibre pads in transfer buffer.
• Prepare the gel sandwich.
  o Place one pre-wetted fiber pad on the black side of the cassette.
  o Place 2x sheets of filter paper on the fiber pad.
  o Place the gel on the filter paper, either in full or cut at specific molecular weight for the required target.
  o Place the pre-wetted membrane on the filter paper.
  o Gently roll to remove any air bubbles.
  o Place a filter paper on the gel.
  o Complete the sandwich by placing the fiber pad on the filter paper.
  o Remove any air bubbles which may have formed with the small rolling tool by gently going over the transfer fiber pad horizontally and vertically.
  o Close the cassette firmly, being careful not to move the gel and filter paper.
sandwich.
  o Lock the cassette with the white latch and align black to black within the red and black transfer unit.
  o Add an ice block and fill the tank with 1X transfer buffer.
  o Put on lid, plug the cables into the power supply, and run at 0.02 mA per tank for 60-90 minutes.
  o After the allotted transfer time, remove the blotting sandwich, trim the membrane and place in a clean petri dish for antibody binding and subsequent development.

Membrane Blocking and Incubation
  • Block membrane with TBST and 5% w/v skim milk for at least 1 hr. at room temperature with gentle agitation.

Antibodies
  • Dispose of blocking solution, rinse the membrane with TBST for 15 min. and add the appropriate 1° Antibody- either frozen stock or prepared fresh, 1:1000 in TBST and 5% BSA or skim milk
  • Incubate with gentle agitation at 4°C overnight.
  • The following day, remove the 1° Antibody using a transfer pipette and store at -80°C until next use.
  • Rinse the membrane 2x 15 min. in TBST
  • Add the appropriate 2° Antibody (dependent on 1°) in TBST with 1% w/v skim milk
  • Leave the membrane to shake/rock at room temp for 1 hr.
  • Wash 2x15 min with TBST and 1x 10 min. with TBS

ECL (Enhanced Chemiluminesence)
  • Pour out all TBS from the petri dish.
  • Combine Western Lightning ECL (PerkinElmer) solutions in equal fractions as per directions (~4 mL/membrane, 2 mL of each clear or brown solution) in a separate container. Pour over membrane and incubate with agitation for 30 sec.
  • Transfer the membrane to plastic wrap on the tray within the AlphaInnotech imager, lay flat and ensure no air bubbles are present.
  • Adjust light and image settings using gross and fine-tune knobs and appropriate exposure times. Collect 5-10 stacked frames per blot, and save the image to the desktop.
  • Quantify the band density using AlphaInnotech software.
Appendix VI: Maximal Glucose Uptake

REAGENTS
1. Weekly Buffer (Base solution for M1, M2, & M3)
   - Krebs Henseleit Buffer
   - 0.1% Bovine Serum Albumin

2. Medium 1 (Pre-Inc, pregassed with 95% O₂ 5% CO₂)
   - 8 M Glucose
   - 32 M Mannitol

3. Medium 2 (Wash, pregassed with 95% O₂ 5% CO₂)
   - 36 M Mannitol
   - 4mM Pyruvate

4. Medium 3 (Uptake Buffer, pregassed with 95% O₂ 5% CO₂)
   - 8 mM 3-O-Methyl-D-Glucose
   - 28 mM Mannitol
   - 4 mM Pyruvate
   - 0.5 µL/ mL Medium 3 of ³H-Methyl-O-Glucose (1 mCi/ 1000 µL)
   - 1.0 µL/ mL Medium 3 of ¹⁴C-Mannitol (250 µCi/ 1250µL)

** Make just prior to addition; insulin is more stable with 0.1% BSA.
** Add 15 µL of Insulin stock/ 2 mL buffer (final [ ] =10 mU/ mL)
** Add insulin to all vials for insulin stimulation (M1, M2 & M3)

PROTOCOL
1. Incubate muscle strips (~10-15 mg) for 30 minutes in 2 mL Medium 1 (+/- insulin)
2. Wash muscle strips two times, 10 minutes each in Medium 2 (+/- insulin)
3. Incubate muscles for 20 minutes (+ insulin) or 40 minutes (- insulin) in Medium 3 (+/- insulin)
4. Blot muscle dry, cut tendons and weigh
5. Solubilize muscles by boiling for 10 minutes in 1 mL of 1 M NaOH. Vortex 1-2 times during solubilization and keep the vial covered
6. Sample 200 µL of the muscle digest into scintillation vials and add 5 mL of cocktail. Prepare duplicates for each sample
7. Allow samples to quench overnight in a dark place
8. Count samples, 5 minutes per sample
Appendix VII: RNA Extraction & Reverse Transcription

1. Add 1mL TRIzole (Invitrogen) reagent to a homogenization tube, add sample, and homogenize for 60sec in FastPrep machine (MP Biomedicals).
   - TRIzole contains phenol and should be handled in the fume hood
2. Centrifuge at 12 000 x g for 10min at 4°C.
   - Removes large amounts of proteins, polysaccharides, & fats from sample
3. Transfer supernatant to a new 1.5mL tube and incubate at RT for 5min.
4. Add 20 μL chloroform to each sample for every 1mL of TriPure used and shake vigorously by hand for 10-15sec.
5. Incubate at RT for 15min, centrifuge at 12 000 x g for 15min at 4°C, and transfer supernatant to a new 1.5mL tube.
   - The clear upper phase (supernatant) contains DNA & RNA
6. Add 500μL isopropanol for every 1mL to TriPure used. Invert 5-6 times to mix.
   - The RNA is precipitated from the TRIzole by the isopropanol
7. Transfer 700uL of mixture onto a pink Qiagen spin column (RNeasy Kit, Qiagen) and centrifuge at 12 000 x g for 15sec.
8. Discard flow-through. Add remaining sample mixture (~700 μL) onto the same column, and repeat spin.
   - The column can hold up to 100ug of RNA
9. Discard flow-through. Add 350μL of Wash Buffer RW1 to the column and centrifuge at 12 000 x g for 15sec.
10. Discard flow-through.
11. Transfer column to a new collection tube, wash with 500uL of Buffer RPE. Centrifuge at 12 000 x g for 15sec.
12. Discard flow-through, repeat wash with 500uL of Buffer RPE, and spin at 12 000 x g for 2min at 4°C.
13. Transfer column to a new tube.
14. Elute the RNA in 30μL of RNase-free H₂O, incubate for 1min at RT. Centrifuge at 12 000 x g for 1min.
   - The H₂O removes the RNA from the column
15. Set pipette to 50 uL, take bottom volume and pipette over the whole column filter again. Incubate for 1-min at RT and centrifuge at 12,000xg for 1min.
16. Throw out pink tube. RNA is in the bottom eppendorf.
17. RNA is extracted, next steps are for DNA removal:
18. Centrifuge DNA kit contents for 1 min.
19. Add 0.1x volume of 10X DNA buffer (Assume sample volume = 30 μL, so add 3 μL). Mix gently using a pipette.
20. Add 1 μL of rDNase. Incubate tubes for 30 minutes at 37° C.
21. Add 0.1x volume DNAse inactivation reagent, centrifuge for 1.5 minutes at 10 000 x g. Label another set of tubes.
22. Remove clear supernatant without touching the bottom pellet, add to fresh tubes (set pipette to 50 μL).
23. Determine RNA concentration using nanodrop.
Appendix VIII: Intraperitoneal Glucose Tolerance Protocol (IPGTT)

1. Give each rat a clean cage and begin fasting them (6 hour fast) with water ad libitum.

2. The next morning, or the afternoon before testing, weigh each rat (weight will determine the amount of glucose to inject).

3. Glucose solution:
   - 50% glucose: 9 mL H2O + 5g D-glucose. Then adjust to 10 mL with ddH2O.
   - This dilution will get 0.5 mg/mL.
   - Multiply the body weight in g by 4 to obtain a desired dose of 2g/kg in µL volume, i.e. for a rat weighing 300g, need to inject glucose: 300g x 4 = 1200uL.

4. Set up your bench with the followings:
   - Gauze; A pair of scissors (or razor blades) to cut the tails;
   - 2 glucometers; timers; insulin injection syringes;
   - Table of rat order for recording keeping track of values;

5. Calculate the dose of glucose required for each rat.

6. At the end of the 6 hour fast, when you are completely set up and ready to begin, measure blood glucose (t= 0 min.) and then inject the mouse with the diluted glucose following the steps below:
   - Fill the syringe with the correct dose of diluted glucose Fill your syringe for ALL cages first.
   - Inject intraperitoneally after restraining and comforting the rat using a large cloth
   - Measure the blood glucose after 15, 30, 45, 60, 120min.
   - As soon as you inject the rat start your timer counting up from 0:00 and record the time and blood glucose.
   - Continue these steps until you have taken blood glucose and injected all of your rats.
   - Make sure you record all injection times and the blood glucose for each mouse.

7. At the end of 120 min., return rats to their normal cages and make sure there is adequate food and water.
Appendix IX: The integration between insulin and lipolysis signaling pathways

**The integration between insulin and lipolysis signaling:** when insulin binds to its receptor on the plasma membrane (shown, skeletal muscle), this causes a downstream signaling cascade of metabolites and enzymatic activation. Docking of PI3K with IRS1 causes conversion of PIP2 to PIP3 and subsequent activation of PDK1/2. This causes phosphorylation of Akt, or PKB, on two sites (Ser473 and Thr308), triggers AS160, or TBC1D4, to stimulate translocation of GLUT4 vesicles to the membrane, thereby facilitating glucose uptake. Simultaneously, insulin will act to suppress the process of TAG breakdown, or lipolysis in lipid droplets (shown, skeletal muscle or adipose tissue). In the presence of Epi or other lipolytic activators, AC will be activated and covert ATP to second messenger cAMP, thereby triggering PKA catalytic activity. This protein will cause phosphorylation and activation of key enzyme ATGL and recruit its co-activator protein CGI-58. PKA will also phosphorylate HSL on two residues (Ser660 and Ser563), and cause production of three FFAs and one glycerol backbone. Importantly, non-esterified FFAs can travel back across the plasma membrane and negatively impart effects on insulin binding and signaling in peripheral tissues.