Sex Differences in Endothelial FoxO1 Response To Nutrient and Oxidative Stress

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A THESIS SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF MASTER OF SCIENCE

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCE

YORK UNIVERSITY

TORONTO, ONTARIO

December 2024

Abstract

Endothelial cells (ECs) are crucial for maintaining vascular homeostasis, as they regulate blood flow, facilitate the exchange of gases and nutrients, and contribute to immune responses. Dysfunction of ECs, however, leads to impaired vascular function and is implicated in the pathogenesis of various diseases, including type 2 diabetes, atherosclerosis, hypertension, and chronic inflammation. Notably, these vascular dysfunctions exhibit sex-specific differences in their manifestation and progression. Previous studies from our lab have highlighted sex differences in the response of adipose tissue endothelial cells (ATECs) to the prolonged metabolic stress induced by a high-fat diet, along with response to oxidative stress stimulus, with females generally exhibiting more favorable outcomes. Moreover, female ECs were found to express higher levels of Forkhead Box O1 (FoxO1) compared to their male counterparts. This raised the hypothesis that sex differences in FoxO1 regulation might contribute to the observed differential responses to metabolic and oxidative stress in male and female ATECs. To investigate this, male and female ECs were treated with varying conditions encapsulating metabolic and oxidative stress. Whole cell and subcellular fractionation methods were used to measure sex differences in FoxO1 sub-cellular localization and post-translational modifications (PTMs) in responses to stressors. My results show that male ECs exhibit increased sensitivity to FoxO1 phosphorylation and a greater cytoplasmic localization of FoxO1. Female ECs however, demonstrate a stronger capacity to retain FoxO1 in the nucleus in response to oxidative stressors. Higher nuclear FoxO1 in females provides a strong argument for an enhanced FoxO1 transcriptional potential. These findings suggest that the regulation of FoxO1 is sex-dependent, providing new insights into the molecular mechanisms underlying sex differences in vascular physiology.

Acknowledgements

I would like to thank my supervisor Dr. Tara Haas for allowing me the opportunity to be part of her lab and conduct research. She is an expert in her field and an amazing supervisor who always goes out of her way to discuss and guide her students. Her tireless efforts at any time of the day ensured a successful thesis project.

I would also like to thank my current and past lab mates Alex, Mark, George, and Christian who always guided me when I needed help. Their expertise and willingness to help was always very generous and collaboration with them was always rewarding.

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Abbreviations

AKT - Protein kinase B

AMPK - AMP-activated protein kinase

ATEC - Adipose tissue endothelial cells

BH4 - Tetrahydrobiopterin

CD31 - Cluster of differentiation 31

CVD - Cardiovascular disease

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle Medium

DHT - Dihydrotestosterone

DTT - Dithiothreitol

E2 - Estradiol

EC - Endothelial cell

eNOS - Endothelial nitric oxide synthase

ERK - Extracellular signal-regulated kinase

ETC - Electron transport chain

ET-1 - Endothelin-1

FBS - Fetal bovine serum

FoxO1 - Forkhead box O1

FVB/J - Friend Virus B NIH Jackson

Gadd45 - Growth arrest and DNA damage-inducible protein

HAEC - Human aortic endothelial cells

H2O2 - Hydrogen peroxide

H2S - Hydrogen sulfide

H3 - Histone H3

HUVECS - Human umbilical vein endothelial cells

ICAM-1 - Intercellular adhesion molecule 1

IL-1β - Interleukin-1 beta

IL-6 - Interleukin-6

JNK - c-Jun N-terminal kinase

LPS - Lipopolysaccharides

mnSOD - Manganese superoxide dismutase

MST1 - Mammalian STE20-like kinase 1

mtDNA - Mitochondrial DNA

NADPH - Nicotinamide adenine dinucleotide phosphate

NFκB - Nuclear factor-κB

NO - Nitric oxide

NOX - Nicotinamide adenine dinucleotide phosphate oxidase

O2 - Molecular oxygen

O_2^- - Superoxide ion

$OH^\cdot$ - Hydroxyl radical

ONOO- - Peroxynitrite

P38 - p38 mitogen-activated protein kinase

PBS - Phosphate-buffered saline

PGI2 - Prostacyclin

PTM - Post-translational modification

PVDF - Polyvinylidene difluoride

RCF - Relative centrifugal force

ROS - Reactive oxygen species

SDS - Sodium dodecyl sulfate

SKMEC - Skeletal muscle endothelial cells

SOD - Superoxide dismutase

TBHP - tert-butyl hydroperoxide

TNF-α - Tumor necrosis factor-α

TTBS – Tris buffered saline with tween

VCAM-1 - Vascular cell adhesion molecule-1

VEGF - Vascular endothelial growth factor

O-GlcNAc - O-glcnacylation

Chapter 1: Literature Review

1.1 Vascular System

The vasculature system is a highly dynamic and intricate network responsible for the distribution of blood, nutrients, oxygen, and hormones throughout the body, while simultaneously removing metabolic waste products. The vasculature system is composed of three types of vessels: arteries, veins, and capillaries, each composed of differing morphology conducive to its respective function. Arteries transport oxygen rich blood from the heart to tissue and are thick vessels composed of multiple layers of smooth muscle to withstand the high pressure generated by the pumping of the heart. Once at the levels of tissue, capillaries facilitate the exchange of gasses and waste. Being the smallest structure void of any smooth muscle, their single cell layer thickness is conducive for fast and efficient exchange of materials. In contrast to arteries, veins are thinner vessels composed of less smooth muscle, conducive to their function in transporting deoxygenated blood back to the heart in a low-pressure environment. Together, these vessels ensure adequate blood flow to the body and are responsible for maintaining homeostasis blood pressure.

1.2 Endothelium

Although blood vessels differ in their morphology, endothelial cells form the foundation of all vascular structures. Endothelial cells line the inner surface of all blood vessels and are responsible for a myriad of functions in maintaining vascular homeostasis, one of which includes the regulation of the vascular tone. Vascular tone refers to the contractile state of blood vessels and is a pivotal determinant of resistance to blood flow (Jackson, 2000), enacting an important role in regulating blood pressure. Proper functioning of vascular tone regulation is essential for

allowing vessels to vasodilate (relax) to deliver adequate blood supply and ensure non-turbulent blood flow. Through production of vasodilatory molecules such as nitric oxide (NO), prostacyclin (PGI_2), and hydrogen sulfide (H_2S), endothelial cells are able to stimulate smooth muscle relaxation and vasodilation (X.-Y. Liu et al., 2022; Mitchell et al., 2008). Additionally, endothelial cells can initiate angiogenesis, the formation of new capillaries from pre-existing ones to ensure sufficient blood flow to expanding or oxygen-deprived tissues, as seen in processes like adipogenesis, muscle hypertrophy, or in response to ischemic conditions (Corvera et al., 2022; Kanazawa et al., 2019; Olfert et al., 2010). In addition to these functions, endothelial cells are vital players in immune responses as they can express adhesion markers, facilitating recruitment of immune cells to sites of injury (Amersfoort et al., 2022), while serving as an important barrier between the bloodstream and tissue.

Healthy endothelial cells are the cornerstone in maintaining vascular homeostasis, a role that becomes particularly clear when their dysfunction leads to disease. Endothelial dysfunction occurs when the healthy functions of endothelial cells are disrupted and is often the impetus of many vascular-related diseases, including atherosclerosis, hypertension, diabetes, and stroke (Cai & Harrison, 2000; Little et al., 2021; Shaito et al., 2022). One of the prominent stimuli that elicits endothelial dysfunction is oxidative stress, characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses (Pizzino et al., 2017). This imbalance can be triggered by both external factors, such as smoking, and internal issues like mitochondrial dysfunction, ultimately leading to endothelial dysfunction.

1.3 Oxidative Stress in the Vasculature

Oxidative stress can be defined as a state of heightened cellular stress caused by an imbalance between reactive oxygen species (ROS) production and the antioxidant defense system's capacity to neutralize these molecules (Pizzino et al., 2017). ROS are free radicals naturally generated during cellular metabolic activities in the mitochondria, and their levels can also rise due to harmful cellular responses. This imbalance leads to oxidative damage to cellular components such as proteins and DNA, contributing to various pathological conditions, including cardiovascular diseases (CVD) (Moris et al., 2017); in fact, oxidative stress plays a critical role in the progression endothelial dysfunction, which then promotes diseases like atherosclerosis and hypertension (Shaito et al., 2022), resulting in overall negative vasculature health.

1.3.1 Sources of Oxidative Stress in Endothelial Cells

Reactive oxygen species (ROS) consist of various molecular types, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$). While these molecules are mainly produced in the mitochondria, other cellular sources include NADPH oxidase, xanthine oxidase, and uncoupled endothelial nitric oxide synthase (eNOS).

Mitochondria are a major source of ROS production through complex I and III of the electron transport chain (ETC) as they carry out oxidative phosphorylation to produce ATP. While this is a healthy physiological process that results in a normal amount of ROS production necessary for redox signaling, leakage of electrons from the ETC can interact with molecular oxygen (O_2), forming superoxide (O_2^-), the primary ROS generated by mitochondria.

NADPH oxidases (NOX) are membrane-bound enzyme complexes that directly generate ROS by transferring electrons from NADPH to oxygen, forming superoxide. Within endothelial cells, NOX family enzymes, particularly NOX1, NOX2, and NOX4, are key sources of ROS (Konior et al., 2014). Certain factors such as hypertension, hyperglycemia, metabolic syndrome, and inflammation can all upregulate NADPH activity, leading to excessive ROS production.

One of most important regulators of endothelial cell homeostasis is endothelial nitric oxide synthase (eNOS). eNOS is responsible for the production of the most potent vasodilator, nitric oxide (NO), which regulates vascular tone, inhibits platelet aggregation, and prevents leukocyte adhesion (Moncada & Higgs, 2006). In pathology, uncoupling of eNOS occurs when there are insufficient levels of its cofactor tetrahydrobiopterin (BH4) or its substrate L-arginine, causing electron transfer within eNOS to be diverted toward molecular oxygen, forming superoxide rather than NO. Uncoupled eNOS thus shifts from being a vasoprotective enzyme to being a source of ROS, contributing to endothelial dysfunction.

1.4 Mechanisms of ROS induced Endothelial Dysfunction

Increased levels of reactive oxygen species (ROS) in endothelial cells are extremely harmful, leading to disturbances in multiple pathways, resulting in endothelial cell dysfunction. This dysfunction not only triggers issues in vascular cells but also creates a persistent positive feedback loop, where the dysfunctional endothelial cells contribute to additional problems and promotion in cardiovascular diseases such including atherosclerosis, hypertension, systemic inflammation (Cai & Harrison, 2000; Little et al., 2021; Shaito et al., 2022). The following explanation will shed light on various ways endothelial cell dysfunction can arise due to oxidative stress.

1.4.1 Reduced NO bioavailability:

A defining feature of endothelial dysfunction is insufficient NO production, which leads to a pro-inflammatory, pro-thrombotic, and vasoconstrictive endothelial state, contributing to cardiovascular diseases (CVDs) (Naseem, 2005). The reduction in NO bioavailability due to reactive oxygen species (ROS) can occur through various mechanisms. For instance, superoxide ions produced by mitochondria can react with NO, rendering it inactive and resulting in the formation of peroxynitrite (ONOO-) (Beckman & Koppenol, 1996). Another major pathway involves eNOS uncoupling, where instead of NO, superoxide ions are produced (Bauersachs & Schäfer, 2005). Notably, this creates a harmful cycle of decreased NO bioavailability and ROS as superoxide produced from uncoupled eNOS reacts with the otherwise functional NO. This reaction diminishes NO levels and forms peroxynitrite ($ONOO^-$). Additionally, $ONOO^-$ can lead to eNOS uncoupling by converting the active eNOS dimer into monomers, which produce superoxide instead of NO. $ONOO^-$ also oxidizes tetrahydrobiopterin (BH_4), a key cofactor for eNOS, into dihydrobiopterin (BH_2). In the presence of BH2, eNOS transfers electrons to molecular oxygen, generating superoxide rather than NO (Bauersachs & Schäfer, 2005). $ONOO^-$ has also been shown in bovine aortic endothelial cells to lead to mitochondrial dysfunction, resulting in elevated mitochondrial H_2O_2 production (Zorov et al., 2000), resulting in a net increase oxidative stress and prolonged endothelial cell dysfunction.

1.4.2 Inflammation

Endothelial dysfunction is a major contributing factor to chronic systemic inflammation. Excess ROS induces activation of nuclear factor kappa B (NF-κB), a protein that acts as a master regulator of inflammation through upregulating multiple pro-inflammatory factors such as

interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) and cell adhesion molecules like vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Davies et al., 1993). TNF-α, IL-6, and IL-1β can contribute to insulin resistance in the vasculature and promote monocyte infiltration across the endothelium, leading to endothelial cell damage. NO production typically inhibits the NF-κB signaling pathway, preventing the secretion of adhesion molecules to the cell membrane. Thus, reduced NO production or bioavailability leads to increased NF-κB activation and amplifies inflammation (Spiecker et al., 1998).

1.4.3 Mitochondrial dysfunction

Pathologies in mitochondrial functioning denoted by excessive superoxide production by the ETC results in damage to lipids, proteins, and DNA within the endothelium, resulting in endothelial dysfunction (Tang et al., 2014). Superoxide also reacts with NO to produce peroxynitrite (ONOO-), which lowers (NO) availability. Peroxynitrite can also directly damage electron transport chain (ETC) proteins (Davidson & Duchen, 2006), causing increased reactive oxygen species (ROS) production instead of ATP. ROS can also damage mitochondrial DNA, disrupting the protein synthesis of enzymes involved in the oxidative phosphorylation (Capt et al., 2016), such as cytochrome C oxidase and NADH subunits, all crucial components of the ETC. Importantly, pathology in the ETC and ATP production can also result in excessive ROS generation from complexes I and III, resulting in greater oxidative stress in the endothelium. The interconnected effects of ROS and ONOO- on mitochondrial dysfunction have been shown by Ballinger et al., in vascular endothelial cells. Induction of ROS via hydrogen peroxide resulted in mtDNA damage and consequentially decreased mtRNA transcription, protein synthesis, and

mitochondrial ATP production (Ballinger et al., 2000). They also found ONOO- induction to be effective in causing significant mtDNA damage, reduced mtRNA transcription and protein synthesis.

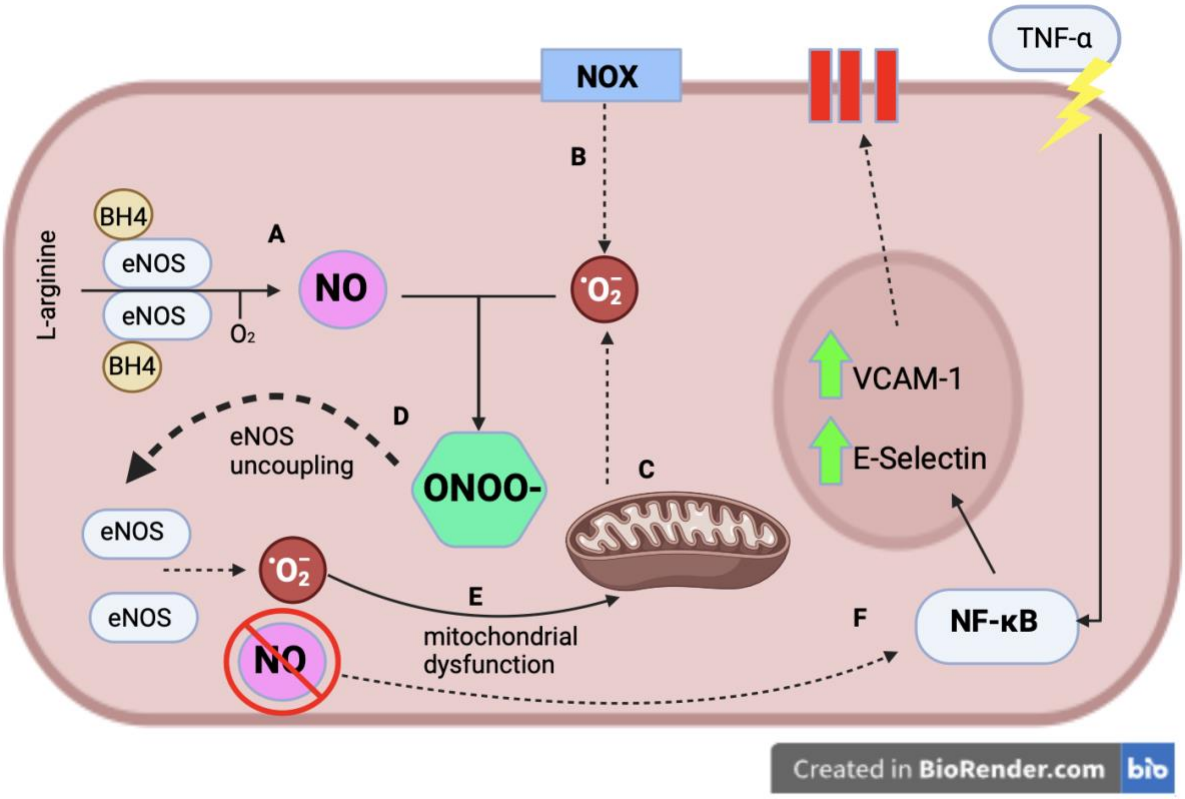

Figure 1.1: Schematic depicting effects of ROS on nitric oxide bioavailability and endothelial cell dysfunction. (A) In a healthy state, eNOS relies on co-factor BH4 and uses substrates L-arginine and molecular oxygen to produce nitric oxide (NO), necessary for healthy endothelial functioning. Superoxide, a form of ROS can be generated from NADPH oxidases (NOX) (B), as well as from the mitochondria (C); (D) this superoxide can interact with NO to produce peroxynitrite (ONOO), which in turn can cause eNOS uncoupling, resulting in eNOS monomers that produce more superoxide instead of NO, (E), contributing to mitochondrial damage and further elevations in ROS. (F) NO otherwise blocks TNF-alpha induced NF-κB activation, resulting in inhibition of cell adhesion molecules and less inflammation; decreased NO resulting from uncoupled eNOS mitigates this inhibitory effect, increasing susceptibility and responses to inflammatory stimuli.

1.5 Sex Differences in Endothelial Cells

One of the relatively understudied areas of vascular physiology is the influence of sex on endothelial cell function. Sex differences can arise from a combination of genetic and hormonal factors. Genetic variations, such as those on sex chromosomes (X and Y), can influence cell biology. In males, 693 genes are unique to the Y chromosome (Rhie et al., 2023), and not found

in females. For example, the SRY gene is unique to the Y chromosome, and results in male sex determination and development (Fechner, 1996). In females, which possess two X chromosomes, one X chromosome is randomly inactivated in the early process of embryogenesis with the purpose of achieving equal expression of X-linked genes in males and females, a process called dosage compensation (Meyer, 2022). However, 15-20% of the X-linked can escape inactivation in humans (Balaton et al., 2015), which gives rise to differences in gene expression in males and females. Epigenetic changes can also introduce another variable in genetic differences as histone modifications, DNA methylation can vary between males and females, impacting gene expression. Notably, epigenetic modifications are not exclusive to sex chromosomes, but can occur across the genome and can contribute to sex-specific susceptibilities to various pathologies (Hall et al., 2014; Maschietto et al., 2017; Migliore et al., 2021). Hormonal differences also play a large contributing factor to sex differences as the presence of estrogen, progesterone, and testosterone will exert specific effects on cell biology (Mank & Rideout, 2021), effects that become increasingly clear when they change from a physiological level such as in menopause.

Multiple studies provide evidence of sex- dimorphism in vascular function, with men exhibiting a higher risk of cardiovascular diseases compared to age-matched women (Dev et al., 2022; Rodgers et al., 2019; Maas & Appelman, 2010). Notably, this disparity decreases in postmenopausal women (Robert, 2023), suggesting a potential protective effect of female sex hormones. There exists ample data showing sex differences in gene expression specifically in endothelial cells that could result in an array of differences such ATP production pathways, insulin sensitivity, or response to cellular stressors. For example, RNA sequencing analysis reveals 1695 differently expressed genes between male and female mouse aortic endothelial cells (MAECs) (Shin et al., 2024). In adipose tissue endothelial cells, differential gene expression

analysis revealed 1,281 differentially expressed genes between the males and females (Rudnicki et al., 2023). Moreover, when it comes to immune responsiveness, such as the response to inflammatory stimuli, female endothelial cells exhibit an enrichment of genes that support this process (Lorenz et al., 2015).

Estrogen has been shown to provide vasoprotective effects on the endothelium. For instance, estrogen stimulates flow mediated dilation (FMD) via its activation of eNOS and subsequent production of NO (Davezac et al., 2021; Holder et al., 2019). Estrogen, also was shown to induce vasorelaxation via production of hydrogen sulfite (H_2S) in both HUVECs and HAECs (K. Zhou et al., 2013). Estrogen also decreases vasoconstrictors such as endothelin-1 (ET-1). This is evident in the fact that ovariectomized rats had enhanced levels and activity of ET-1, which was significantly reduced with estradiol (E2) treatment (Rodrigo et al., 2003; Tan et al., 2003). In addition to in vivo effects, cultured HUVECs also have a significant reduction in ET-1 upon E2 treatment (Bilsel et al., 2000). Along with regulating vascular tone, estrogen has also been reported to reduce expression of NADPH oxidase and ROS formation in HUVECs (Wagner et al., 2001). However, there are conflicting data that show the contrary. Shin et al., observed higher intracellular ROS in female HAECs compared to males, accompanied with higher mitochondrial damage, in both badsal conditions along with post Lipopolysaccharides (LPS) (Shin et al., 2024).

Elucidating the role of sex hormones in inflammation regulation, estrogen has been shown to reduce pro-inflammatory cytokine and consequentially tumor necrosis factor alpha (TNFα)-induced inflammation (Rodríguez et al., 2002). HUVECs treated with E2 also displayed decreased leukocyte adherence to endothelial cells as a result of inhibition in vascular cell adhesion molecule-1 induction (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-

selectin expression (Caulin-Glaser et al., 1996). These data can be complemented by findings from the Haas lab which showed male endothelial cells to have higher gene expression of cell adhesion molecules and to be more prone to stress when treated with TNFα (Rudnicki et al., 2023). Notably, under atherosclerotic conditions, female rats have also exhibited lower vascular ROS compared to males (Miller et al., 2007). However, Shin et al., reported female MAEC to have higher expression of ICAM1 and VCAM1(Shin et al., 2024), as well as higher levels of oxidative stress and apoptosis in atherosclerosis.

The male sex hormone testosterone has a wide range of effects in the vasculature which appears to differ based upon the context. On one hand, several studies indicate it has vasodilatory properties, as it promotes the activity of eNOS and increases NO levels (Hotta et al., 2019). However, in contrast to estrogen, testosterone treatment in HUVECs raises the levels of ET-1, which promotes vasoconstriction (Pearson et al., 2008). Further, testosterone treatment also results in reduced expression of prostacyclin PGI2, another vasodilator, both in vivo and in vitro (Jiang et al., 2021). Effects of androgens however are contradictory on inflammatory regulation. Testosterone and dihydrotestosterone were reported to inhibit TNFα induced VCAM-1 expression in HAECs, TNFα- induced VCAM-1 expression in HUVECS, and LPS-induced ICAM-1 expression in HUVECS (Hatakeyama et al., 2002; Norata et al., 2006). However, other researchers showed an enhancement of TNFα induced expression of E-selectin and VCAM-1 in HUVECs following testosterone administration (Zhang, Wang, et al., 2002). Further, women treated with testosterone exhibited elevated expression of pro-inflammatory cytokines IL-6 and TNFα as well as VCAM1 and E-selectin (Iannantuoni et al., 2021).

Female endothelial cells have been reported to display enhanced proliferation and migratory ability compared to males, along with higher expression of VEGFA and an overall pro angiogenic response (Addis et al., 2014; Rudnicki et al., 2018). Female adipose tissue vascularization in a high fat diet context has also been reported to be more robust and result in healthier outcomes whereas male adipose angiogenesis was supressed (Rudnicki et al., 2018). Further, in the context of a high-fat diet, adipose endothelial cells from male mice exhibited increased expression of genes associated with inflammatory responses compared to those from females. In vitro, when treated with the inflammatory stimulus tumor necrosis factor-alpha (TNF-alpha), male endothelial cells displayed greater expression of the adhesion molecule ICAM-1 (Rudnicki et al., 2023) which could potentially result in further recruitment of stress related factors. Overall, female endothelial cells displayed a less inflammatory phenotype. It is important to mention that other studies mentioned earlier (Shin et al., 2024) do report to the contrary as sometimes females are reported to have higher oxidative stress as well as a more robust inflammatory profile. Here again, difference in context may explain discrepancy in results as Shin et al. (2024), conducted their experiments in human aortic endothelial cells (HAEC) which due to their high-pressure setting have a greatly different phenotype, contrasted to the mouse adipose tissue endothelial cells utilized by Rudnicki et al. (Rudnicki et al., 2023); highlighting the cell-specific diversity in response.

In conclusion, the differences in endothelial cell physiology between sexes are extensive yet perplexing. Varying contexts, including different cell lines, generation of mouse models, and handling of hormones/drug administration, lead to inconsistent findings. The choice of hormonal manipulation seems to be a big factor in differing results. Many sex studies examining the role of testosterone often use dihydrotestosterone (DHT) interchangeably with testosterone, leading to

inconsistent results. DHT is significantly more potent, binding to receptors with greater strength and for a longer duration (Kinter et al., 2022). Additionally, unlike testosterone, DHT cannot be converted into estrogen (Kinter et al., 2022). These differences contribute to the observed discrepancies in the effects of sex hormones, particularly in males. The lack of a clear consensus on the impact of sex on vascular physiology highlights the need for more rigorous studies with standardized conditions to produce definitive conclusions.

1.6 Forkhead box protein O1

Forkhead box 1 (FoxO1) is a transcription factor belonging to the forkhead box O (FoxO) family. In mammals, there are four distinct FoxO genes: FoxO1, FoxO3, FoxO4, and FoxO6 (Calnan & Brunet, 2008). FoxO1 is essential for numerous cellular processes including metabolism, oxidative stress response, cell cycle regulation, DNA repair, and apoptosis management (Calnan & Brunet, 2008; Greer & Brunet, 2005; Tran et al., 2002; Y et al., 2005). Consequently, it is often referred to as a "tumor suppressor gene" and is one of the targets of research in oncology. FoxO1 acts primarily as a transcription factor that is modified by phosphorylation, acetylation, ubiquitinating, and methylation (Matsuzaki et al., 2005; Yamagata et al., 2008; Zhang, Gan, et al., 2002). These modifications influence the cellular localization and transcriptional activity of FoxO1. FoxO1 is expressed ubiquitously and performs various functions depending on the context, with especially critical roles in maintaining vascular homeostasis.

1.6.1 Role of FoxO1 in Vascular Development

FoxO1 has been discovered early on as being a crucial component in development as it plays an imperative role in vasculogenesis. Emphasizing its importance in healthy vascular development, two highly influential papers published in 2004 detailed the degenerative and lethal consequences of FoxO1 deficiency. These studies demonstrated that FoxO1 is essential during embryonic development for proper vascularization (Furuyama et al., 2004; Hosaka et al., 2004). In this study, FoxO1 knockout mice died around day 11 of embryonic development due to impaired vascular development. This was further confirmed by Dharaneeswaran et al., who replicated these findings, showing that FoxO1 knockout mice exhibited defective vascularization and development during embryogenesis (Dharaneeswaran et al., 2014). Among the mammalian FoxO family, FoxO1 is the most critical for vascular development, as mice lacking either FoxO3 or FoxO4 were viable and showed no significant differences compared to their controls (Hosaka et al., 2004). It is important to note that the embryonic lethality observed in mice is due to a total FoxO1 knockout. However, it becomes evident that the potential underlying cause is specifically the loss of FoxO1 in endothelial cells, as endothelial-specific FoxO1 knockout mice also exhibit the same degenerative phenotype seen in total FoxO1 knockout mice (Dharaneeswaran et al., 2014), a finding also replicated in invitro in HUVECs. To contrast the necessity of endothelial FoxO1, embryonic development remains unaffected in mice with lineage-specific deletions of FoxO1 in bone (Rached et al., 2010), liver (Matsumoto et al., 2007), heart (Sengupta et al., 2011), suggesting the embryonic lethality in the complete knockout is mainly attributed to the loss of FoxO1 in the endothelium. To emphasize the importance of endothelial specific FoxO1 in development, endothelial FoxO1 re-expression in the total knockout was able to rescue the mice (Dharaneeswaran et al., 2014).

The requirement of FoxO1 for normal vascular development may depend on its connection to vascular endothelial growth factor (VEGF). FoxO1 has been shown to be essential for proper VEGF signaling, a powerful angiogenic factor that promotes endothelial cell growth, proliferation, and migration (Furuyama et al., 2004; Hosaka et al., 2004). In HUVECs, FoxO1 silenced endothelial cells displayed decreased migratory and proliferative activity in response to VEGF (Dharaneeswaran et al., 2014). Indeed, active, nuclear FoxO1 is responsible for vessel sprouting and tip cell formation (important initiating steps for vessel formation) in response to VEGF (Miyamura et al., 2024). However, contradicting findings have also been reported to show that the necessity of FoxO1 in VEGF mediated vessel growth is not ubiquitous, rather it is context dependant. Liver endothelial cells taken from FoxO1/3/4 knockout mice actually displayed greater proliferation and growth when stimulated with VEGF (Paik et al., 2007). Potente et al., also found HUVECs with FoxO1 silencing displaying more proliferative and migratory activity in tube formation assays (Potente et al., 2005), illustrating the dynamic nature of FoxO1 in varying contexts.

While FoxO1 is essential for embryonic development and vasculogenesis, its impact on vascular processes such as angiogenesis begins to change postnatally. In these adult stages, it can enact anti-angiogenic roles due to its ability to induce quiescence in endothelial cells, thereby maintaining them in a low-energy, non-proliferative state. This is highlighted by Wilhelm et al., where it was demonstrated that in HUVECs, FoxO1 was responsible for reducing glycolysis (the primary energy pathway for endothelial cells), mitochondrial respiration, and cell cycle activators like Cyclin D1 (Wilhelm et al., 2016). FoxO1 also downregulates c-Myc, a key regulator of growth and metabolism (Dang, 2013; Wilhelm et al., 2016). Highlighting the

metabolic role of FoxO1, research from the Haas lab demonstrated that the depletion of FoxO1 specifically in endothelial cells led to an increase in their glycolytic capacity, which subsequently promoted greater endothelial cell proliferation and growth (Rudnicki et al., 2018). Given its inhibitory effect on endothelial cell metabolism, growth, and proliferation, FoxO1 has been termed a "gatekeeper" of endothelial cell quiescence (Wilhelm et al., 2016). The complexity of FoxO1 behaviour underscores the need to study FoxO1 in various conditions to fully understand its complex behavior.

1.6.2 FoxO1 role in oxidative stress:

FoxO1 also acts as a regulator of the stress response to reactive oxygen species and oxidative stress. The role of FoxO1 in stress response includes the elevation of antioxidant proteins, facilitating the detoxification of reactive oxygen species (ROS) and enhancing stress resistance (Klotz et al., 2015). Among the antioxidant genes transcribed by FoxO1 is Mn-SOD (manganese superoxide dismutase; SOD2) (Kajihara et al., 2006), a mitochondrial antioxidant protein. By deterring the accumulation of free radicals produced by the electron transport chain, SOD2 diminishes the release of ROS from the mitochondria (Holley et al., 2011), one of the largest sources of ROS within the cell. FoxO1 not only scavenges ROS produced by the mitochondria but also indirectly reduces the initial production of mitochondrial ROS. In HUVECs, overexpression of FoxO1 led to a decrease in both mitochondrial oxidative phosphorylation and ROS levels (Wilhelm et al., 2016). FoxO1 is also responsible for upregulation of other anti-oxidant genes such as superoxide dismutase (SOD1), peroxidases, and catalases (Krafczyk & Klotz, 2022). These defense proteins work in conjunction to dismutase free radicals (ROS) into its intermediate, hydrogen peroxide, and eventual dissociation into water

(H2O) and molecular oxygen (O2). FoxO1 not only promotes the expression of genes that directly counteract reactive oxygen species (ROS), but it also plays a role in repair processes and apoptosis triggered by oxidative stress. For example, Gadd45, a downstream target of FoxO proteins, is upregulated in response to cellular stress, aiding in the repair of DNA damage caused by ROS (Brunet et al., 2004).

The importance of FoxO1 in protecting against oxidative stress is highlighted by Akasaki et al., in human chondrocytes. Upon FoxO1 and FoxO3 silencing, there is a greater increase in ROS generation when stimulated with tert-butyl hydroperoxide (TBHP), a generator of ROS (Akasaki et al., 2014). This may be attributed to the observed downregulation of oxidative defense proteins like glutathione peroxidase and catalase, even though SOD2 was unaffected (Akasaki et al., 2014). FoxO1 knockout mice have also shown reduced levels of mn-SOD and catalase (Kandula et al., 2016). One major shortcoming of current FoxO1 knowledge pertaining to oxidative stress is the lack of endothelial cell specific studies. When discussing the role of oxidative stress, the term "FoxO" is generally stated without specificity to the exact FoxO factor. Additionally, there is a limited amount of direct research on FoxO1 and oxidative stress specifically in endothelial cells. Given the context-dependent nature of FoxO1 in other areas, this raises questions about its role in the vasculature under stress. Therefore, my project will focus on directly examining the effects of oxidative stress on FoxO1.

1.7 FoxO1 influence on Mitochondria

It has been established that FoxO1 is involved in transcriptional regulation of nuclear encoded genes that can repress mitochondrial oxidative phosphorylation in endothelial cells, specifically through suppression of c-MYC (Wilhelm et al., 2016). Wilhelm et al. showed that

FoxO1 overexpression reduced the oxygen consumption rate, decreased ATP production and decreased ROS production, showing how FoxO1 indirectly impacts ROS production by virtue of decreases in oxidative phosphorylation, while simultaneously limiting accumulation of ROS through its targets such as catalase and superoxide dismutase (Holley et al., 2011). These functions occur via the actions of nuclear FoxO1.

Interestingly, there is evidence indicating that FoxO1 can localize to mitochondria and bind to mitochondrial DNA (mtDNA) in adipocytes (Lettieri-Barbato et al., 2019); here, they reported that FoxO1 localized to mitochondria under high glucose conditions, and it delocalized and shuttled to the nucleus during nutrient-restriction. Further, they reported that FoxO1 was indeed able to bind to the D-loop region of mitochondrial DNA under basal conditions, which was then diminished under a nutrient restrictive state. They reported that mitochondrial encoded genes MTCO1 (cytochrome X oxidase subunit 1 of respiratory complex IV) and ATP6 (ATP synthase F0 subunit 6) were upregulated following nutrient restriction (resulting in mitochondrial export of FoxO1), implying that FoxO1 had an inhibitory function on the transcription of mtDNA. Another member of the FoxO family that shares close similarities with FoxO1, FoxO3, also was shown to localize to the mitochondria where it bound to the D-loop region (Celestini et al., 2018; Jerome et al., 2022; Peserico et al., 2013). However, in skeletal muscle cells, fibroblasts, and cancer cells, low glucose availability was shown to induce FoxO3 mitochondrial localization through an AMP-activated protein kinase (AMPK) and extracellular signal-regulated kinase (ERK) dependant pathway. Unlike FoxO1, FoxO3 was shown to upregulate transcription of mitochondrial encoded genes upon mtDNA binding; protein levels of ND6 and COX I (mitochondrial encoded OXPHOS subunits) were also upregulated (Peserico et al., 2013). Variations in FoxO1 related outcomes in mitochondria warrants further research. Importantly,

these studies have not been attempted in endothelial cells, so it remains an unanswered question if FoxO1 can localize to mitochondria in endothelial cells, and further, influence mitochondrial functioning at a transcriptional level. For this reason, my research will help illuminate the role of endothelial FoxO1 in mitochondrial functioning.

1.8 FoxO1 Post Translational Modifications

The regulatory influence of FoxO1 on cellular processes and responses is determined by its subcellular localization. When FoxO1 is localized to the nucleus and in thus in close proximity to nuclear DNA, it can act as a transcription factor in upregulating target genes. Cytoplasmic shuttling and localization are widely known to inhibit FoxO1 activity due to its inability to act as a transcription factor as it detaches from the DNA. However, there have been reports where cytoplasmic FoxO1 enacts specific functions independent of its transcriptional activity. In human lung cancer cell lines, cytoplasmic FoxO1 has been shown to bind to and activate Atg7, (a protein involved in autophagy and cell death) resulting in cell death (Zhao et al., 2010). This was one of the first and very few studies illustrating the role of cytoplasmic FoxO1. The dynamic nature of FoxO1 shuttling and localization is key mechanism in determining cellular response to external stimuli and transcriptional potential. This localization is tightly regulated by various post-translational modifications (PTMs), which either promote nuclear import or cytoplasmic retention of FoxO1, thus modulating its activity through activation or inhibition, respectively. The movement of FoxO1 between the nucleus and the cytoplasm is facilitated by proteins like the 14-3-3 chaperone. Inside the nucleus, the 14-3-3 protein binds to FoxO1 and facilitates its transport it to the cytoplasm (Tzivion et al., 2011). For FoxO1 nuclear import, however, it must first detach from the cytoplasmic 14-3-3 chaperone. The modification

of FoxO1 leads to a highly intricate network of regulatory mechanisms and functional outcomes, as each (PTM) can be triggered by diverse cellular conditions, ultimately affecting FoxO1 localization and transcriptional activity. Among the numerous PTMs, phosphorylation, acetylation, and O-GlcNAcylation are key regulators of FoxO1 behavior. These three PTMs form the basis of my thesis project and will be discussed in detail, with an emphasis on their regulatory pathways and their effects on FoxO1 localization and function.

1.8.1 Phosphorylation:

Phosphorylation is one of the most studied and well-defined PTMs that FoxO1 can undergo. There is an array of kinases responsible for FoxO1 phosphorylation, some of which are implicated in altering FoxO1 subcellular localization, altering its subcellular localization, DNA binding and consequently its transcriptional capability. The primary pathway responsible for the nuclear exclusion, and thus downregulation in the transcriptional ability of FoxO1 is phosphorylation via the PI3K/AKT pathway (Biggs et al., 1999). This pathway stimulates protein kinase B (AKT) and serum/glucocorticoid inducible kinase (SGK), which then phosphorylate cytoplasmic and nuclear FoxO1 on Ser256 and Thr24, resulting in its translocation into the cytoplasm where it can then be ubiquitinated and thus targeted for degradation (Matsuzaki et al., 2003). A key external stimulus that activates this pathway is insulin. Conversely, under nutrient-restricted conditions that diminish AKT signaling, the lack of phosphorylation at Ser256 and Thr24 facilitates the nuclear import and retention of FoxO1, allowing it to bind to its target genes.

Stress kinases such as MST1, JNK, and P38 phosphorylate FoxO1 resulting in its nuclear localization and enhanced transcriptional capability (Brown & Webb, 2018). FoxO1 residues targeted by these stress kinases include: er, Ser295, Ser326 (Zhang et al., 2021). The nuclear localization of FoxO1 achieved by these stress kinases can be attributed to their active disruption of the 14-3-3 chaperone proteins which otherwise sequester FoxO1 to the cytoplasm (Tzivion et al., 2011). These stress kinases are pivotal in the oxidative stress response as their modifications to FoxO1 results in increased nuclear localization, and in the upregulation of FoxO1-target oxidative defense genes such as SOD2 and catalases (Almeida, 2011), establishing a negative feedback loop to counteract the initial stimuli that activated JNK and MST1. Extracellular signal-regulated kinase (ERK) is another member of the MAP kinase family, like JNK, which is also implicated in oxidative stress response and FoxO1 phosphorylation. ERK can be activated by oxidative stress (Guyton et al., 1996), and directly phosphorylates FoxO1 on multiple sites; however this does not seem to influence FoxO1 localization. Adenosine monophosphate-activated protein kinase (AMPK) is another kinase that is responsible for FoxO1 phosphorylation and is particularly important in oxidative stress response. Foxo1 can be directly phosphorylated at Thr649, resulting in nuclear localization of FoxO1, and upregulation in anti-oxidant genes such as superoxide dismutase and catalase (Yun et al., 2014). Notably, AMPK, in addition to directly phosphorylating FoxO1, can also increase the activity of a histone deacetylase, sirtuin 1 (SIRT1), which consequently leads FoxO1 deacetylation and activation (Cantó et al., 2009).

1.8.2 Acetylation:

Acetylation is another significant post-translational modification (PTM) of FoxO1 that influences its localization and transcriptional activity, depending on the cell's nutritional state and

oxidative stress levels. When the cell is in a regular fed state, or stimulated with insulin, this typically activates the acetyltransferases involved in acetylation, while diminishing the activity of histone deacetylases (HDACS). Oxidative stress however works in an opposite fashion where HDAC activity is increased, leading to deacetylation.

FoxO1 can be acetylated at a myriad of amino acid residues, and this is achieved by an acetyltransferase called Calcium response element-binding protein (CBP)/p300 (Daitoku et al., 2004). Acetylation of FoxO1 by CBP/p300 results in a decreased DNA-binding capacity of FoxO1, thus reducing its transcriptional capability (Matsuzaki et al., 2005). By inhibiting the sensitivity of FoxO1 to HCADs, the CBP/p300 complex acetylates FoxO1, reducing its transcriptional ability, and resulting in the downregulation of target genes such as glucose 6-phosphatase in liver hepatocytes (K. Li et al., 2019), for example. CBP/p300 has been shown to be activated by insulin stimulation (He et al., 2009; Wan et al., 2017; X. Y. Zhou et al., 2004) and AKT phosphorylation (Huang & Chen, 2005; Y. Liu et al., 2006).

FoxO1 can be deacetylated by histone deacetylases (HDACs) called sirtuins, which are located in the nucleus, cytoplasm, and mitochondria. Sirtuin 2 (SIRT2), predominantly located in the cytoplasm, can deacetylate cytoplasmic FoxO1, resulting in its nuclear import (Daitoku et al., 2011). Inhibiting SIRT2 mediated deacetylation has been shown to heighten FoxO1 acetylation and cytoplasmic retention (Jing et al., 2007). In the nucleus, SIRT1 deacetylates FoxO1, which increases its DNA binding ability and thus transcriptional capacity (Matsuzaki et al., 2005). Contrary to acetylation, HDACs and deacetylation are stimulated by cellular conditions associated with nutrient restriction (Beharry et al., 2014). Since sirtuins are Nicotinamide adenine dinucleotide (NAD+) dependent, depriving cells of glucose (Picard et al., 2004) can increase NAD+ content, and thus activation of HDACs. Notably, acetylation and

phosphorylation not only share similarity in their inhibitory effect on FoxO1, but they are also proven to be interconnected. Matsuzaki et al. illustrated in HepG2 cells that FoxO1 acetylation in the nucleus not only reduced DNA binding of FoxO1, but also resulted in a heightened AKT phosphorylation, and a subsequent nuclear exclusion of FoxO1; highlighting the synergistic relationship between different PTMs (Matsuzaki et al., 2005). Thus, acetylation and phosphorylation work in tandem and are stimulated in a fed state; Conversely, upon nutrient restrictive conditions, or states of high oxidative stress, acetylation and phosphorylation will be repressed, while increased HDAC activity will result in increased FoxO1 nuclear localization, and transcriptional capability.

1.8.3 O-GlcNAcylation

One of the least studied PTMs of FoxO1 is GlcNAcylation. This glycosylation pathway is activated by the hexosamine biosynthesis pathway where OGT (O-GlcNAc transferase) transfers O-GlcNAc (a monosaccharide) to FoxO1 (Housley et al., 2008). High glucose conditions result in an increase in uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is the donor molecule required for O-GlcNAcylation. The GlcNAc molecule from UDP transferred to the protein of interest by OGT. This modification has been shown to increase FoxO1 transcriptional potential (Kuo et al., 2008), resulting in increases in its downstream genes such as SOD and catalase (Housley et al., 2008). In contrast to acetylation and phosphorylation, it remains unclear whether glycosylation affects the nuclear and cytoplasmic shuttling of FoxO1. While O-GlcNAcylation can induce nuclear-cytoplasmic shuttling in other transcription factors (Andrali et al., 2007), this has not yet been observed with FoxO1. So far, FoxO1 modulation by O-GlcNAcylation appears to be independent of its cellular localization (Kuo et al., 2008). However,

there is a significant lack of research on this topic, and the role of FoxO1 O-GlcNAcylation in endothelial cells needs further investigation.

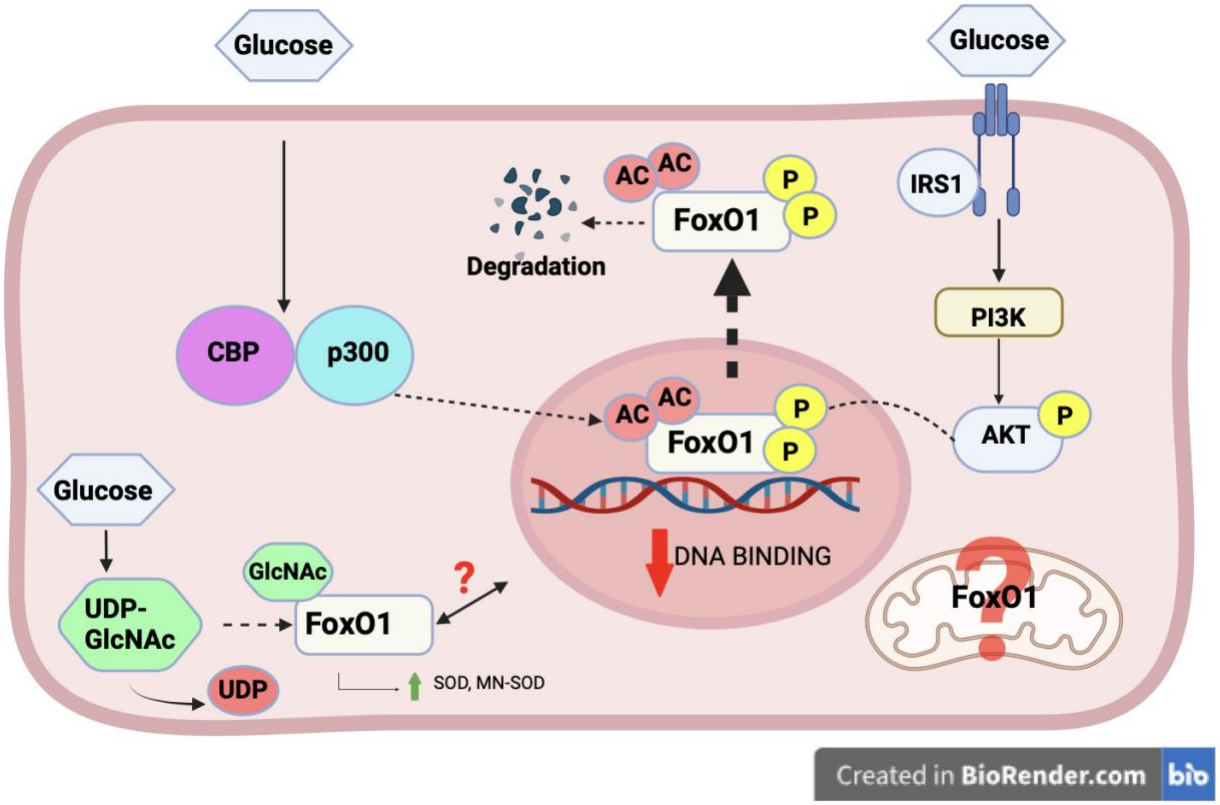

Figure 1.2: Schematic illustrating signals that promote FoxO1 nuclear export.
Insulin stimulation activates the PI3K/AKT pathway, resulting in FoxO1 phosphorylation and its translocation from the nucleus to the cytoplasm. This can result in its degradation upon targeting by ubiquitination proteins. In fed states, as well as insulin stimulation, acetyltransferase CBP/p300 is activated and acetylates nuclear FoxO1, resulting in decreased binding and nuclear export. The hexosamine biosynthesis pathway is activated by glucose, resulting in FoxO1 O-GlcNAcylation; the influence of this on FoxO1 localization is unclear. Whether or not FoxO1 can localize to the mitochondria, and the impact PTMs have on this is also unestablished.

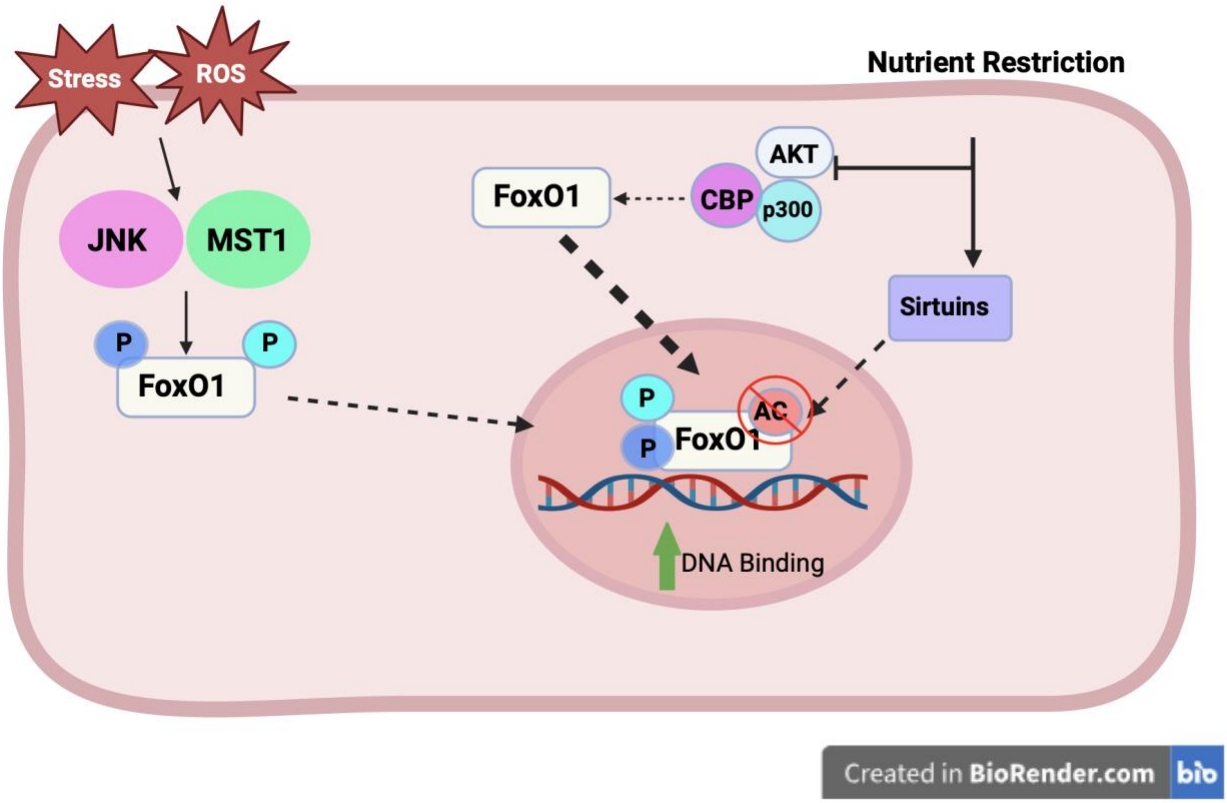

Figure 1.3: Schematic illustrating signals that promote Foxo1 nuclear import.
With nutrient restriction, the actions of AKT and CBP/p300 are inhibited, resulting in nuclear import of FoxO1 due to lack of acetylation/phosphorylation. Simultaneously, nutrient restriction activates different HDACs called sirtuins which deacetylate FoxO1 to cause nuclear import of and increased DNA binding. Further, oxidative stress can activate stress kinases JNK and MST1 which phosphorylate FoxO1 on different residues resulting in nuclear import and transcriptional activation.

1.9 FoxO1: Potential Target for Sex Differences in Endothelial Cells

Upon transcriptome analysis, it was revealed that FoxO1 protein and mRNA expression in female endothelial cells was higher compared to males both in vivo and in vitro(Rudnicki et al., 2023). Knowing that FoxO1 represses inflammatory markers such as NF-kB, whose downstream targets are ICAM-1, for example (Feinberg, 2013; Kim et al., 2008; Yu et al., 2014)

while also deterring accumulation of cytoplasmic and mitochondrial derived ROS via its induction of catalases and MnSOD, respectively, it has been hypothesized that FoxO1 contributes to the enhanced stress-protection in female endothelial cells.

The elevated levels of FoxO1 in female compared to male endothelial cells suggests a differential regulation of FoxO1 between the sexes. Unpublished data from our lab show that deleting FoxO1 in female endothelial cells leads to vascular abnormalities and adipose tissue issues, whereas its deletion in males appears to have beneficial effects. This points to the possibility that FoxO1 also may have distinct functional roles in males and females. Investigating potential sex differences in post-translational modifications (PTMs) of FoxO1 in response to various stressors may help explain why FoxO1 levels are elevated in females and reveal how FoxO1 contributes to the different outcomes observed in male and female endothelial cells under cellular stress.

The overall goal of my thesis is to establish whether oxidative stressors and nutrient stressors differentially affect FoxO1 modifications and sub-cellular localization in male and female endothelial cells. Given the limited information on the role of FoxO1 in influencing mitochondrial transcription in endothelial cells, my research also aims to uncover whether FoxO1 can localize to the mitochondria of microvascular endothelial cells.

1.10 Hypothesis: Distinct patterns of FoxO1 protein post-translational modifications and subcellular localization contribute to sex differences in FoxO1 transcriptional activation.

Objective 1: To assess and compare FoxO1 subcellular localization in male and female endothelial cells in responses to different nutrition and oxidative stress stimuli. 1) Test if nuclear levels of FoxO1 are higher in female than male EC; and 2) Establish if FoxO1 can localize to mitochondria, and if this differs between male and female ECs.

- Using primary cultures of skeletal muscle or adipose tissue endothelial cells from male and female mice, I will induce changes in FoxO1 sub-cellular localization by manipulating:

1) Fed states of the cell: using serum starvation, insulin-stimulation. These stimuli induce well-documented changes in FoxO1 cytoplasmic/nuclear localization.
2) Low vs. high oxidative stress: ROS sources: hydrogen peroxide (H_2O_2) and Rotenone. These ROS sources are selected to encapsulate both cytosolic and mitochondrial sources of oxidative stress.

- Nuclear and mitochondrial fractionation methods will be used to separate nuclear, cytoplasmic, and mitochondrial proteins, followed by western blotting to assess the relative amounts of FoxO1 within each subcellular fraction.

Objective 2: Examine post-translational modifications of FoxO1 (acetylation and phosphorylation) to elucidate: 1) Whether post-translational modifications differ between male

and female ECs; and 2) If these modifications influence the extent of nuclear or mitochondrial localization.

- Using the conditions outlined in Objective 1, I will quantify the relative amounts of FoxO1 post translational modifications (acetylation, Akt-dependent phosphorylation) in whole cell extracts of male and female primary skeletal muscle and adipose endothelial cells by western blotting using modification-specific antibodies.
- I will use nuclear and mitochondrial extraction protocols to assess the post translational modifications of FOXO1 that are contained within each fraction.

Chapter 2: Methods

2.1 Endothelial cell isolation:

Visceral and subcutaneous adipose tissues, along with hindlimb skeletal muscles were collected from male and female wildtype FVB/J mice. Muscles and adipose tissue were pooled separately and were first mechanically digested with scissors and scalpel. Adipose tissue was enzymatically digested with type I collagenase (#LS004197, Worthington Biochemical, USA) at 37°C for 30 minutes, while skeletal muscle with type II collagenase (#LS004177, Worthington Biochemical, USA) at 37°C for 45 minutes. The homogenates were spun at 4000 RCF for 5 minutes; the resulting pellet was suspended in PBS and filtered through a 100 µm cell strainer. This mixture of cells was plated in a T25 flask coated with gelatin. Once confluent, the mixture of cells were incubated with biotinylated rat anti-mouse CD31 (#553371; BD IMag, USA) antibody conjugated to streptavidin magnetic beads (#557812; ThermoFisher, Scientific, USA). These endothelial cells were plated in a gelatin coated T75 flask, grown in media consisting of: high-glucose (4.5 g/L) DMEM (#19960044, Gibco, USA) supplemented with 20% fetal bovine serum (FBS, #10082147, Gibco, USA), Penicillin-Streptomycin (100µg/mL, #15140122, Gibco, USA), GlutaMAX™ L-glutamine (2 mM, #35050061, Gibco, USA), and Sodium Pyruvate (1 mM, #11360070, Gibco, USA). Cells used in the experiments were in passages 4-6 and were passage-matched for comparisons between males and females.

2.2 Cell culture conditions:

Cells were grown using high glucose growth media consisting of the following: high-glucose (4.5 g/L) DMEM (#19960044, Gibco, USA) 20% (FBS,#10082147, Gibco, USA), Penicillin-Streptomycin (100µg/mL, 15140122, Gibco, USA), GlutaMAX™ L-glutamine (2

mM, #35050061, Gibco, USA), and Sodium Pyruvate (1 mM, #11360070, Gibco, USA). To induce nutrient deprivation, cells were serum starved for 2 hours in media without FBS and sodium pyruvate. Insulin treated cells were incubated with 10% FBS high glucose (4.5 g/L) media and insulin (25 μU/ml]) for 30 minutes. Control cells were incubated in 10% FBS high glucose media on the morning of cell treatment. To induce oxidative stress, two exogenous sources of stress were used: rotenone (#557368; EMD Millipore, USA) and hydrogen peroxide (H_2O_2) (# L14000.AP; ThermoFisher Scientific, ON, Canada). Rotenone (2 μM) and H_2O_2 (300 μM) treatments were carried out for 1 hour in 10% FBS high glucose media.

2.3 Protein Extraction

A) Whole cell lysis

Approximately 200,000 ECs were plated in 6 well plates at a confluency of approximately 70% a day prior to extraction. The following day, cells were treated as described in 2.2, and protein samples collected for whole cell lysates were extracted with a RIPA lysis buffer consisting of 50 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, phosphatase inhibitor tablets (PhosSTOP™) and protease inhibitor tablets (cOmplete™ Protease Inhibitor Cocktail, Sigma-Aldrich, USA). Cells were collected with a scraper and centrifuged for 20 minutes at 12,000 RCF. The supernatant was collected and stored in a -80℃ freezer.

B) Nuclear and Cytoplasmic protein extractions

Approximately 3 million cells were plated in T75 flasks per condition at a confluency of approximately 100% a day prior to the extraction. To obtain nuclear and cytoplasmic cellular fractions, the Rockland Immunochemical “Nuclear and Cytoplasmic Extract Protocol” was used.

Briefly, cells were collected and lysed in a cytoplasmic extraction buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP-40, 1mM DTT and 1 mM PMSF) for 3 minutes on ice. Following a 4 minute spin at 1500 RPM, a cytoplasmic extract was obtained. The nuclear pellet was solubilized in a nuclear extraction buffer (20 mM Tris Cl, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM PMSF and 25% (v/v) glycerol) with 10 minute incubation on ice with vortex agitation. Following a 17,000 RCF centrifugation for 10 minutes, the soluble portion was retained as the nuclear extract.

C) Mitochondrial protein extraction

Approximately 10 million cells per condition were used for mitochondrial isolation a day prior to extraction. The following day, endothelial cells were treated as described in 2.2, and scraped from 150 mm plates in 1X PBS and collected in 15ml tubes. Cells were pelleted by centrifugation at 500 RCF for 5 minutes. Cell pellets were resuspended in a mitochondrial extraction buffer consisting of 0.25M sucrose, 0.2mM EDTA, 10mM HEPES, pH 7.4). Suspension was transferred to a dounce homogenizer and homogenized with pestle a and b 8-10 times. Following different centrifugation parameters, mitochondrial pellet was separated from the cytoplasm. After multiple washes, the mitochondrial pellet was resuspended in 30 uL of mitochondrial extraction buffer and lysed by freeze thawing in dry ice 5 times.

2.4 Protein Quantification:

Quantification of all protein extracts was conducted with a bicinchoninic acid assay (BCA) kit (Pierce, Fisher ThermoScientific, ON, Canada). A 96 well plate was used to generate triplicates of protein lysates diluted in double distilled water (ddH2O). Reagents A and B from

the BCA kit were mixed at a 49:1 ratio, respectively, and added to each triplicate sample. Serial dilutions of bovine serum albumin (BSA 0-2000 µg/mL, Pierce, ThermoFisher Scientific, ON, Canada) were made to create a standard curve, for comparison to the samples. The absorbance of the samples was analyzed at 562 nm using a Cytation3 microplate reader (Biotek, Vermont, USA).

2.5 Western Blot:

Proteins (25 µg per sample) were mixed with 4x DTT (Dithiothreitol) loading buffer (Tris 151 mM; Dithiothreitol 260 mM; SDS 15%; glycerol 20%; bromophenol blue 0.012%) and denatured for 5 minutes in a 95 ℃ heat block and were loaded and electrophoresed through a 12% SDS-Polyacrylamide gel. Following separation, a wet transfer technique was used to transfer proteins to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, EMD Millipore, ON, Canada) in transfer buffer consisting of 25 mM Tris-Base, 191.82 mM Glycine, and 20% v/v methanol, for 1 hour at 100 volts, powered by PowerPac™ HC High-Current Power Supply (Bio-Rad, ON, Canada). To confirm transfer, a Ponceau stain (0.2% ponceau in 3% acetic acid) was used. Membranes were then blocked for 1 hour in 5% non-fat dry milk diluted in 0.05% Tween Tris-Buffered saline solution (TTBS) with agitation. Membranes were then incubated overnight in different antibodies at 4 ℃ with agitation. After 24 hours, membranes were washed 3 times, for 5 minutes each, in 0.05% TTBS at room temperature. Then, they were incubated for 1 hour in 5% non-fat dry milk diluted in 0.05% (TTBS) containing a secondary antibody. Following this was 3 more washes for 5 minutes each with 0.05% TTBS. Finally, membranes were incubated with SuperSignal™ West Pico PLUS Chemiluminescent Substrate ECL (ThermoFisher, Scientific, USA) for 5 minutes. The ECL reaction was detected using the

iBright 1500 Imaging System (#A44114, ThermoFisher Scientific, ON, Canada). In order to probe for multiple proteins of interest on one membrane (such as the case in different FoxO1 PTMs), a stripping protocol was used as followed: membrane was incubated at 50°C r for 7 minutes in western stripping buffer, consisting of: 62.5 mM TRIS HCL, 2% sodium dodecyl sulfate (SDS), and 0.7% Beta mercaptoethanol, pH 6.75. Stripped membranes were washed in 0.05% TTBS 3 times for 20 minutes each. They were then blocked for 1 hour in 5% non-fat dry milk diluted in 0.05% TTBS, and incubated with primary antibody overnight.

The primary and secondary antibodies used included: anti-FoxO1 (1:1000; #2880, Cell Signaling, ON, Canada); anti-Phospho-FoxO1 (Ser256) (1:1000; #9461, Cell Signaling, ON, Canada); anti-Acetyl-FOXO1 (Lys245) (1:1000; #STJA000593, St. John's Laboratory, London, England); anti-O-GlcNAc (1:1000; #82332, CellSignaling, ON, Canada); anti-B-Actin (1:5000; #sc- 47778, Santa Cruz Biotechnology, TX, USA); anti-RCC1 (1:1000; #3589, Cell Signaling, ON, Canada); anti-Histone H3 (1:1000; #9717, Cell Signaling, ON, Canada); anti-GAPDH (1:1000; #5174, Cell Signaling, ON, Canada); anti-α/β-Tubulin (1:1000; #2418, Cell Signaling, ON, Canada); anti-TOM20 (1:1000; #42406, Cell Signaling, ON, Canada); goat anti-mouse IgG, peroxidase-conjugated(1:10000; #115-035-003) and goat anti-rabbit IgG, peroxidase-conjugated (1:10000; #111-035-003; Jackson ImmunoResearch Laboratories Inc., USA).

2.6 Immunofluorescence:

Approximately 20,000 ECs were plated per well in an 8-well chamber slide coated with type I rat tail collagen (12μg/cm^2) diluted in ELISA coating buffer. ECs were washed 3 times for 5 minutes each with PBS, and were then fixed with 4% paraformaldehyde for 20 minutes at

room temperature. ECs were then washed with PBS again, followed by blocking and permeabilization for 30 minutes with blocking solution that consisted of 0.1-0.3% Triton-X and 5% donkey serum diluted is PBS. After removing the blocking solution, ECs were incubated with either FoxO1 primary antibody (1:50-1:100; #2880, Cell Signaling, ON, Canada) or Phospho-FoxO1 (Ser256) (1:100; #9461, Cell Signaling, ON, Canada) antibody diluted in blocking buffer overnight at 4℃. The next day, primary antibody was removed, and ECs were washes 3 times for 5 minutes with PBS, followed by incubation with Alexafluor 488-conjugated donkey anti-rabbit secondary antibody (1:200; #711-545-152, Jackson ImmunoResearch Inc., USA) diluted in blocking buffer for 1 hour at a concentration of 1:200. Then, ECs were once again washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) for 5 minutes. Cells were mounted with a cover slip using anti-fade mounting media (Vector laboratories #H-1700) and imaged using a Zeiss Axiovert 200M Inverted fluorescence microscope or a Zeiss Spinning Disc Z1 confocal microscope.

2.7 Immunoprecipitation:

Whole cell lysates (25 µg) were pre-cleared with 40 µL Protein A/G magnetic beads (#B23202; Selleckchem, ON, Canada) for 1 hour on a rotisserie at 4 ºC to eliminate non-specific protein binding to the beads. Separate Protein A/G magnetic beads consisting of 20 µL bead slurry was washed 3 times with PBS for 10 minutes and were then conjugated with FoxO1 primary antibody (1:100; #2880, Cell Signaling, ON, Canada) for 2 hours. FoxO1-conjugated magnetic beads were incubated with the cell lysates overnight at 4ºC on a rotisserie. The following day, the protein A/G magnetic beads were washed for 3 times for 10 minutes each with a wash buffer (50 mM Tris, 150 mM NaCl, 0.1%-0.5% Triton X-100, pH 7.5). After the last wash, 50 µL of 1X

DTT was added to the samples and beads were eluted by heating at 95ºC for 5 minutes. Tubes were put on a magnetic stand and supernatants were then subjected to Western Blot to detect FoxO1 and O-GlcNAc.

2.9 Statistical Analysis:

Results are presented as Mean ± standard error of mean (SEM). The effects of the nutritional and oxidative stress stimuli on male and female EC FoxO1 localization and PTMs were analyzed using a two-way ANOVA (Treatment X Sex). All two-way ANOVA analyses were followed by Sidak post hoc tests (Prism8; GraphPad Software Inc; La Holla, CA, USA). In all statistical tests, $p < 0.05$ was defined as statistically significant.

Chapter 3: Results

3.1. Whole Cell *O*-GlcNAcylation of Skeletal Muscle Endothelial Cells in response to Glucose

O-GlcNAcylation is a very understudied PTM of FoxO1, yet it has been implicated in modulating FoxO1 stress responses. To expand our understanding of FoxO1 O-GlcNAcylation, the initial goal was to establish whether FoxO1 undergoes O-GlcNAc modification and how this is impacted with varying glucose concentrations, which would provide a foundation for investigating its potential functional implications. Because *O*-GlcNAcylation is reported to be a nutrient-sensitive modification, I first used varying levels of serum and glucose to evaluate whole cell patterns of *O*-GlcNAcylation in male and female SKMECs. (Fig. 3.1A). An initial result was observed where female SKMECs displayed higher levels of *O*-GlcNAcylation compared to males (Fig. 3.1B). There is no commercial antibody that detect O-GlcNac modified FoxO1, thus I attempted to assess the modified protein using immunoprecipitation. I initially did an immunoprecipitation using anti-FoxO1 and western blotted the extraction with an O-GlcNAc antibody, but many non-specific bands were detected (Fig 3.1C). Varying amounts of protein as well as antibody concentrations were manipulated to yield a more favourable outcome, but non-specific bands were detected in each case (Fig. 3.1D). I confirmed the efficacy of my protocol using an AKT antibody, which resulted in successful detection of AKT (Fig. 3.1E). Last, I tried immunoprecipitating using O-GlcNAc antibody, and then blotted using a FoxO1 antibody (Fig. 3.1F); however, non-specific bands were detected. I suspect that the FoxO1 antibody being used may not be effective for this protocol. Future work will need to be done to create a path for assessing FoxO1-O-GlcNAc modifications.

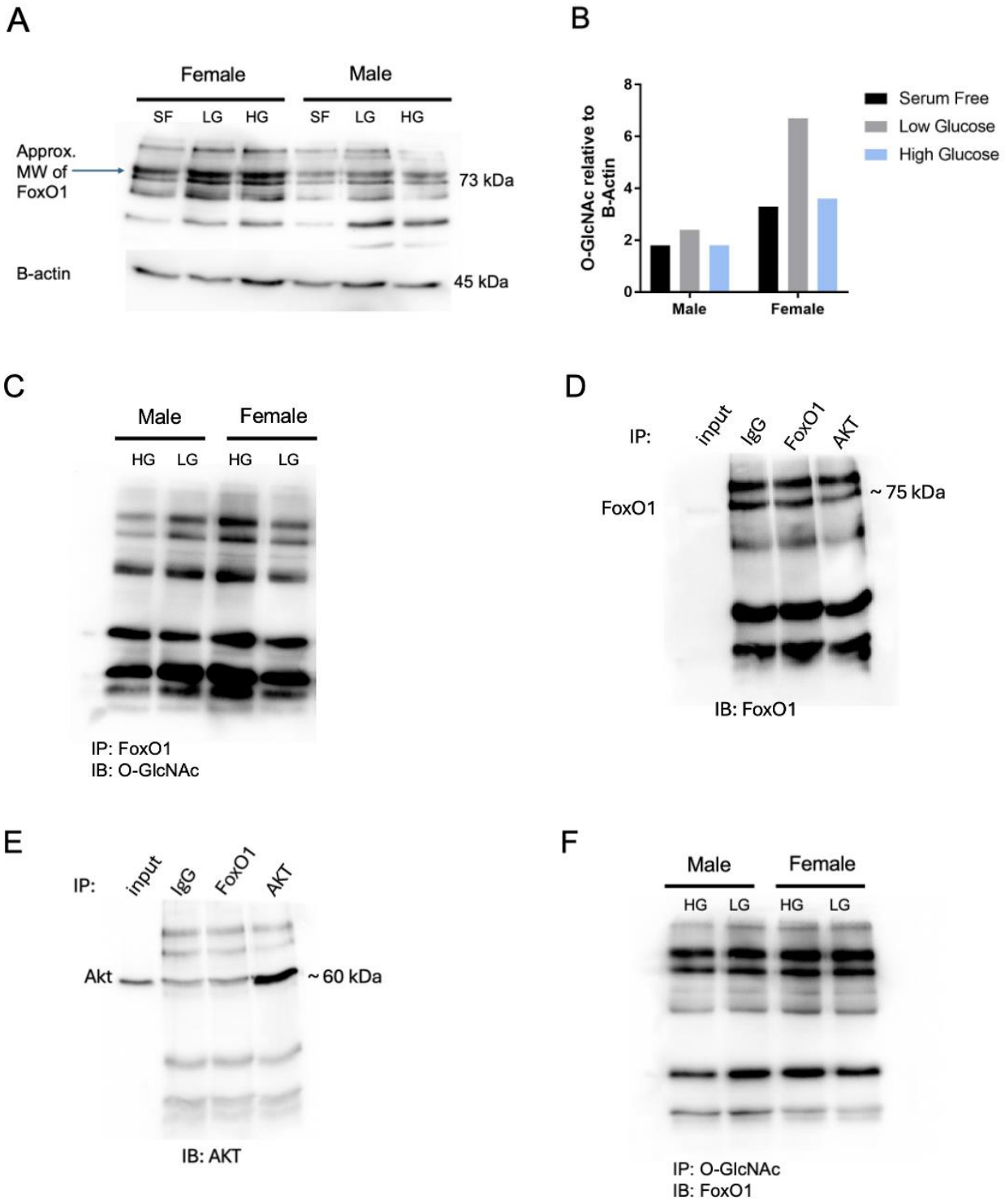

Figure 3.1. FoxO1 whole cell *O*-GlcNAcylation and immunoprecipitation of male and female skeletal muscle endothelial cells. (A) Male and female whole cell SKMEC *O*-GlcNAcylation response to 6 hours serum starvation, 24 hour low glucose (1g/L), and high glucose (4.5g/L). (B) Quantification of *O*-GlcNAcylation is represented in a bar graph. n=1. (C-

F) Different attempts demonstrating immunoprecipitation for FoxO1 *O*-GlcNAcylation. SF= serum free, HG= high glucose, LG= low glucose, IgG= Immunoglobulin G

3.2 Visualizing FoxO1 Localization and Phosphorylation with Immunofluorescence Imaging

The main objective of this study was to establish sex differences in FoxO1 localization and modification in ECs. To elucidate these differences, the initial step was immunofluorescence staining of SKMECs under the contrasting conditions of serum starvation versus short-duration nutrient stimulation (serum plus insulin). However, this proved to be challenging as FoxO1 imaging was of very poor quality, possibly due to poor antibody efficacy for staining purposes. Imaging at 20x magnification proved futile as the FoxO1 signal was extremely weak. FoxO1 imaging however was improved when using 63x magnification on a confocal microscope. A clear overlap of FoxO1 signal with the nucleus (indicated by DAPI staining) was observed, portraying strong nuclear FoxO1 localization with serum starvation (Fig. 3.2A). This FoxO1 signal became more dispersed and elongated outside the boundaries of DAPI signalling with insulin stimulation, consistent with nuclear export of FoxO1, and cytoplasmic localization. I also stained cells with phospho-FoxO1 antibody. A clear juxtaposition between FoxO1 phosphorylation was seen between the two conditions as the robust phospho-FoxO1 signal with insulin plus serum was greatly diminished with serum starvation (Fig. 3.2B). It is worth noting that phospho-FoxO1 signal overlapped with the DAPI signaling here, which was an unexpected result given the current understanding of FoxO1 phosphorylation resulting in nuclear export. However, quality and outcomes of immunofluorescence trials were generally poor. I made various attempts with different FoxO1 antibodies and modifications to cell fixing or Triton X-100 percentages, but the resulting immunofluorescence images were not adequate in confidently discerning FoxO1 localization or phosphorylation. Thus, I pivoted to focus on analysis of sub-

cellular fractionations and western blotting of proteins to better elucidate the differential patterns of FoxO1 localization and PTMs in male and female ECs.

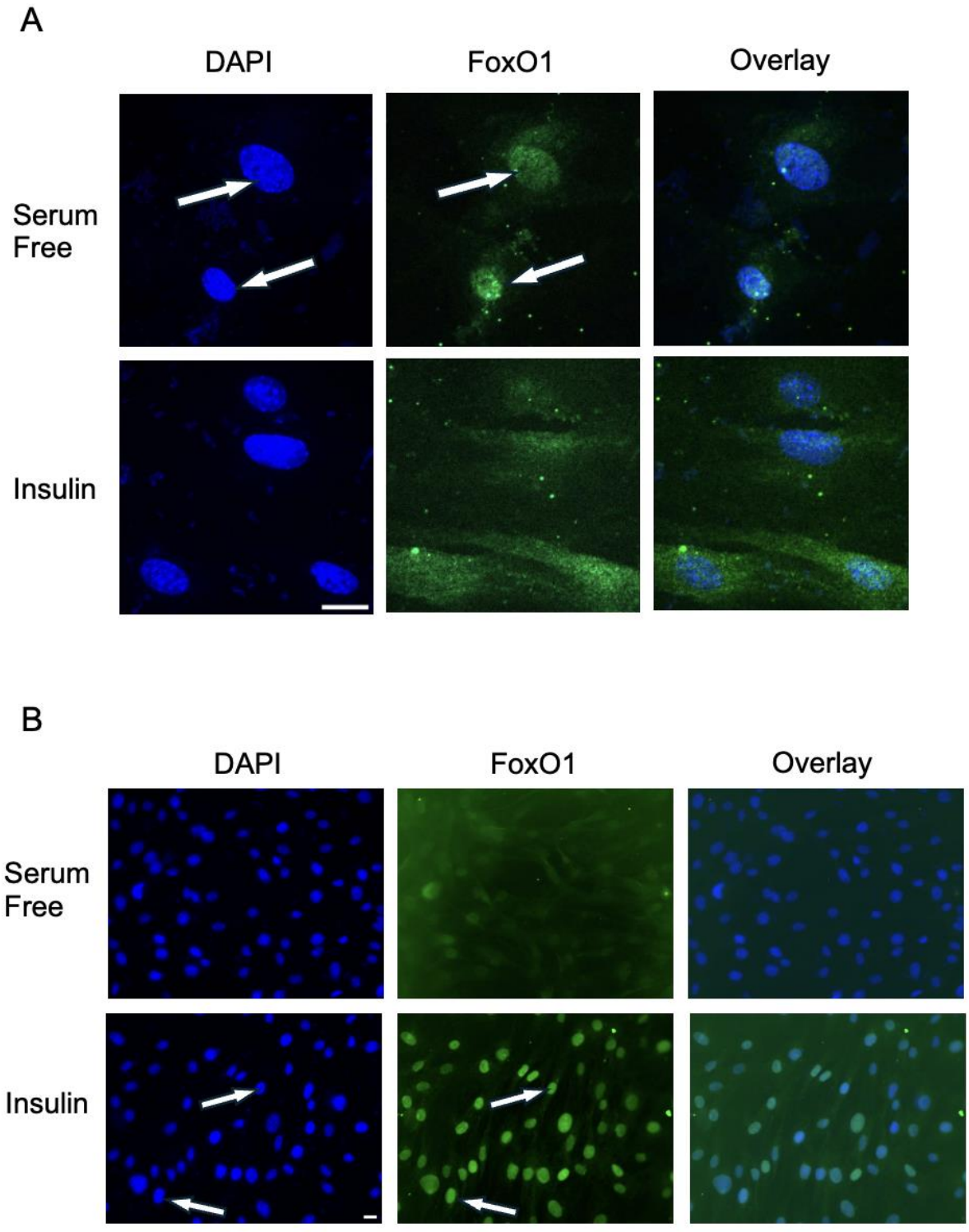

Figure 3.2. Immunofluorescence staining of FoxO1 and phospho-FoxO1 in female skeletal muscle endothelial cells. Female SKMEC were treated with 2 hours of serum starvation or 40 minutes of 10% FBS serum plus insulin stimulation (25 μU/ml). (A) Confocal images of FoxO1 at 63x magnification. (B) Phospho-FoxO1 immunostaining at 20x magnification. Cells were co-stained with DAPI (blue) to delineate the nuclei. Images at 20x magnification (B) were taken with the ZEISS Axiovert 200M microscope, while confocal images at 63x magnification (A) were taken with the Zeiss Spinning Disk Z1 microscope. Scale bar is 20 μm.

3.3 Whole Cell Skeletal Muscle EC Sex Differences in FoxO1 Modifications in Response to Nutritional Stimuli

Before assessing FoxO1 subcellular localization patterns, it was pertinent to first establish a whole cell response to assess sex differences in FoxO1 sensitivity to stimuli, and consequentially, modifications. To achieve this, western blot analysis was performed on whole cell extracts from male and female SKMECs (Fig. 3.3A). FoxO1 levels did not differ between male and female ECs and were not affected by 30 minutes nutrient stimulation (Fig. 3.3B). Insulin plus serum stimulation led to a significant increase in FoxO1 phosphorylation on serine256 in both male and female SKMECs relative to serum starved cells (Fig. 3.3C-D). Male SKMECs exhibited higher levels of FoxO1 phosphorylation in response to insulin plus serum stimulation compared to female SKMECs, while no sex-specific differences were observed under serum starvation (Fig. 3.3C-D). Although FoxO1 acetylation on lysine 245 was detected, there were no differences due to insulin plus serum stimulation or sex differences (Fig. 3.3E-F).

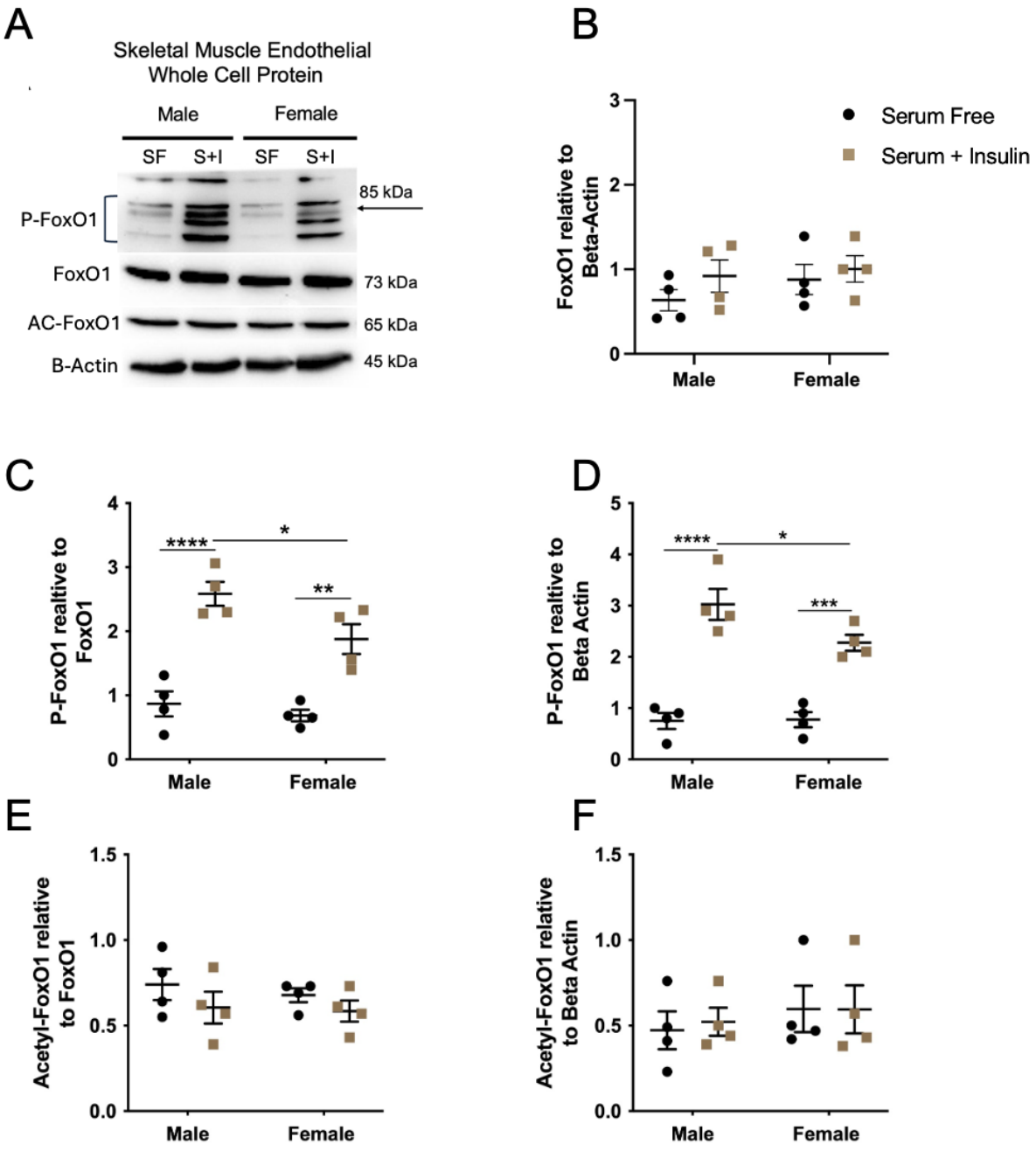

Figure 3.3. The effects of nutritional stimuli on FoxO1 PTMs in whole cell extracts from male and female skeletal muscle endothelial cells. Male and female SKMEC whole cell protein extracts were collected from cells following 2 different treatments: 2 hours of serum starvation or 30 minutes of 10% FBS serum plus insulin stimulation (25 µU/ml). (A) Representative western blots of FoxO1 phosphorylation and acetylation. **(**B) Total levels of FoxO1 in male and female SKMECs. Phosphorylated and acetylated FoxO1 were normalised to FoxO1 (C and E, respectively) and to beta actin (D and F, respectively). The bracket in western blot image (A) delineates the bands that were quantified for FoxO1 phosphorylation. SF= serum free, S+I= serum plus insulin, P-FoxO1= phosphorylated FoxO1, AC-FoxO1= acetylated FoxO1.

n= 4. Data are presented as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significance was detected. *P<0.05, **P<0.005, ***P<0.001, ****P<0.0001

3.4 Skeletal Muscle EC Sex Differences in FoxO1 Nuclear and Cytoplasmic Localization and Modifications in Response to Nutritional Stimuli

To determine whether the measured sex differences in Foxo1 phosphorylation would correspond with differences in nuclear and cytoplasmic localization, sub-cellular fractionation was performed. No significant sex differences in nuclear FoxO1 were detected, and nutrient stimulation did not result in significant differences in nuclear FoxO1 in either sex (Fig. 3.4C). Insulin plus serum treatment caused an increase in cytoplasmic FoxO1 only in male SKMECs (Fig. 3.4D). Male SKMECs also had significantly higher cytoplasmic FoxO1 in response to insulin compared to females (Fig. 3.4D).

FoxO1 modifications were normalized to both total FoxO1 levels and the loading control specific to each subcellular fraction. This normalization was performed to ensure accurate protein quantification, as shifts in the overall localization of FoxO1 protein could otherwise distort the results, leading to misleading interpretations. Insulin plus serum stimulation significantly increased nuclear FoxO1 phosphorylation relative to total FoxO1 protein, as indicated by the main effect of the treatment (Fig. 3.4E). An overall sex difference in nuclear FoxO1 phosphorylation was detected, with females exhibiting higher phospho-FoxO1 levels, although this did not reach post-hoc significance (Fig. 3.4E). When phospho-FoxO1 was quantified relative to RCC1, no sex differences were detected (Fig. 3.4F), though insulin stimulation significantly elevated nuclear phospho-FoxO1 in males, with a near-significant trend in females (P=0.06) (Fig. 3.4F). Cytoplasmic FoxO1 phosphorylation levels exhibited a near

significant (P=0.0575) main effect in sex, with male SKEMCs having higher cytoplasmic phospho-FoxO1 (Fig. 3.4G). Insulin plus serum stimulation resulted in significantly higher levels of phospho-FoxO1 in male SKMECs, compared to females (Fig. 3.4H). Overall, male SKMECs appear to be more insulin sensitive based on higher cytoplasm levels of FoxO1 phosphorylation compared to females.

Normalising nuclear acetyl-FoxO1 to total nuclear FoxO1, there were no significant changes between sex or treatment (Fig. 3.4I). But when normalised to loading control RCC1, nuclear FoxO1 acetylation on lysine 245 analyses revealed a significant treatment effect (P 0.01), with insulin plus serum stimulation increasing FoxO1 acetylation (Fig. 3.4J). Normalising cytoplasmic acetyl-FoxO1 to total cytoplasmic FoxO1, a near significant (P=0.06) main effect of sex was observed in cytoplasmic FoxO1 acetylation with males having higher FoxO1 acetylation (Fig. 3.4K). But quantifying acetyl-FoxO1 to cytoplasmic loading control GAPDH showed no changes between sex or treatment (Fig.3.4L).

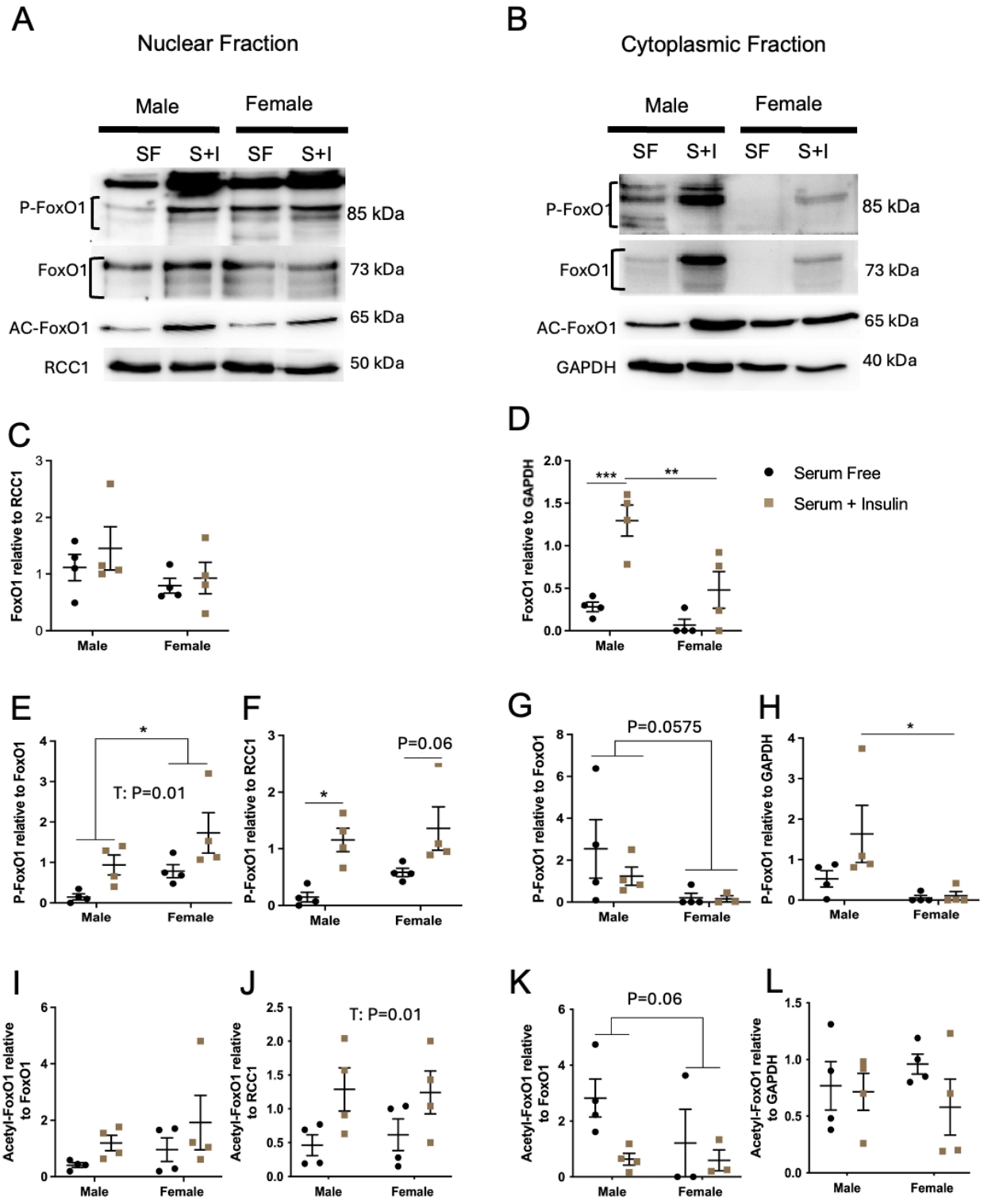

Figure 3.4. The effects of nutritional stimuli on FoxO1 nuclear and cytoplasmic localization, and modifications in skeletal muscle ECs. Nuclear and cytoplasmic fractions were generated from male and female SKMECs following 2 different treatments: 2 hours of serum starvation, or serum starvation followed by 30 minutes of serum + insulin stimulation (25 μU/ml). Representative western blots for nuclear and cytoplasmic fractions are shown in A and

B, respectively. Relative levels of FoxO1 were normalised to the loading controls of each fraction, RCC1 and GAPDH (C and D). Phosphorylated and acetylated FoxO1 were normalised to FoxO1 (E and G) and the loading controls for each fraction (F,H,J,L). The brackets in western blot images (A and B) delineates the bands that were quantified for FoxO1 phosphorylation. SF= serum free, S+I= serum plus insulin, P-FoxO1= phosphorylated FoxO1, AC-FoxO1= acetylated FoxO1. n= 4. Data are presented as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significance was detected. *P<0.05, **P<0.005, ***P<0.001. "T" represents significant main effect of treatment, serum free vs insulin.

3.5 Sex Differences in FoxO1 Modifications in Response to Oxidative Stress in Whole cell Skeletal Muscle ECs

FoxO1 localization and PTMs in response to oxidative stress have been studied in various cell types but endothelial cell specific studies are scarce. Published findings of sex disparities in handling oxidative stress warrants investigation into regulatory mechanism that may be responsible, such as FoxO1. Thus, male and female SKMECs were exposed to oxidative stress stimuli, and sex differences in PTMs were assessed. Hydrogen peroxide and rotenone, which elevate cytoplasmic and mitochondrial sources of ROS, respectively, were used as stimulants of oxidative stress (N. Li et al., 2003; Schafer & Buettner, 2001) and these were compared to a control condition consisting of culture media containing 10% FBS. Important to note, Cell ROX, a fluorogenic probe, was utilized to confirm an uptake in cellular ROS, but this staining method also failed to provide substantial results. FoxO1 levels and its modifications first were detected by western blotting of whole cell extracts (Fig. 3.5A). Total FoxO1 levels did not differ between sexes, or with treatment (Fig. 3.5B). No effects of either oxidative stress source was found when measuring phosphorylated FoxO1 relative to FoxO1 (Fig. 3.5C). Phospho-FoxO1 level in response to rotenone stimulation was higher in males compared to females (Fig. 3.5D) when normalized to loading control Beta Actin. However, hydrogen peroxide stimulation revealed enhanced FoxO1 phosphorylation in females compared to males (Fig. 3.5D). Within each sex,

effects of ROS sources were found only in female SKMECs where FoxO1 phosphorylation with hydrogen peroxide treatment was higher compared to control and rotenone conditions (Fig. 3.5D). Neither source of oxidative stress resulted in differences in FoxO1 acetylation (Fig. 3.5 E and F).

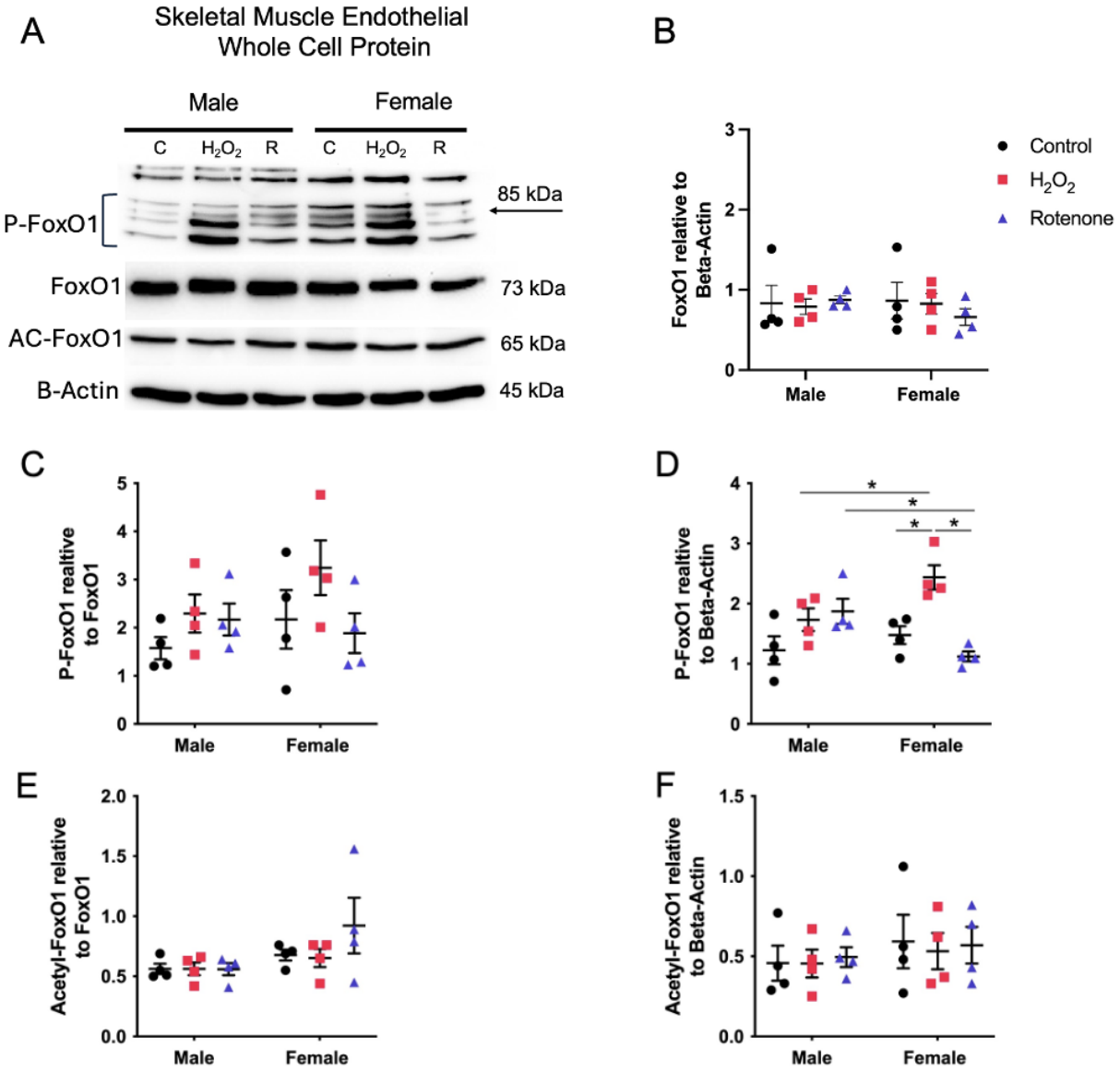

Figure 3.5. The effects of oxidative stress on FoxO1 PTMs in male and female skeletal muscle endothelial cells. Male and female SKMEC whole cell protein was collected following treatment with 10% FBS media, hydrogen peroxide (300 µM), and rotenone (2µM). (A) Representative western blots of FoxO1 phosphorylation and acetylation. **(**B) Total levels of FoxO1 in male and female SKMECs. Phosphorylated and acetylated FoxO1 were normalised to

FoxO1 (C and E, respectively) and to beta actin (D and F, respectively). The bracket in western blot image (A) delineates the bands that were quantified for FoxO1 phosphorylation. C= control media, H_2O_2 = hydrogen peroxide, R= rotenone, P-FoxO1= phosphorylated FoxO1, AC-FoxO1= acetylated FoxO1. n= 4. Data are presented as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significance was detected. *P<0.05.

3.6 Sex Differences in FoxO1 Nuclear and Cytoplasmic Localization and PTMs in Response to Oxidative Stress

Subcellular fractionation was implemented to resolve nuclear and cytoplasmic localization of FoxO1, and its modified forms, in response to the oxidative stressors hydrogen peroxide and rotenone. Representative western blots are shown in Figure 3.6 A and B. Sex differences in FoxO1 nuclear localization were observed, as female EC had higher FoxO1 compared to males, in both the control conditions and following hydrogen peroxide stimulation (Fig. 3.6C). However, no treatment effect of either stressors was observed in either male or female SKMECs. In the cytoplasmic fraction, oxidant stimulation resulted in decreased FoxO1 levels in females (trend with hydrogen peroxide, P=0.09; significant with rotenone) (Fig. 3.6D). Cytoplasmic FoxO1 in males was increased with hydrogen peroxide treatment, with no change with rotenone stimulation (Fig. 3.6D). Male SKMECs had higher cytoplasmic FoxO1 compared to females with hydrogen peroxide treatment (Fig. 3.6D), juxtaposing the higher nuclear FoxO1 in females compared to males with this treatment. Cytoplasmic FoxO1 was also higher in males compared to females following rotenone treatment (Fig. 3.6D).

There were minimal differences in nuclear FoxO1 phosphorylation between sex or with treatment (Fig. 3.6E). Only when phospho-FoxO1 was normalized to RCC1 was there an apparent higher level of phospho-FoxO1 in females compared to males with hydrogen peroxide stimulation (Fig. 3.6F). No changes in phospho-FoxO1 were detected in the cytoplasmic fraction

when normalized to FoxO1 (Fig. 3.6G) in either sex. However, when normalized to the loading control GAPDH, hydrogen peroxide treatment induced higher FoxO1 phosphorylation in male compared to female SKMEC (Fig. 3.6H).

No differences were measured in nuclear FoxO1 acetylation between sex or with treatment (Fig. 3.6I). Only when normalising to RCC1, was there a main effect of sex in nuclear FoxO1 acetylation, with higher acetyl-FoxO1 in females compared to males (Fig. 3.6J). This outcome was reversed in the cytoplasm where male SKMECs had higher FoxO1 acetylation compared to females, as indicated by a main effect of sex (Fig. 3.6L). Normalising acetyl-FoxO1 to cytoplasmic FoxO1 revealed only a treatment effect in females where rotenone stimulation increased cytoplasmic FoxO1 (Fig. 3.6K).

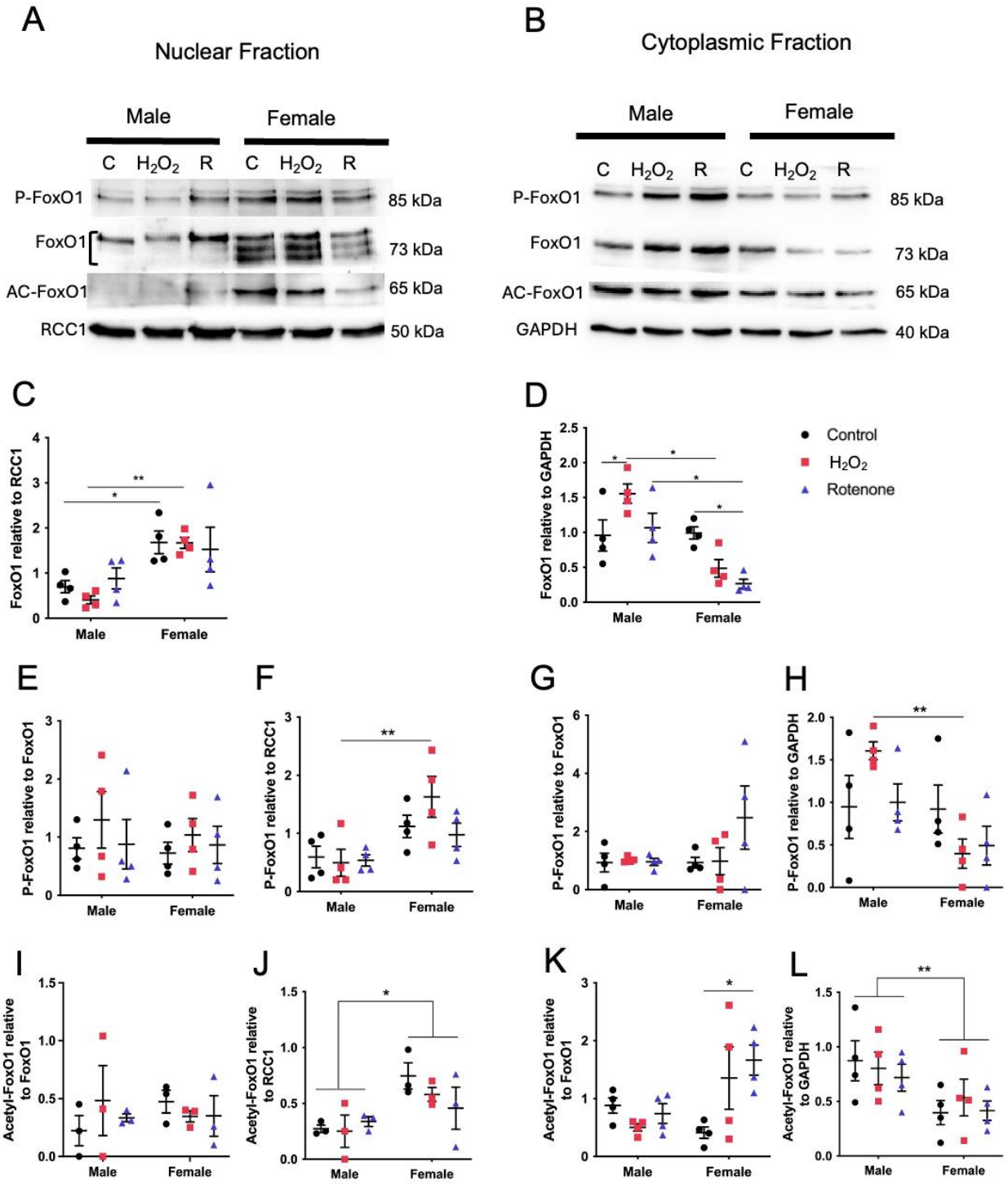

Figure 3.6. The effects of oxidative stressors on FoxO1 nuclear and cytoplasmic localization, and modifications in skeletal muscle ECs. Nuclear and cytoplasmic fractionations from male and female SKMECs following treatment with 10% FBS media, hydrogen peroxide (300 µM), and rotenone (2µM). Representative western blots for nuclear and cytoplasmic fraction are shown in A and B, respectively. Relative levels of FoxO1 were normalised to the loading controls of each fraction, RCC1 and GAPDH (C and D). Phosphorylated and acetylated FoxO1 were normalised to FoxO1 (E and G) and the loading controls for each fraction (F,H,J,L).

The brackets in western blot image (A) delineates the bands that were quantified for FoxO1 phosphorylation. C= control media, H_2O_2 = hydrogen peroxide, R= rotenone, P-FoxO1= phosphorylated FoxO1, AC-FoxO1= acetylated FoxO1. n= 4. Data are presented as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significance was detected. *P<0.05.

3.7 Sex Differences in Adipose Tissue EC FoxO1 Nuclear and Cytoplasmic Localization and Modifications in Response to Nutritional Stimuli

Endothelial cells exhibit considerable phenotypic heterogeneity, which is influenced by the distinct microenvironmental conditions present in various tissues (Gifre-Renom et al., 2022). This heterogeneity enables endothelial cells to adapt to the specific functional requirements of their respective tissue types. Additionally, adipose tissue and skeletal muscle are known to exhibit differential responses in terms of glucose uptake and insulin sensitivity (Fazakerley et al., 2018). Thus, it was important to contrast EC FoxO1 behaviour across different tissues. To achieve this, the fractionations conducted on SKMECs were replicated on adipose tissue ECs. Using the same nutritional treatments performed on SKMECs, ATECs were treated and fractionated (Fig. 3.7A and B). Nuclear FoxO1 measurements resulted in no significant changes with treatment of between sex (Fig. 3.7C). However, female ATECs did display a consistent pattern of decreased nuclear FoxO1 with insulin plus serum (Fig. 3.7C). Cytoplasmic FoxO1 measurements revealed a main effect of treatment; in males, however, insulin plus serum stimulation showed a trend (P= 0.08) for higher cytoplasmic FoxO1 compared to serum starvation (Fig. 3.7D).

A trend (P=0.08) for a treatment effect in nuclear FoxO1 phosphorylation was observed when quantifying phospho-FoxO1 to FoxO1 (Fig. 3.7E). When quantified relative to RCC1, male ATEC had significantly higher FoxO1 phosphorylation compared serum starvation, and higher

phospho-FoxO1 than females with insulin plus serum starvation (Fig. 3.7F). Cytoplasmic FoxO1 phosphorylation measurements revealed a near significant (P=0.057) main effect of the treatment (Fig. 3.7H), but no sex differences.

Nuclear acetyl-FoxO1 measurements showed no changes with treatment or between sex when normalised to total FoxO1 (Fig. 3.7I). Quantifying nuclear acetyl-FoxO1 to RCC1, insulin plus serum stimulation resulted in increased FoxO1 acetylation in both male and females, while no sex differences were apparent (Fig. 3.7J). Cytoplasmic FoxO1 acetylation measurements revealed a main effect of treatment where insulin plus serum stimulation decreased acetyl-FoxO1, which was near significant (post-hoc P=0.06) for males (Fig. 3.7K). Cytoplasmic FoxO1 acetylation however did not show any significant changes when normalised to GAPDH (Fig. 3.7L).

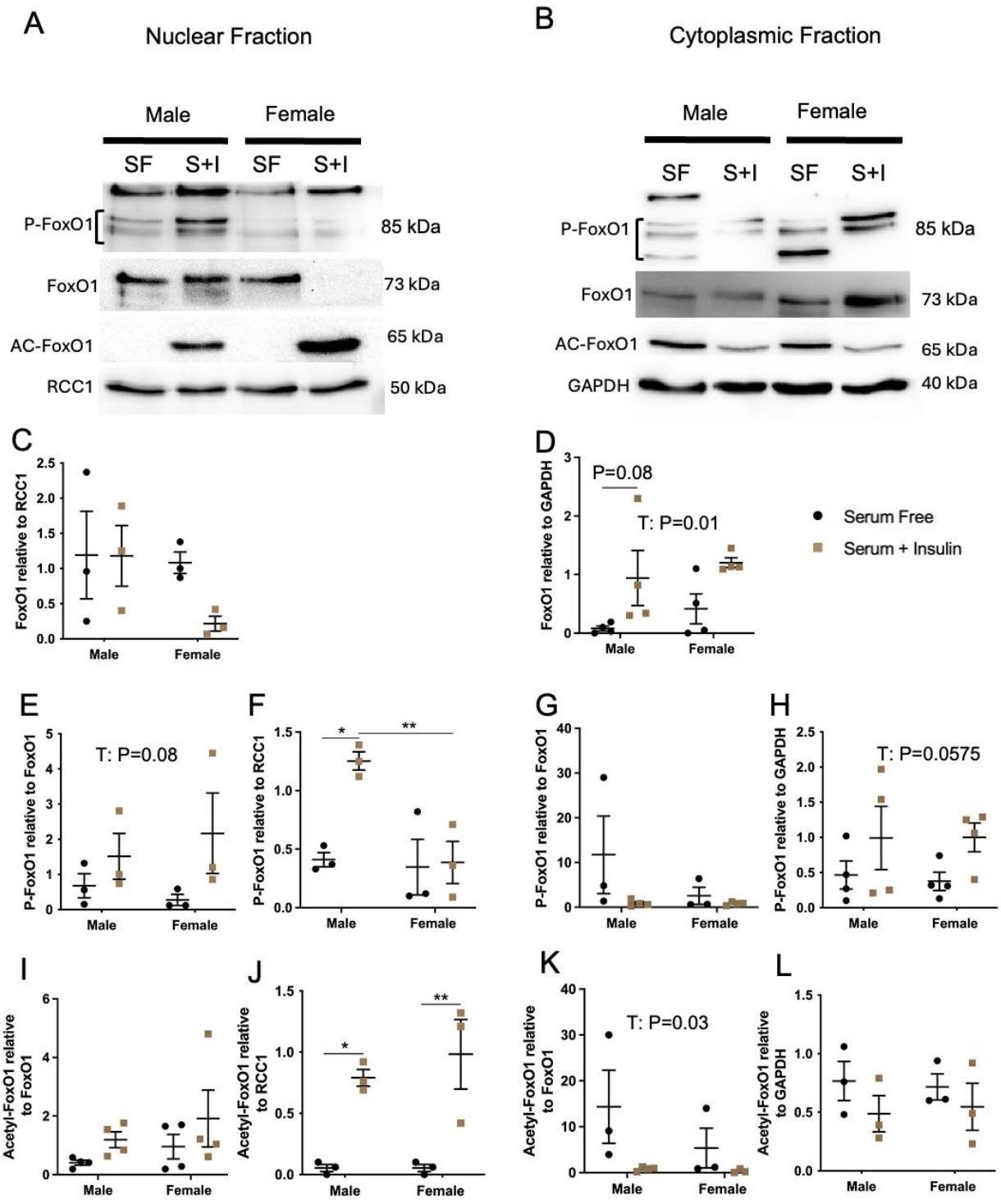

Figure 3.7. The effects of nutritional stimuli on FoxO1 nuclear and cytoplasmic localization, and modifications in adipose tissue ECs. Nuclear and cytoplasmic fractionations from male and female ATECs treated with 2 hours of serum starvation or 30 minutes of serum + insulin stimulation (25 μU/ml). Representative western blots for nuclear and cytoplasmic fractions are shown in A and B, respectively. Relative levels of FoxO1 were normalised to the

loading controls of each fraction, RCC1 and GAPDH (C and D). Phosphorylated and acetylated FoxO1 were normalised to FoxO1 (E and G) and the loading controls for each fraction (F,H,J,L). The brackets in western blot images (A and B) delineate the bands that were quantified for FoxO1 phosphorylation. SF= serum free, S+I= serum plus insulin, P-FoxO1= phosphorylated FoxO1, AC-FoxO1= acetylated FoxO1. n= 4. Data are presented as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significance was detected. *P<0.05, **P<0.005. "T" represents significant main effect of treatment, serum free vs insulin.

3.8 Sex Differences in Adipose Tissue EC FoxO1 Nuclear and Cytoplasmic Localization and Modifications in Response to oxidative stress

To investigate FoxO1 subcellular localization in response to oxidative stress in ATECs, an analogous treatment model as done with SKMECs was implemented and western blotted (Fig. 3.8A and B). No sex or treatment differences in nuclear FoxO1 levels were detected (Fig. 3.8C). In the cytoplasm, there was a significant increase of FoxO1 only in males with oxidant stimulation (significantly with rotenone, and a trend P=0.08 with hydrogen peroxide) (Fig.3.8D).

A trend (P=0.06) for a sex difference was detected in nuclear levels of FoxO1 phosphorylation (relative to FoxO1), with higher phosphorylation in male versus female ATECs (Fig. 3.8E). No differences across conditions were detected for cytoplasmic phospho-FoxO1 (Fig. 3.8G-H). A trend (P=0.08) for greater nuclear FoxO1 acetylation (relative to RCC1) was observed in males compared to females (Fig. 3.8I). No differences across conditions were detectable for cytoplasmic levels of acetylated FoxO1 (Fig. 3.8K-L). Overall, the ATECs displayed high across different experiments as compared to skeletal muscle ECs.

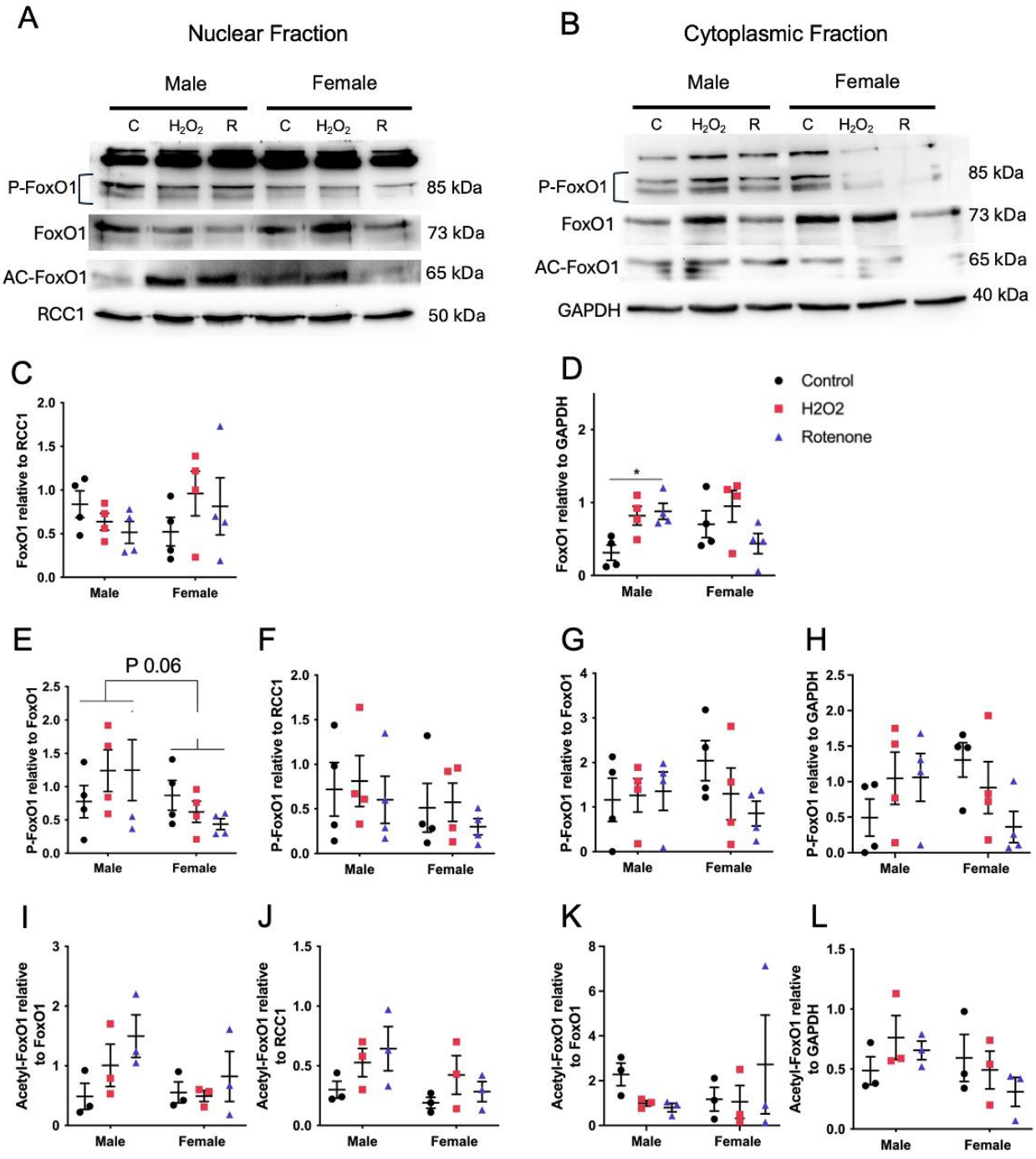

Figure 3.8. The effects of oxidative stressors on FoxO1 nuclear and cytoplasmic localization, and modifications in adipose tissue ECs. Nuclear and cytoplasmic fractionations from male and female SKMECs following treatment with 10% FBS media, hydrogen peroxide (300 µM), and rotenone (2µM). Representative western blots for nuclear and cytoplasmic fraction are shown in A and B, respectively. Relative levels of FoxO1 were normalised to the loading controls of each fraction, RCC1 and GAPDH (C and D). Phosphorylated and acetylated FoxO1 were normalised to FoxO1 (E and G) and the loading controls for each fraction (F,H,J,L). The brackets in western blot images A and B delineate the bands that were quantified for FoxO1

phosphorylation. C= control media, H_2O_2 = hydrogen peroxide, R= rotenone, P-FoxO1= phosphorylated FoxO1, AC-FoxO1= acetylated FoxO1. n= 4. Data are presented as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significance was detected. *P<0.05.

3.9. FoxO1 Mitochondrial Localization in response to Nutritional and Oxidative Stress

Thus far, there have been no studies published showing FoxO1 mitochondrial localization in ECs. Thus, measuring mitochondrial FoxO1 in ECs would be a major breakthrough in de-coding the FoxO1-mitochondrial relationship in ECs. To achieve this step, a mitochondrial fractionation technique was used and refined over the course of months to achieve a pure mitochondrial isolate. The various trials were optimized in order to minimize cross contamination of the cytoplasmic and mitochondrial extracts. The mitochondrial loading control TOMM20 was not detected in the cytoplasmic fraction, indicating purity of this fraction. A small signal from the cytoplasmic fraction loading control, alpha-tubulin, was measured in the mitochondrial extraction (Fig. 3.9A). However, alpha tubulin has been shown to be in the mitochondrial membrane (Carré et al., 2002), which may explain its continued signal in the mitochondrial fraction.

Western blot analysis was used to compare male and female SKMEC FoxO1 mitochondrial (Fig. 3.9A) and cytoplasmic (Fig. 3.9C) localization in response to nutritional stress: consisting of either serum starvation or insulin plus serum stimulation; and oxidative stress: 10% FBS growth media or 10%FBS growth media with rotenone. Mitochondrial FoxO1 levels were not different between serum starvation and insulin plus serum conditions in either sex (Fig. 3.9C). While no sex differences in FoxO1 levels were detected in the mitochondria, cytoplasmic FoxO1 with insulin stimulation was significantly higher in females compared to males (Fig. 3.9D).

When comparing samples that were in regular growth media with those that were rotenone-stimulated, a main effect of sex on FoxO1 mitochondrial localization was detected (Fig. 3.9E). Male SKMECs did show a trend (P=0.09) of higher mitochondrial FoxO1 in control conditions compared to females (Fig. 3.9E). Cytoplasmic levels of FoxO1 were not affected by rotenone stimulation in either male or female EC (Fig. 3.9F).

Although of interest, I was not able to blot for phosphorylated or acetylated FoxO1 in these samples due to the small amount of protein obtained in these extracts.

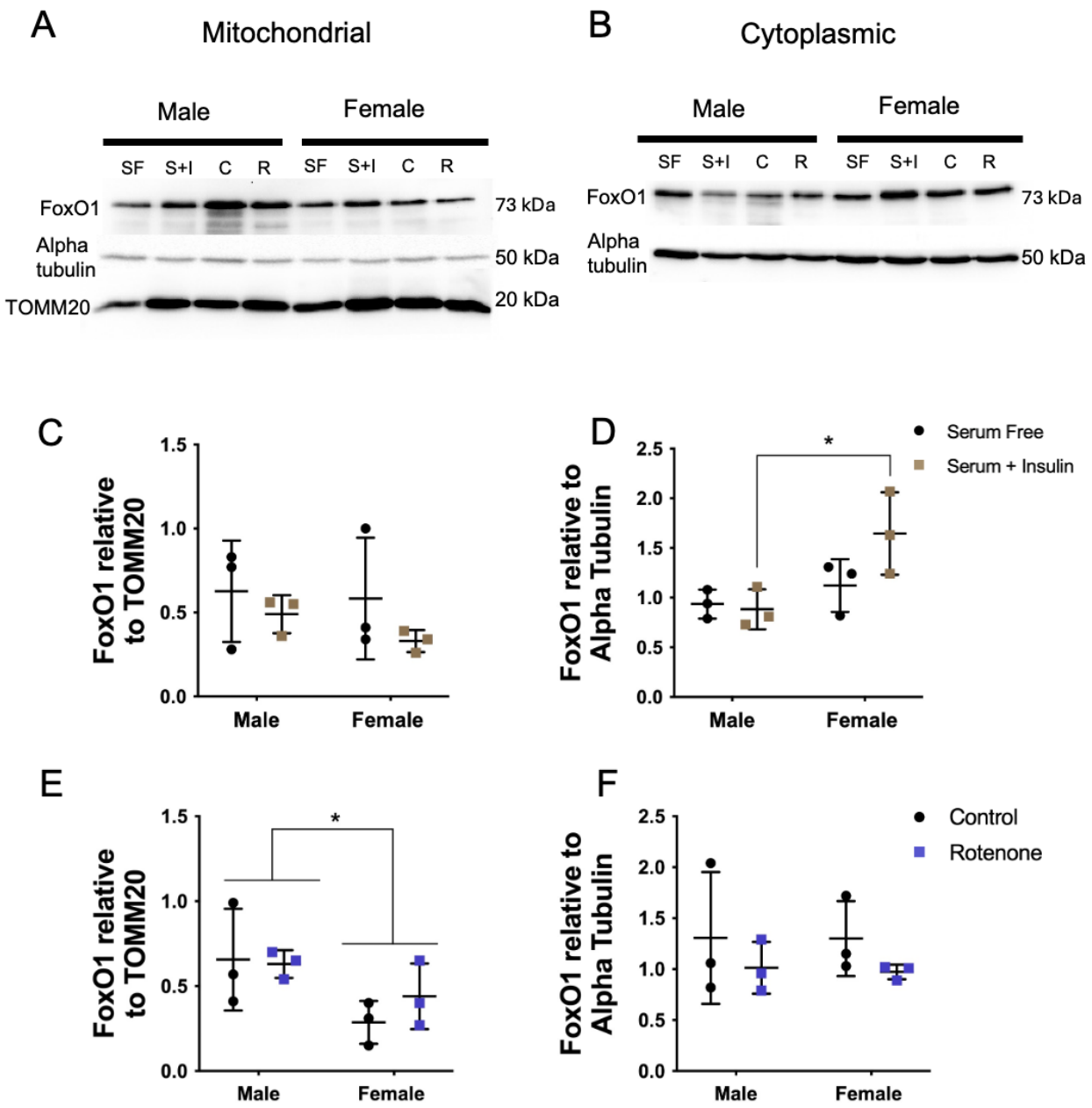

Figure 3.9. Mitochondrial and cytoplasmic FoxO1 in male and female skeletal muscle endothelial cells. Relative levels of FoxO1 protein in the mitochondria (A) and cytoplasm (D) were assessed in response to nutrient stimulation: 2 hour serum starvation, or 30 min of serum plus insulin treatment (25 µU/ml), and stress stimulation: 10% FBS control media, or1 hour rotenone treatment (2µM). TOMM20 and Alpha tubulin were used as loading controls for mitochondrial and cytoplasmic fractions, respectively. FoxO1 was normalized to its corresponding fraction's loading control using ImageJ software. SF= serum free, I= insulin, C= control, R= rotenone. n=3 Data are presented as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significance was detected. *p < 0.05.

Chapter 4: Discussion

The aim of my thesis was to discern sex specific patterns of FoxO1 modifications and localizations in response to nutritional and oxidative stress in adipose tissue and skeletal muscle endothelial cells. Little has been published on FoxO1 direct regulation on oxidative stress response specifically in endothelial cells. Furthermore, no current research has been established in elucidating sex-based differences in FoxO1 functioning, an area of pivotal importance that can help understand sex differences in vascular health outcomes. Thus, FoxO1 levels and PTMs were measured in different sub-cellular compartments of male and female ECs in repones to nutritional and oxidative stress stimuli. Male SKMECs proved to be more sensitive to phosphorylation, which resulted in higher cytoplasmic FoxO1 localization compared to females. Female SKMECs however showed to retain higher levels of FoxO1 in the nucleus in response to oxidative stress. These findings discern differential FoxO1 sub-cellular regulation which can imply sex-specific responses to stress.

Increased Insulin Sensitivity in Male SKMEC Resulting in FoxO1 Nuclear Exclusion

Insulin and growth factor stimulation are well established to activate PI3K/AKT, leading to the phosphorylation of FoxO1-Ser256. While insulin stimulation leading to nuclear export of FoxO1 to the cytoplasm is a widely known phenomenon across a myriad of cell types (Calnan & Brunet, 2008), no reports thus far have established if these effects on FoxO1 regulation are dependent on sex. Findings from my study shed light on sex differences in FoxO1 regulation in response to nutritional stress, which are summarized in Table 4.1. Notably, male SKMECs exhibited significantly higher levels of FoxO1 phosphorylation compared to females in whole cell and cytoplasmic measurements, suggesting a heightened sensitivity to insulin signaling in

males. This increased phosphorylation coincided with elevated cytoplasmic localization of FoxO1 in males following insulin stimulation, supporting the idea that phosphorylated FoxO1 may be more readily exported from the nucleus in male ECs. However, in females, the insulin induced phosphorylation of FoxO1 did not correlate with a marked increase in cytoplasmic localization, suggesting a different regulatory mechanism of FoxO1 shuttling that may be more tightly controlled in females to ensure nuclear entrapment of FoxO1 under metabolic stresses. This discrepancy suggests that male ECs may exhibit higher sensitivity to insulin, promoting a more significant exclusion of FoxO1 from the nucleus in response to the nutrient stimuli. Herein a new sex dimorphic regulatory response of FoxO1 has been uncovered.

My current findings of enhanced insulin sensitivity in males are unexpected as previous established literature generally finds females to be more insulin sensitive (Geer & Shen, 2009; Greenhill, 2018; Riccio et al., 2024). This difference is often attributed to female sex hormones, as females typically display greater insulin sensitivity before menopause, which then declines post-menopause (D'Eon et al., 2005; Moran et al., 2008). Additionally, men lacking estrogen synthesis or activity are known to develop insulin resistance (Herrmann et al., 2002; Smith et al., 1994).

The observation of enhanced insulin sensitivity in males in this study was therefore unexpected. The observed sex differences in insulin sensitivity may be driven by distinct FoxO1 nuclear shuttling dynamics and cytoplasmic FoxO1 retention between males and females. Specifically, we found that while males demonstrate enhanced insulin sensitivity, this may stem from a temporal disparity in FoxO1 phosphorylation and its subsequent translocation from the nucleus to the cytoplasm, while also negating any opportunity of cytoplasmic FoxO1 to be transported to the nucleus. For example, FoxO1 in males could be entirely exported from the

nucleus within 30 minutes of insulin stimulation, whereas females may require a longer duration to elicit a similar response. Previous studies support the notion that FoxO1 shuttling dynamics vary depending on the stimulus (Lasick et al., 2023), thus it is entirety plausible that FoxO1 nuclear shuttling dynamics are also sex dependant. Thus, while AKT phosphorylation, in response to insulin signaling, may occur in both sexes, additional FoxO1 regulatory mechanisms might differentially influence nuclear exclusion, impacting insulin sensitivity outcomes. This explanation is substantiated when you consider FoxO1 is phosphorylated in females, yet the prevailing outcome of that being nuclear exclusion is abrogated, relative to that of males.

It was unexpected to observe no difference in whole cell acetylation patterns in response to insulin because the effects of nutrient stimulation on FoxO1 have been well studied in promoting acetylation (K. Li et al., 2019). However, changes in FoxO1 acetylation in response to nutrient stimulation became more apparent within subcellular fractionations. Male ECs showed higher cytoplasmic FoxO1 acetylation than females. Knowing that acetylation of FoxO1 promotes its nuclear exclusion (K. Li et al., 2019; Matsuzaki et al., 2005), it was not surprising to see that males also had comparatively higher levels of total cytoplasmic FoxO1 than females suggesting that sex differences in FoxO1-acetylation leads to sex differences in FoxO1 localization. These acetylation patterns, along with phosphorylation data, imply that male SKMECs may be more sensitive to nuclear exclusion FoxO1 modifications following insulin stimulation. It is important to note that the higher levels of phosphorylation and acetylation observed in male ECs could potentially work in a synergistic manner rather than independent PTMs. Matsuzaki et al., had demonstrated in HepG2 cells that FoxO1 acetylation increases sensitivity towards subsequent phosphorylation (Matsuzaki et al., 2005); which could imply

enhanced cytoplasmic FoxO1 levels as a result of acetylation. Overall, the enhanced nuclear exclusion PTMs along with increased cytoplasmic FoxO1 in males could lead to greater eventual loss of FoxO1 protein levels as this trend could potentially extend to other outcomes not measured in this study, such as ubiquitination and degradation. Thus, these sex differences in FoxO1 could hinder the ability of males to counter cellular actions that requires FoxO1 transcription.

Table 4.1. **Summary of SKMEC Nuclear and Cytoplasmic Fractionation in Response to Nutrient Stress**

Protein Fraction	FoxO1 modification	Serum Free	Serum Plus Insulin
Nuclear	FoxO1	-	-
	P-FoxO1	F > M	F > M, M: ↑ vs SF
	AC-FoxO1	-	-
Cytoplasmic	FoxO1	-	M > F, M:↑ vs SF
	P-FoxO1	M > F	M > F
	AC-FoxO1	-	-

Enhanced Nuclear FoxO1 Retainment in Females in Response to Oxidative Stress

Whole cell and subcellular fractionation revealed that oxidative stress influences the distribution of FoxO1 between the nucleus and cytoplasm in a sex dependent manner. Additionally, the source of ROS affects FoxO1 outcomes. My findings, summarized in Table 4.2, revealed that oxidative stress elicits a sex dependent regulation of FoxO1, with males displaying higher phosphorylation of FoxO1 at Ser256 in response to mitochondrial ROS generated by

rotenone, compared to females. This suggests that mitochondrial ROS may restrict the transcriptional activity of FoxO1 more in male ECs, potentially compromising antioxidant defense (considering nucleus encodes for antioxidant genes). Notably, the elevated whole cell phosphorylation in males seems primarily driven by cytoplasmic FoxO1, as nuclear FoxO1 levels remain relatively stable between sexes, reinforcing that males predominantly utilize a cytoplasmic, rather than nuclear, regulatory mechanism in response to mitochondrial oxidative stress. Conversely, when exposed to hydrogen peroxide, which generates cytoplasmic ROS, females show increased FoxO1 Ser256 phosphorylation; however, this failed to cause nuclear export.

These patterns indicate a distinct, sex specific response to ROS sources where males may suppress FoxO1 activity when facing mitochondrial stress, while females appear better equipped to respond to cytoplasmic ROS challenges. These findings may support a more active FoxO1-mediated antioxidant response in females in the presence of mitochondrial ROS, as shown by higher nuclear FoxO1 levels. These findings align with a study by Borrás et al., which report increased antioxidant gene expression, including glutathione peroxidase and Mn-SOD, in mitochondria from female cells compared to males, leading to enhanced resistance to oxidative damage (Borrás et al., 2003). Interestingly, this could be potentially linked to estrogen, as this effect was absent in ovariectomized females, although estradiol treatment restored the effect. Similarly, Farhat et al. observed that female rat skeletal muscle mitochondria exhibit greater resistance to ROS than those of males (Farhat et al., 2017). Although FoxO1 transcriptional activity was not directly measured in this study, the observed FoxO1 phosphorylation and localization patterns support a model in which females may enhance antioxidant defenses against mitochondrial ROS through FoxO1.

With a cytoplasmic source of ROS, hydrogen peroxide, there is greater FoxO1 Ser256 phosphorylation in females, in whole cell and nuclear compartments, yet this results in greater nuclear FoxO1, while males have higher cytoplasmic phospho-FoxO1 and total FoxO1. While only one PTM and its respective kinase is implicated in this response, this stimulates the idea that PTMs and their kinases could potentially influenced differently depending on the source of ROS. It is known that stress stimuli induce phosphorylation on various residue of FOXO1 via MST1/JNK; thus, I was surprised to see that high levels of Akt-dependent serine256 phosphorylation were detected. Serine 256 phosphorylation on FoxO1 generally results in a decrease in FoxO1 transcriptional potential, on the contrary, oxidative stress has been reported to increase FoxO1 activity and nuclear import via phosphorylation on Ser184, Ser212, Ser284 (Zhang et al., 2021). A certain level of serine 256 phosphorylation was expected with the oxidative stress experiments due to serum being included in the control media, thus introducing growth factors that activate insulin signaling pathway. However, this was very significant with hydrogen peroxide.

The reported effects of H_2O_2 on cellular pathways are complex as published findings have demonstrating insulin mimetic actions by transiently increasing AKT activity, serine 256 phosphorylation of FoxO1, and FoxO1 nuclear exclusion; however, these effects dissipate with extended H_2O_2 exposure (Frescas et al., 2005; Wang et al., 2010). This finding of H_2O_2 induced FoxO1 phosphorylation on serine256 has also been reported in rat aortic endothelial cells (Y. Wang et al., 2010). Interestingly, H_2O_2 has been found to induce AKT activation in multiple cell types in a time dependant manner, with the height of activation being close to 30 minutes, and returning back to baseline at 60 minutes (X. Wang et al., 2000). Considering that ECs were

treated with H_2O_2 for 60 minutes, serine256 FoxO1 phosphorylation in response to H_2O_2 was elevated differently in male and female SKMEC. This suggests that sex differences in the temporal pattern of H_2O_2 signaling could also influence the observed differences in FoxO1 localization, with males showing higher cytoplasmic levels and females exhibiting greater nuclear levels of FoxO1. In males, prolonged H_2O_2 exposure might be required to achieve nuclear FoxO1 retention, potentially reducing serine 256 phosphorylation over this extended time course. This variance could represent a protective advantage for females, where greater nuclear retention of FoxO1 may enhance transcriptional activity under oxidative stress. A key consideration in these experiments, similar to those involving nutritional stimuli, is the selection of treatment time courses. By assessing FoxO1 functional changes at only a single time point, the analysis may not fully capture the dynamic range of responses that could emerge over different intervals. This limitation suggests that multiple time courses might be necessary to comprehensively understand the progression and regulation of FoxO1 under these conditions.

FoxO1 acetylation did not vary significantly with different ROS sources; rather, it exhibited generalized sex-based differences with increased cytoplasmic acetylation in males. Previous studies in immortalized cell lines such as HEK293T and HeLa cells have shown that FoxO acetylation can be induced in response to oxidative stress (Daitoku et al., 2011). FoxO acetylation patterns seem to be inconsistent with considerable variability as some studies show FoxO1 acetylation resulting in enhanced transcriptional activation (Motta et al., 2004; Perrot & Rechler, 2005; Yang et al., 2005), whereas other studies as mentioned in the review widely state the inhibiting effect of acetylation on FoxO1. This complexity likely stems from the numerous

acetylation sites and the variety of responsible kinases, making it challenging to draw definitive conclusions based upon the specific site chosen in this study.

Overall, oxidative stress appears to enhance nuclear FoxO1 retention in females, whereas males exhibit greater cytoplasmic FoxO1 levels, supporting the hypothesis that FoxO1 dynamics may contribute to stronger oxidative stress defenses in females. Preliminary findings, including my master's research, also point to higher nuclear FoxO1 in females, contributing to an existing body of evidence that females possess greater overall FoxO1 levels, possibly underlying more favorable stress responses in females. To further support the argument for sex-based differences in nuclear FoxO1 retention, examining pathological changes post menopause, when female sex hormone levels decline and oxidative stress susceptibility increases, could be informative (Martínez de Toda et al., 2023; Robert, 2023). This phenomenon suggests that sex hormones could influence FoxO1 nuclear localization, implying unknown mechanisms that counteract nuclear export signals.

Table 4.2. Summary of SKMEC Nuclear and Cytoplasmic Fractionation in Response to Oxidative Stress

Protein Fraction	FoxO1 modification	Control	H_2O_2	Rotenone
Nuclear	FoxO1	F > M	F > M	-
	P-FoxO1	-	F > M	-
	AC-FoxO1	F > M	F > M	F > M
Cytoplasmic	FoxO1	F: ↑ vs R	M > F, M: ↑ vs C	M > F
	P-FoxO1	-	M > F	-
	AC-FoxO1	M > F	M > F	M > F

Sex Differences in FoxO1 Mitochondrial Localization in Response to Different Stimuli

Thus far there have been no studies showing FoxO1 mitochondrial localization in ECs. A key discovery from my study is the identification of FoxO1 localization within mitochondria. Therefore, my findings contribute novel insights to this field, as FoxO1 localization within mitochondria was clearly observed in both male and female skeletal muscle endothelial cells. Statistical analysis revealed a significant main effect, with male SKMECs showing higher mitochondrial FoxO1 levels compared to females. This finding highlights potential sex-based differences in FoxO1 localization that warrant further investigation. However, there was no evidence that mitochondrial localization was influenced by the specific treatments used in this study. The presence of FoxO1 within mitochondria could facilitate its binding to mitochondrial DNA, while reducing its nuclear localization and subsequent binding to nuclear DNA. This phenomenon was reported by Barbato et al., where FoxO1 mitochondrial localization in adipocytes allowed for mtDNA binding, an effect that was diminished with nutrient deprivation, resulting in FoxO1 shuttling back to the nucleus (Lettieri-Barbato et al., 2019). This distinction is noteworthy because mtDNA encodes for proteins and enzymes essential for the electron transport chain (ETC) (Taanman, 1999) rather than antioxidant defense mechanisms, which are typically regulated by nuclear DNA (Holley et al., 2011).

Knowing this difference in transcripts of mtDNA and nuclear DNA, it is noteworthy to point out how male SKMEC have higher mitochondrial and lower nuclear FoxO1 levels compared to females under both control and stress conditions. This might imply that females have a more robust protective response to oxidative stress as nuclear encoded antioxidant genes would be potentially targeted, whereas males would favour increase in mitochondrial encoded

genes. Perhaps this could generate a question of whether male mitochondrial localization is another regulatory mechanism to adapt to stress, in that upregulating ETC components could overcome the otherwise ROS induced damage ETC complex. However, the precise effects of mitochondrial FoxO on mtDNA transcription under baseline conditions remain unclear and warrant further study as different outcomes have been reported. For example, FoxO1 has shown to induce negative effects on mtDNA transcription in adipocytes (Lettieri-Barbato et al., 2019), whereas FoxO3 mtDNA binding resulted in enhanced ETC subunits in fibroblasts and skeletal myotubes (Peserico et al., 2013).

While I was unable to investigate the functional impact of nuclear-mitochondrial FoxO1 localization, preliminary research from the Haas lab has suggested that FoxO1 may inhibit mtDNA transcription, as FoxO1 inhibition has been associated with increases in mitochondrial gene transcripts. This aligns with findings by Barbato et al., who also reported an inhibitory effect of FoxO1 on mitochondrial gene transcription (Lettieri-Barbato et al., 2019). Though more research is necessary to clarify sex differences in FoxO1 function within endothelial cell mitochondria, my findings of FoxO1 mitochondrial localization provide a foundation for understanding sex specific roles in mitochondrial regulation.

Conclusion

These findings provide new insights into the sex specific regulation of FoxO1 within ECs, revealing distinct patterns in post translational modifications and subcellular localization. These observations also generate new possibilities where FoxO1 may not only be regulated by pathways like insulin signaling but also by also by other cellular pathways that may not be synonymous between both sexes, resulting in different FoxO1 shuttling dynamics, modifications,

and interactions with kinases. In females, FoxO1 shows a greater propensity to remain nuclear under oxidative stress. Molecularly, this may reinforce transcriptional potential due to proximity to DNA, implying enhancement of downstream antioxidant genes. Conversely, in male ECs, FoxO1 localizes more to the cytoplasm after insulin and stress stimulation. This shift may enhance its role in nutrient-sensitive signaling but could reduce its effectiveness in responding to stress. A novel mitochondrial localization in ECs was also established, providing the basis for assessing FoxO1 transcriptional regulation of mtDNA.

Significance

This study sheds light on sex-based differences in FoxO1 localization and post translational modifications within endothelial cells, an area with considerable implications for understanding vascular health disparities between males and females. By analyzing responses to both nutritional and oxidative stress, these findings are the first to reveal that FoxO1 activity and localization follow distinct sex specific patterns, underscoring differences in endothelial function. The enhanced insulin sensitivity in males may indicate a different regulatory response to metabolic stress, potentially offering temporary protection against vascular dysfunction and related diseases, such as atherosclerosis and hypertension. However, this does not imply males have an overall advantage, as they may still be predisposed to faster onset of insulin resistance under prolonged or excessive metabolic stress. However, this heightened insulin sensitivity, along with reduced nuclear FoxO1 under stress, may limit the capacity of males to activate a comparable stress response. In contrast, my data imply that females may demonstrate a more resilient adaptation to oxidative stress, a characteristic linked to vascular dysfunction and aging.

While the role of FoxO1 in oxidative stress is well established amongst other cell types, endothelial cell specific studies are scarce. These findings provide pivotal first steps in recognizing sex differences in FoxO1 stress response specifically in endothelial cells, something that needs to be expanded with future work to mitigate disparities in endothelial dysfunction and consequentially CVDs. Investigating FoxO1 modifications and their effects on localization may help explain some of the mechanisms driving sex differences in stress resilience and metabolic health.

Limitations

A primary limitation was the technical challenge of performing consistent cell fractionation, which impacted the ability to establish reliable patterns of FoxO1 localization in adipose tissue endothelial cells (ATECs). Despite multiple attempts with Western blotting, variability in FoxO1 distribution in ATECs limited a direct assessment of the hypothesis in this cell type. However, to address this, I adjusted the experimental approach by including skeletal muscle endothelial cells as a complementary model. The SKMEC data helped to support certain trends initially observed in ATECs, providing valuable insights and an opportunity to compare responses across endothelial cells from two distinct tissue types. This not only allowed progress despite technical challenges but also opened new avenues for understanding tissue specific endothelial cell responses to stressors.

Oxidative stress experiments showed considerable variability as inconsistent FoxO1 localization outcomes to the same stimulus were measured. On re-evaluation, I can attribute this to the asynchronous handling of male and female endothelial cell passages due to differing growth patterns. Each trial (n) of a set of experiments was conducted on the same passage

number of EC for both male and female cells. Example: male EC passage 4 and female EC passage 4 were fractionated and compared. However, due to unequal growth speeds, male and female ECs could not always be plated for experiment and then fractionated simultaneously. Thus, variations in the handling of cells and in buffers made the day of extraction could have resulted in inconsistent baseline parameters between male and female ECs.

An additional limitation in evaluating the response of FoxO1 to oxidative stress was the inability to verify an increase in intracellular ROS in response to the applied stress stimuli. Although fluorogenic probes were used to stain cells for ROS measurement, the visual results were consistently similar across conditions and did not indicate differences in ROS levels. This lack of detectable variation persisted even when using elevated concentrations of oxidative agents to test the method's validity.

In this study, I was only able assess two PTMs of FoxO1 as the third, O-GlcNAcylation, was unsuccessful. Due to poor antibody availability, an immunoprecipitation (IP) of FoxO1 was required to assess the glycosylation modification. Despite numerous attempts to develop an effective FoxO1 pulldown protocol, these efforts were unsuccessful. An IP protocol would have also allowed me to use other techniques such as mass spectrometry to accurately assess a variety of FoxO1 PTMs for which antibodies are not available such as MST and JNK phosphorylation. Another concern I have is regarding accurate measurement of FoxO1 acetylation. Concerns about the antibody used to detect acetylated FoxO1 arose when the molecular weight observed on the blots differed considerably from expected values. This inconsistency suggests that the antibody may not have accurately detected FoxO1 acetylation as reported in the literature. With

an effective IP protocol, I could have isolated FoxO1 and used an acetylation antibody to assess its acetylation, an approach commonly published in studies investigating FoxO1 acetylation.

Although not technically a limitation, it is also important to differentiate the two methods I used to measure modified FoxO1 in nuclear and cytoplasmic fractions. Modified FoxO1 was always normalized to the total FoxO1 within that fraction for the same stimuli, while also being quantified to the loading control for that respective fraction, RCC1 or GAPDH. One of the reasons this was done was due to avoid misinterpreting PTM levels in the absence of total FoxO1. For instance, in certain female ATEC nuclear western blots, phosphorylated FoxO1 signal in response to insulin was apparent even in the absence of any total FoxO1 signal. Normalizing a modified protein such as phosphorylated FoxO1 to the total protein within the same subcellular fraction like nuclear FoxO1 provides insight into the proportion of the protein that is modified, allowing assessment of phosphorylation status relative to the total nuclear FoxO1 pool. This approach is commonly used to determine whether a stimulus induces a specific modification. Normalizing to loading controls lets us know the level of modified FoxO1 in comparison to other cellular proteins within that condition, also allowing for comparisons between other samples by comparing measurements to a stable marker, such as RCC1, that is not affected by different conditions. Each type of normalization offers complementary insights, allowing for more accurate interpretation of post-translational modifications and localization changes.

Future Directions

A key next step is to investigate the temporal dynamics of FoxO1 nuclear and cytoplasmic localization, enabling a more nuanced understanding of sex differences in FoxO1

shuttling in response to nutritional and oxidative stress. Conducting time course experiments across multiple time points will allow for detailed observation of FoxO1 subcellular responses in both sexes. Furthermore, examining upstream kinase activity presents a promising approach to understanding sex differences in FoxO1 PTMs and localization. For example, assessing AKT levels and phosphorylation following insulin treatment could help clarify whether sex based differences in insulin sensitivity exist or if differential FoxO1 phosphorylation is influenced by other factors. Similarly, evaluating the activation of FoxO1 related kinases such as MST1/JNK in response to oxidative stress would provide insights into sex specific signaling dynamics in endothelial cells.

The influence of FoxO1 on mitochondrial function and DNA binding remains a challenging yet critical area of exploration. While initial progress has been made in establishing FoxO1 mitochondrial localization, further experiments are necessary to refine the protocol for isolating pure mitochondrial fractions, which will help validate and strengthen these findings. Subsequently, DNA binding assays could be employed to determine if FoxO1 binds to mitochondrial DNA in ECs and to assess the functional consequences, whether it is positive or negative on mitochondrial function. These steps will be pivotal in uncovering the complex role of FoxO1 in sex-biased endothelial cell phenotypes and in broadening our understanding of the full scope of FoxO1 functions in endothelial cells.

A key future direction will involve refining the nutritional stimuli treatment method to improve the interpretability of results. In retrospect, the inclusion of serum in the media during insulin stimulation introduced a significant limitation. My initial objective was to evaluate the nuclear export of FoxO1 in response to insulin. However, a review of the data highlighted a

methodological flaw: a fully nuclear localization of FoxO1 had not been established as the baseline prior to insulin treatment, making it difficult to assess nuclear export accurately. By starting with some cytoplasmic FoxO1 already present, it was not accurate to measure a genuine nuclear export effect induced by insulin. To address this, future experiments will establish a nuclear enriched baseline by using serum starvation across all conditions. Following this, insulin stimulation will be applied to one condition to enable a clear assessment of FoxO1 nuclear export in response to insulin.

Undoubtedly the next steps in elucidating the role of FoxO1 in EC oxidative stress response would be measuring its transcriptional regulation of genes such as superoxide dismutase, catalase, and peroxidases in response to stressors. This can also be expanded then to contract the outcomes in male and female ECs to discern any differences in oxidative stress response. Expanding upon this, silencing FoxO1 would be an essential step in establishing a direct FoxO1 regulatory requirements on these antioxidant target genes. Knockdown of FoxO1 would allow us to assess the magnitude of its impact within and between both sexes. Although I initially aimed to undertake this work for my thesis, various experimental setbacks and lengthy optimization efforts delayed my progression and did not permit the execution of these experiments.

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