SEX DIFFERENCES IN SKELETAL MUSCLE ENDOTHELIAL CELLS

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Abstract

The proper functioning of endothelial cells (ECs) is often impaired during obesity-related disorders. Our lab's previous data demonstrated sex differences in mitochondrial functioning and angiogenic capacity in adipose microvascular ECs. The goal of this study was to identify potential sex differences in lipid handling and metabolic capacities of skeletal muscle ECs.

To achieve this, ECs were isolated from hindlimb skeletal muscle of mice and treated in culture to mimic obesogenic-like conditions. I found higher proliferation and lipid storage in female ECs in control conditions. In control and obesogenic-like conditions, females expressed higher levels of lipid handling genes, while males displayed greater *Hk2* expression. Obesogenic-like conditions resulted in increased expression of *Pfkfb3* in females, and decreased insulin sensitivity in male ECs only.

These data provide evidence of sex-related differences in skeletal muscle ECs which can help to identify how their response to altered nutrient environment may in turn affect tissue health.

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Contents Abbreviations:

3PO – 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one

Akt – Protein kinase B

Ang1 – Angiopoietin-1

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

CD36 – Cluster of differentiation 36

Cdk – Cyclin-dependent kinase

CPT1α – Carnitine palmitoyltransferase 1α

Dll4 – Delta like ligand 4

dNTP – Deoxynucleotide triphosphate

EC – Endothelial cell

ECM – Endothelial cell matrix

EdU – 5-ethynyl-2'-deoxyuridine

eNOS – Endothelial nitric-oxide synthase

FA – Fatty acid

FABP4 – Fatty acid binding protein 4

FABPpm – Fatty acid binding protein plasma membrane

FAO – Fatty acid oxidation

FATP – Fatty acid transport protein

FFA – Free fatty acid

FGF – Fibroblast growth factor

FoxO – Forkhead transcription factor

FoxO1 – Forkhead transcription factor 1

G6P – Glucose-6-phosphate

GLUT1 – Glucose transporter 1

GLUT4 – Glucose transporter 4

GPIHBP1 – Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1

HK2 – Hexokinase 2

HUVEC – Human umbilical vascular endothelial cell

IR – Insulin receptor

IRS1/2 – Insulin receptor substrate proteins 1/2

KLF2 – Krüppel-like factor 2

LPL – Lipoprotein lipase

NO – Nitric oxide

NOS3 – Nitric oxide synthase 3

pAkt – Phosphorylated protein kinase B

PECAM – Platelet endothelial cell adhesion molecule

PFKFB3 – 6-phosphofructo-2-kinase/fructose-2,6

PI3K – Phosphatidylinositol 3-kinase pathway

PPARγ – Peroxisome proliferator-activated receptor-γ

ROS – Reactive oxygen species

T2D – Type 2 diabetes

TAG – Triglyceride

TBP – TATA-Box binding protein

TCA – Tricarboxyl acid

Tie2 – Tyrosine kinase receptor 2

VE-cadherin – Vascular-endothelial cadherin

VEGFA – Vascular endothelial growth factor A

Chapter 1: Literature Review

1.1 Microvasculature

The survival of cells is dependent on the delivery of oxygen and nutrients, as well as the removal of metabolic by-products. Blood vessels are able to achieve these functions through their ubiquitous distribution throughout the body, resulting in a highly organized and complex branching pattern (Bassingthwaighte et al., 1989; Carmeliet, 2000). The cardiovascular system consists of the macrovasculature, large-capacity vessels responsible for the transport of blood towards or away from organs, and the microvasculature, smaller-diameter vessels that comprise a vascular network within tissues to allow for the exchange of solubles (Yuan and Rigor, 2011).

Large-diameter arteries rapidly deliver large volumes of oxygenated blood from the heart to all areas of the body, including peripheral limbs and vital organs (Miller and Gal, 2017). Next, arteries branch into smaller blood vessels of the microvasculature known as arterioles, which will penetrate and extend the vasculature into tissues. Arterioles are essential for the regulation of local blood flow into tissues by altering lumen diameter through either relaxing or contracting smooth muscle cells that line the membrane (Emerson and Segal, 2000). From arterioles, blood flow branches into capillaries where the exchange of solubles occurs between plasma and tissue (Oellerich and Potente, 2012). These capillaries empty into venules, which are the main site of immune cell recruitment and transmigration during inflammation (Pober and Sessa, 2015). Once blood is collected into the venules, it travels into large-capacity blood vessels, known as veins, which leave the tissue and return back to the heart (Warnes, 2006).

1.2 The Endothelium

The endothelium forms the inner cell lining of all blood vessels including capillaries (Montesano et al., 1983). Here, quiescent endothelial cells (ECs) are connected using junctional molecules such as vascular-endothelial (VE) cadherin, claudins, and platelet endothelial cell adhesion molecules (PECAM), to form a continuous monolayer (Carmeliet and Jain, 2011). These endothelial-cell junctions present a complex network of adhesion proteins that are linked to intracellular cytoskeletal and signaling partners (Cerutti and Ridley, 2017). The luminal side of endothelial cells express surface plasma membrane proteins such as shear sensors and receptors for various ligands, whereas the basolateral side expresses cell surface integrins that attach ECs to the extracellular matrix comprising the basement membrane (Pompili et al., 2021; Daneman and Prat, 2015). The basement membrane consists of collagen type IV and glycoprotein laminin, as well as other molecules such as nidogens and heparin sulphate proteoglycan, depending on the type of microvessel (Kalluri, 2003; Hohenester and Yurchenco, 2013). It also plays a crucial role in contributing to the structural stability of the capillaries, and acts as a barrier by controlling the movement and transport of selective solutes (Hallmann et al., 2005).

1.3 Skeletal Muscle

Skeletal muscle is regarded as one of the most dynamic and plastic tissues in the body. It comprises 40-50% of total body weight and contains 50-70% of the body's proteins (Biewener, 2016; Frontera and Ochala, 2015). The most apparent functions of skeletal muscle are to generate forces required to produce movement and to provide structural support to help maintain body posture. However, it also plays roles in physiological processes including thermogenesis, metabolism, and endocrine signaling through myokine secretion (Rowland et al., 2015; Pedersen

and Febbraio, 2012) and is a key site for insulin-mediated glucose uptake (Kelley and Mandarino, 2000). Skeletal muscle is composed of myofibers that are arranged in parallel to numerous capillaries supplied from one or more arterioles of the microcirculatory system (Hudlicka, 2011). These myofibers are ensheathed by connective tissue that provides structural stability and support to cells such as satellite cells and mesenchymal cells (Haun et al., 2019; Kjaer, 2004). Importantly, muscle fibers also have different contractile and metabolic properties that are divided into glycolytic (fast) or oxidative (fast and slow) fiber types (Zierath and Hawley, 2004). Most muscles are composed of both fiber types, with only a few consisting of predominantly oxidative or glycolytic fibers.

1.4 Capillarization in Skeletal Muscle

Given that skeletal muscle is an energy demanding tissue, adequate nutrient delivery is crucial for its proper functioning (Baker et al., 2010). There are several factors that can limit the nutrient transport process. One of the most important elements is capillarization, which refers to both the number and surface area of capillaries. Increased muscle capillary density results in a larger muscle-to-blood exchange surface area, a shorter oxygen diffusion distance, and higher red blood cell transit time (Joyner and Casey, 2015). This in turn results in a faster rate of delivery to the tissue and a greater overall capacity for nutrient and oxygen transport. Alternatively, if capillarization is insufficient, soluble transport becomes less efficient and may result in deficient nutrient delivery. Importantly, reduced capillary number in skeletal muscle is correlated with decreased peripheral glucose utilization as well as whole-body insulin resistance (Sylow et al., 2021).

A second limiting factor of nutrient transport is blood flow. As such, during times of elevated metabolic activity, hypoxia or nutrient deficiency, vasodilation of arterioles can rapidly increase blood flow to enhance the delivery of nutrients and/or oxygen to that muscle to restore homeostasis (Joyner and Casey, 2015).

1.4.1 Endothelium of Skeletal Muscle

ECs are heterogenous in terms of their gene expression, morphology, and function depending on the tissue in which they reside (Marcu et al., 2018). These differences are demonstrated by three major types of capillaries that exhibit varying levels of permeability: continuous, fenestrated, and sinusoidal (Sarin, 2010). Since skeletal muscle contains capillaries with a continuous endothelium this will be the focus of my thesis. Continuous capillaries are found in most organs and are characterized by an uninterrupted endothelial lining that allow only smaller molecules, such as water and ions, to pass through the endothelial junctions connecting ECs to one another (Daneman and Prat, 2015). Therefore, in addition to capillarization and blood flow, the transport of solutes across the endothelial barrier also presents a limiting factor in nutrient delivery.

1.5 Types of Transport Across the Continuous Endothelium in Skeletal Muscle

1.5.1 Paracellular Transport

There are two primary routes of transport across the endothelium of skeletal muscle (Figure 1.1). The first is paracellular transport, which is the main route of fluid movement and thus determines the permeability of the endothelium. In paracellular transport, water and small water-soluble molecules travel through the intercellular clefts between adjacent endothelial cells (Vandenbroucke et al., 2008). The size of these clefts is dictated by the types of junctional

molecules connecting ECs to each other (Wessel et al., 2014). The two groups of endothelial junctions responsible for regulating molecular permeability of the intercellular clefts are adherens and tight junctions. Adherens junctions are the more permeable of the two, with VE-cadherin as the dominant protein that mediates cell-cell adhesion (Lampugnani et al., 2018; Harris and Nelson, 2010). In contrast, tight junctions consist mainly of claudins and occludins that tightly connect adjacent cells, thus restricting both the passage of ions and small molecules and establishing cell polarity (Bauer et al., 2014; Wallez and Huber, 2008). Importantly, the expression level of tight junctions varies greatly between tissues, thus leading to tissue-specific endothelial permeability. Occludin expression level, for example, is highest in endothelial cells of the central nervous system, which has a largely restrictive endothelial barrier compared to skeletal muscle (Hirase et al., 1997). Moreover, the expression of tight junction protein claudin-5, varies across the microvascular tree with higher levels in arterioles compared to venules in human dermal microvascular ECs (HDMECs) (Kluger et al., 2013).

1.5.2 Transcellular Transport

Large molecules are unable to fit through intercellular clefts and instead move across the endothelium by transcellular transport. This type of transport involves the movement of solutes from the plasma, through the endothelial cell and into the tissue. This process can occur by three different processes: active transport, passive transport, and transcytosis. In active transcellular transport, molecules are moving from an area of low concentration to an area of high concentration either by using ATP (primary), or by traveling down the electrochemical gradient, to produce energy and force the transport of another molecule (secondary) (Stokum et al., 2016).

The second process of transcellular transport is passive, where molecules move from an area of high to low concentration without exerting energy (Skou, 1990). This can occur by three different mechanisms. Passive diffusion involves the unassisted movement of small, non-polar molecules or lipid-soluble solutes from high to low concentration across a permeable membrane, which is seen in the gas exchange between oxygen and carbon dioxide for example (Kreuzer, 1970). However, due to the hydrophobic nature of the fatty acid tails of the phospholipids that make up the lipid bilayer, polar hydrophilic molecules cannot freely diffuse across the cell membrane; therefore, they require facilitation, also known as facilitated diffusion. This can occur using carrier or transport proteins such as fatty acid transport proteins (FATPs) for lipid transport, that are specific for a particular ion or molecule and transport solubles across by altering their conformation, such as seen with glucose or amino acids (Saier, 2003).

The final mechanism is transcytosis, which is the transport of macromolecules, such as hormones and proteins, across the interior of the cell (Williams et al., 1984). In transcytosis, macromolecules are captured in vesicles on one side of the cell, drawn across the cell and ejected on the other side (Ghitescu et al., 1986). The cellular process by which substances are brought into the cell is referred to as endocytosis (Bârzu et al., 1985). In endocytosis, the material to be internalized is surrounded by an area of the cell membrane, which then buds off inside of the cell to form a vesicle that contains the ingested material (Marsh et al., 2001). In some cases, endocytosis is non-receptor mediated (also referred to as pinocytosis or fluid-phase endocytosis), where small particles suspended in the extracellular fluid are brought into the cell through an invagination of the cell membrane (Kirkham and Parton, 2005). This non-specific process results in a suspension of the particles within a small vesicle inside of the cell (Conner and Schmid,

2003; Pulgar, 2019). Though pinocytosis takes place in most cell types, its role in ECs in capillaries has been recognized as an important trans-endothelial pathway for proteins, lipids, and lipoproteins (Simionescu et al., 2002). Endocytosis can also occur as a receptor-mediated process, in which molecules that are bound to cell surface receptors are internalized by the inward budding (invagination) of the plasma membrane, (Marsh and McMahon, 1999). These formed vesicles contain the receptor and the associated ligand. The transport of insulin for example, is known to use this pathway to cross the endothelial barrier via binding to the insulin receptor (King and Johnson, 1985).

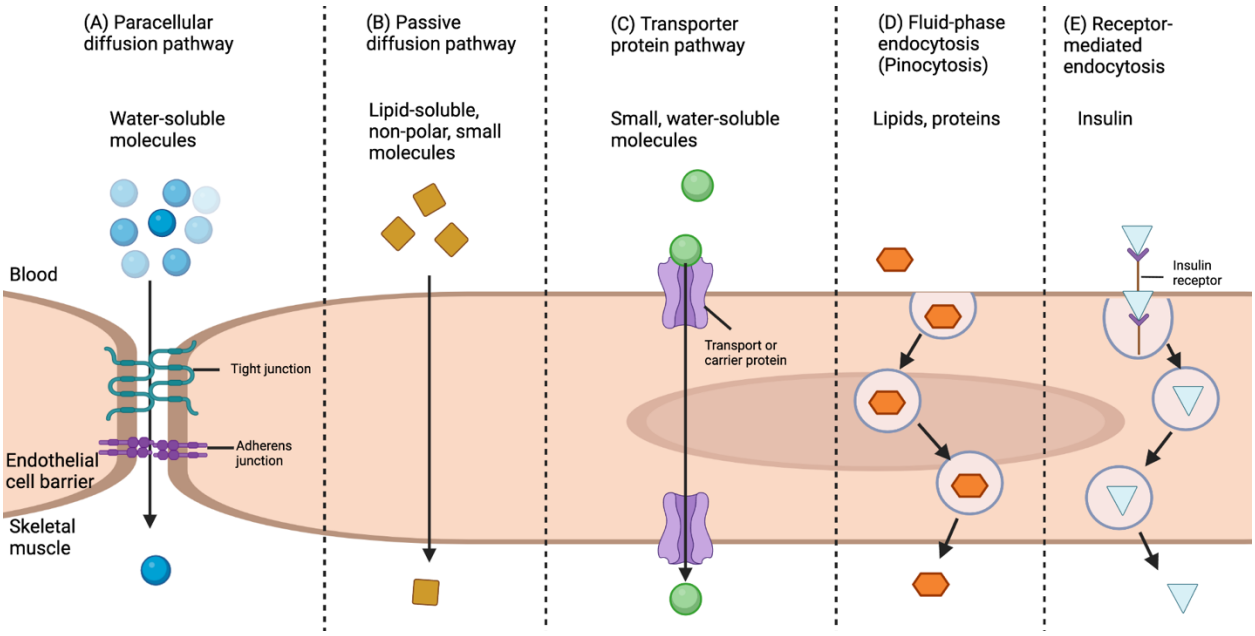

Figure 1.1: Schematic representation of types of transport across the endothelial barrier. A) Water-soluble molecules can travel between the endothelial cells via the intercellular clefts formed at endothelial junctions (ie. Adherens or tight junctions); B) Lipid-soluble solutes, small, polar molecules can use passive diffusion; C) Transport or carrier proteins present on the cell membrane facilitate crossing of water-soluble molecules such as glucose and amino acids; D) Lipids and proteins that do not require receptors can be transported across the membrane by fluid-phase endocytosis or pinocytosis; E) Regulated movement of molecules, such as insulin, can occur via receptor-mediated endocytosis. Schematic adapted from McAdams and Juul (2012) and created using Bioredner.com.

1.6 Nutrient Transport into Skeletal Muscle

Diet composition determines the nutrient availability for transport into the skeletal muscle. Although skeletal muscle uses a variety of substrates to fulfill its energy demands, glucose, and fatty acids (FAs) are the two most preferred sources, and are therefore the focus of this thesis (Figure 1.2).

1.6.1 Lipid Uptake

FAs are the building blocks of lipids, and can be classified by the length of carbons in the molecule. For example, short-chain FAs consist of five or fewer carbons, medium-chain FAs have 6-12 carbons, long-chain FAs are composed of 12-21 carbons, and very long chain FAs contain 22 or more carbons (Cifuentes, 2013; Beermann et al., 2003). However, I will limit my discussion to long-chain FAs since they represent most dietary lipids. Long-chain FAs are large, neutral lipids that are poorly soluble in water (De Carvalho and Caramujo, 2018). Therefore, they are packaged as triglycerides (TAGs) within lipoproteins (i.e., low density lipoprotein), which allows for transportation in the blood stream. ECs in capillaries use lipoprotein lipases (s) to breakdown plasma triglycerides into FAs and glycerol to be transported inside (Rinninger et al., 1998; Davies et al., 2010). LPLs are tethered to the EC luminal surface by glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1) (Young et al., 2011). FA uptake into ECs then occurs either through passive diffusion, or by facilitative transfer using fatty acid transfer protein 1 or 4 (FATP1/4) or CD36 (fatty acid translocase) (Schwenk et al., 2010).

Once inside the cell, FAs bind to fatty acid binding protein 4 (FABP4), where they can then undergo one of three possible fates (Vorum, et al., 1992; Harjes, et al., 2016). FAs can be oxidized for energy production, or be transported into surrounding tissues (Morales, et al., 2017). During times of exposure to excessive amounts of FA, ECs also can esterify FAs into triglyceride-rich lipid droplets (Kuo et al., 2017). This process is ultimately a protective mechanism from lipotoxicity. These stored FAs can be hydrolyzed from lipid droplets through lipolysis to be used as a source of energy when needed (Chen and Huang, 2021).

1.6.2 Glucose Uptake

Despite the long history of investigating glucose uptake into skeletal muscle tissues, the mechanisms underlying its transport across the endothelial barrier remain poorly understood. Since the size of glucose is relatively small (approximately 1nm) and is water soluble, it can travel through the EC adherens junctions using paracellular transport (Dissanayake et al., 2018). However, the most dominant method of transport for glucose is transcellular via glucose transporter 1 (GLUT1) (Thorens and Mueckler, 2010). GLUT1 is a transmembrane protein responsible for facilitating the diffusion of glucose across the membrane, a process driven by the concentration gradient of glucose (Navale and Paranjape, 2016). In some tissues, importantly the brain, once intracellular, glucose is rapidly phosphorylated into glucose-6-phosphate (G6P) (Mergenthaler et al., 2013). This ensures unidirectional glucose uptake since G6P cannot be transported by GLUT1. However, the extent to which this mechanism exists in other tissues is not established. For example, in cultured ECs from the retina only 30% of glucose was found to undergo this process (Betz et al., 1983).

Inside the endothelial cell, glucose can be metabolized to generate energy. One pathway for glucose metabolism is the hexosamine biosynthetic pathway, a side branch of glycolysis, that is responsible for generating glycosylations that are necessary for protein functions (De Queiroz et al., 2019). However, it has also been reported that ECs prefer glucose consumption and generate more than 80% of their ATP through glycolysis (De Bock et al., 2013). As such, glucose can either undergo oxidative or non-oxidative metabolism. Glucose can be broken down into pyruvate through glycolysis where it can subsequently be transported into the mitochondria and decarboxylated into acetyl-CoA (McCommis and Finck, 2015). Alternatively, during times of limited oxygen, pyruvate can be converted into lactate to allow the continuation of glucose metabolism and thus energy production. Alternatively, glucose may be transported across the endothelium and into surrounding tissues (Veys et al., 2020). This glucose efflux out of the endothelium is driven by abluminal membrane glucose transporter density and the glucose concentration gradient between cell and interstitial space. Therefore, ECs within mature vessels function to tightly balance the transport of glucose to tissues with consumption to fuel their own metabolic need.

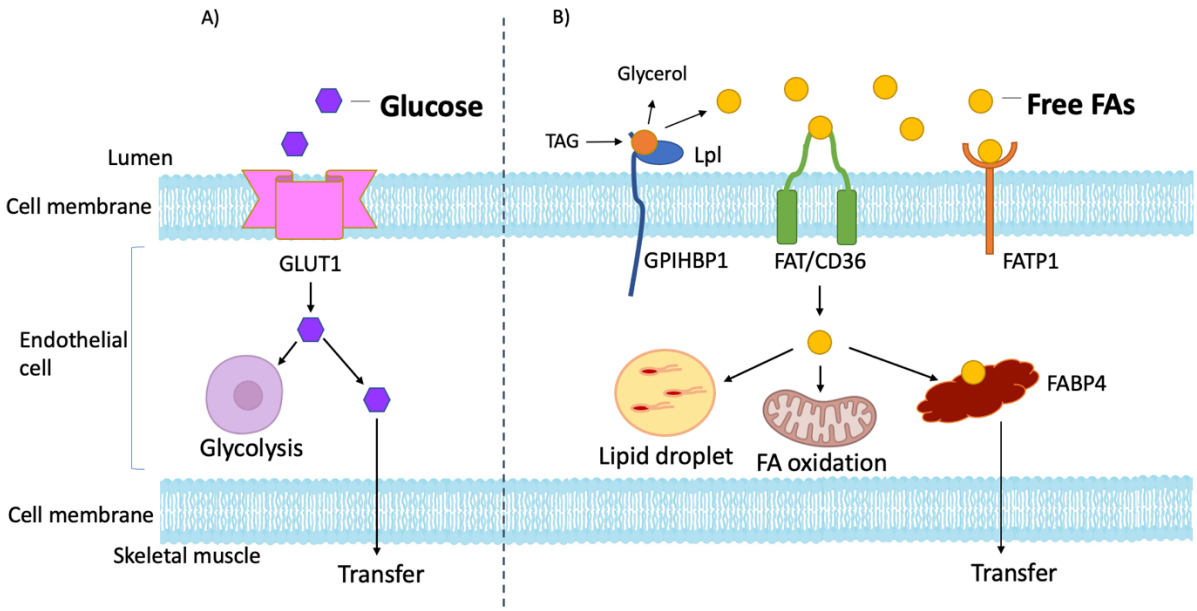

Figure 1.2: **Schematic of glucose and FA transport into ECs.** (A) Glucose transport across the EC membrane occurs using transport protein GLUT1. Once inside the cell, glucose can be metabolized via glycolysis, or be transported into the surrounding tissue. (B) Lpl is bound to the cell membrane by tethering protein GPIHBP1, where it breaks down triglycerides into free FAs (FFA) and glycerol so they can be taken up by skeletal muscle. FA transport across the membrane occurs by protein-mediated mechanisms either with CD36 or FATPs. Once in the cell, FAs either remain free (FFA) or are bound to FABP4 for transportation. FFAs can then either be stored as lipid droplets or used as fuel in FAO to generate energy. GLUT1, glucose transporter 1; TAG, triacylglycerol; Lpl, lipoprotein lipase, CD36, cluster of differentiation 36; FATP1/4, fatty acid transport protein 1/4; FABP4, fatty acid binding protein 4. Created using Biorender.com

1.7 The Role of Insulin in Nutrient Homeostasis

Skeletal muscle takes up approximately 30% of ingested glucose. Insulin is a major regulator of glucose uptake into skeletal myocytes (Kelley and Mandarino, 2000). Indeed, glucose uptake in skeletal muscle is 85% of total whole body glucose uptake during a hyperinsulinemic-euglycemic clamp, highlighting the importance of insulin action in skeletal muscle to support whole-body glucose homeostasis (DeFronzo et al., 1981).

Insulin is a peptide hormone produced by beta cells of the pancreas in response to high levels of blood glucose. By enhancing glucose uptake by specific cells such as myocytes, insulin promotes the lowering of blood sugar levels to maintain homeostatic levels. Insulin delivery to skeletal

myocytes is dependent on crossing the endothelial barrier. As mentioned earlier when discussing nutrient delivery, this is limited by: (1) blood flow to skeletal muscle; (2) surface area for capillary insulin exchange; and (3) capillary endothelial permeability to insulin. Therefore, adequate perfusion as a result of high capillarization can ensure the proper functioning of metabolism and insulin sensitivity in skeletal muscle (Barrett et al., 2011).

1.7.1 Insulin Transport Across Skeletal Muscle Endothelial Barrier

To date, the method by which insulin crosses the endothelium in muscle remains controversial. Earlier studies have shown that the transport of insulin was not saturable, which suggests that insulin receptors were not needed for trans-endothelial transport (Steil et al., 1996; Brunner and Wascher, 1998). In contrast, other work has shown that insulin does indeed co-localize with its receptor on ECs with the involvement of caveolin to transport insulin into these cells (Wang et al., 2006). However, those studies demonstrated this pathway in vitro, and thus further studies are required to ensure the presence of this co-localization in vivo. Thus, the mechanisms involved in regulating insulin transport across the endothelium still remain unclear and further investigation is required.

1.7.2 Insulin Signaling in Skeletal Myocytes

Once across the endothelial barrier, insulin can stimulate glucose uptake in skeletal myofibers, where it is a key regulator of glucose metabolism. Here, the insulin signaling pathway in skeletal myocytes begins by binding to the alpha-subunits of the insulin receptors (IRs) along the membrane, resulting in the activation of the insulin receptor substrate (IRS) and in turn activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Upon this activation of the

IRS/PI3K pathway, GLUT4 will be translocated to the membrane surface, where it promotes glucose uptake and storage as glycogen (Chang et al., 2004; Pereira and Lancha, 2004). Specifically, insulin-mediated glucose uptake is important in hyperglycemia, where it works to remove excessive amounts of sugars from the blood. Simultaneously, insulin suppresses lipid uptake, leading to a decline in the rate of lipid oxidation (Abdul-Ghani and DeFronzo, 2010). This ability of skeletal muscle to switch between glucose and lipids as sources of fuel can be referred to as metabolic flexibility (Kiens, 2006). However, in the event of insulin resistance, skeletal muscle will increase lipid uptake while decreasing glucose uptake at the same time, resulting in chronically higher levels of glucose in the blood (Turcotte and Fisher, 2008). Therefore, understanding the mechanism behind insulin sensitivity in ECs and glucose metabolism in skeletal muscle can provide insight into potential therapeutic approaches.

1.7.3 Insulin Signaling in ECs

Insulin signaling in ECs results in the phosphorylation of the tyrosine kinase of IRS1/2 and activates the phosphatidylinositol 3-kinase (PI3K)/Akt/eNOS or 'metabolic' pathway (De Nigris et al., 2015; Petersen and Shulman, 2018; Boucher et al., 2014). In ECs, the activation of this pathway regulates a variety of cellular processes including metabolism and proliferation; ultimately promoting cell survival (Świderska et al., 2018; Abeyrathna and Su, 2015). PI3K/Akt activation also causes phosphorylation of endothelial nitric-oxide synthase (eNOS) and leads to the production of nitric oxide (NO). In the endothelium, nitric oxide plays a crucial role in regulating vascular tone inducing vasodilation (Steinberg et al., 2000). This will in turn, increase both blood flow and perfusion in the tissue which plays an important role in promoting nutrient delivery (Förstermann and Sessa, 2012).

Additionally, it is possible that the PI3K pathway may also be involved in signaling to promote insulin-mediated glucose uptake (Huang et al., 2018; Świderska et al., 2018). For example, mice with EC-specific knockout of IRS-2, which prevents insulin-dependent activation of the PI3K pathway, showed impairment in insulin-mediated glucose uptake (Kubota et al., 2011). However, this study did not report whether this was due to impaired transport of insulin across the endothelium, or the result of cellular events instigated by decreased signaling via the PI3K pathway (Kolka and Bergman, 2013). Therefore, further research on this the role of PI3K in glucose uptake in skeletal muscle through an insulin-dependent mechanism, as well as additional functions of insulin are required to understand the specific signaling mechanisms underlying this processes.

1.8 Endothelial Cell States

1.8.1 Quiescence

In healthy adults, endothelial cells are in a primarily quiescent state defined by minimal or absent endothelial proliferation. EC are held in this state by numerous regulatory pathways that suppress the cell cycle. Notably, the endothelial cell cycle and metabolism are interconnected, and it has been shown that ECs will rewire their metabolism to switch from a proliferative to a quiescent state upon exiting the cell cycle (Zecchin et al., 2017). Moreover, upon inhibition of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6 (PFKFB3), proliferating ECs lower their glycolytic flux by up to 40% and display a quiescent phenotype (De Bock et al., 2013). FoxO1, a member of the Forkhead (FoxO) family of transcription factors, is one of the most potent regulators of EC quiescence. FoxO1 suppresses MYC signaling, which is a known driver

of metabolism and proliferation in endothelial cells (Wilhelm et al., 2016). FoxO1 also suppresses cyclin D1 expression (a positive regulator of cell cycle progression) and in turn increases levels of the cell cycle repressors p21 and p27, which are cyclin-dependent kinase (Cdk) inhibitors (Schmidt et al., 2002). Additionally, the Notch signaling pathway regulates EC quiescence by repressing MYC expression via microRNA-218 (Sun et al., 2021). Further, Notch activation is reported to increase as ECs enter quiescence and vessels begin to stabilize (Henderson et al., 2001; Taylor et al., 2002). This may be mediated by Angiopoietin-1/Tie 2 (Ang1/Tie2) receptor signaling, which upregulates Notch ligand delta like ligand 4 (Dll4) (Zhang et al., 2011). Lastly, shear stress is a biomechanical stimulus that maintains the quiescent state of endothelial cells (Sun and Feinberg, 2015). Shear stress sensors induce the expression of transcription factor Krüppel-like factor 2 (KLF2), which in turn orchestrates a network of genes that elicit a quiescent endothelial cell phenotype such as upregulation of Ang1/Tie2 and repression of the glycolytic enzyme PFKFB3 (Dekker et al., 2006; Parmar et al., 2006; Doddaballapur et al., 2015).

1.8.2 Angiogenesis

ECs become activated in response to various physiological and pathological stimuli (Gimbrone and García-Cardeña, 2016; Potente et al., 2011). Though the most well-known is endothelial growth factor VEGF-A, angiogenesis can also be prompted by a variety of pro-angiogenic stimuli such as fibroblast growth factor (FGF) and angiotensin 2 (Carmeliet, 2000; Gorman et al., 2014). Once activated, ECs proliferate and form new blood vessels from pre-existing vasculature, a process known as angiogenesis (Carmeliet, 2000; Fischer et al., 2006). This growth of the capillary network ensures proper functioning of the tissue when there is an

increase in metabolic demand (such as in muscle tissue during exercise) or during times of tissue expansion (such as in adipose tissue during obesity) (Herbert et al., 2012; Papetti and Herman, 2002). Importantly, the production and maintenance of these vessel networks require an intricate progression of events and signaling pathways to control the angiogenic pathway (Herbert et al., 2012).

There are two types of angiogenesis. The first is abluminal or sprouting angiogenesis, where the endothelial sprouts to give rise to new capillaries (Carmeliet, 2000). The second type is intussusceptive or non-sprouting angiogenesis, where the pre-existing vessel splits using transcapillary pillars of the extracellular matrix (ECM), resulting in two parallel vessels (Makanya et al., 2009). However, this type of angiogenesis requires much lower rates of EC proliferation, and the involved molecular mechanisms remain poorly understood compared to sprouting angiogenesis (Makanya et al., 2009; Spiegelaere et al., 2012). Therefore, the focus in this literature review will be on sprouting angiogenesis. During sprouting angiogenesis, activated ECs shift their phenotypes into three distinct morphological subtypes: tip cells, stalk cells, and quiescent phalanx cells. Each cell type has a specific role in angiogenesis. Tip cells are highly migratory and are responsible for navigating new sprouts in the correct direction; stalk cells are largely proliferative and elongate the newly migrated sprouts; and quiescent phalanx cells line the matured, newly established blood vessel (Gerhardt, 2003; Potente et al., 2011). There is a growing body of evidence supporting the role of EC metabolism in regulating vessel sprouting and tip/stalk cell phenotype, wherein EC phenotypes (tip, stalk, and phalanx cells) determine energy requirements and thus, the metabolic state of endothelial cells (De Bock et al., 2013;

Schoors et al., 2015). Therefore, understanding this relationship can provide further insight into the role of metabolism and the angiogenic capacity of ECs.

1.9 Endothelial Cell Metabolism

ECs are metabolically flexible and are able to appropriately respond to shifts in the diet composition (Goodpaster and Sparks, 2017; Smith et al., 2018). Although ECs use several types of metabolic pathways to support their functions, the two most common are glycolysis and fatty acid oxidation (FAO) (Figure 1.3).

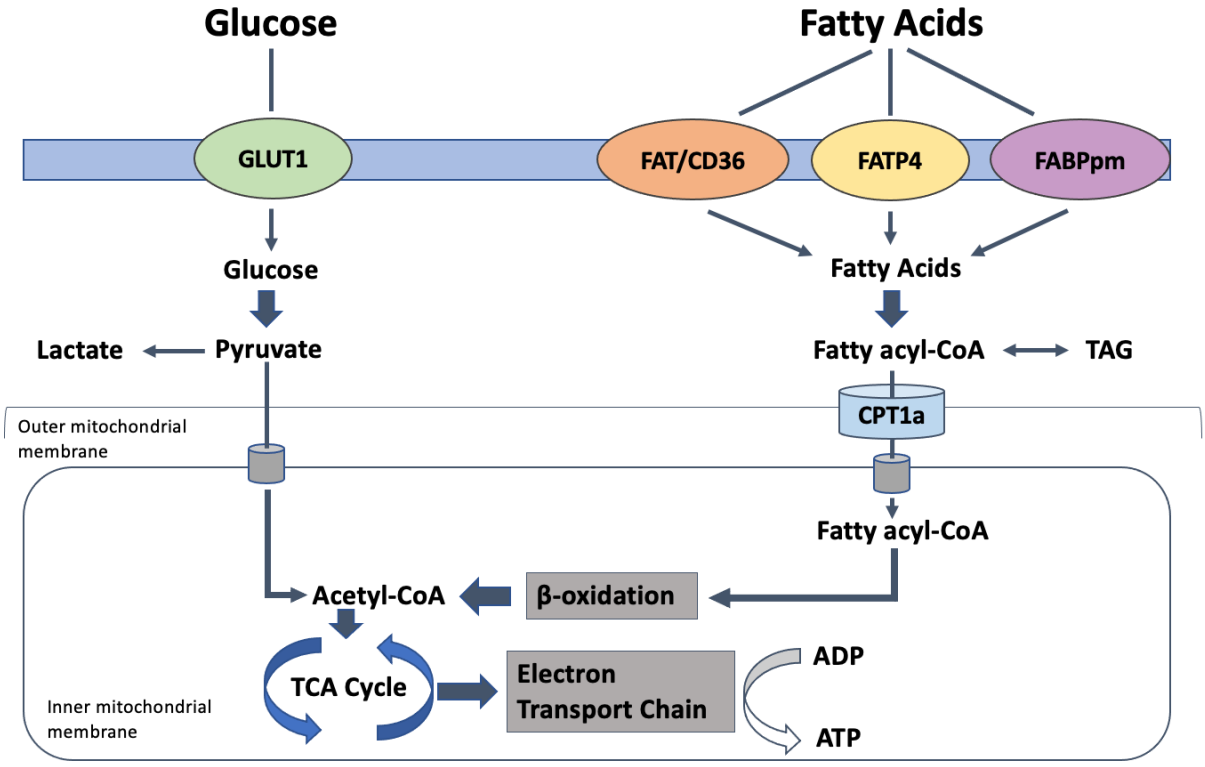

Figure 1.3: Schematic representation of glycolysis and FA metabolism in the endothelial cell. Glucose uptake into the EC occurs through glucose transporter1 (GLUT1). Once inside, glucose can be broken down into pyruvate through glycolysis where it is subsequently transported into the mitochondria and decarboxylated into acetyl-CoA. Pyruvate can alternatively be converted into lactate via anaerobic glycolysis. FAs are taken up through fatty acid transporter (FAT/CD36), fatty acid transport protein 4 (FATP4), and plasma membrane fatty acid binding protein (FABPpm). Once inside, FAs form fatty acyl-CoA and can either be esterified into TAGs or enter the mitochondria via CPT1α). Fatty acyl-CoA enters the beta-oxidation pathway, forming acetyl-CoA. Glucose or FA-derived acetyl-CoA then goes into the tricarboxyl acid (TCA) cycle with entry of reducing equivalents to the electron transport chain and oxidative phosphorylation, with the formation of ATP. Adapted from van den Brom et al., (2013) and created using Biorender.com

1.9.1 Glycolysis

In comparison to other cells in the body, ECs rely much more heavily on glycolysis as the primary source of energy (Culic et al., 1997; Krützfeldt et al., 1990). ECs rely dominantly on glycolysis even under quiescent conditions when energy needs are low, whereas other cell types instead prefer a more energy efficient process such as oxidative phosphorylation (Verdegem et al., 2014; Eelen et al., 2018). Furthermore, glycolysis has also long been recognized as the driving force of angiogenesis, generating approximately 85% of the ATP that is required by EC for vessel sprouting (Krützfeldt et al., 1990; DeBock et al., 2013). Glycolysis may also be especially important for the filopodia of tip cells during the revascularization of ischemic tissue given that it has been postulated that at increasing distances from a capillary, interstitial levels of oxygen drop faster than that of glucose levels (De Bock et al., 2013; Gatenby and Gillies, 2004).

Glycolysis is regulated by two rate-limiting enzymes: 6-phosphofructo-2-kinase/fructose-2,6 biphosphate 3 (PFKFB3) and Hexokinase 2 (HK2). The role of PFKFB3 in regulating EC glycolysis has been studied intensively. For example, Xu et al. (2014) demonstrated that overexpression of PFKFB3 increased EC proliferation by 85-90%, and augmented tube-like formation in vitro. A separate study by De Bock et al. (2013), showed that EC-specific deletion of PFKFB3 caused vascular defects and diminished number of branch points in the mouse retina, ultimately leading to vascular regression. Indeed, reducing glycolysis in ECs through the deletion of PFKFB3 has been shown to result in reduced tip cell migration and stalk cell proliferation (Vandekeere et al., 2015; Rohlenova et al., 2018). In addition, laminar shear stress generated by blood flow is also known to suppress PFKFB3 expression through KLF2 signaling, and

ultimately prevent EC activation (Doddaballapur et al., 2015). Angiogenic growth factors have also shown to have a profound role in regulating glycolysis. For example, VEGF has been shown to promote PFKFB3 transcription, whereas FGF is known to upregulate the MYC family of transcription factors, as well as its downstream target HK2 to promote angiogenesis (Li et al., 2017). Alternatively, angiogenic inhibitor Forkhead box transcription factor O1 (FoxO1) is a known inhibitor of glycolysis through suppressing MYC, and thus decreasing HK2 transcription (Wilhelm, et al. 2016).

1.9.2 Fatty Acid Oxidation

Endothelial cells also use FAO to meet their metabolic requirements. The rate-limiting step of FAO is carnitine palmitoyltransferase 1α (CPT1α), which shuttles long-chain FAs across the inner mitochondrial membrane and into the mitochondria (Sebastián et al., 2009). Once in the mitochondria, FAs can then be oxidized to produce acetyl-CoA, which can be further reduced in the tricarboxyl acid (TCA) cycle as well as the electron transport chain to generate ATP (Martínez-Reyes and Chandel, 2020). Apart from generating ATP, the TCA cycle also provides precursors for the synthesis of biomass precursors for dNTPs, which are required for DNA replication in proliferating stalk cells (Schoors et al., 2015; Sebastián et al., 2009). EC-specific inhibition of CPT1α not only causes a reduction in energy production, but it also impairs de novo nucleotide synthesis, resulting in a depletion of dNTP stores in ECs (Schoors et al., 2015). These findings highlight the requirement of FAs and FAO in dNTP production in ECs during proliferation, and in turn vessel sprouting.

Lipid metabolism is promoted by peroxisome proliferator-activated receptor-γ (PPARγ) and Notch signalling, which both increase the transcription of genes encoding lipid uptake and oxidation in ECs (He et al., 2003; Jabs et al., 2018). PPARγ is a master transcriptional regulator of lipid metabolism by controlling the expression of Lpl, CD36, and FABP4 (Goto et al., 2013; Kadegowda et al., 2009). EC-specific PPARγ deletion in mice results in increased concentrations of FFAs in the plasma as a result of impaired FAs uptake in skeletal muscle. Notch signaling has also recently emerged as a regulator of metabolism. By inhibiting Notch signaling in the endothelium of adult mice, transendothelial transport of FAs to myocytes via CD36 and FABP4 was inhibited, which in turn resulted in higher triglyceride and FFA levels in the blood (Jabs et al., 2018). Additionally, blocking the Notch signalling pathway in quiescent ECs decreased CPT1α expression and the rate of FAO, thus, identifying the Notch signaling pathway as an upregulator of this metabolic process (Kalucka et al., 2018).

1.10 Metabolic Disorders and Endothelial Dysfunction

In obesity-related disorders, ECs are often subjected to metabolic stressors such as increased levels of plasma glucose, FAs, and circulating insulin. This in turn disturbs the metabolic processes of ECs and can lead to the development of insulin resistance and ultimately endothelial dysfunction, which underpins vascular pathologies and inflammation (Rajendran et al., 2013; Hiatt et al., 2015). In diabetic individuals, high levels of glucose in the bloodstream result in increased endothelial reactive oxygen species (ROS) production, eNOS uncoupling and mitochondrial dysfunction (Du et al., 2003). Conversely, elevated levels of FAs in plasma can lead to an induction of EC apoptosis and dysfunction by impairing NO-mediated vasodilation, which in turn promotes oxidative endoplasmic reticulum stress, and pro-inflammatory signaling

(Theodorou and Boon, 2018). This may promote EC dysfunction and cause in a decrease in the amount of nutrient transport, which can ultimately result in the needs of the tissue not being met.

Insulin resistance in skeletal muscle ECs impairs glucose uptake while concomitantly increasing lipid uptake (Muniyappa and Sowers, 2013; Wilcox et al., 2005). This impairment can result in hyperglycemia and further impair insulin sensitivity in the cells, which thereby results in a destructive metabolic cycle and the development of diabetes and obesity related disorders (Wilcox, 2005). Since skeletal muscle represents a key site for glucose uptake, improving insulin sensitivity here provides a therapeutic opportunity for obesity-related disorders (Klaman et al., 2000). Additionally, since insulin is a known stimulator of glucose uptake, it is plausible that the insulin signaling pathway may also regulate glycolysis in ECs (Boucher et al., 2014; Peterson et al., 2004). However, there is minimal research on this potential relationship between insulin sensitivity and EC metabolism in skeletal muscle ECs.

1.11 Sex Differences in Metabolism

The majority of research on EC metabolism and nutrient transport has been conducted on males, without considering possible sex differences. However, nearly all aspects of metabolism are regulated in a sex-dimorphic manner (Mauvais-Jarvis, 2015; Clegg and Mauvais-Jarvis, 2018). In humans, females are at a greater risk of obesity compared to males due to their increased tendency to gain fat (Kanter and Caballero, 2012). Nonetheless, females have also reported to be more resistant to FA-induced insulin resistance, despite having higher levels of circulating lipids in basal conditions (Frias et al., 2001). Sex-based differences are also present in the regulation of glucose homeostasis and development of insulin resistance in skeletal muscle (Fatima et al.,

2017; Rich-Edwards et al., 2018). Indeed, it has been reported that females have higher insulin sensitivity, by displaying greater insulin-mediated glucose uptake within skeletal muscle (Nuutila et al., 1995). These sex differences are the summation of hormonal effects and of intrinsic differences based on sex chromosomes (Reue and Wiese, 2022). Additionally, hormonal effects can include epigenetic modifications on transcriptional differences in the metabolic pathway of males and females (Maher et al., 2009).

1.11.1 Sex Differences in Endothelial Cells

Sex differences have also been found in endothelial cells. Lorenz et al (2015) reported that genes involved in migration, proliferation, and shear stress in human umbilical vascular endothelial cells (HUVECs) were regulated in the opposite direction for males and females in absence of sex hormones. Female ECs have also exhibited higher rates of proliferation, migration, and capillary-like tube formation in vitro (Addis et al., 2014; Cattaneo et al., 2017; Boscaro et al., 2020). Moreover, a growing amount of research has looked at inherent sex differences in endothelial cells independent of the effects of estradiol. As such, several studies have shown higher migratory capacity, transcription of pro-angiogenic genes in response to shear stress, expression of eNOS and cell viability after 20 hours of serum starvation and tube formation capacity in females (Hoffmann et al., 2017; Mehta et al., 2007). Indeed, intrinsic sex differences in ECs may be the cause for differences in vascularization with significantly higher proliferation, migration, and both mRNA and protein expression of NOS3 in female HUVECs (Addis et al., 2014).

Other studies have illustrated the existence of sex differences in metabolism, in terms of substrate utilization in male and female ECs. Using EC spheroids, Cattaneo et al. (2017) found

greater sprouting inhibition in males when cultured in media stripped of all FAs, indicating a greater dependence of male ECs on lipids for proliferation and angiogenesis in vitro compared to females. However, the underlying mechanisms for these observed differences in male and female ECs are yet to be identified. It is possible that differences in metabolic pathways such as glycolysis or FA utilization contribute to the sex differences in EC behaviour and angiogenic potential. The logical hypothesis is that sexual dimorphism observed in angiogenesis is caused by differences in the angiogenic capacity of male and female ECs. However, little research has been conducted on this topic to date.

1.12 Study Rationale

Previously, our lab found that female mice had greater adipose tissue vascularity, as shown by increased microvascular endothelial area and greater vessel diameters, and preserved adipose function compared to males, when placed on a high fat diet for 16 weeks (Rudnicki et al., 2018). In recent experiments, differential gene expression data from RNA-sequencing of adipose tissue EC of mice on high fat diet extended these observations, by documenting that female ECs showed an enrichment for genes associated with cell proliferation, whereas males were associated with genes promoting inflammation (Rudnicki et al., In review). Notably, that data-set also pointed to sex differences in genes related to lipid handling. Female EC showed enrichment for genes associated with lipid oxidation, whereas males EC were enriched in genes associated with lipid uptake. These data suggest that females may have higher lipid consumption as a metabolic fuel, whereas males may prioritize the storage of lipids in AT.

Although skeletal muscle is a major regulator in whole-body homeostasis, tissue-specific functions of ECs in skeletal muscle have not yet been investigated. Since, ECs display heterogeneity and organ specificity to support their specific tissue functions (Marcu et al., 2018), it is likely that the sex differences in skeletal muscle will differ from findings in AT. Additionally, little is known about the potential differences in lipid handling and metabolism in male and female ECs in skeletal muscle. It is possible that sex differences in ECs that may affect their proliferative capacity and their response to altered nutrient environment, which in turn will affect tissue function. Seeing as how skeletal muscle undergoes significant structural, metabolic, and functional changes under obesogenic conditions (Teng and Huang, 2019), understanding potential sex differences in EC characteristics provides a novel therapeutic approach.

Additionally, it is important to understand how sex differences in skeletal muscle EC functions may affect insulin sensitivity. In the Rudnicki et al. study (2018), females on a high fat diet maintained higher insulin sensitivity in skeletal muscle and better whole-body glucose disposal. This suggests that females were able to preserve healthy skeletal muscle endothelial function, but direct evidence of this is lacking. Also, the effects of chronic lipid exposure on these features of skeletal muscle ECs remain largely undefined. Since elevated levels of FAs are known to contribute to insulin resistance and EC dysfunction, understanding how insulin sensitivity affects both tissue and EC functions can provide a better knowledge of the role of insulin in maintaining tissue health.

1.13 Objectives

Scientific Question: Do female skeletal muscle ECs handle lipids differently than males?

1.13.1 Objective 1

Goal: To determine lipid handling properties in male and female skeletal muscle microvascular endothelial cells under control and obesogenic-like conditions.

Hypothesis: Female ECs will exhibit superior capacity to uptake and handle lipids in control conditions, which will be exaggerated under obesogenic-like conditions.

1.13.2 Objective 2

Goal: To evaluate the proliferative capability of male and female skeletal muscle microvascular endothelial cells under control or obesogenic-like conditions, as well as their reliance on fatty acids versus glucose to promote cell proliferation.

Hypothesis: Female ECs will have higher levels of proliferation in comparison to male ECs that is dependent on lipid metabolism.

1.13.3 Objective 3

Goal: To assess insulin sensitivity in male and female skeletal muscle ECs under control and obesogenic-like conditions post-insulin stimulation.

Hypothesis: No sex differences in insulin responsiveness in ECs will be found under control conditions, however female ECs will show increased insulin responsiveness compared to male ECs under obesogenic-like conditions.

Chapter 2: Methods

2.1 Ethical Approval

All animal studies were approved by York University Committee on Animal Care (#2017-20).

2.2 Endothelial Cell Isolation

Male and female microvascular endothelial cells were extracted from hindlimb skeletal muscle pooled from male and female C57BL/6 mice (n=2-4 mice per sex). For cell isolation, skeletal muscle was first digested using Type II Collagenase (0.5%, #17101015, ThermoFisher Scientific, USA) or Type D Collagenase (2mg/mL, #11088882001, Millipore Sigma) at 37 °C with agitation for 30 minutes or 2 hours respectively. Cells were then centrifuged (4000xg for 5 minutes), resuspended and passed through a cell strainer (100 μm) before being incubated with biotinylated rat-anti mouse CD31 antibody coated streptavidin (BD IMag, USA) -coupled Dynabeads (ThermoFisher, Scientific, USA). Isolated endothelial cells were next plated on gelatin-coated plates and maintained in high-glucose (4.5g/L) DMEM (#19960044, Gibco, USA) with 20% fetal bovine serum (FBS, #10082147, Gibco, USA), Penicillin-Streptomycin (100μg/mL, 15140122, Gibco, USA), GlutaMAX^{TM} L-glutamine (2 mM, #35050061, Gibco, USA), and Sodium Pyruvate (1 mM, #11360070, Gibco, USA). All cells were subjected to estradiol (4.32 pg/mL), Progesterone (<12 pg/mL) and Testosterone (<2 ng/mL) found in the FBS. Cells used for experiments were passage-matched and between passages 2-6.

2.3 Cell Culture Conditions

For gene expression experiments, male and female ECs were plated in high glucose, 20% FBS DMEM at 70-80% confluency in 12-well plates. Cells either remined in control conditions or

were placed in obesogenic-like conditions by treating with the fatty acid palmitate (200 μM) conjugated to bovine serum albumin (BSA), and insulin (25 mU/mL) for 48 hours prior to lysis for RNA analysis. An equivalent volume of BSA dissolved in 50% ethanol was used as the vehicle control.

For insulin stimulation experiments, ECs were either pre-treated with palmitate-conjugated BSA (200μM) for four days or remained in control conditions, by adding equivalent volume of BSA with ethanol as a negative control, prior to plating in 6-well plates at a density $5x10^6$ cells per well. The following day, wells were incubated with serum-free, high glucose DMEM for 2 hours followed by stimulation with insulin (25 mU/mL) or control for 15 minutes prior to protein extraction.

2.4 RNA Extraction

Endothelial cells were lysed using Qiazol reagent (Qiagen #79306), before adding chloroform and centrifuging to separate RNA from DNA and protein. RNA was extracted by collecting the upper aqueous phase of the lysate and, precipitating with isopropyl alcohol, and washing with 75% ethanol before being resuspended in 40μL of RNase-free water. RNA concentrations and purity were quantified by spectrophotometer.

2.5 q-RT PCR

For each sample, 300 ng of RNA was reversed transcribed to cDNA in a two-step polymerase chain reaction (PCR) on a Thermal cycler (2720 cycler, Applied Biosystems, CA, USA) using dNTP (#N0447S, New England Biolabs, ON, Canada), Oligo dT (#100002344, Invitrogen, CA,

USA), Ribolock RNase inhibitor (#EO038, ThermoFisher Scientific, MA, USA), Random hexamers (#100026484, Invitrogen, CA, USA), M-MuLV reverse transcriptase and buffer (New England BioLabs, ON, Canada). cDNA was diluted in 80 µL of Rnase free water. 2 µL cDNA was combined with Taqman® Fast Advanced Master Mix (#4444557, Applied Biosystems, Thermo Fisher Scientific, CA, USA) and TaqMan® FAM-Labelled probe sets for murine *Cd36*, *Fabp4*, *Lpl, Hk2, Pfkfb3*. q-RT PCR was performed using the PCR cycler Rotor-Gene Q system (Qiagen, ON Canada) in the following thermal conditions: 60 °C for 30 minutes, 95 °C for 10 minutes for a total of 40 cycles. Samples were assessed in duplicate. To determine the relative amount of target sample mRNA, the average cycle threshold (C_T) was calculated and compared to the average C_T of the housekeeping gene TATA-box binding protein (*Tbp*) for the same samples with the following formula: ΔC_T= Average C_T (gene of interest) - Average C_T (housekeeping gene). The relative amount of target genes was calculated using the formula $2^{-\Delta CT}$.

2.6 Protein Extraction

Proteins were extracted from cells using RIPA lysis buffer (50 mM Tris base, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing dissolved PhosSTOP™ and cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich, USA).

2.7 Protein Quantification

Protein extracts were quantified by bicinchoninic acid assay (BCA) (Pierce, Fisher ThermoScientific, ON, Canada). Pure protein lysates were loaded in triplicates on 96 well plates with working reagent and compared to a standard curve using serial dilutions of bovine serum albumin (BSA: 0-2000 µg/mL, Pierce, ThermoFisher Scientific, ON, Canada). Sample

absorbance at 562 nm was measured using the Cytation3 microplate reader (BioTek, Vermont, USA).

2.8 Western Blot

Western blots were performed using 10-20 μg of total protein per sample prepared in 5X DLB denaturing loading buffer (DLB) containing 0.72M β-mercaptoethanol, 69.4 mM sodium dodecyl sulfate, 30% (v/v) glycerol, 14.29 μM bromophenol blue, 62.4 mM Tris pH 6.8, and RIPA lysis buffer as a diluent . Samples were heated to 99 °C for 5 minutes, centrifuged and placed on ice prior to loading. The prepared lysates were then loaded (10-20 μg per lane) and separated by electrophoresis through 10% SDS-Polyacrylamide gels under reducing conditions. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, EMD Millipore, ON, Canada) using wet transfer at 100 V for 90 minutes. Membranes were then blocked for one hour with 5% milk in a 0.05% Tween Tris-buffered saline solution (TTBS) with agitation. After blocking, membranes were incubated with primary antibodies Phospho-Akt-ser473 (1:1000; #4058, Cell Signaling, ON, Canada) and Akt (1:1000; #972, Cell Signaling) in 5% BSA (bovine serum albumin) (Bioshop, Canada, Inc, ON, Canada) in 0.05% TTBS overnight at 4 °C with rocking. The following day membranes were washed with TTBS and incubated with secondary antibody (1:1000 goat anti-rabbit IgG-horseradish peroxidase; #111-035-003, Jackson ImmunoResearch Laboratories Inc., USA) in 5% BSA for 1 hour at room temperature with rocking. To measure phosphorylated protein and total protein on the same membrane, membranes were stripped using a Western Blot Stripping Buffer comprised of 0.7% β-mercaptoethanol (14.3 M), 62.5 mM Tris pH 6.75, 2% sodium dodecyl sulfate at 50 °C for 8 minutes, then washed for 1 hour in TTBS before being blocked and incubated in the antibody as

described above. The antibodies used were detected by enhanced chemiluminescence (Pierce, Fisher Thermoscientific, ON, Canada) using Microchemi DNA Bio-imaging system (Neve Yamin, Israel). Blots were quantified using densitometry analysis and ImageJ Analysis Software.

2.9 Cell Proliferation Assay

ECs were then plated in quadruplicate wells of a 96-well plate at a density of $8x10^3$ cells per well. To assess cellular proliferation, EC were incubated with 10 μM EdU (5-ethynyl-2'-deoxyuridine; Abcam #ab146186) for 3 hours the following day, and were fixed with 4% paraformaldehyde. Proliferating cells were marked based on the incorporation of EdU into the DNA strand during the S phase of the cell cycle. EdU was subsequently detected by a 'Click' reaction, in which fluorophore-azide reacts with the incorporated EdU using a Cu(I)-catalyzed cycloaddition. The 'Click' Solution was comprised of 10% Tris pH 8.5 (1M), 4% copper sulfate (26.1 mM), 1% Azide Fluor545 (10mM), 65% double-distilled H2O, and 100mM ascorbic acid. All cell nuclei were stained with DAPI (0.1μg/mL) (D3571, Molecular probes, OR, USA). Imaging was performed using a fluorescence microscope at 10x objective. The numbers of EdU-positive and DAPI-positive cells were counted separately using ImageJ Analysis Software, and the percentage of proliferating cells was expressed as (EdU/DAPI) x 100. The average of 20 fields of view (5 fields of view per well x 4 wells) was used to represent the percentage of proliferating cells in each condition.

To assess cell proliferation under obesogenic-like conditions, some ECs were pre-treated under the same treatment conditions described above in cell culture conditions for protein expression.

The following day, ECs from control and pre-treated obesogenic-like conditions were then plated to evaluate cell proliferation using the protocol described above.

The effect of pathway inhibition on cell proliferation was assessed by plating ECs at a density of $8x10^3$ cells per well, and incubating overnight with PFKFB3 inhibitor 3PO (3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) (10 μM, #525330, EMD Millipore, MA, USA) or with CPT1α inhibitor Etomoxir (EMD Millipore, MA, USA) using various methods based on previous reports in the literature (1, 25 or 50 μM; Kalucka et al., 2018; Kim et al., 2020). An equal volume of dimethyl sulfoxide (DMSO) (D2650, Sigma, MO, USA) was used as the vehicle control. EC proliferation was then assessed following the same protocol as described above

2.10 BODIPY 493/503 Lipid Stain

Male and female skeletal muscle ECs were plated in quadruplicate, at a density of $30x10^3$ cells per well in low glucose, serum-free DMEM in an 8-well chamber glass slide for 48-hours. ECs were then fixed using 4% paraformaldehyde and lipid droplets were detected using BODIPY 493/503 (5μM) in all wells. Cell nuclei in all wells were stained with DAPI. Imaging was performed using a microscope at 40x objective. Using ImageJ Analysis Software, the intensity of lipid droplets in each field of view was determined by measuring the mean signal intensity of BODIPY in the green (FITC) channel. To determine the average lipid uptake for sex, the average of 4 fields of view per well for 4 wells were averaged.

2.11 Statistical Analysis

Results are presented as Mean ± standard error of the mean (SEM). The effect of obesogenic-like conditions on mRNA expression was analyzed using a two-way ANOVA repeated measures followed by Bonferroni post hoc tests when measuring mRNA expression of glycolytic enzymes (Prism8; GraphPad Software Inc; La Holla, CA, USA). Insulin responsiveness and cell proliferation assays in treated experiments were also analyzed using a two-way ANOVA but was followed by Bonferroni post hoc tests. Endothelial cell proliferation assays under basal conditions and lipid droplet staining were analyzed using a two-tailed Student's *t*-test. In all cases, $p < 0.05$ was considered to represent statistical significance.

Chapter 3: Results

3.1 Relative mRNA Expression of Lipid Handling Machinery and Glycolytic Enzymes in Male and Female ECs

I assessed mRNA levels of genes associated with different aspects of lipid handling and glycolysis in male and female skeletal muscle EC in control conditions and following 48-hour treatment with palmitate and insulin, to mimic the obesogenic-like conditions induced by high fat diet. The expression of *Lpl*, a gene associated with triglyceride catabolism on the EC surface, was significantly higher in female than male ECs irrespective of the treatment condition (Fig. 3.1A). Expression of *Cd36,* a gene involved in fatty acid translocation across the plasma membrane, was minimal in both male and female ECs regardless of the condition (Fig. 3.1B). Lastly, the expression of *Fabp4* mRNA was evaluated as a marker of fatty acid intracellular transportation. Similar to *Lpl,* female skeletal muscle ECs displayed significantly higher levels of *Fabp4* mRNA in both control and palmitate/insulin conditions (Fig. 3.1C). Taken together, these results demonstrate that female EC display higher levels of gene expression for lipid handling machinery in skeletal muscle.

The expression of *Hk2*, a gene involved in the first step of glucose metabolism, was significantly higher in male ECs compared to females irrespective of the treatment condition (Fig. 3.1D). The expression of *Pfkfb3,* a critical regulator of glycolysis, displayed an interaction between sex and treatment. There was higher expression in palmitate-treated female ECs compared to both their control counterparts and to palmitate-treated male ECs (Fig. 3.1E). This suggests that enzymes of the glycolytic pathway may be regulated differently in male and female ECs in skeletal muscle in control and high-fat conditions.

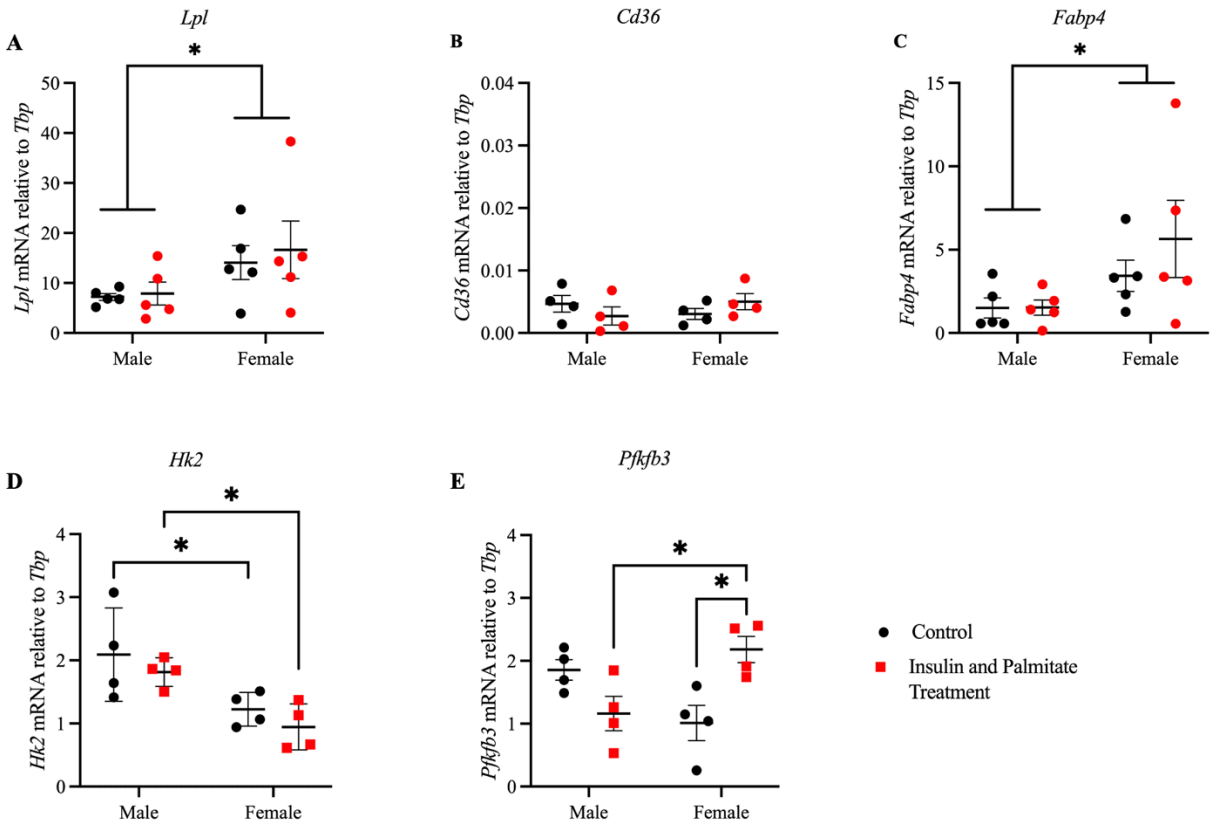

Figure 3.1: mRNA Levels of Lipid Handling Machinery and Glycolytic Enzymes in Endothelial Cells. Male and female ECs were cultured in control media or with addition of insulin (25 mU/mL) and palmitate (200 μM) for 48 hours prior to RNA extraction for analysis using qPCR. The mRNA levels of lipid handling related markers **(A)** *Lpl*; **(B)**; *Cd36;* and **(C)** *Fabp4* were quantified relative to *Tbp* mRNA, $n = 5$. The mRNA levels of **(D)** *Hk2*; **(E)** *Pfkfb3*, genes involved in the glycolytic pathway, were quantified relative to *Tbp* mRNA, $n = 4$. All data were expressed as mean ± SEM and analyzed using two-way ANOVA repeated measures with Bonferroni *post hoc* analysis when significant effects were detected. *Main effect of sex ($P < 0.05$) in **(A)** and **(C)**. In **(D)**, main effect of sex ($P = 0.007$), with post hoc significance as indicated by *$P < 0.05$. **(E)** Main effect of interaction between sex and treatment ($P = 0.01$), with post hoc significance as indicated by *$P < 0.05$.

3.2 Lipid Content in Skeletal Muscle ECs

To confirm the finding of higher gene expression of lipid handling machinery in females, I assessed lipid content inside of ECs. This was done by measuring the mean intensity of BODIPY found in lipids (Fig. 3.2A). Female ECs displayed significantly higher intracellular lipids compared to the male ECs, based on BODIPY intensity (Fig. 3.2B). This suggests that greater amounts of lipid content is seen in female ECs in this cell culture condition.

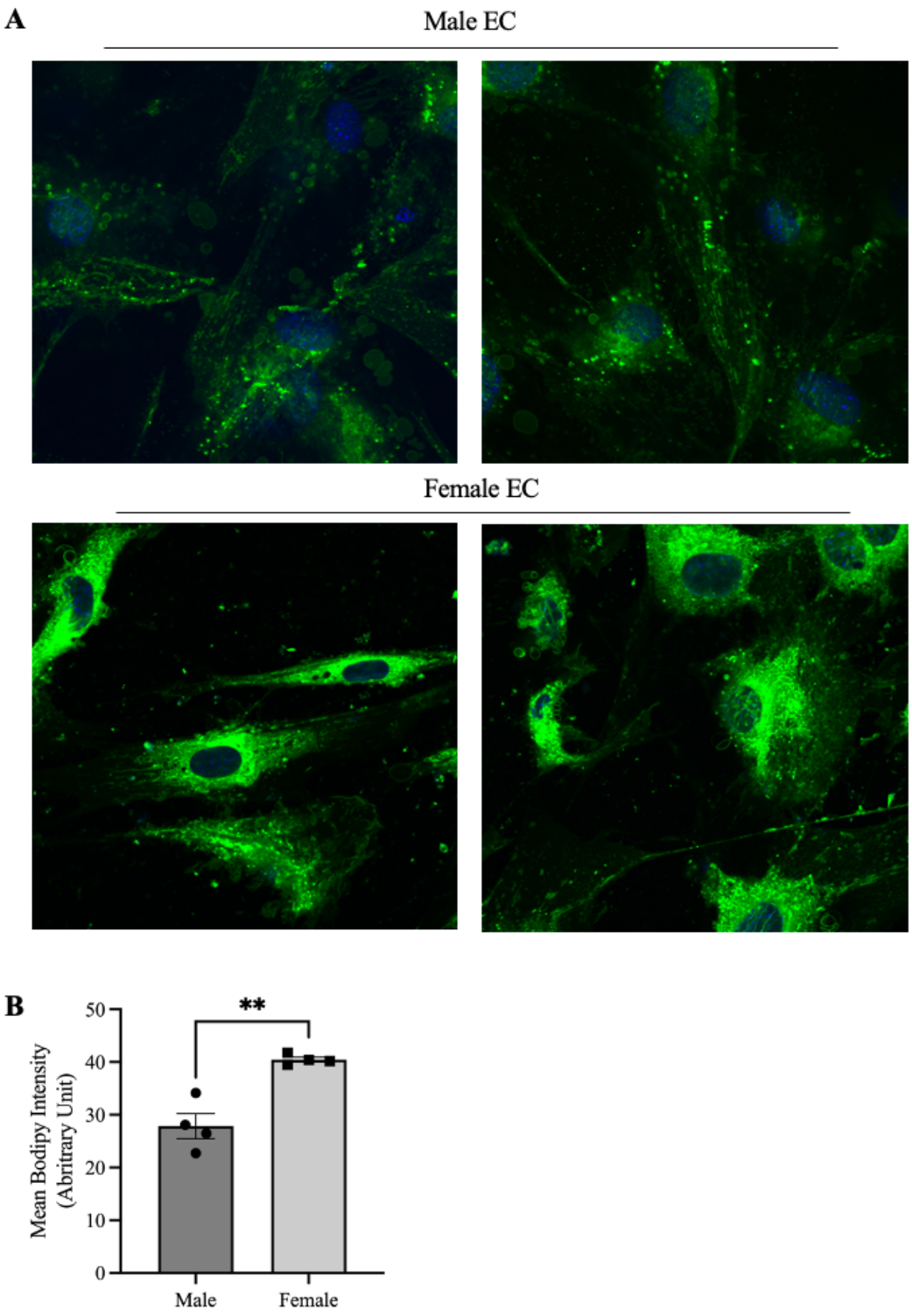

Figure 3.2: Lipid Staining and Quantification in Skeletal Muscle ECs. (A) Representative images showing lipid staining in male (M-EC) and female (F-EC) skeletal muscle ECs under low glucose, serum-starved conditions. Lipid droplets are stained with BODIPY (green), and nuclei stained with DAPI (blue). **(B)** Quantification of lipid droplets expressed as the average of mean bodipy intensity of four fields of view per well; $n = 4$ wells of ECs. Data expressed as mean ± SEM, and analyzed using paired student t-test, $*P < 0.01$. Scale bar=10μm.

3.3 Higher Proliferation in Female Skeletal Muscle ECs

Our lab previously documented that female ECs from adipose tissue proliferate at a higher rate than their male counterparts (Rudnicki et al., In review). Here, I tested if this sex difference can also be observed in ECs from skeletal muscle. Proliferating ECs were detected based on the incorporation of EdU (Fig. 3.3A). There were significantly more EdU+ female skeletal muscle ECs compared to male (Fig. 3.3B), demonstrating a greater rate of proliferation in female ECs compared to males under basal conditions. Since prolonged fat exposure has been associated with reduced cell proliferation in HUVECs (Broniarek et al., 2016), I also examined the effect of chronic palmitate exposure on the rate of EC proliferation in both sexes. Females were again found to maintain significantly higher proliferation than male ECs under control conditions. Palmitate treatment did not affect EC proliferation in either sex; however, sex differences were no longer detected in palmitate pre-treated conditions (Fig. 3.3C).

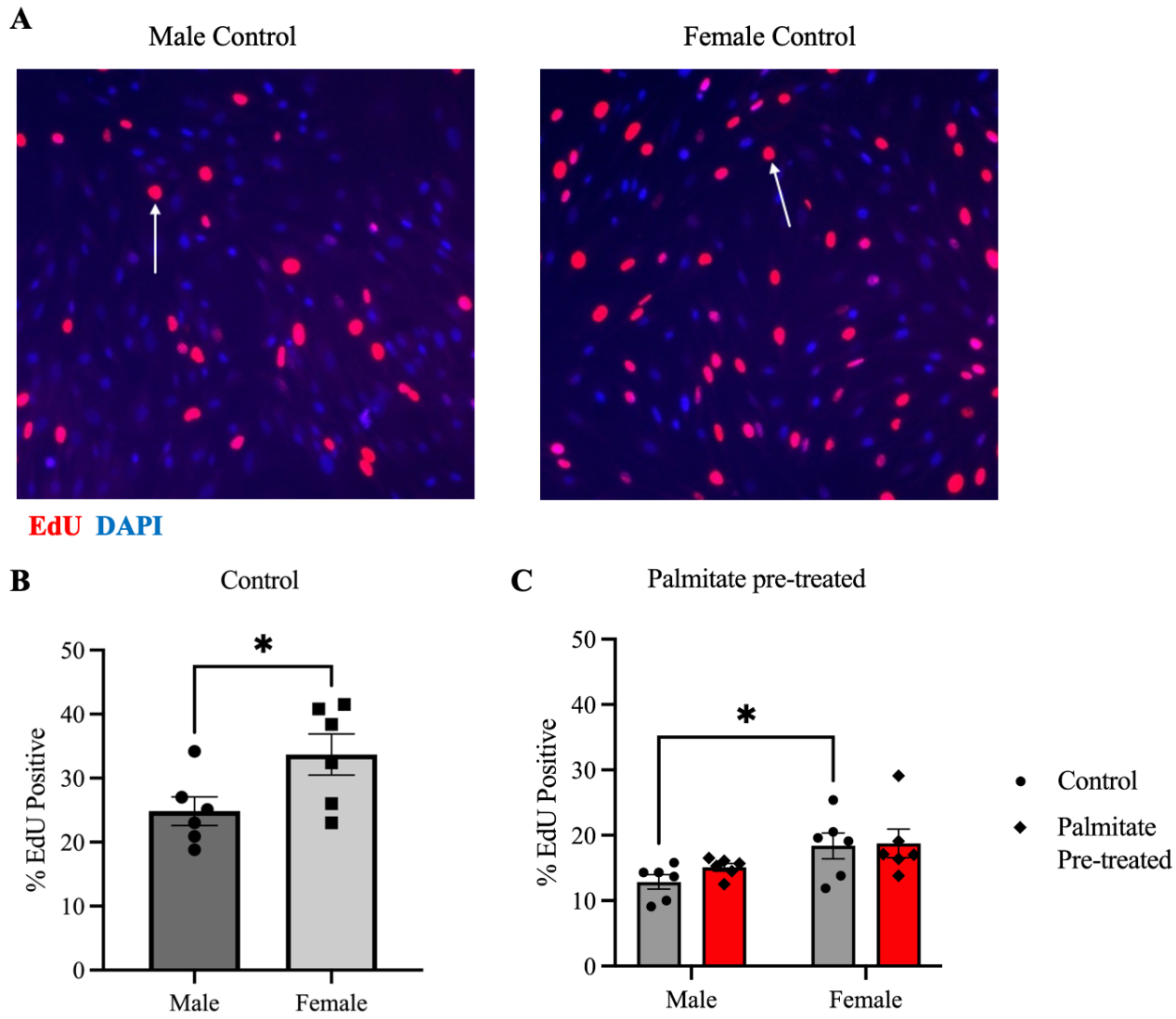

Figure 3.3: Proliferation of Male and Female ECs. (A) Representative images showing proliferation assay with male (M-EC) and female (F-EC) skeletal muscle ECs under control conditions. White arrows indicate EdU-positive cells (red- Alexa Fluor 545). All nuclei stained with DAPI (blue). **(B)** Quantification of proliferating cells (3 hours EdU incubation), expressed as the percent of EdU-positive cells; $n = 6$. **(C)** Quantification of proliferating cells (3 hours EdU incubation) following four-day palmitate pre-treatment of ECs, expressed as the percent of EdU-positive cells, $n = 6$. Data expressed as mean ± SEM, and analyzed using paired student t-test, $^*P < 0.05$ **(B)** and two-way ANOVA repeated measures; main effect of sex ($P = 0.05$), $^*P < 0.05$ Bonferroni *post hoc* analysis **(C).**

3.4 Metabolic Preferences in Male and Female ECs

I next assessed potential sex differences in reliance on metabolic pathways to support proliferation. Inhibition of glycolysis using PFKFB3 inhibitor 3PO (10 μM) did not alter the rates of cell proliferation in males or females compared to their respective controls. However, the 3PO-treated male ECs showed significantly greater EdU+ incorporation compared to their treated female counterparts, as indicated by a significant interaction between sex and the drug treatment (Fig. 3.4A, B). These results suggest that male ECs may rely less on glycolysis to support proliferation, compared to female ECs.

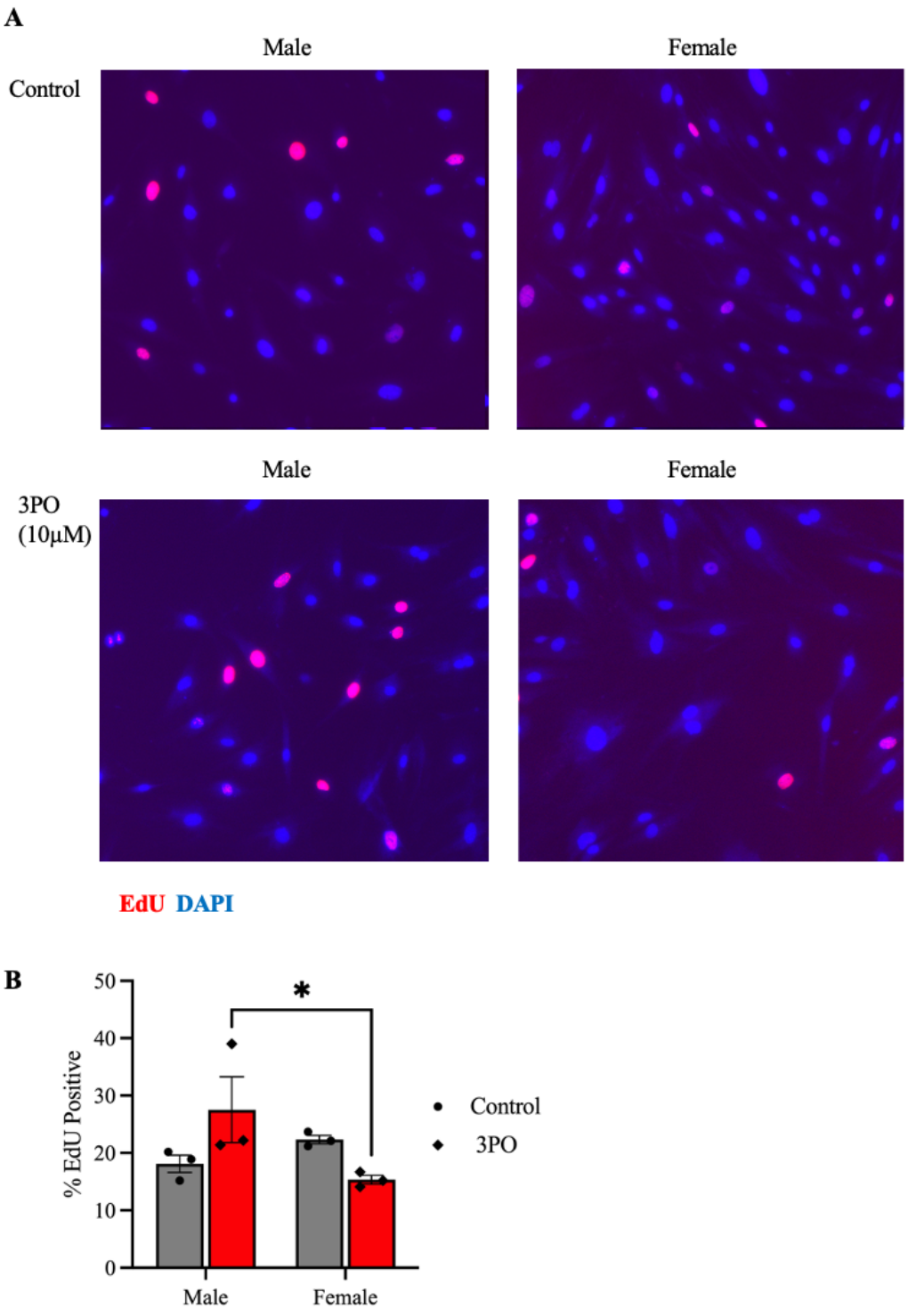

Figure 3.4: Effect of PFKFB3 Inhibition on Male and Female EC Proliferation. (A) Representative images showing proliferation assay with male (M-EC) and female (F-EC) skeletal muscle ECs after 24-hours of PFKFB3 inhibition (3PO; 10 μM) and control conditions. EdU-positive cells (red- Alexa Fluor 545). All nuclei stained with DAPI (blue). **(B)** Quantification of proliferating cells, expressed as the percent of EdU-positive cells after 3 hours of EdU incubation. Data expressed as mean ± SEM, and analyzed using two-way ANOVA repeated measures; interaction between sex and treatment ($P = 0.05$); *$P < 0.05$, Bonferroni *post hoc* analysis, $n = 3$.

I also tested effects of inhibiting fatty acid oxidation by inhibiting CPT1α (a key enzyme in fatty acid oxidation) using Etomoxir. Although there was a trend for a main effect of sex, there was no treatment effects of Etomoxir (Fig. 3.5A, B).

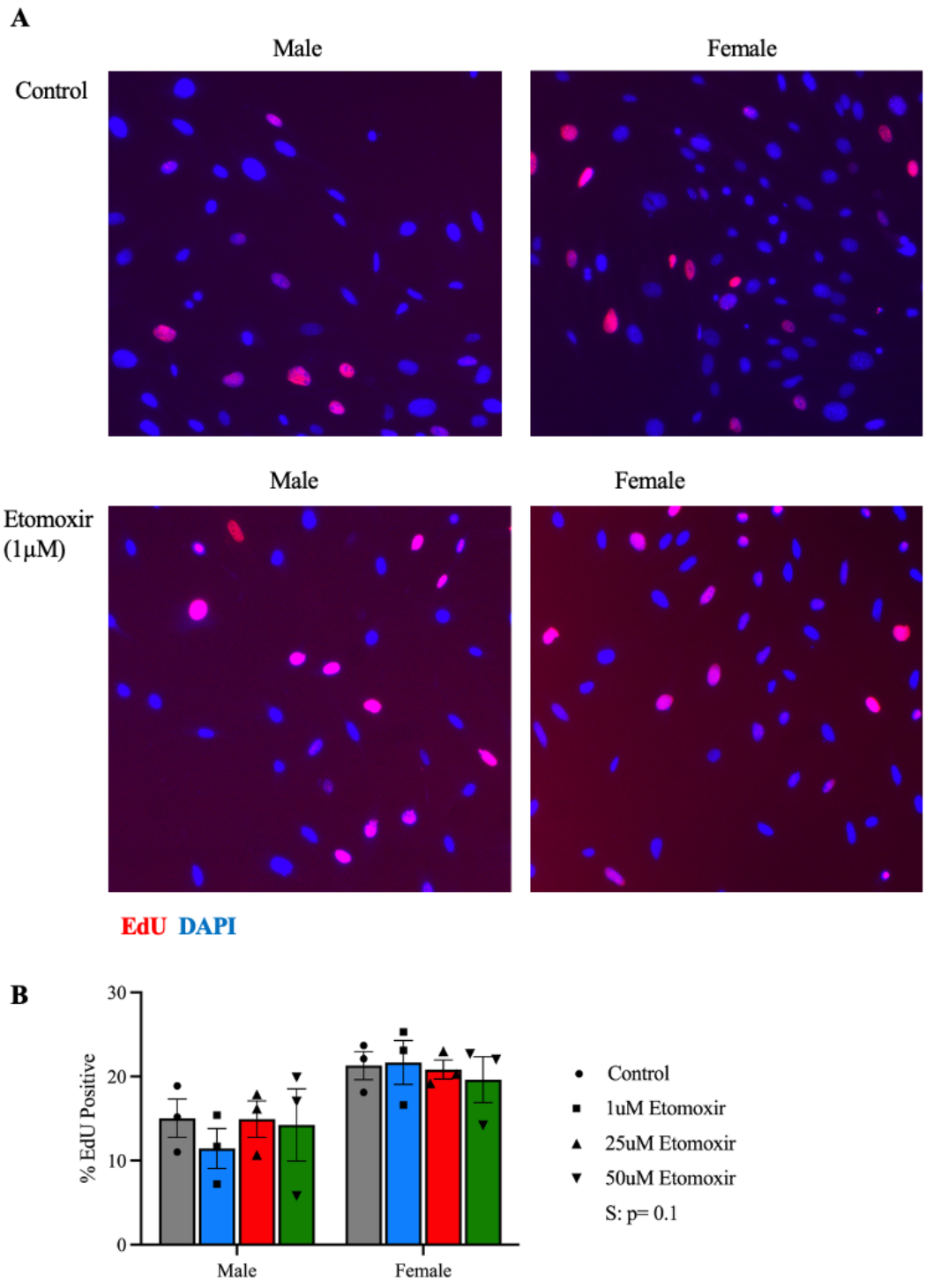

Figure 3.5: Effect of CPT1α Inhibition on Male and Female EC Proliferation. (A) Representative images showing proliferation assay with male (M-EC) and female (F-EC) skeletal muscle ECs following 24-hours of CPT1α inhibition (Etomoxir; 1 μM) and control conditions. EdU-positive cells (red- Alexa Fluor 545). All nuclei stained with DAPI (blue). **(B)** Quantification of proliferating cells treated with Etomoxir- 1, 25, 50 μM and control conditions, expressed as the percent of EdU-positive cells after 3 hours of EdU incubation. Data expressed as mean ± SEM, and analyzed using two-way ANOVA repeated measures. Trend for a main effect of sex (S); P =0.1 , n = 3.

3.5 Insulin Sensitivity in Male and Female ECs

Although sex differences in insulin sensitivity have been found in skeletal muscle (Haider et al., 2021; Broussard et al., 2021), it remains unclear if ECs themselves display sex differences in insulin sensitivity. Since insulin signaling is an important regulator of glucose and lipid handling in skeletal myocytes, it could possibly influence these processes in ECs. To determine responsiveness to insulin, I assessed the phosphorylation of Akt (on Serine 473), which occurs downstream of insulin receptor activation. Both male and female ECs showed significant increases in Akt phosphorylation after insulin stimulation, with no differences in levels of pSer473-Akt between sexes (Fig. 3.6A, B). This indicates that insulin sensitivity does not differ between male and female ECs under control culture conditions.

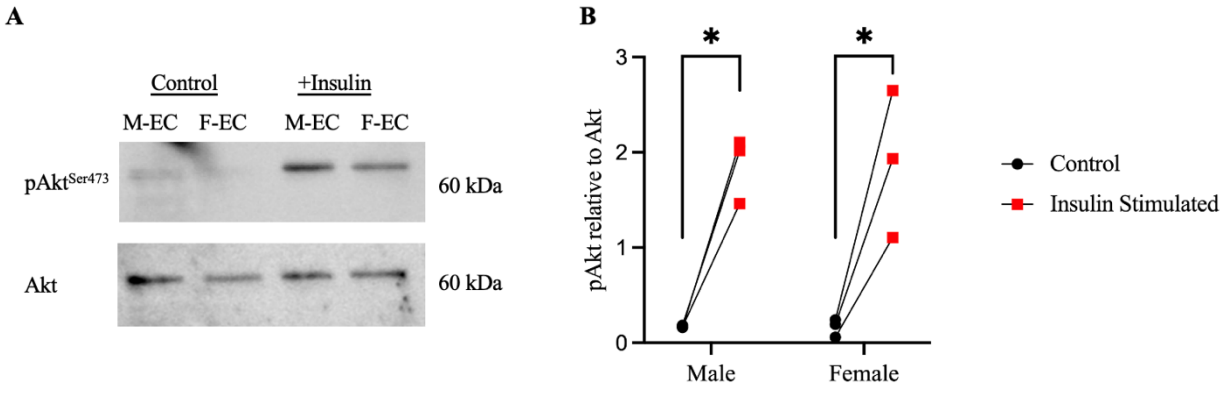

Figure 3.6: Levels of PhosphoSer473-Akt in Male and Female ECs Post-Insulin Stimulation. Western blot **(A)** and protein quantification of pSer473-Akt relative to total Akt protein in male (M-EC) and female (F-EC) skeletal muscle Ecs under basal (control) or insulin stimulated (25 mU/mL for 15 minutes) conditions. **(B)** Data analyzed using repeated measures two-way ANOVA, main effect of treatment ($P = 0.0015$); *$P < 0.05$, Bonferroni *post hoc* analysis, $n = 3$.

Based on the previous finding of higher insulin sensitivity in skeletal muscle of female compared to male high-fat diet fed mice (Rudnicki et al., 2018), I tested if pre-treatment with palmitate would alter insulin responsiveness in male or female ECs. In the male ECs, the insulin-stimulated increase in pAkt was significantly attenuated in palmitate pre-treated cells (Fig. 3.7A, C). Female ECs showed a trend to increase Akt phosphorylation upon insulin stimulation ($p=0.06$), but in contrast to the males, no significant variation in phosphorylation levels between control and palmitate pre-treatment was detected (Fig. 3.7B, D). These results indicate that prolonged palmitate exposure lowers insulin responsiveness in males, whereas insulin sensitivity in females may not be affected by palmitate treatment.

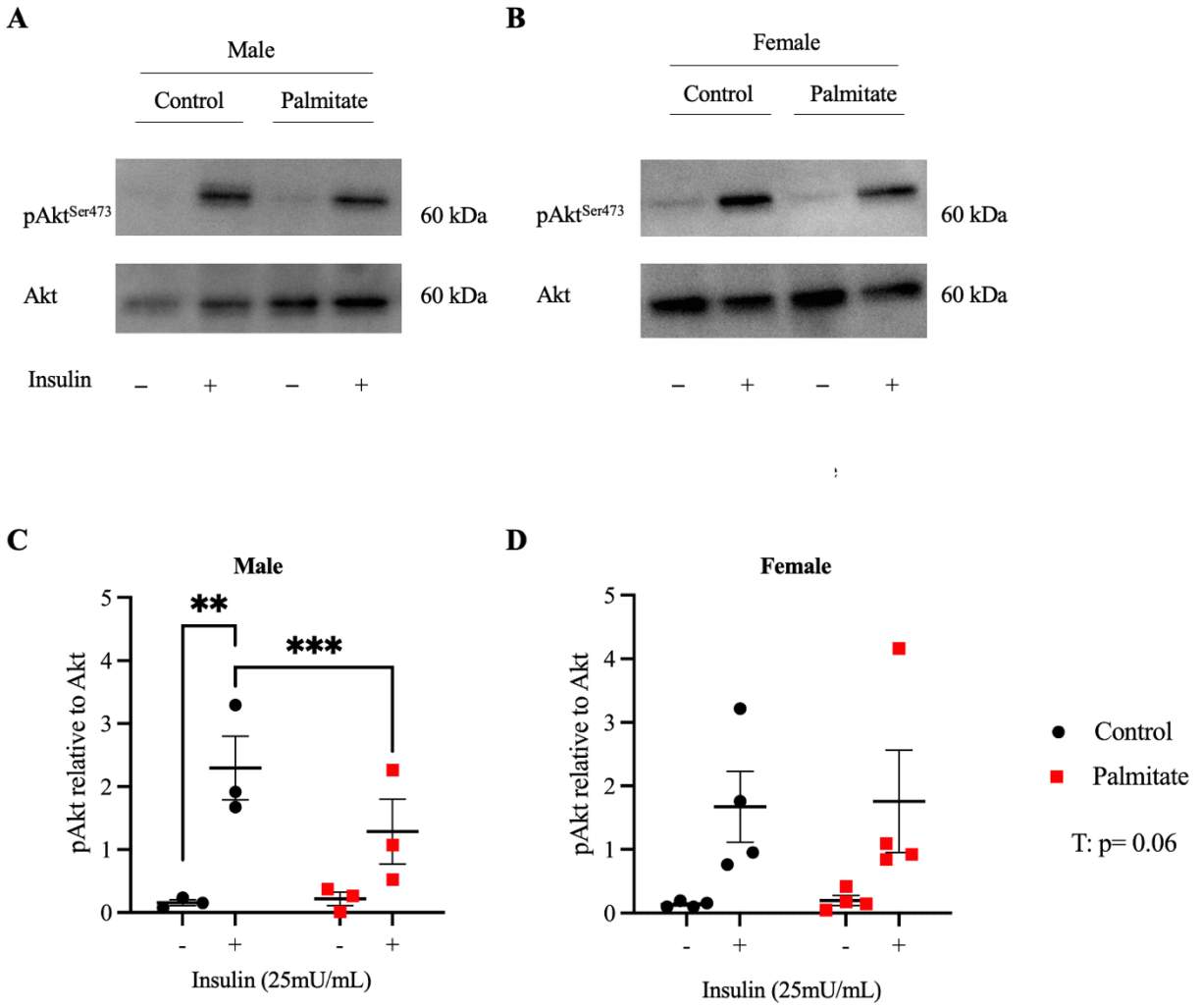

Figure 3.7: Effect of Palmitate Pre-Conditioning on Insulin-Dependent Phosphorylation of Akt in Male and Female ECs. Western blots for male **(A)** and female **(B)** ECs in control or palmitate pre-treated conditions (4-day palmitate pre-treatment). Protein quantification of pAkt-Ser473 relative to total Akt protein in male **(C)** and female **(D)** skeletal muscle ECs, under basal (-) or insulin (25 mU/mL for 15 minutes) conditions. Data expressed as mean ± SEM and analyzed using repeated measures two-way ANOVA. **(C)** Main effect of Insulin ($P = 0.0352$), treatment ($P = 0.0016$), and interaction between insulin and treatment ($P = 0.001$). $^{**}P$ <0.01, $^{***}P < 0.001$, with Bonferroni *post hoc* analysis, $n = 3$. **(D)** Trend for a main effect of insulin treatment (T); $P = 0.06$, $n = 4$.

Chapter 4: Discussion

4.1 Overview of findings

The goal of my thesis was to assess potential sex differences in lipid handling capacities and metabolic preferences in skeletal muscle ECs. My gene expression data showed that under both control and obesogenic-like conditions females expressed higher levels of genes involved in lipid handling, while males displayed greater levels of the glycolytic gene *Hk2.* Further, female ECs in obesogenic-like conditions demonstrated increased expression of *Pfkfb3*, a gene involved in regulating glycolysis. Complementing this gene expression data, I found that female ECs store more lipids, suggesting that they could be taking up and storing larger amounts of lipids compared to males under specific cell culture conditions. I also found that female ECs had higher rates of cell proliferation compared to male ECs in vitro. Moreover, I found sex differences in the reliance on metabolic pathways to promote cell proliferation, where males ECs showed a greater reliance on the glycolysis. Interestingly, no sex difference in insulin-dependent phosphorylation of Akt was seen under control conditions; however, after prolonged exposure to fatty acids, insulin sensitivity in females was unchanged, while males decreased their responsiveness. This could suggest that male ECs are more susceptible to dysfunction in insulin sensitivity when exposed to high levels of fatty acids such as seen in diabetes- and obesity-related disorders.

4.2 Sex Differences in Lipid Handling Machinery and Glycolytic Enzymes

In measuring gene expression of lipid handling machinery, I observed that female ECs displayed significantly higher levels of expression in *Lpl* and *Fabp4*. Although both genes are involved in lipid uptake into EC, they each have specific functions. Lpl is responsible for the breakdown of

plasma triglycerides into FAs and glycerol for transport into tissues, whereas Fabp4 binds to FAs inside of the EC to allow for movement within the cell (Mehrotra et al., 2014). The higher expression of both genes in female ECs, implies that females may have a greater capacity to uptake lipids regardless of condition. However, previous work in our lab indicated that male ECs from adipose tissue had greater expression of genes involved in lipid uptake (Rudnicki et al., In review). This brings evidence for potential sex differences that are tissue-specific, and promotes greater understanding EC characteristics based on sex and tissue environment. In measuring the expression of genes involved in lipid uptake, I also assessed *Cd36*, which had very low levels of expression regardless of sex or condition. Although *Cd36* is responsible for translocating FAs across the cell membrane, low gene expression of *Cd36* have been shown in our lab previously in cultured ECs. This may reflect a greater reliance of FAs on binding proteins or diffusion to enter the cell, and minimal reliance on transport proteins in cell culture conditions. Moreover, Cd36 is also a receptor for thrombospondin-1 (TSP-1), which functions as a negative regulator of angiogenesis (Dawson et al., 1997; Jiménez et al., 2000). Therefore, it is possible that the low expression of *Cd36* may be due to higher rates of proliferation and angiogenesis in these cells.

The results of the lipid droplet formation experiment demonstrated that female ECs stored greater amounts of lipids compared to males in serum-free, low-glucose conditions. This supports my prior gene expression data where females expressed higher levels of genes involved in lipid uptake. However, it still remains unclear if females are truly taking up more lipids as my experiment was not an uptake assay, just a snapshot of the cells at the present moment. In addition, it is unknown if these results can be repeated in other cell culture conditions. Moreover, the fatty acid uptake by ECs is not understood clearly (Mallick and Duttaroy, 2022).

Additionally, it is possible that the lipids seen in the cell will be used as fuel rather than stored for later use. This is in line with previous study by Rudnicki et al. (In review), where female ECs were found to be enriched in genes involved in fatty acid oxidation and the mitochondria; therefore, it can be suggested that these cells were taking up greater amounts of lipids to support the higher level of FAO. This hypothesis is in agreeance with several studies where females took up higher amounts of lipids (Olefsky et al., 1974; Mittendorfer et al., 2002), with one study indicating that skeletal muscle had the highest level of lipid uptake compared to adipose tissue in the females (Horton et al., 2002). Future research should be done to investigate potential sex differences in lipid uptake in ECs. Since the fate of fatty acids remains unknown once taken up, fatty acid oxidation should be measured in both sexes to determine a possible sex difference in the fates of lipids.

The lipid staining also differed between males and females based on the dispersion of the cytoplasm and the presence unknown structures. More specifically, male ECs showed greater dispersion of cytoplasm in the images, whereas females stored lipids in a more compact manner that is limited to the area immediately surrounding the nucleus. Additionally, males demonstrated the presence of a variety of unknown vesicular structures both inside and around the cells. It is possible that these unknown structures are the result of a stress response from the cells after being in serum-free, low glucose media for an extended period of time. However, future work must be done to determine the identify of these structures as well as how they affect male and female ECs.

In contrast to the genes associated with lipid uptake, I found that male skeletal muscle ECs expressed higher levels of *Hk2*, which is the enzyme involved in regulating the first step of glycolysis. This sex difference was independent of the culture conditions. Interestingly though, female ECs treated with palmitate and insulin demonstrated higher expression of *Pfkfb3* mRNA compared to their male counterparts. Pfkfb3 encodes the main enzyme involved in regulating glycolysis. These results were surprising as I had anticipated that male ECs would express higher levels of both glycolytic enzymes. Moreover, although my data does suggest sex differences in metabolism in skeletal muscle ECs, further work needs to be done to understand the broad picture, as this gene expression data is novel and has never been reported before in skeletal muscle ECs. For example, the metabolic rate of glycolysis and FAO in ECs should be assessed using the Seahorse method to help reveal potential sex differences in these pathways. Additionally, ECs from adipose tissue males displayed greater expression of genes involved in lipid uptake and no sex differences were found in glycolytic genes (Rudnicki et al., In review). This data is in contrast with my results, which suggests possible tissue-specificity.

4.3 Proliferative Capacity of Male and Female Skeletal Muscle ECs

My finding that female skeletal muscle ECs have higher rates of proliferation compared to male ECs in vitro agrees with several other studies that used ECs. For example, Addis et al., (2014) also found this observation in human umbilical vascular endothelial cells (HUVECs), while Rudnicki et al. (In review) found greater rates of proliferation in female ECs from adipose tissue from mice. However, the opposite has also been demonstrated where male ECs displayed higher rates of proliferation compared to female ECs from skeletal muscle tissue of rats (Huxley et al., 2018). It is important to mention that the rate of cellular proliferation can be affected by many

factors related to the cell culture conditions. For example, in the Huxley study, cell culture media contained 10% fetal bovine serum (FBS), which is lower than the 20% in the cell culture media that was used in my experiments. Thus, higher levels of lipids and growth factors present in my cell culture conditions may have enabled higher proliferative rates. In these two studies, cell proliferation was also measured differently and thus reflects a difference in the sensitivity of assessing cell proliferation. In my study, I measured cell proliferation based on EdU incorporation. This is a superior method since it enables you to measure the amount of newly synthesized DNA, which must occur prior to cell division, thus making it a highly accurate measurement of cell proliferation. In comparison, Addis used the MTT assay, which measures metabolic activity of the cells and Huxley measured fluorescence intensity of DNA content compared to a standard curve; therefore, neither method assesses DNA synthesis per cell. Finally, the density at which ECs are plated at prior to measuring cell proliferation can also impact the rate of cell proliferation. If proliferation is measured when cells have a greater level of confluency (as in the Huxley paper), it is likely that contact inhibition has occurred, in which case proliferation has slowed or ceased (Eagle and Levine, 1967).

In the cells that were pre-incubated with excess palmitate, I anticipated that ECs would be affected in one of two ways: (1) increase levels of proliferation due to greater abundance of lipids to metabolize; or (2) ECs would decrease their levels of proliferation due to excessive amounts of lipids. Based on my earlier finding of higher lipid handling gene expression in females, as well as previous data in our lab, I hypothesized that females would demonstrate increased levels of proliferation when pre-treated with lipids, while male EC proliferation would not be impacted by these conditions (Rudnicki et al., In review; Rudnicki et al., 2018).

Therefore, when higher amounts of lipids are available, females can better metabolize greater amounts of lipids and use fatty acid oxidation to in turn promote cell proliferation. Surprisingly, my data showed no difference in the rate of cell proliferation in male or female ECs when pre-incubated with excess lipids compared to their respective control counterparts. In detail, female ECs did not display higher levels of cell proliferation when treated with pro-longed lipid exposure as was hypothesized. However, the overall lower rates of proliferation in these cells suggests that the pre-treatment time window lowered their proliferative capacity potentially due to contact inhibition signals; and thus, it is difficult to draw conclusions.

4.4 Reliance on Metabolic Pathways to Promote Cell Proliferation

Based on my gene expression and lipid content assessments, I hypothesized that females prefer FAO, while males rely more on glycolysis for proliferation. In these experiments, the CPT1α inhibitor Etomoxir was administered in a dose response to assess its efficacy in inhibiting fatty acid oxidation to support cell proliferation. However, I observed no drug effect in my data. This could be the result of using doses that are ineffective, or that FAO inhibition does not alter EC proliferation in these conditions. In contrast, when inhibiting glycolysis, my results showed that there was a significant interaction between sex and treatment, however there was no drug effect. Therefore, I am reluctant to draw conclusions and future research will be required to provide more certainty.

4.5 Insulin Sensitivity in Male and Female Skeletal Muscle ECs

In ECs, insulin signaling is vital in promoting several different biological pathways including glucose uptake and the activation of eNOS (Steinberg et al., 2000). However, if the cell becomes

resistant to insulin these processes can be impaired, and may result in the cell becoming dysfunctional. Therefore, measuring potential insulin responsiveness in ECs can provide a greater understanding into the health of both the cell and the tissue. Importantly, this is the first study to directly assess insulin sensitivity of ECs specifically. In contrast, other studies have investigated in vivo, where insulin sensitivity is largely dependent on the vascular network and blood flow, which may affect the results. Under control conditions, I did not detect sex differences in EC responsiveness to insulin, as both male and female ECs had similar levels of Akt phosphorylation after insulin stimulation. This result is supported by similar findings in the skeletal muscle tissue (Nuutila et al., 1995; Lundsgaard and Kiens, 2014). In pre-treating ECs with excess lipids, I found a reduction in pAkt levels in the male ECs. This is in agreeance with prior studies that reported EC dysfunction and insulin resistance under high-fat diet conditions in males specifically (Rudnicki et al., 2018). Conversely, I hypothesized that females would not show this decline in Akt phosphorylation, based on previous findings of female ECs displaying greater insulin sensitivity after high-fat diet in skeletal muscle (Rudnicki et al., 2018). However, my results were not clearly interpretable as female ECs were not affected by lipid pre-treatment conditions. These findings were not as I had expected since the insulin stimulation did not exert a significant effect in the females, which makes it difficult to assess the impact of the palmitate pre-treatment conditioning.

4.6 Limitations

4.6.1 Cell Culture

Since my experiments were performed *in-vitro*, the results I observed may not provide a full explanation for sex-related differences in skeletal muscle ECs *in-vivo*. When the cells are

cultured, they are removed from their native micro-environment. ECs therefore are not exposed to different stimuli such as sex hormones, growth hormones, and cytokines, as well as secretions form parenchymal cells, that are normally present in vivo. In addition, ECs are also not exposed to shear stress in culture, which is known to coordinate multiple signaling networks that can lead to alterations in cell morphology, gene expression, and functional responses in the ECs (Davis et al., 2001). An additional challenge with in vitro experiments is knowing the appropriate media conditions to use, such as the amount of serum, pre-conditioning time, glucose levels, etc. For example, the glucose levels used in the cell culture media were likely higher than that seen in vivo. This may have exposed the cells to hyperglycemia-like conditions and in turn impacted their metabolic state. This can then affect cellular behaviour as well as influence the outcomes that are seen. Alternatively, by performing experiments *in-vitro,* I was able to focus specifically on ECs and tightly regulate the cell culture environment without the confounding contribution of additional stimuli as seen in vivo.

4.6.2 Palmitate Treatment

I had expected greater consequences of the palmitate treatment on the ECs in all of my palmitate treatment experiments. Based on previous literature, I anticipated that ECs would become dysfunctional due to increased levels of inflammation (Xing et al., 2019), impaired insulin signaling (Vazquez-Jimenez et al., 2016), and increased oxidative stress (Chen et al., 2019). Instead, I detected only modest effects on insulin-dependent activation of Akt in male ECs. Since my treatment did not impair EC health or function as effectively as expected, it is possible that palmitate I used was degraded. This might have been due to repeated freeze-thaw cycles, considering that the palmitic acid I had used was made in the years previous. In the event of

multiple freeze-thaw cycles, the conjugation of palmitate to BSA can become degraded, which prevents the proper interaction of the FAs with the cells (Angelini et al., 2022). In the future, fresh conjugates of palmitate to BSA will be made, and aliquoted to prevent multiple freeze-thaw cycles and the use of old fatty acids.

4.6.3 Inhibition of Glycolysis and Fatty Acid Oxidation

To assess EC reliance on different metabolic pathways to promote cell proliferation, I used pharmacological inhibitors to inhibit either glycolysis or fatty acid oxidation. One potential concern with pharmacological inhibitors is that they can have off target-effects that may in turn affect the results. For example, Etomoxir, used to inhibit Cpt1α, has also been shown to inhibit complex I of the electron transport chain and in turn induce oxidative stress (Yao et al., 2018; O'Connor et al., 2018). Additionally, the efficacy of Etomoxir in inhibiting Cpt1α must also be confirmed since it had no effect on proliferation in my experiments. This can be done by measuring lipid accumulation in ECs, as Etomoxir has been shown to increase lipid accumulation (DeZwaan-McCabe e al., 2017). Moreover, it is possible that 3PO, used to inhibit the enzyme Pfkfb3, has lost its efficacy and stability over time as I had been using an aliquot made in previous years despite 3PO only remaining stable for one year.

4.7 Significance

My thesis provides evidence for a higher proliferative capacity of female compared to male skeletal muscle ECs in culture conditions. Additionally, my findings support the potential for sex differences in EC lipid handling and metabolism. I showed that females may have higher lipid uptake capacity and storage, suggesting that they might handle excess lipids better without

developing dysfunction. Although my data lay the foundation to indicate sex differences in metabolic pathways used for cell proliferation, further work must be done to establish these potential sex differences. Future experiments also need to be done to assess the effect of obesogenic-like conditions on insulin sensitivity specifically in female skeletal muscle ECs.

In conclusion, sex differences in both lipid handling and metabolism in male and female ECs in skeletal muscle remains largely unknown in both control and obesogenic-like conditions. Through these investigations, we can understand how different EC characteristics may affect their proliferative capacity and their response to a high-fat diet environment. Future research will help to identify potential sex-differences in EC metabolic capabilities in skeletal muscle, and how their response to altered nutrient environment may affect tissue function, which may in turn lead to a greater understanding of the physio-pathological differences in male and female cardio-metabolic function. Moreover, a greater appreciation of sex-related differences in skeletal muscle ECs may suggest new targets for preventative and therapeutic approaches in treating obesity and metabolic-related disorders such as cardiovascular disease and type 2 diabetes.

4.8 Future Work

In continuing my current research, I will assess EC metabolism directly to identify any potential sex differences. Although my data showed the potential for some sex differences in the reliance on glycolysis versus fatty acid oxidation to promote cell proliferation, it is unconvincing on its own. The next step will be to use the seahorse assay to measure the rate of glycolysis and fatty acid oxidation in ECs directly, under several different conditions, such as control, obesogenic-like, or under inhibition of glycolysis or fatty acid oxidation. This procedure would present a full picture of EC metabolism and potential sex differences in their reliance on glycolysis and FAO

under different conditions. In addition, since I am assessing EC reliance on metabolic pathways to promote proliferation, the next step will be to induce a pro-angiogenic stimuli on the cells in culture. This will allow me to directly assess how EC metabolism affects the rate of proliferation.

Since leptin and insulin signalling pathways are complimentary to regulate glucose and energy homeostasis, I will also investigate the potential for sex differences in leptin sensitivity in skeletal muscle ECs. Leptin is a hormone that regulates insulin as it has been shown to increase insulin sensitvity, and is associated with fatty acid oxidation in skeletal myocytes (Kamohara et al., 1997; Minokoshi et al., 2012). One study has also found that circulating leptin levels may be higher in females, which in turn resulted in increased leptin signaling and increased fatty acid oxidation (Steinberg et al., 2002; Steinberg et al., 2003; Lundsgaard and Kiens, 2014). Therefore, it is possible that leptin signaling is regulated differently in males and females, which may result in the greater rate fatty acid oxidation seen in female ECs from adipose tissue (Rudnicki et al., 2018). Moreover, by studying the leptin signaling pathway in conjunction with insulin, it will provide a greater understanding how these hormones regulate EC metabolism, as well as how ECs can develop resistance to leptin and insulin which is often seen in obesity- and diabetes-related disorders.

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