ABSTRACT

Title of Document:	ASSOCIATION BETWEEN INCREASED HEPATIC LIPID STORAGE AND IMPAIRED HEPATIC MITOCHONDRIAL FUNTION IN OVARIECTOMIZED MICE
	Ana Patricia Valencia, M.A., 2012
Directed By:	Espen Spangenburg, Ph. D, 2012

Reduced ovarian function is associated with development of the metabolic syndrome (MetS). Increased risk for MetS is strongly linked to hepatic metabolic dysfunction. However, at this time few studies have examined metabolic function of hepatic tissue under conditions of reduced ovarian function. The purpose of this study was to determine if ovariectomy (OVX) impaired hepatic mitochondrial function and its potential association with sirtuin (SIRT) function. Female C57BL/6 mice were divided into two groups (SHAM, OVX). Hepatic mitochondrial function was measured by assessing oxygen consumption, reactive oxygen species (ROS) production, and mitochondrial protein content. In addition, mitochondrial acetylation status and SIRT protein content was determined. The OVX group exhibited increased ROS production compared to the SHAM group. However, no differences were detected in oxygen consumption, mitochondrial protein content, acetylation status, or total SIRT content between groups. The data shows that ovariectomy increases mitochondrial ROS production, which suggests a novel mechanism to consider.

ASSOCIATION BETWEEN INCREASED HEPATIC LIPID STORAGE
AND IMPAIRED HEPATIC MITOCHONDRIAL FUNTION IN
OVARIECTOMIZED MICE

By

Ana Patricia Valencia

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of the Arts
2012

Advisory Committee:
Espen Spangenburg, Ph.D, Chair
James Hagberg, Ph.D
Stephen Roth, Ph. D

Acknowledgements

I am profoundly grateful to my advisor, Dr. Espen Spangenburg, for providing me with excellent support and guidance throughout the past two years to complete my Master's thesis. I also would like to show my sincere gratitude to the members of my committee, Dr. James Hagberg and Dr. Steve Roth, for their valuable advise and input for this project. Last but not least, I would like to thank all the members of my program for their unconditional support, particularly Katie Jackson and Lindsay Wohlers, as my thesis would not have been possible without their help.

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ACC	Acetyl-CoA Carboxylase
ArKO	Aromatase knockout
ATP	Adenosine triphosphate
BMI	Body mass index
CPT	Carnitine palmitoyltransferase
CVD	Cardiovascular disease
DAG	Diacylglycerol
DGAT	Diglyceride acyltransferase
FA	Fatty acid
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase/ cluster of differentiation 36
FFA	Free fatty acid
GS	Glycogen synthase
GSK	Glycogen synthase kinase
LCAD	Long chain acyl-CoA dehydrogenase
MCAD	Medium chain acyl-CoA dehydrogenase
MetS	Metabolic syndrome
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steaohepatitis
IRS	Insulin receptor substrate
OLETF	Otsuka Long Evans Tokushima Fatty
OVX	Ovariectomy
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species
SCD-1	Stearoyl-CoA desaturase
SIRT	Sirtuin
SREBP-1c	Sterol regulatory element binding protein 1c
TAG	Triacylglycerol
VLCAD	Very long chain acyl-CoA dehydrogenase
ER	Estrogen receptor
ERKO	Estrogen receptor knockout

Introduction

Metabolic-related diseases such as cardiovascular disease and type 2 diabetes are currently among the leading causes of mortality in the Western world. The majority of metabolic diseases are associated with obesity, making them a major concern in the United States, where over 30% of Americans are classified as obese and another third as overweight (Menifield et al, 2008). Both a sedentary lifestyle and increased caloric consumption contribute to the onset of obesity and increase the susceptibility to a constellation of conditions such as hypertension, dyslipidemia, insulin resistance, and hyperglycemia. The onset of any of these conditions increases the risk for mortality, and when at least three of the afore-mentioned conditions are present it becomes known as the metabolic syndrome (MetS). Epidemiological studies show that post-menopausal women are at a higher risk for developing the MetS than their pre-menopausal counterparts, resulting in a substantial increase in the mortality risk in this population (Polotsky & Polotsky, 2010). Further, estrogens appear to play a key role in the regulation of a number of metabolic processes that would be predicted to protect women from metabolic disease. Thus, any condition that results in the attenuation or ablation of estrogen function would likely increase the risk of developing metabolic disease.

Independent of obesity, individuals with the MetS have up to five-fold more stored lipid in the liver than those without the MetS (Kotronen et al., 2008). Accordingly, increased liver fat content is strongly associated with the development of insulin resistance, which is a characteristic of the MetS (Eckles et al., 2005). Enhanced lipid deposition in the liver decreases hepatic insulin sensitivity, resulting in increased gluconeogenesis and VLDL secretion, all of which exacerbate the risk for the MetS (Choi & Ginsberg, 2011). Reduced

ovarian function in post-menopausal women is associated with metabolic abnormalities that increase the susceptibility for insulin resistance, central obesity, and dyslipidemia (Qureshi et al., 2007; Chow et al., 2011). Recent data suggest that the loss of estrogen function leads to insulin resistance in the hepatic tissue, suggesting that estrogens play a key role in defining hepatic metabolic function (Stubbins et al. 2011). Unfortunately, we have a poor understanding of the key factors contributing to the development of insulin resistance in the liver under conditions of reduced estrogen function.

Individuals diagnosed with the MetS often exhibit non-alcoholic fatty liver disease (NAFLD), a condition characterized by the accumulation of hepatic fat making up more than 5% of the total liver weight. NAFLD is the most prevalent form of liver disease worldwide and affects 95% of obese individuals (Basaranoglu et al., 2010). The increased fat storage can result in the development of insulin resistance (Satia-Abouta et al., 2002), and if left untreated NAFLD can progress into non-alcoholic steaohepatitis, fibrosis, cirrhosis, and hepatic carcinoma (Day, 2002). Recent data have shown that the incidence of NAFLD is most frequent in older sedentary men, with growing evidence suggesting that fatty liver develops in women who are in states of estrogen deficiency (Lin et al., 2006). We have previously found that the surgical removal of the ovaries in the mouse contributes to increased hepatic lipid accumulation (Jackson et al., 2011), which is confirmed by similar findings by Stubbins et al. (2011), Kamada et al., (2011), and Rogers et al., (2009). This occurs even though the animals were maintained on a low-fat rodent chow, indicating the importance of estrogens in the maintenance of liver metabolic function in females. Furthermore, the Aromatase knockout male mouse (ArKO), which is unable to produce estrogen, has increased adiposity and enhanced hepatic lipid storage, both of which are

reversed by the administration of either exogenous estradiol (Hewitt et al., 2004) or an estrogen receptor agonist (Chow et al., 2011). In humans, 43% of female cancer patients developed hepatic steatosis within two years of Tamoxifen treatment, which is an estrogen receptor antagonist (Nishino et al., 2003). Collectively, these studies provide compelling evidence that the loss of ovarian hormone function is associated with abnormalities in hepatic metabolism.

Under conditions of reduced ovarian function, one of the contributing factors to hepatic fat accumulation is the onset of visceral adiposity. Studies show that free fatty acids (FFAs) released via visceral adipose tissue lipolysis are the predominant source of stored hepatic lipid in patients with NAFLD (Thorne et al., 2010). Unlike the subcutaneous fat depot, FFAs derived from visceral adipose tissue have direct access to the liver through the hepatic portal vein. We have previously shown that there is a substantial increase in visceral adiposity in the ovariectomized (OVX) mouse model when compared to the age-matched controls (Wohlers & Spangenburg, 2010). This increase in visceral adiposity is also seen in other models of reduced estrogen function such as the ArKO (Chow et al., 2011) and estrogen receptor alpha knockout (ERKO) (Bryzgalova et al., 2006). Our data also indicate that OVX mice have a loss of regulatory control over basal lipolytic function (Wohlers et al., 2011), resulting in enhanced FFA release from the visceral depot into the portal vein where the FFAs have direct access to the hepatic tissue. Therefore, it is likely that the enhanced rate of basal lipolysis in the OVX animal increases the rate of FFA transport through the portal vein, ultimately resulting in increased lipid deposition in the liver. The development of fatty liver due to increased lipolysis in visceral adipose tissue is referred to as the *portal vein hypothesis* (Basaranoglu et al., 2010). This

hypothesis could explain why most of the obese population exhibits NAFLD, and why increased visceral fat mass in postmenopausal women puts them at a higher risk for NAFLD.

At this point, there is a relatively poor understanding of how estrogens regulate lipid metabolism within hepatic tissue. Under conditions of reduced estrogen function, the accumulation of hepatic lipid could be due to either enhanced lipid storage or poor lipid utilization. The formation of triacylglycerol (TAG), which comprises most of the lipid stored in the liver, can either be derived from FFAs in circulation or by synthesis of FA through *de novo* lipogenesis (DNL). Lipogenesis, or the assembly of FAs from glucose derivatives, is enzymatically regulated by Acetyl CoA Carboxylase (ACC) and Fatty Acid Synthase (FAS). Pighon et al., (2011) found that the hepatic mRNA expression of ACC and FAS as well as their transcription factor, sterol regulatory element-binding protein (SREBP-1c) were enhanced in OVX rats compared to SHAM rats (Pighon et al., 2011). Furthermore, ArKO mice have increased hepatic FAS mRNA and protein expression (Chow et al., 2011). In contrast, my group has failed to see any changes in the protein content of ACC or FAS in livers from OVX mice compared to SHAM mice. Further, we failed to detect any difference in the lipogenic index (16:0/18:2n-6), suggesting the accumulation of TAG in the liver of the OVX mice is not the result of enhanced lipogenesis (Jackson et al., 2011). The discrepancies between our data and those of Pighon et al. (2011) may be explained by the species-specific differences in the response to OVX, specifically, rats significantly increase their food intake in response to OVX (Paquette et al., 2007), while mice do not (Rogers et al, 2009). Thus, it is possible that the changes in FAS and ACC protein content in OVX rats may be more due to increased

caloric consumption rather than a loss of ovarian function. In addition, we have also failed to see changes in mRNA expression of TAG assembly proteins such as diacylglyceol transferase (DGAT-1) and (DGAT-2) (Jackson et al., 2011), suggesting that the increase in hepatic TAG content is not a result of increased esterification of FFA.

However, we have found significant differences in the FA composition of TAG isolated from hepatic tissue of OVX mice compared to SHAM mice. We have shown that OVX mice have proportionately higher levels of the monounsaturated FAs, palmitoleic (16:1) and oleic (18:1) acids, than the saturated FAs palmitic (16:0) and stearic (18:0) acid (Jackson et al., 2011). The ratio between the monounsaturated to the saturated forms of FAs is known as the *desaturase index*, which is used as a measure of the conversion of saturated FAs into monounsaturated FAs (Kotronen et al., 2009). This conversion is regulated by the enzyme stearoyl Coa desaturase (SCD-1). Increased hepatic SCD-1 activity is associated with increased lipid storage in the liver, as well as hepatic insulin resistance (Cohen et al., 2003; Choi et al., 2011). Our lab has found a significant increase in hepatic SCD-1 protein content in OVX mice, suggesting the increased TAG in the desaturase index is the result of an upregulation in SCD-1 protein content (Jackson et al., 2011). Furthermore, we found that by providing the OVX mice access to voluntary running wheels we were able to completely prevent the increases in SCD-1 content, desaturase index, and hepatic TAG content when compared to sedentary OVX mice. At this time it is unclear why exercise prevented an increase in SCD-1 activity, however we have hypothesized this could be a result of increased oxidation of the FFA's by the hepatic mitochondria ,thus providing less substrate to SCD-1.

Therefore, if exercise can prevent increased hepatic lipid storage in OVX mice, it might be speculated that hepatic mitochondrial dysfunction is a contributor to accumulation of hepatic TAG accumulation. This speculation is based on data by others showing that modest amounts of exercise training increase hepatic mitochondrial function and prevent the onset of NAFLD in animals that are extremely susceptible to metabolic diseases (Rector et al., 2008). These authors suggested that the prevention of NAFLD is mediated by improvements in mitochondrial function. However, at this time there are few data examining hepatic mitochondrial function in animal models of reduced estrogen function, with the exception of Paquette et al. (2009) who demonstrated a 34% reduction in oxidation of 9,10-[3-H] palmitate in isolated hepatocytes of OVX rats. Thus, it is critical to undertake more studies to dissect the possibility that mitochondrial dysfunction may exist in hepatic tissue under conditions of reduced ovarian function.

A challenging issue in studying the OVX mouse model is that it often does not mimic other animal models of obesity when examining alterations in metabolic mechanisms. Thus, it has been difficult to derive hypotheses based on traditional literature searches. However, a recent publication described the development of the sirtuin3 knockout mouse (SIRT3 KO), which has a remarkably similar hepatic phenotype to what we have found in the OVX mouse model. Specifically, the SIRT3 KO mouse not only develops hepatic lipid accumulation, but it also has a similar gene profile to our OVX mouse model (Hirschey et al., 2011). The development of metabolic dysfunction in the SIRT3 KO mouse is a result of impaired mitochondrial function that occurs as a result of hyperacetylation of mitochondrial proteins.

Sirtuins (SIRT) are a family of deacetylases, which are involved in the post-translational modification of proteins through the removal of acetyl groups (Finkel et al., 2009; Lombard et al., 2007). Acetylation is a form of post-translational modification mediated by the addition of acetyl groups to lysine residues within the protein sequence. The activities of SIRT1 and SIRT3 are mediated by changes in NAD^+ levels, and thus it is largely affected by changes in nutrient flux to the cell (Imai et al., 2000; Lombard et al., 2007). For example, in cases of caloric excess, the decrease in the NAD^+/NADH ratio suppresses SIRT3 activity resulting in increased acetylation of proteins (Kendrick. et al., 2011), while fasting increases SIRT3 activity leading to deacetylation (Hirschey et al., 2010). When mitochondrial protein acetylation is increased, enzymes involved in lipid oxidation (i.e. long chain acylCoA dehydrogenase and succinate dehydrogenase) decrease their function, resulting in an impaired abolity to oxidize lipid (Hirschey et al., 2010).

In a similar fashion to the OVX mouse, SIRT3KO mice develop normally, but eventually develop visceral adiposity, fatty liver, and glucose intolerance (Hirschey et al., 2011). Also, like the OVX mouse SIRT3KO mice exhibit significant increases in hepatic SCD-1 levels with no changes in regulators of hepatic de novo lipogenesis (Hirschey et al., 2011). Instead, the SIRT3KO exhibit abnormalities in mitochondrial function that are mediated by hyperacetylation of specific mitochondrial proteins (Lombard et al., 2007; Hirschey et al., 2011; Kendrick et al., 2011). To date, one hundred and thirty three different mitochondrial proteins have been identified as regulated by acetylation including enzymes in the TCA cycle, gluconeogenesis, and β-oxidation (Hirschey et al., 2010; Kim et al., 2006). For example, acetylation of succinate dehydrogenase (SDH) leads to a reduction in its activity, thus hyper-acetylation results in a decrease in metabolic function

(Cimen et al., 2010). Acetylation is increasingly being recognized as a cellular regulatory mechanism that plays a role in metabolic disorders. For instance, chronic high fat feeding induces hypercetylation of mitochondrial hepatic proteins, a reduction in SIRT3 activity, and the development of fatty liver and glucose intolerance (Kendrick. et al., 2011) (Hirschey et al., 2011).

These data suggest that acetylation and SIRT3 activity play an important role in mitochondrial function, however to the best of our knowledge no link has ever been found between estrogen status and SIRT regulation of mitochondrial function in hepatic tissue. Due to the qualitative similarities between the OVX model and the SIRT3KO mice ,it is possible that ovarian hormones affect hepatic mitochondrial function by regulating protein acetylation patterns. Thus, it is possible that any changes in mitochondrial function in the OVX model may be mediated by alterations in acetylation due to changes in SIRT function.

Our previous studies show the increase in lipid accumulation in hepatic tissue is not mediated by increased lipogenesis or FA esterification (Jackson et al., 2011). This could suggest that the increased hepatic TAG accumulation in OVX mice is not the result of increased rates of lipid storage, but of decreased rates of lipid utilization by the mitochondria that could be associated with increased acetylation of mitochondrial proteins. At this time, few data exist which examine the effects of ovarian hormones on hepatic mitochondrial function and acetylation patterns. Therefore, the objective of these experiments was to determine whether the surgical removal of the ovaries resulted in changes in hepatic mitochondrial function, in order to better define why mice have accelerated rates of lipid accumulation in the liver following ovariectomy.

Overall Purpose: The purpose of this study was to determine if the loss of ovarian function in female mice results in impaired hepatic mitochondrial function.

OVERALL HYPOTHESIS: Ovariectomy leads to hepatic lipid accumulation due to the development of mitochondrial dysfunction when compared to intact SHAM controls.

SPECIFIC AIM 1: To determine if OVX mice exhibit reduced hepatic mitochondrial function compared to SHAM mice.

RATIONALE: The accumulation of lipid within a tissue in the form of TAG is either the result of enhanced anabolic processes or reduced catabolic processes. Previous data from our lab failed to demonstrate an upregulation of mechanisms that would be expected to contribute to increased TAG formation, thus we are hypothesizing the increases in TAG content are due to mitochondrial dysfunction. To the best of my knowledge, no data have been published measuring mitochondrial function in the liver from the OVX mouse model. Thus, this study seeks to determine if an increase in hepatic TAG content is associated with mitochondrial dysfunction, which is defined as reduced respiration rates coupled with increased reactive oxygen species (ROS) production when provided with adequate metabolic substrate.

HYPOTHESIS 1:   Livers from OVX mice will exhibit increased lipid storage compared to SHAM mice.

HYPOTHESIS 2:   Mitochondria isolated from hepatic tissue of OVX mice will exhibit reduced respiration rates compared to mitochondria from hepatic tissue in SHAM mice.

***HYPOTHESIS 3*:**   Mitochondria isolated from hepatic tissue of OVX mice will

exhibit increased ROS production rates compared to

mitochondria from hepatic tissue in SHAM mice

SPECIFIC AIM 2: To determine if acetylation patterns of hepatic mitochondrial

proteins differ in OVX mice compared to SHAM mice.

RATIONALE: Qualitatively, the OVX mouse model has a similar hepatic phenotype and

gene expression profile to the SIRT3KO mouse. Specifically, both accumulate hepatic

TAG and have impaired glucose tolerance without changes in food intake. The increased

TAG content in the SIRT3KO mouse is the result of impaired lipid oxidation that results

from the hyperacetylation of mitochondrial proteins. Due to the similarities between the

OVX and SIRT3KO mice, it is possible for hepatic mitochondrial acetylation to be altered

under states of estrogen deficiency. Therefore, OVX mice may have alterations in hepatic

mitochondrial function resulting from alterations in acetylation and SIRT function. To

date, we are not aware of any published experiments testing this hypothesis.

***HYPOTHESIS 1*:**   Mitochondria isolated from hepatic tissue of OVX mice will

exhibit hyperacetylation compared to mitochondria from

hepatic tissue in SHAM mice.

***HYPOTHESIS 2*:**   Hyperacetylation in OVX hepatic mitochondrial proteins will

be inversely related to sirtuin protein content.

Methods

Animal care: Twenty-six eight-week-old C57BL/6 female mice were divided into two groups. One group underwent bilateral ovariectomy (OVX), and the other underwent SHAM surgery. Animals were housed in a temperature-controlled room on a twelve-hour light and dark cycle, and were provided with ad libitum access to water and standard rodent chow (Purina Laboratory Rodent Diet 5001: 23% protein, 4.5% fat, 6% fiber). After eight weeks we have found that the OVX group manifests significant differences in body weight, glucose tolerance, and hepatic TAG composition compared to the SHAM group (Jackson, et al., 2011). Thus, tissue was collected eight weeks after the OVX or SHAM surgery. On the day of tissue collection, the mice were fasted for 4 hours prior to euthanasia. For one set of mice (n=6/group) the whole liver was surgically removed and used to isolate mitochondria as described by Frezza et al. (2007). After isolation, mitochondria were immediately used to assess mitochondrial oxygen consumption and H_2O_2 production. In a second set of experiments, livers were surgically removed from another set of SHAM and OVX mice (n=8). One half of the liver was snap frozen in liquid nitrogen, and the other half was used to isolate mitochondria. The mitochondrial pellet was then isolated and stored at -80°C for subsequent immunoblotting. All aspects of this study were approved by the University of Maryland Animal Care and Use Committee Review Board.

Hepatic TAG analysis: Using a small section of liver, FA's were measured as previously described (Jackson et al., 2011) through a previously described extraction procedure (Folch et al., 1957).

Mitochondrial Isolation: Liver tissue was collected and immediately placed in ice-cold isolation buffer (IBc) containing 10mM Tris-MOPS, 1mM EGTA/Tris, and 0.2M sucrose (pH 7.4). All procedures were performed in an ice bath to prevent mitochondrial degradation. The liver was washed with IBc until the solution was clear. Then it was minced with scissors and gently homogenized using a 30ml glass/Teflon potter Elvehjem tissue grinder. The homogenate was transferred to a conical tube and centrifuged at 600g for 10 minutes at 4°C. The supernatant was transferred to a glass centrifuge tube and spun at 7,000g for 10 minutes at 4°C. The pellet was re-suspended in 5ml IBc and centrifuged at 7,000g for 10 minutes at 4°C. The resulting supernatant was discarded. Then the pellet was loosened with a ice-cold glass rod and transferred to a microcentrifuge tube. Any excess buffer solution was removed. The protein concentration of the pellet was determined using the Pierce BSA protein assay. Only freshly isolated mitochondria were used to measure oxygen consumption and H_2O_2 production, and the remaining mitochondria were stored at -80°C.

Mitochondrial H_2O_2 production: Hydrogen peroxide production by isolated mitochondria was measured fluorimetically with the oxidation of nonfluorescent Amplex red into fluorescent resorufin (530nm excitation, 590 emission) (Schonfeld,

et al., 2009) using a Synergy H1 plate reader (BioTek). All of these assays were measured at 37°C in a black-bottom 96-well plate. Mitochondria (~0.2mg protein) was added to respiration buffer (125mM KCl, 2mM K_2PO_4, 10mM HEPES, 1mM MgCl, 0.1mM EGTA, 1%BSA, pH 7.4) with horseradish peroxidase 0.1 U/ml and 50μM Amplex Red reagent. Mitochondrial H_2O_2 production was assessed in the presence of 500μM glutamate, 25μM malate, 300μM ADP (stage 3 respiration), or in the presence of 40μM oligomycin (stage 4 respiration). The content of hydrogen peroxide was calculated using a standard H_2O_2 curve in respiration buffer and normalized to the total mitochondrial protein in each well. Four to five replicates were measured from each isolated mitochondrial sample from both groups.

Mitochondrial oxygen consumption: Oxygen consumption was measured polarographically using an Oxytherm (Hansatech) as described by Garcia-Cazarin et al. (2011). Freshly isolated mitochondria (~150µg) from each group were placed in the calibrated electrode chamber containing 500µl of respiration buffer (125mM KCl, 2mM K_2PO_4, 10mM HEPES, 1mM MgCl, 0.1mM EGTA, 1%BSA), 0.5mM glutamate/0.025mM malate and 300μM ADP (Sigma) as substrates to induce state 3 or maximal respiration. After 3 minutes, oligomycin (Calbiochem) was added for a final concentration of 40μM to induce state 4 (i.e. basal respiration) for 3 minutes. This was repeated at least eight times for each sample. To ensure mitochondrial integrity, samples were only used when the respiratory control ratio (RCR= state 3 VO_2/ state 4 VO_2) fell between 2.49 and 6.0 (Valle et al., 2007). All mitochondria measures with RCR values outside this range were discarded.

Immunoprecipitation of Acetylated Long Acyl Chain CoA Acetyl Dehydrogenase

(LCAD): 250μg of protein from the mitochondrial pellet was diluted in a modified

Mueller buffer (100mM KCl, 50mM HEPES, 5mM MgSO$_4$, 1mM EGTA) and

incubated in 1:100 dilution of long-chain acyl-CoA dehydrogenase (LCAD) antibody

rocking overnight in 4°C. High affinity sepharose beads (Sigma) were washed two

times in 1ml of Mueller buffer and centrifuged at 8,000g for 2 minutes at 4°C. The

protein-antibody complex was then added to 30μl of washed beads and left rocking

overnight in 4°C. Beads bound with the antigen-protein complex were then washed

three times with Mueller buffer and centrifuged at 13,000g for 5 minutes at 4°C,

followed by the addition of 6.25μl of sample buffer (25mM Tris, 192mM Glycine,

0.1%SDS). The protein was then loaded in a Mini-PROTEAN®TGX™ Precast Gel

(BioRad) at 180V for 45 minutes, and transferred onto a PDVF membrane. The blot

was then incubated in Acetylated-Lysine (1:1000) antibody overnight (diluted in

5%BSA-TBS-T), and washed with 0.05%TBS-T prior to an hour-long incubation

with horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (1:1000) in

a buffer of 3% nonfat dry milk and TBS-T. Membrane was then visualized as

described below. To assess for LCAD content the blot was stripped by incubating

membrane in strip buffer at 50°C while rotating at 125RPM for 30 minutes. The blot

was then probed for LCAD as described below.

Western Blot Analysis of ETC enzymes, protein acetylation, and sirtuin content:

Western blotting was used to quantify protein content of electron transport chain

(ETC) enzymes (MitoSciences) (1:250), acetylated-lysine proteins (1:1000) (Cell Signaling), SIRT3 and SIRT1 (1:1000) (Cell Signaling), and LCAD, very long chain acyl-Coa dehydrogenase (VLCAD), and medium chain acyl-Coa dehydrogenase (MCAD) (1:2500) (kind gift of Dr. Gerald Vockley, University of Pittsburgh). Equal amounts of protein (30-35μg) were run in a Mini-PROTEAN®TGX™ Precast Gel (BioRad) and transferred onto a PDVF membrane. Blots were blocked for one hour using 3% nonfat dry milk dissolved in Tris-buffered saline with 0.05% or 0.01% Tween (TBS-T). Following, the membrane was incubated in the appropriate primary antibody diluted in TBS-T and 5% BSA overnight in 4°C. The membrane was then washed with TBS-T and incubated for one hour with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 for Cell Signaling antibodies and 1:2000 for ACAD antibodies) in a buffer of 3% nonfat dry milk and TBS-T. Membranes were incubated for one minute in enhanced chemiluminescence reagent (PierceProtein Research). Membranes were visualized with a chemiluminescence imager (Syngene, Frederick, Md., USA) and quantified with Image J software (National Institutes of Health, Bethesda, MD).

Statistical Analysis: To compare means between SHAM and OVX, a one-tailed t-test was used for detecting significant differences between groups. Statistical significance was set at a level of p≤0.05.

Results

Anatomical characteristics of SHAM and OVX groups

The OVX group had 14% higher body weight compared to the SHAM group (Table 1). In addition, the OVX group exhibited a significant three-fold higher visceral fat mass and 121% higher inguinal fat mass compared to the SHAM group. These differences in fat mass were still present even after adjusting to total body weight (p < 0.01). The liver mass was not different between groups, but it was significantly different when adjusted to body weight (P < 0.05). This trend was also seen when adjusting heart mass to total body weight (p <0.01).

Table 1: Anatomical characteristics for SHAM and OVX groups

	SHAM n=14	OVX n=14	P-value
Body Weight (g)	22.7±0.5	25.8±0.8*	< 0.01
Heart (mg)	134±4	126±4	0.097
Heart/BW	0.006±0.0001	0.005±8.5E-05*	< 0.01
Visceral Fat (mg)	260±24	842±69*	< 0.01
VCF/BW	0.011±0.001	0.032±0.003*	< 0.01
Inguinal Fat (mg)	233±14	514±48*	< 0.01
ING/BW	0.010±0.001	0.020±0.001*	< 0.01
Liver (g)	1.08±0.05	1.08±0.08	0.494
Liver/BW	0.047±0.001	0.042±0.003*	<0.05

Values presented as means ± SEM
* Significant with a p-value < 0.05

Hepatic TAG content

There were no significant differences in hepatic TAG storage between the two groups (Fig. 1). However, OVX mice tended to store more hepatic TAG (10.5±1.8 μg/mg) than SHAM mice (7.8±1.2μg/mg) (p=0.119).
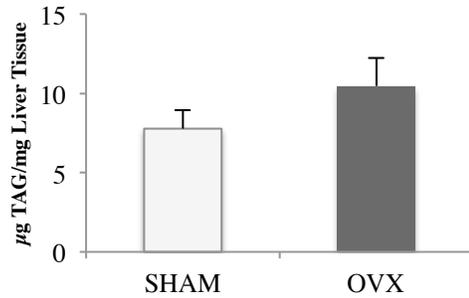
Figure 1: Hepatic TAG content was not significantly different between SHAM and OVX group (p= 0.119). Bars represent means ± SEM of 5 animals per group.

Oxygen consumption of hepatic mitochondria

The rate of mitochondrial oxygen consumption was measured using glutamate and malate as substrates (Fig. 2A). ADP was added to induce state 3 respiration (i.e. maximal respiration), followed by the addition of oligomycin to induce state 4 respiration (i.e. minimal respiration). No statistical differences were detected in state 3 or state 4 respiration rates (Fig. 2b), or in the average RCR values (VO_2 state 3/ VO_2 state 4) between SHAM and OVX groups (data not shown).

2a
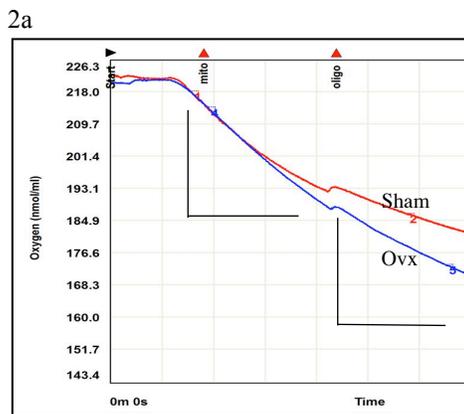
Figure 2: Example output of hepatic mitochondrial oxygen consumption using a Hansatech Oxytherm (Fig. 2a). Maximal oxygen consumption (State 3) was measured when mitochondria were provided with 0.5mM glutamate, 0.125mM malate, and 300μM ADP. Basal oxygen consumption (State 4) was induced with 40μM oligomycin. Ovariectomy had no effect on hepatic mitochondrial O_2 consumption during state 3 nor state 4 respiration (Fig. 2b). Bars represent means ± SEM of 5 animals per group.

2b
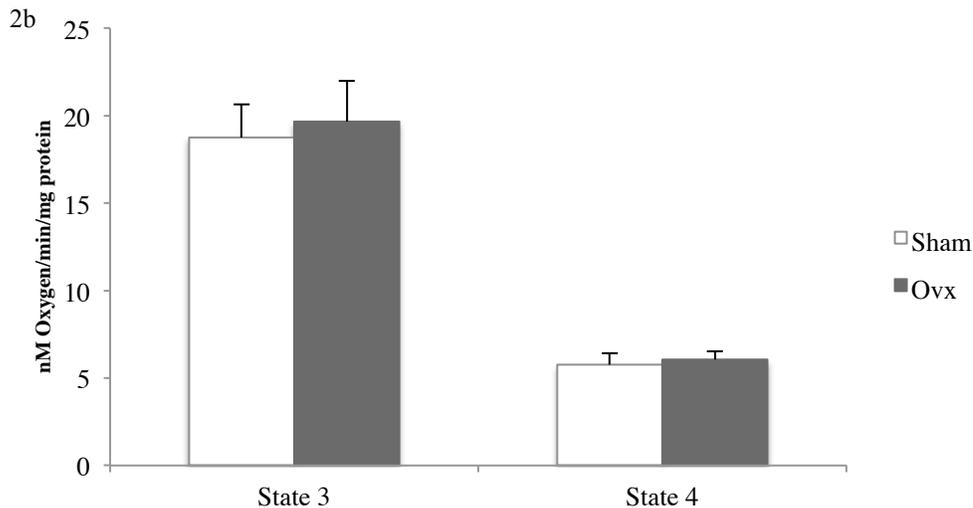
Mitochondrial hydrogen peroxide production

Hepatic mitochondria from the OVX mice had increased rates of H_2O_2 production (Fig.3) in state 3 ($p = 0.07$) and in state 4 ($p < 0.05$).
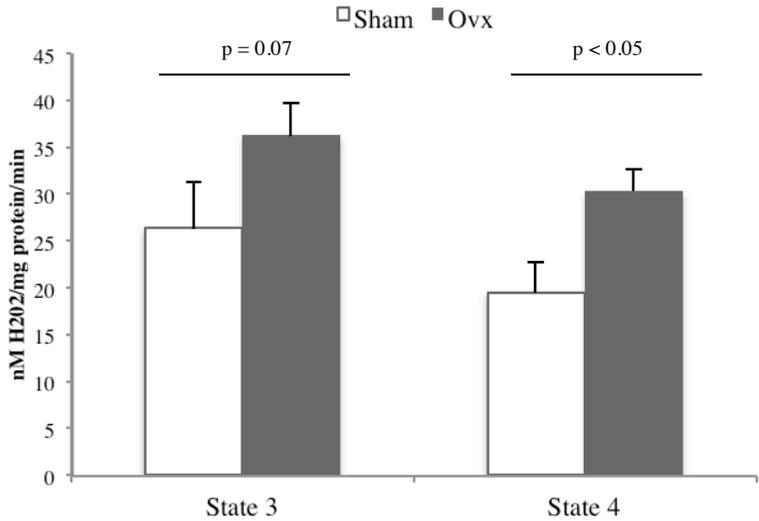
Figure 3: Effect of ovariectomy on H_2O_2 production by hepatic mitochondria. Mitochondria were incubated with Amplex Red reagent, 0.5mM glutamate/0.125 malate, $300\mu M$ ADP (state 3), and $40\mu M$ oligomycin (state 4) for 30 minutes. Bars represent means ± SEM of 5 animals. *Represents significance p<0.05.

Mitochondrial protein content

There were no differences detected between the SHAM and OVX mice regarding the protein content of LCAD, MCAD, and VLCAD, which are critical enzymes involved in β-oxidation (Fig. 4). There were also no differences in the protein content of the five complexes in the ETC (Fig. 5).
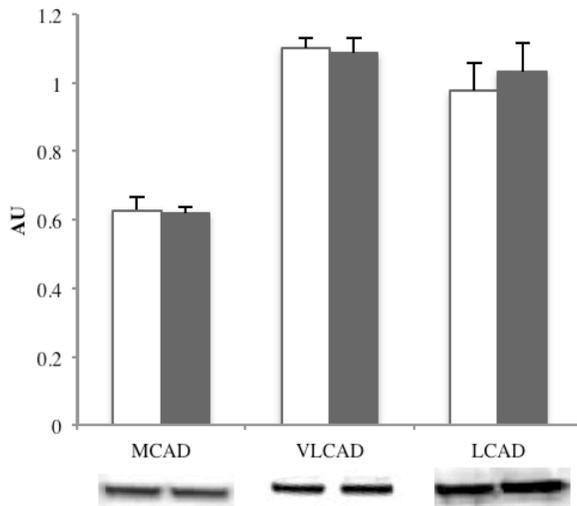
Figure 4: Ovariectomy (closed bars) had no effect on the content of MCAD, VLCAD, or LCAD proteins in isolated hepatic mitochondria (n=8). Bars represent means ± SEM.
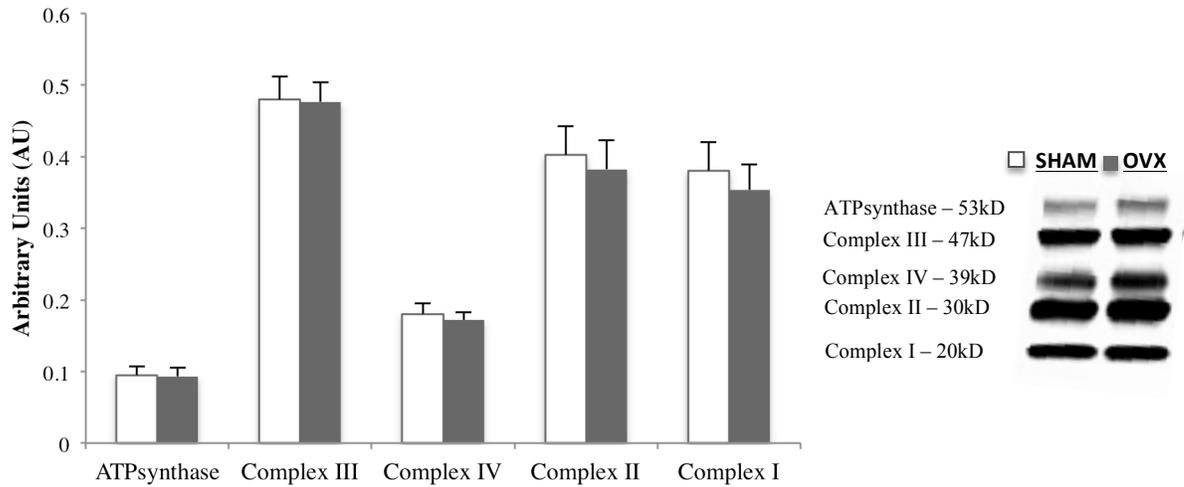
Figure 5: Ovariectomy (closed bars) had no effect on the content of electron transport chain (ETC) proteins in isolated hepatic mitochondria (n=8). Bars represent means ± SEM.

Acetylation of mitochondrial proteins

Acetylation of lysine residues regulates enzymatic activity of numerous mitochondrial enzymes. No differences were detected in global acetylation patterns of mitochondrial proteins (Fig. 6). In addition, no significant differences were detected in key deacetylase proteins Sirt1 and Sirt3 (Fig. 7) or in the acetylation of LCAD (Fig. 8).
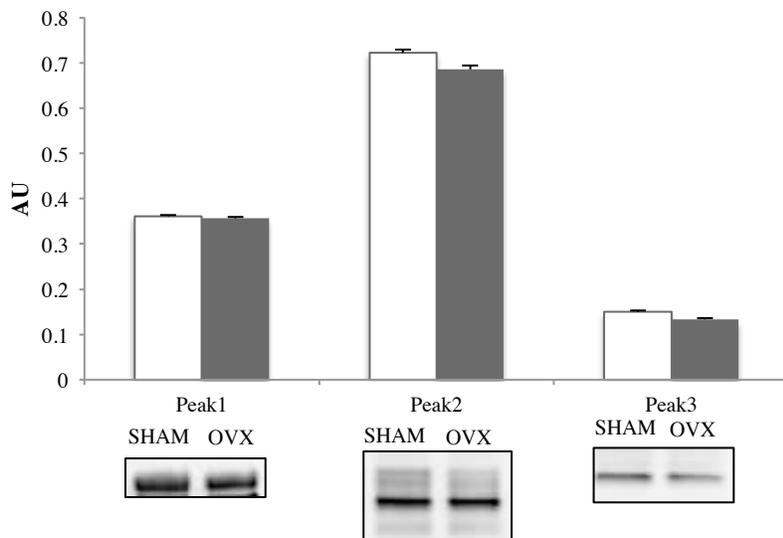
Figure 6: Ovariectomy (closed bars) had no effect on global acetylation of mitochondrial proteins in isolated hepatic mitochondria (n=8). Acetylation was detected on proteins ~155kDa (Peak 1), ~40kDa (Peak 2), and ~22kDa (Peak 3). Bars represent means ± SEM.
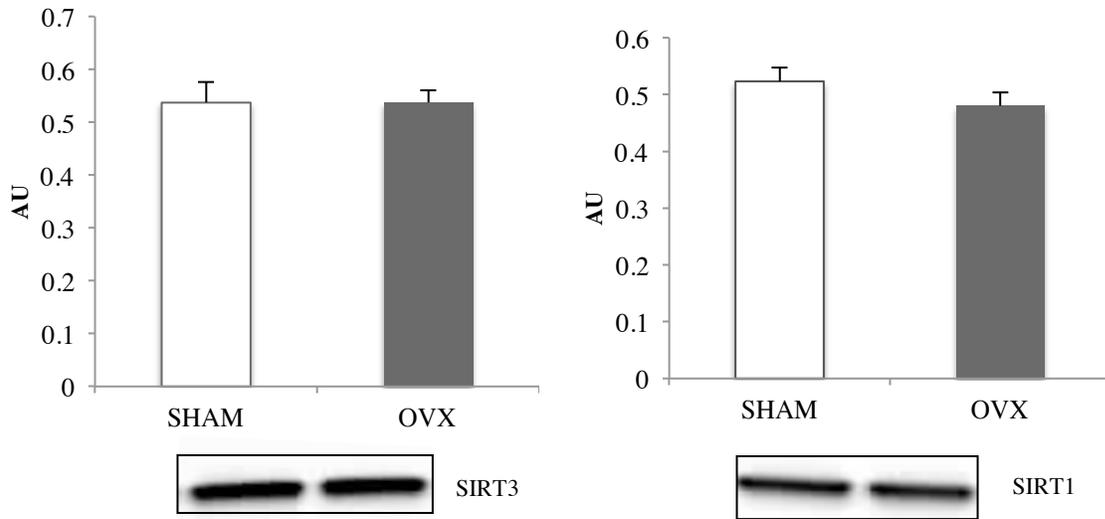
Figure 7: Ovariectomy (closed bars) had no effect on SIRT3 content (7a) and SIRT1 content (7b) in isolated hepatic mitochondria (n=8). Bars represent means ± SEM.
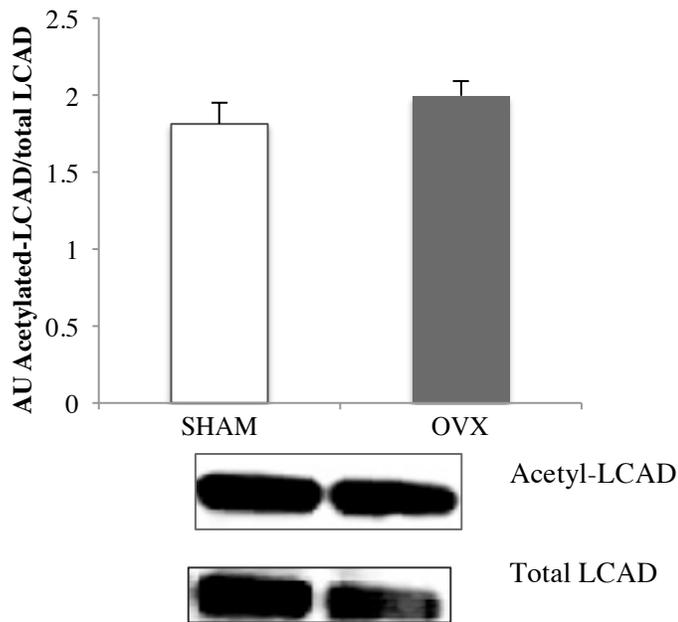
Figure 8: Ovariectomy (closed bars) had no effect on LCAD acetylation in isolated hepatic mitochondria (n=3). Bars represent means ± SEM.

Discussion

Numerous studies including our own have shown that OVX mice develop significant alterations in metabolic function in multiple peripheral tissues (Chen et al., 2009; Mauvais-Jarvis, 2011; Wohlers et al., 2011; Jackson et al., 2011). Although the liver is a key tissue for the regulation of whole-body metabolic function, there are few published studies examining the role of ovarian hormone deficiency on hepatic metabolic function. In this study, hepatic mitochondria isolated from OVX mice produced higher levels of reactive oxygen species (ROS) during State 4 respiration and a trend for increased ROS production in State 3 compared to hepatic mitochondria isolated from SHAM mice. However, there were no differences in any other measures of mitochondrial function or in hepatic TAG content despite a two-fold increase in visceral fat mass in the OVX animals. These data suggest that increased ROS production may contribute to the previously documented onset of hepatic steatosis in the OVX animals (Rogers et al., 2009). The data further indicate that metabolic changes in the OVX model occur in a time-dependent manner, and thus, in the future more time point based experiments are necessary to completely elucidate the mechanisms contributing to the onset of metabolic disease in the OVX animals.

My data demonstrate for the first time that ROS production is increased in mitochondria isolated from hepatic tissue of OVX animals when compared to SHAM animals. Although there was no indication of hepatic steatosis in these animals eight weeks following OVX, other groups have reported the development of hepatic steatosis at later time points (i.e. 12 weeks and later)(Rogers et al., 2009; Paquette et

al. 2009). The tendency for elevated hepatic TAG content in the OVX mouse was quantitatively similar to previous findings from my laboratory group (Jackson et al., 2011). Thus, it appears that the animals would continue to accumulate hepatic TAG if they were allowed to age for a longer period of time following OVX (Rogers et al., 2009). The eight-week time point was chosen based on previous data where my laboratory group has found significant increases in visceral fat mass in the OVX animals (Wohlers et al., 2010) coupled with development of glucose intolerance (data not published). At this time, I am unaware of any other published data demonstrating increased mitochondrial ROS production in hepatic tissue from OVX mice. Since the increase in ROS production precedes hepatic TAG accumulation in this current study, it is possible that ROS production is a critical signal for the development of mitochondrial dysfunction that would ultimately contribute to increases in hepatic TAG accumulation at later timepoints in the OVX group.

Mitochondria utilize an intermembrane hydrogen ion gradient created by the movement of reducing equivalents (i.e. NADH and/or FADH) through complex I and/or II of the electron-transport chain (ETC) to ultimately drive ATP synthesis in the cell. The movement of the hydrogen ions against the electrochemical gradient is possible due to a transfer of electrons across the various complexes of the ETC with oxygen (O_2) acting as the final electron acceptor. Thus, O_2 consumption by the mitochondria is necessary for optimal function of the organelle. In this study, I defined mitochondrial dysfunction as reduced respiration rates (as measured by mitochondrial O_2 consumption) coupled with increased mitochondrial ROS production. Based on this definition, ovariectomy induced some signs of

mitochondrial dysfunction as evidenced by increased ROS production. Surprisingly, my data did not demonstrate any differences in the hepatic mitochondrial O_2 consumption in the OVX group when compared to age-matched SHAM animals. However, mitochondrial function encompasses more processes than O_2 consumption by the electron transport chain (ETC), such as the activity of β-oxidation and the Krebs cycle. Other published data have suggested that hepatic mitochondria from OVX animals lack the ability to oxidize fatty acids compared to SHAM animals (Paquette et al., 2009; Campbell et al., 2003). These data are somewhat conflicting with my own, but are likely due to the different experimental approaches. My approach directly addressed function of the ETC, while the other published approaches have examined the function of upstream processes of FA oxidation (i.e β-oxidation and Krebs Cycle function). Specifically, Paquette et al. (2009) found deficiencies in oxidation of radiolabeled palmitate in isolated hepatic mitochondria from OVX animals compared to SHAM animals, as determined by reduced release rates of radiolabeled CO_2. These data suggest that deficiencies in β-oxidation may have specifically developed under conditions of reduced ovarian hormone function; however, these measures do address ETC function. On the other hand, my data directly addressed hepatic mitochondrial ETC function and suggest that there is no deficiency eight weeks following OVX. It is possible that an estrogenic effect acting on enzymes specific to β-oxidation is responsible for the discrepant findings, since the impact of estrogens on ETC enzymes is unknown (Campbell et al., 2003; Toda et al., 2001). However, I was unable to detect any differences in the protein content of mitochondrial acyl-CoA dehydrogenases LCAD, MCAD, or VLCAD (key regulators

of β-oxidation), between the SHAM and OVX animals. However, measuring the total protein content does not account for the activation level of these proteins, thus, it is possible that their activity is reduced independent of the protein content.

Acetylation is a recently described post-translational regulator of mitochondrial function (Hirschey et al., 2010). SIRT1 and SIRT3 are the major deacetylases that modulate mitochondrial function in response to changes in energetic demand (i.e. increases in the NAD^+/NADH ratio) by removing acetyl groups bound to lysine residues in a given mitochondrial protein sequence (Lombard et al., 2007). Growing evidence supports that excessive acetylation decreases the activity of proteins involved in lipid oxidation in the mitochondria (Hirschey et al., 2010; Cimen et al., 2010; Kendrick et al., 2011). In addition, Hirschey et al. (2011) found an association between increased acetylation of mitochondrial proteins and the development of hepatic steatosis and impaired glucose homeostasis. In similar fashion to our previously published data in the OVX model, the SIRT3KO model develops hepatic steatosis without changes in the function of known lipogenic regulators (e.g. SREBP-1c, ACC-1, FAS) (Jackson et al., 2011; Hirschey et al, 2010). The SIRT3KO mouse also exhibits impaired palmitate oxidation due to decreased activity of LCAD (Hirschey et al., 2010). Thus, based on the number of striking similarities between the SIRT3KO mouse and the OVX mouse model, acetylation and hepatic SIRT content were measured as a potential mechanism for explaining differences in mitochondrial function in the OVX mouse model. However, no differences were detected in total acetylation of mitochondrial proteins or in the specific acetylation of LCAD between the SHAM and OVX groups. In addition, I

found no differences in total hepatic SIRT1 and SIRT3 content. These observations suggest that at eight weeks following OVX there are minimal changes in SIRT function and, thus, it appears that changes in deacetylase function do not contribute to hepatic TAG accumulation.

It has been previously observed that impaired mitochondrial function is often associated with hepatic steatosis and insulin resistance. For example, a decline in hepatic mitochondrial function predisposes elderly lean subjects to accumulate intrahepatic lipid, contributing to the development of hepatic insulin resistance (Petersen et al., 2003; Petersen et al., 2004). This effect corresponds with an increase in intrahepatic lipid content, which is associated with a reduction in mitochondrial rates of oxidative phosphorylation. However, it is difficult to confirm the order of events based on these studies, in that it is hard to determine whether mitochondrial dysfunction occured prior to intrahepatic lipid accumulation or vice versa. It is for this reason that the implementation of animal studies has allowed researchers to gain a better understanding of regulation of hepatic TAG dynamics by the mitochondria.

A number of animal studies demonstrate that impaired fat oxidation actually precedes the development of hepatic steatosis and insulin resistance. Bruce et al. (2009) showed that the offspring from mice maintained on a chronic high-fat diet exhibit significant declines in mitochondrial function prior to developing hepatic steatosis. Similar findings were published by Rector et al. (2010) in the OLETF rat model, which develops severe NAFLD due to hyperphagia. This study demonstrated that insulin sensitivity was not impaired in OLETF rats until twelve weeks after the development of hepatic steatosis, suggesting that the presence of lipid in hepatic

tissue contributed to the pathology of insulin resistance in this model (Rector et al., 2010). Importantly, at only 5 weeks of age (before there are any signs of hepatic lipid accumulation) these animals manifest a significantly reduced ability to oxidize lipid (Rector et al., 2010). Collectively, these studies indicate that a loss of normal mitochondrial function appears to act as a precursor in the development of hepatic steatosis and insulin resistance in obesity.

Thus, my data suggest that excessive ROS production could potentially be the precipitating event that contributes to the development of overt mitochondrial dysfunction, ultimately leading to the significant accumulation of lipid in hepatic tissue. Using other experimental models, a few studies have shown that increased ROS levels induce mitochondrial dysfunction in hepatocytes due to excessive lipid peroxidation. Lipid peroxidation leads to altered permeability of the mitochondrial membrane, contributing to the leakage of matrix enzymes and a subsequent alteration in the electrochemical gradient that is critical for the synthesis of ATP (Carini et al., 1992). Increased levels of ROS also play a role in the etiology of alcohol-induced fatty liver disease (AFLD). Ethanol induced- mitochondrial ROS production in cultured rat hepatocytes led to a 66% increase in lipid peroxidation, which resulted in significant mitochondrial dysfunction (Devi et al., 1993). Collectively, these studies suggest that ROS-induced mitochondrial dysfunction is an important component of the pathology of fatty liver disease, and thus ROS production could be one of the initial signals leading to NAFLD in the OVX model.

To the best of my knowledge, this is the first study that has shown increased ROS production in hepatic mitochondria from OVX animals. However, other studies

have found increased ROS production in in other tissues when estrogen function is reduced (Duckles et al., 2010; Strione et al., 2005; Razmara et al., 2008). It is suggested that estrogen down regulates ROS production by increasing the expression of ROS-scavenging enzymes such as catalase (Toda et al., 2001) and glutathione (Valle et al., 2007). Further, estrogen treatment increased levels of ROS-scavenging enzymes in brain endothelial cells from OVX mice (Razmara et al., 2008) so it is possible that OVX results in excess ROS production by hepatic mitochondria due to a loss in the activity of ROS-scavenging enzymes.

Ovarian dysfunction or inhibition of estrogen function in women increases the risk for NAFLD, insulin resistance, and ultimately the MetS. An estimated 20-30% of middle-aged women have MetS, but the prevalence escalates significantly after the onset of menopause, resulting in increased mortality risk in this population (Janssen et al., 2008; Ervin, 2009). The data presented here show that ovarian dysfunction is associated with an increase in ROS production by hepatic mitochondria, which I have hypothesized to be a potential signal for the development of hepatic metabolic dysfunction under conditions of reduced ovarian function. Due to the importance of the liver in the regulation of metabolic function, increased ROS production may be a significant contributor to the development of overt metabolic disease in post-menopausal women. Therefore, if we can target the single initial metabolic change that is induced by ovarian dysfunction, such as a rise in hepatic mitochondrial ROS production, we may drastically improve and even prevent the consequential metabolic changes that predispose middle-aged women to the MetS.

Limitations and Delimitations

This study used eight-week old C57Bl/6 mice to study the effects of a reduction in female sex hormones on metabolic function. This design does not consider the effect of aging, which is yet another factor affecting the rate of hepatic lipid accumulation. For this reason, my research approach is appropriate to study how reduced ovarian function affects hepatic metabolism without aging as a confounding variable; however, I also cannot account for the effect of age on hepatic lipid accumulation using this model.

Moreover, studies suggest that a reduction in female sex steroid hormones increases appetite in some animals, like the OVX rat, which may affect hepatic metabolism differently. Based on unpublished data from my lab group and previous publications (Rogers et al., 2009), the OVX mouse model does not show signs of hyperphagia, but does show significant loss of spontaneous physical activity levels that could influence my results, independent of the ovarian hormone status. Physical activity is also a critical regulator of hepatic mitochondrial function in the liver, thus this is a likely key factor that needs to be considered (Rector & Thyfault, 2011). Furthermore, estrogen can affect numerous different tissues, such as skeletal muscle and adipose tissue, both of which may influence whole body metabolism as well as hepatic metabolism. Therefore, by using this model I could not account for the effects of estrogen in tissues other than the liver.

Finally, evidence suggests that this OVX model reproduces a number of metabolic similarities when compared to the human with estrogen dysfunction.

However, it is not clear if the mechanisms affected would be the same in mice and humans, especially when there are inconsistencies in the effects of ovariectomy between different types of rodents. Nevertheless, this is a starting point for defining targets of interest to follow-up in human based studies.

Future direction

In this study I provided evidence for hepatic mitochondrial ROS production to be the initiating factor for further mitochondrial dysfunction that predisposes the liver to fat accumulation and insulin resistance in the OVX model. However, the OVX model exhibits other confounding variables such as increased visceral fat mass and decreased physical activity that could also affect hepatic mitochondrial function. In order to isolate the effects of estrogen function to the liver alone, it would be an appropriate next step to genetically knock out the estrogen receptor in hepatic tissue (LERKO). I would hypothesize that LERKO animals would have normal body weight, visceral fat mass, and physical activity levels at an early stage when hepatic metabolism is not fully impaired. This approach would allow me to examine mitochondrial function and hepatic TAG content at different time points in LERKO mice independent of secondary changes seen in the OVX model that could influence hepatic tissue function. This study would then provide insight as to the effects of estrogen on hepatic mitochondrial function and to determine whether ROS production is a critical step that prompts changes in hepatic metabolism that lead to hepatic steatosis.

Review of Literature

The metabolic syndrome increases the rate of mortality

The rate of mortality is increasing in the United States partially due to the high incidence rates of cardiovascular disease, cancer, and type 2 diabetes (Centers for Disease Control and Prevention [CDC], 2010; Lakka et al., 2002). These conditions are linked to the Metabolic Syndrome (MetS). Currently, it is estimated that over a third of the population over the age of 20 has the MetS (Nedungadi & Clegg, 2009), making it a critical risk factor for mortality in the American population.

Insulin resistance is a main characteristic of the metabolic syndrome

A cluster of metabolic abnormalities gives rise to the MetS, including visceral adiposity, insulin resistance, dyslipidemia, and hypertension (NCEP, 2001). However, the most accepted and unifying hypothesis to describe the pathophysiology of the MetS is the development of insulin resistance (IR) (Eckel et al., 2005; Grundy et al., 1999), which is characterized by defective insulin action resulting in a dysregulation in blood glucose levels.   Insulin resistance predisposes multiple organs to metabolic abnormalities, such as increased glucose and lipoprotein secretion by the liver (Austin et al., 1996; Haffner et al., 1995), increased secretion of fatty acid (FA) by adipose tissue (Mostazza et al., 1998), and impaired vasodialation of endothelial cells (Steinberg et al., 1996). Therefore, the metabolic abnormalities that give rise to the MetS are also consequences of insulin resistance.

Insulin resistance is associated with visceral adiposity

The risk of becoming insulin resistant is strongly associated with the development of visceral adiposity. It was reported that the strongest predictor for common manifestations of insulin resistance (i.e. increased circulating glucose, insulin, and triglycerides) was the waist-to-hip ratio (Evans et al., 1984). Which suggests that the distribution of fat in the abdominal area versus gluteal region increases the risk for insulin resistance (Evans et al., 1984). In addition, abdominal adiposity is associated with developing impaired fasting glucose over a 5-year period (Guthrie et al., 2001). Moreover, it was suggested that in order for visceral fat to promote insulin resistance in women, visceral fat mass had to reach a certain threshold that was comparable to the levels found in men (De Nino et al., 2001). Therefore, the volume of fat in the visceral region is highly associated with the development of insulin resistance.

Visceral adiposity is associated with increased hepatic lipid storage

Obesity is often associated with the development of non-alcoholic fatty liver disease (NAFLD), which is characterized by increased hepatic triacylglycerol (TAG) accumulation (>5% of total liver mass) in the absence of excess alcohol consumption (Rector & Thyfault, 2011). NAFLD is the most common chronic liver disease worldwide, affecting more than 75% of all obese individuals (Thorne et al., 2010). Because of the high prevalence of NAFLD in obesity, it is suggested that visceral adiposity, is a strong predictor for hepatic fat content (Parks et al., 2007). Moreover,

most of hepatic TAG (~60%) in NAFLD patients comes from circulating FFA

derived from visceral adipose tissue (Donnelly et al., 2005). This association was

evident when comparing adipose tissue distribution through CT scans and hepatic

lipid storage using liver biopsies from healthy human subjects (Parks et al., 2007).

This study found that some of the predictors for hepatic steatosis were BMI, increased

alanine aminostrasferase (ALT), low levels of high-density lipoprotein (HDL), and

the ratio between visceral to subcutaneous fat. However, the strongest predictor for

risk and degree of hepatic steatosis in both men and women was the mass of visceral

fat, which led to the conclusion that visceral fat is an independent risk factor for

hepatic steatosis in both men and women.

Hepatic lipid storage is a strong predictor for insulin resistance

Current studies using non-invasive imaging techniques support the idea that

intrahepatic fat (IHF) is a stronger predictor of insulin resistance than visceral fat

(Hwang et al., 2007; Gentile et al., 1993; Kotronen et al.,2008). This concept is

further supported in patients with type 2 diabetes, as the content of intrahepatic fat

(IHF) was closely correlated with the sensitivity to endogenous insulin, insulin dose,

and it often explained the variation in insulin requirements between subjects (Gentile

et al., 1993). Also, IHF was the only independent predictor of insulin mediated

glucose disposal when compared to visceral adipose tissue and intramyocellular lipid

content (IMCL) (Koska et al., 2008). Collectively, the data suggest that the

accumulation of fat in the liver has severe implications in whole-body insulin sensitivity.

Loss of ovarian function increases the risk for the metabolic syndrome

Women over the age of 40 who have the MetS are at a much higher risk for not only cardiovascular-mortality, but all-cause mortality as well (Lin et al., 2010). More women are affected by MetS than men once they reach the age of 60 (Ervin, 2009), an age in which women are menopausal (Reyes, 1977). In a longitudinal study, it was estimated that 20%-30% of middle aged women have the MetS, but it was observed that the incidence rate of the MetS increased steadily after the onset of menopause (Janssen et al., 2008). This is evidenced by the higher fasting insulin and glucose levels reported in post-menopausal women when compared to premenopausal women (Poehlman et al., 1997; Razay et al., 1992; Lynch et al., 2002). Lindheim et al. (1994) also found that postmenopausal women had decreased insulin sensitivity compared to age-matched premenopausal women, which suggest that the onset of metabolic defects are a direct result of menopause instead of age alone. The metabolic syndrome was also highly prevalent in women with bilateral oophorectomy (Dorum et al., 2007), and recent data also show that oophorectomy before the age of 40 in overweight women were more than twice as likely to die from CVD than same-age non-obese women with intact ovaries (McCarthy et al., 2012). Collectively, the data suggest that ovarian dysfunction increases the risk for MetS and mortality in women.

Loss of ovarian function increases visceral adiposity

Obesity is more prevalent among middle-age women than in men, with approximately two thirds of women between 40 to 60 years of age being overweight or obese (Ogden, 2006). One of the major contributing factors to the development of adiposity during a woman's life is the onset of menopause (Polotsky, 2010), which is a period when ovarian hormone production is substantially reduced (Reyes, 1977). Pre-menopausal women tend to store more subcutaneous fat, but women exhibit significant increases in visceral fat mass with the onset of menopause (Janssen et al., 2008); however, these data are based from cross-sectional studies, and their findings are not fully consistent with those reported by Lovejoy et al. (2008). In a longitudinal study of over a hundred women, it was reported that visceral fat increased throughout the four years prior to the onset of menopause, and it also remained relatively stable after the first two years of menopause (Lovejoy et al., 2008). However, the increase in adiposity was associated with decreased levels of circulating estradiol before menopause, which is consistent with the hypothesis that a decrease in estrogen influences the gain in visceral adiposity (Lovejoy et al., 2008; Sowers et al., 2005).

Due to the multifactorial nature of the onset of obesity in women, various animal models have been employed to study the effects of female sex steroids on metabolism. These animal models allow for better control over other factors including, genetics, food intake and age that may contribute to the development of the MetS. A number of animal models have been employed including: ovariectomized female mice (OVX), estrogen receptor knockouts (ERKO), and aromatase knockouts (ArKO), all of which develop striking increases in visceral fat mass. The OVX model

has a 70-90% reduction in circulating estrogens (Gorzek et al., 2007; Sitnick et al., 2006) which is associated with a significant increase in body weight (~25%) and visceral adiposity (over 100%) independent of changes in food intake (Wohlers et al., 2011; Rogers et al., 2009). Similar increases in adiposity are evident in mice lacking the aromatase enzyme (ARKO), which is responsible for endogenous estrogen production (Hewitt et al., 2004), and the ERαKO mice (Bryzgalova et al., 2005). In fact, visceral adiposity is one of the most striking and apparent metabolic changes induced with reduced function of ovarian hormones. Moreover, various studies have supporting evidence that the metabolic changes occur as a result of decreased estrogen function, as treatment with estradiol or estrogen receptor agonists often prevent or even reverse excess visceral adiposity (Brygazlova et al., 2005; Hewitt et al., 2004; D'Eon et al., 2005; Turgeon et al., 2006).

Visceral fat deposits fatty acids into the liver

Most of stored hepatic lipid appears in the form of triacylglycerol (TAG), of which the majority is derived from portal circulation (Donnelly et al., 2005). Increases in circulating FFA are often the result of the hydrolysis of TAG (i.e. lipolysis) in different tissue depots. It is well established that visceral adipose tissue has increased lipolytic activity compared to subcutaneous adipose tissue (Arner, 1995). Thus, the lipolytic activity of visceral fat is related to hepatic fat accumulation, as FFAs released from this tissue have direct access to the liver through the portal vein (Thorne et al.,2010; Park et al., 2007). Membrane bound transporters like fatty

acid transport protein-2 and 5 (FATP2, FATP5) or fatty acid translocase (FAT/CD36) then facilitate FFA entry into the liver from the portal circulation.

There is also evidence that the release of FFA by adipose tissue is more strongly associated with adipocyte cell size rather than fat mass (Laurencikiene et al., 2011; Koska et al., 2008). Larger adipocytes have an enhanced lipolytic rate compared to small adipocytes in basal (un-stimulated) conditions (Laurencikiene et al., 2011). Thus, adipocyte size is strongly linked to the risk for type 2 diabetes and CVD (Koska et al., 2008). Evidence from my lab group has found that the increase in visceral fat mass in the OVX animal model is due to increased adipocyte size and not due to increased adipocyte number (Wohlers & Spangenburg, unpublished). In addition, isolated adipocytes and adipose tissue explants from OVX animals exhibit increased circulating FFA and glycerol under basal conditions and impaired lipolysis when stimulated with catecholamines (D'Eon et al., 2005, Wohlers & Spangenburg, unpublished). Conceptually, these data suggest that under conditions of chronic ovarian dysfunction an increase in visceral fat mass leads to unregulated release of FFA into the portal circulation, which may contribute to increased hepatic lipid deposition.

Loss of ovarian function in women increases their risk for NAFLD

With the onset of menopause the prevalence of NAFLD is higher in women than men (Nomura et al., 1988; Kojima et al., 2006; Park et al., 2006). If untreated, the progression of NAFLD leads to non-alcoholic steaohepatitis (NASH), chronic hepatitis B, and/or cirrhosis, which are most prevalent in men and postmenopausal

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women when looking across the entire population (Massard et al., 2006; Poynard et al., 2001). A population-based study in Japan gave evidence that at the age of 60 women were more likely to have NAFLD than men of the same age (Nomura et al., 1988; Kojima et al., 2006). Similar reports were made in the American population by the NHANES study (Clark et al., 2007). The NHANES study also indicated that the prevalence of NAFLD is greater in postmenopausal women compared to both premenopausal women and postmenopausal women under hormone replacement therapy (HRT). Thus epidemiological data suggest that the menopausal status in women is associated with the risk for developing NAFLD, and that normal ovarian function may protect the liver from excess lipid accumulation that may lead to NAFLD.

Excessive hepatic fat accumulation causes hepatic insulin resistance and impaired glucose homeostasis

NAFLD is commonly referred to as the hepatic manifestation of the MetS (Bruce et al., 2009; Brunt 2004). This concept is supported by evidence showing that obese women with the high amounts of hepatic lipid content also had elevated plasma glucose levels and HOMA index (Thorne et al., 2010). Severe insulin resistance can also be found in lean, young and older subjects, which is associated excess lipid accumulation in the liver (Petersen et al., 2003; Petersen et al, 2004). However, because of the nature of these cross-sectional studies, it is impossible to determine whether hepatic steatosis causes insulin resistance, or whether insulin resistance promotes hepatic steatosis. Fortunately, animal-based studies measuring hepatic TAG

accumulation and glucose homeostasis at different ages and time points have allowed us to gain a better understanding on the series of events causing the relationship between hepatic steatosis and insulin resistance.

Evidence from animal studies suggests that hepatic lipid accumulation precedes insulin resistance (Rector et al. 2010; Bruce et al., 2009). This was evident in rodents with diet-induced obesity that developed insulin resistance 12 weeks after evidence of hepatic steatosis (Rector et al., 2010). In addition, rodents did not exhibit abnormal glucose and insulin homeostasis until 15 weeks after manifesting signs of NASH (Bruce et al., 2009). However, Ibdah et al. (2005) had different findings as insulin resistance overlapped with the development of hepatic steatosis in rodents with impaired hepatic mitochondrial function (Ibdah et al., 2005). However, the reduction of hepatic TAG content in rodents by inducing hepatic fat oxidation, was able to improve both insulin sensitivity in peripheral tissues and whole body glucose homeostasis (Jie et el., 2004). Collectively, these studies suggest that hepatic steatosis is highly associated with insulin resistance and often precedes its manifestation.

Impaired hepatic mitochondrial function precedes TAG accumulation the liver

Most of the FA delivered to the liver are oxidized by the mitochondria, thus a reduced mitochondrial capacity is associated with increased lipid storage in the muscle and liver. Mitochondria regulate FA oxidation through a number of dynamic regulatory pathways. The FA enters the mitochondria through carnitine-palmitoyl transferase 1 (CPT-1), which catalyzes the conversion of FA-CoA into FA-carnitine to be transported into the matrix. The FA-CoA is then broken down into acetyl-CoA

by β-oxidation to be used as a substrate for the Kreb's Cycle. The electron-transport chain (ETC) then utilizes a proton gradient driven by the flow of electrons from reducing equivalents produced by the Krebs cycle (i.e. NADH and $FADH_2$), in order to drive the synthesis of ATP.

The loss of mitochondrial function can occur for a variety of reasons. For example, a build-up of acetyl-CoA can occur if the flux from β-oxidation exceeds the activation of the ETC, which results in the inhibition of CPT-1 (through allosteric inhibition by malonyl-Coa) and halting further fat oxidation. This excess flux can also lead to increased ROS production by the ETC (Fischer-Wellman & Neufer, 2012). Therefore, when the flux of lipid exceeds the capacity for hepatic mitochondria to oxidize it, fatty oxidation is inhibited which ultimately contributes to enhanced hepatic lipid storage.

A reduced enzymatic capacity of mitochondria to oxidize fat has also been associated with increased lipid storage in the muscle and liver. For instance, it was reported that obese individuals had a 3 to 4-fold reduction in ETC activity in skeletal muscle mitochondria compared to age-matched lean subjects. Moreover, in obese subjects with type 2 diabetes, ETC activity was decreased by 7-fold compared to lean subjects (Ritkov et al., 2005). Furthermore, lean individuals with reduced oxidative capacity in mitochondria from skeletal muscle (Petersen et al., 2003) and liver, (Petersen et al., 2004) exhibited TAG accumulation in each respective tissue, as well as severe insulin resistance despite their lean phenotype.

The link between mitochondrial function, tissue lipid accumulation, and insulin resistance is also evident in animal models (Bruce et al., 2009; Thyfault et al,

2009; Ibdah et al, 2005). Impaired mitochondrial function was the primary event mediating fat deposition in the liver in rats selectively bred with low aerobic capacity (Thyfault et al., 2009). These rats manifested hepatic steatosis as well as impaired palmitate oxidation, reduced β-HAD activity, and reduced mitochondrial content. A similar loss in mitochondrial capacity was evident with diet induced-obesity, which triggered the development of hepatic steatosis three weeks after measurable impairments in mitochondrial function (Rector et al., 2009). Moreover, hepatic steatosis was evident in even in the absence of excess abdominal fat, suggesting that lipid accumulation was not due to increased flux from visceral adipose tissue, and rather due to impaired fat oxidation in the liver (Bruce et al., 2009). More strikingly, when these mice were challenged with a chronic high-fat diet, they developed extensive hepatic fat accumulation, impaired glucose homeostasis, and inflammation resulting in a diagnosis of NASH. Moreover, it has been shown that obese rats with hepatic steatosis do not show signs of insulin resistance until 12 weeks after the onset of hepatic TAG accumulation (Rector et al., 2009). These studies provide insight towards the importance of the capacity of mitochondria to oxidize fat as in preventing the development of hepatic steatosis and insulin resistance. Therefore, fat oxidation may be a critical target in the prevention of metabolic disease.

Lipid promotes insulin resistance by promoting the phosphorylation of serine residues of IRS

Excess lipid accumulation promotes insulin resistance via activation of known stress kinases resulting in the hyper-phosphorylation of serine residues in insulin

receptor substrate-1 (IRS-1). For example, lipid metabolites can activate protein kinase C (PKCθ) in skeletal muscle (Kim et al., 2004) and PKC-ε in hepatic tissue (Samuel et al., 2004) resulting in loss of insulin-induced glucose regulation in both tissues. Lipid intermediates such as DAG and fatty-acyl CoA can act as ligands for the induction of PKC in hepatocytes (Neschen et al., 2005). Furthermore, by reducing the DAG content in hepatic tissue it is possible to protect mice from developing NAFLD (Engeli, 2008). Finally, the critical role of PKC was elucidated using mice lacking the PKC gene, which was used to demonstrate that the lack of PKC protected mice from developing fat-induced insulin resistance (Kim et al., 2004). These data suggest that by encouraging complete oxidation of hepatic FA it would be possible to reduce the activation of PKC by any fat metabolites, and maintain insulin sensitivity in the liver.

Hepatic TAG accumulation and insulin resistance occur in states of reduced ovarian function

Evidence suggests that estrogens play a protective role in the liver by attenuating the development of hepatic steatosis (Cole et al., 2010; Maffei et al., 2004; Anezaki et al., 2009). For instance, over 50% of women with normal liver function who were undergoing cancer treatment with tamoxifen, an estrogen antagonist, developed hepatic steatosis, elevated blood triglycerides, and fasting blood glucose (Akhondi-Meybodi et al., 2011). Aromatase deficient male patients, who lack the ability to make endogenous estrogen, exhibit insulin and glucose intolerance, and hepatic steatosis. (Maffei et al., 2004). However, estrogen therapy

induced a significant reduction in hepatic lipid storage and improved insulin sensitivity in these same men (Maffei et al., 2004). Estrogen therapy was also effective in decreasing the incidence of type 2 diabetes even after adjusting for BMI or waist circumference in postmenopausal women participating in the Women's Health Initiative (2002), which Suzuki et al. (2009) suggested was the result of estrogen preventing the development of NAFLD.

This is similar to findings in animal models that accumulate hepatic fat under conditions of reduced estrogen function. For instance, mice treated with tamoxifen develop hepatic steatosis and insulin resistance without changes in food intake or body weight (Cole et al., 2010). Also, both ERKO and ARKO mice developed hepatic steatosis (Bryzgalova et al., 2008; Hewitt et al., 2004), which was prevented by estrogen administration in ArKO mice (Hewitt et al., 2004). A sex dimorphism in hepatic metabolism was also evident in a clinical model of non-alcoholic steaohepatitis (NASH) as female mice had reduced levels hepatic fat accumulation when compared to male mice (Anezaki et al., 2009). This suggests that ovarian function is protective against hepatic lipid accumulation. Furthermore, ovariectomy also results in hepatic steatosis and insulin resistance in rodents. Lavoie et al, (2007) has shown that ovariectomy induces the accumulation of TAG in the hepatic tissue of rats. This occurs independent of the previously reported hyperphagic effect of estrogen in this model (Paquette et al., 2007). After 12 weeks of ovariectomy mice also exhibit evidence the development of hepatic steatosis (Rogers et al., 2009, Jackson et al., 2012). Overall, in both humans and animals the loss of estrogen function leads to an increase in hepatic lipid accumulation resulting in the

development of insulin resistance, which would be expected to contribute to the development of NAFLD and ultimately the MetS.

Mitochondrial function and fat oxidation is impaired in hepatic tissue under conditions of reduced female sex steroid function

Evidence suggests that ovarian hormones enhance the capacity for mitochondria to oxidize fat (Campbell et al., 2005; Valle et al., 2007; Bryzgalova et al., 2005). For instance, Valle et al. (2007) showed that hepatic mitochondria from female mice exhibited increased activity of Complex I and III of the ETC resulting in increased rates oxygen consumption by the mitochondria when compared to male mice. Conversely, ovarian dysfunction also leads to reductions in fat oxidation. For example, OVX rats had a 34% decrease in hepatic palmitate oxidation compared to controls and a 114% increase in liver TAG storage (Paquette et al., 2009). Ovariectomy also induced hepatic steatosis in mice that was associated with decreased oxygen consumption through indirect calorimentry without differences in energy intake compared to SHAM mice (Rogers et al, 2009). Furthermore, ArKO exhibited reductions in MCAD expression, a key enzyme in β-oxidation, which appears to contribute to the development of hepatic steatosis (Toda et al., 2001).

In non-hepatic tissue, evidence also suggests that ovarian function promotes the expression and activity of mitochondrial oxidative enzymes. Estrogen treatment induced a 7-fold increase in CPT-1 transcription in skeletal muscle, increased CPT-1 activity, and restored β-HAD activity (Campbell et al, 2003). As expected, in conditions of reduced estrogen levels CPT-1 and β-HAD activity were also reduced

(Campbell et al., 2001).   Thus, the data suggest that estrogens have a powerful effect on mitochondria, and ovarian function appears to be critical to the maintenance of optimal mitochondrial function.

CONCLUSION: Collectively, the evidence suggests that impaired hepatic mitochondrial function in states of reduced ovarian function promotes TAG accumulation in the liver, resulting in insulin resistance. Currently 20%-30% of middle-aged women have the MetS, and its prevalence escalates with the reduction in ovarian function caused by menopause. This is a critical matter to resolve as MetS increases the risk for CVD, which is the leading cause of death in women. Insulin resistance is one of the main characteristics of the MetS, and evidence suggests that mitochondrial dysfunction may be a pathological cause of insulin resistance by promoting hepatic lipid accumulation. Therefore, the mechanisms involved in mitochondrial dysfunction from the lack of ovarian function require further study.

Appendices

a) Institutional Animal Care & Use Committee letter
b) Animal Protocol

UNIVERSITY OF MARYLAND

GRADUATE STUDIES AND RESEARCH

Institutional Animal Care & Use Committee

W. Ray Stricklin
IACUC Chair
wrstrick@umd.edu
Phone: (301)405-7044

June 17, 2010

Dr. Espen Spangenburg
Department: Kinesiology
University of Maryland
espen@umd.edu
Phone: (301)405-2450

Dr. Spangenburg,

This letter is to inform you that on **June 17, 2010**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the protocol for:

The Role of Ovary in Metabolic Function

R-10-40

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **June 17, 2013**. Federal laws indicate that protocols must be reviewed yearly. Thus, in order to keep your approved protocol active you **MUST** submit a protocol renewal/update by the first of the month of the anniversary of your approval (June 2011 & June 2012). All work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

W. Ray Stricklin
Asst. Dean, College of Ag. & Natural Resources
Chair, IACUC

CC: Doug Powell, Amanda Underwood

APPENDIX B: Animal Protocol

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