2014

Impact of metabolic perturbation on ovarian function

Jackson Nteeba

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Part of the Genetics Commons

Recommended Citation

Nteeba, Jackson, "Impact of metabolic perturbation on ovarian function" (2014). Graduate Theses and Dissertations. 13675.

https://lib.dr.iastate.edu/etd/13675
Impact of metabolic perturbation on ovarian function

by

Jackson Nteeba

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Aileen F. Keating, Major Professor
Drena L. Dobbs
Jason W. Ross
Jeffrey J. Essner
Jodi A. Sterle
Peng Liu

Iowa State University
Ames, Iowa
2014

Copyright © Jackson Nteeba, 2014. All rights reserved.
DEDICATION

I thank the Almighty God for the gift of life, wisdom and this far you have brought me, Ebenezer! I dedicate this dissertation to my aunt Margret Nabiryo and my guardian Dr. Dan Isabirye, who have played such an incredible parental role in my life! It would have been impossible for me to be where I am today without your selfless love and support. May God continue to bless you abundantly! I would also like to dedicate this dissertation to the woman of my life, Melissa Johnson! You have stood by my side, prayed for me and encouraged me through the hills and valleys of graduate school. Thank you so much for your love and support and I will always be grateful to have in my life. Lastly, I dedicate this thesis to all those who will find its content relevant to them.
TABLE OF CONTENTS

DEDICATION ................................................................. ii

LIST OF FIGURES .................................................................. vi

LIST OF TABLES ..................................................................... ix

ACKNOWLEDGEMENTS ................................................................. x

ABSTRACT ........................................................................ xii

ORGANIZATION OF THE DISSERTATION .................................................. xiii

CHAPTER 1: GENERAL INTRODUCTION ................................................................. 1
  The Ovary ........................................................................ 1
  Ovarian folliculogenesis ....................................................... 2
  Steroid hormone production in the ovary .............................. 5
  Impact of ovotoxicant exposure on ovarian function ................. 7
  Xenobiotic biotransformation enzymes .................................... 8
  The phosphatidylinositol-3 kinase signaling pathway ................. 11
  Insulin signaling ................................................................ 14
  Obesity ............................................................................. 16
  Obesity-induced inflammation ............................................. 18
  Hyperthermia .................................................................... 19
  Summary ........................................................................... 20
  References .......................................................................... 21

CHAPTER 2: HIGH FAT DIET INDUCED OBESITY ALTERS OVARIAN PHOSPHATIDYLINOSITOL-3 KINASE SIGNALING GENE EXPRESSION ................................................................. 68
  Abstract ........................................................................... 44
  Introduction ....................................................................... 45
  Materials and Methods ....................................................... 50
  Results ............................................................................. 56
  Discussion ......................................................................... 58
  Conclusions ...................................................................... 65
  Acknowledgements .......................................................... 65
  References .......................................................................... 67

CHAPTER 3: DIET-INDUCED OBESITY ALTERS IMMUNE CELL INFILTRATION AND EXPRESSION OF INFLAMMATORY CYTOKINE GENES IN MOUSE OVARIAN AND PERI-OVARIAN ADIPOSE DEPOT TISSUES ........................................................................... 87
LIST OF FIGURES

CHAPTER 1
Figure 1. Model of the mammalian ovary ................................................................. 43

CHAPTER 2
Figure 1. Obesity does not alter ovarian weight in mice .................................. 80
Figure 2. Obesity decreases ovarian Irs1 mRNA levels in mice .................... 81
Figure 3. Obesity enhances ovarian KITLG/KIT-PI3K/AKT1 signaling pathway in mice ................................................................. 82
Figure 4. Altered pAKTSer473 levels are observed in the ovaries of obese mice .................................................................................. 83
Figure 5. Obesity down-regulates ovarian Cyp2e1 mRNA levels ................. 84
Figure 6. Obesity alters levels of ovarian miR ....................................................... 85
Figure 7. Proposed model of obesity effects on AKT1 signaling .................... 86

CHAPTER 3
Figure 1. Obesity increases adipocyte size in the peri-ovarian fat pad in mice .... 114
Figure 2. Obesity increases the presence of crown-like structures in peri-ovarian adipose tissue ................................................................. 115
Figure 3. Obesity increases mRNA expression of immune cell and pro-inflammatory markers in peri-ovarian adipose tissue ................................. 116
Figure 4. Obesity up-regulates pro-inflammatory cytokine mRNA expression in ovarian tissue ........................................................................... 117
Figure 5. Obesity increases the downstream target of TNFa, RELA ............. 118
Figure 6. Obesity down-regulates levels of miR125b and miR143 associated with inflammatory cytokine signaling in the ovary ....................... 119

CHAPTER 4
Figure 1: Effect of obesity on follicle number .................................................... 160
Figure 2. Obesity alters mRNA expression of ovarian steroidogenic members in mice ............................................................................... 161
Figure 3. Obesity impacts protein abundance of ovarian steroidogenic members…………………………………………………………………………………..162

Figure 4. Ovarian NF-κB pathway member protein levels are affected by obesity…………………………………………………………………………………..163

CHAPTER 5
Figure 1: Obesity temporally increases ovarian mRNA expression of insulin signaling members…………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………
from pre-pubertal gilts…………………………………………………………..266

Figure 2: Impact of HS on ovarian IR or IRS1 mRNA…………………………267

Figure 3: HS-induced effects on ovarian pIRS\textsuperscript{Tyr632} protein……………….268

Figure 4: Impact of HS on ovarian AKT1 or FOXO3 mRNA………………269

Figure 5: Effect of HS on ovarian pAKT1 protein localization and level………270

Figure 6: Impact of HS on ovarian steroidogenic gene mRNA…………………271

Figure 7: HS-induced effects of ovarian STAR protein…………………………………272

Figure 8: Effect of HS on ovarian CYP19a protein……………………………………273

APPENDIX
Figure 1. Impact of genotype on glucose clearance in mice………………298

Figure 2. Obesity decreased glucose clearance rate in mice at
12 weeks of age………………………………………………………………299

Figure 3. Obesity decreased glucose clearance rate in mice at
18 weeks of age………………………………………………………………300

Figure 4. Obesity decreased glucose clearance rate in mice
at 24 weeks of age……………………………………………………………301

Figure 5. Impact of obesity on ovarian energy utilization gene
expression in mice……………………………………………………………302

Figure 6. Impact of HS on porcine ovarian energy utilization gene
expression……………………………………………………………………303
LIST OF TABLES

CHAPTER 2
Table 1. Sequences of primers used........................................79

CHAPTER 3
Table 1. Primer Sequences..................................................113

CHAPTER 4
Table 1. Primer sequences used in this study..........................156

Table 2. Progressive obesity effects on fasting blood glucose
and ovarian weight..........................................................157

Table 3. Impact of progressive obesity on estrous
cyclicity...........................................................................158

Table 4. Impact of progressive obesity on mRNA level of markers
of immune cells, inflammation and NF-κB pathway members....159

CHAPTER 5
Table 1: Primer Sequences used for quantitative RT-PCR...........195

CHAPTER 7
Table 1: Primer Sequences..................................................265
ACKNOWLEDGEMENTS

I extend my sincere thanks to Dr. Aileen Keating, my major Professor for your countless support and guidance from the start to the completion of my studies. Throughout my graduate time, you have given me many opportunities to read through grant proposals, write manuscripts, attend professional conferences and present our fascinating findings. You have been a great mentor and a true inspiration, encouraging me throughout my four years of scientific endeavors. I would not be where I am today without your support. Thank you so much! In the same spirit, I would also like to thank my advisory committee members: Dr. Drena L. Dobbs, Dr. Jason W. Ross, Dr. Jeffrey J. Essner, Dr. Jodi A. Sterle and Dr. Peng Liu for all your invaluable time you dedicated to attend all our meetings. Your scientific suggestions and wisdom have always challenged me to be a better scientist and they will always guide me as a researcher. Dr. Drena L. Dobbs thank you so much for mentoring me during my Preparing for Future Faculty Program. Your guidance will always be invaluable in my entire teaching career.

I would also like to thank Dr. Lance Baumgard for your invaluable support on the heat stress project and Dr. James Perfield for providing us with the samples I used for my high fat diet-induced obesity studies. Special thanks go to the Ugandan community for your moral support and friends at ISU for being great learning partners. Furthermore, I extend my gratitude to the Ames SDA church for your moral and spiritual support. You have always been a blessing and an inspiration to me! I thank Dr. Mukasa, Dr. Dorothy Masinde and Dr. Gail Nonnecke for writing me the letters of support for
this study opportunity. Thank you for having confidence in me that I can excel through the challenges of graduate school. Last but not least, I thank my labmates, Dr. Shanthi Ganesan and Jill A. Madden for all the support and good working environment you accorded me during my studies.
ABSTRACT

Metabolic perturbations including hyperinsulinemia that are induced during obesity and heat stress in humans and production animals are allied with several health hallmarks and impaired fertility. This dissertation research focused on characterizing the impact of changes to central metabolism on ovarian function. We hypothesized that hyperinsulinemia induced during central metabolic perturbations, alters ovarian insulin-mediated PI3K signaling, negatively impacting ovarian folliculogenesis, steroidogenesis and xenobiotic biotransformation. To test this hypothesis, mRNA and protein expression profiles of insulin, PI3K, steroidogenic, inflammatory and chemical metabolism members were quantified using qRT-PCR, Western blotting or immunohistochemistry techniques using three models of hyperinsulinemia: 1) high fat diet (HFD)-induced obesity, 2) a transgenic mouse model of progressive obesity and 3) a porcine model of hyperinsulinemia. Overall, our data demonstrates that the ovarian insulin-KITLG-KIT-AKT signaling pathway is active and upregulated during central metabolic alterations. Perturbations to ovarian insulin-KITLG-KIT-AKT signaling pathway are likely to impact 1) follicle activation, oocyte viability and recruitment, 2) steroid hormone biosynthesis, and 3) xenobiotic biotransformation, potentially accelerating susceptibility to chemical exposure. All of these scenarios could lead to impairment of ovarian function, and may at least partially explain why female fecundity is compromised during altered metabolic states.
ORGANIZATION OF THE DISSERTATION

This dissertation is organized into eight chapters and an appendix. Chapter 1 is the general introduction to the research. Chapter 2 focuses on the impact of high fat diet induced obesity on ovarian phosphatidylinositol-3 kinase (PI3K) signaling gene expression. We hypothesized that obesity induced by high fat diet feeding would alter ovarian PI3 kinase signaling factors involved in regulating both primordial follicular activation and xenobiotic biotransformation in female mice. These experiments showed that diet-induced obesity induces increased PI3K signaling pathway potentially altering the rate of primordial follicle activation and oocyte viability. Additionally, the expression of major xenobiotic biotransformation enzymes, that are important in ovarian response to ovotoxicant exposure, were also changed. In chapter 3, the impact of diet-induced obesity on immune cell infiltration and expression of inflammatory cytokine genes in mouse ovarian and peri-ovarian adipose depot tissues was determined. These data demonstrated that diet-induced obesity elevates expression of inflammatory mediator genes in both the ovary and surrounding adipose depot, potentially negatively affecting ovarian function. Chapter 4 focuses on the impact of progressive obesity on the expression of ovarian pro-inflammatory and steroidogenic pathway members in mice. This study demonstrates that progressive obesity alters the expression of inflammatory and steroidogenesis signaling pathway members in ways which could alter optimal ovarian function. In chapter 5 we examined the impact of progressive obesity on expression profiles of genes involved in ovarian insulin-PI3K/AKT dependent pathway and ovarian xenobiotic biotransformation in mice. These results suggest that, during
progressive obesity insulin and KITLG-KIT signaling members are upregulated, potentially leading to hyperactivation of the PI3K-AKT pathway and consequently resulting in derangements in follicular growth, activation, and oocyte viability, leading to impaired fecundity. In chapter 6, the impact of obesity on ovotoxicity induced by 7,12-dimethylbenz[a]anthracene in mice was examined. These experiments further established that obesity induces increased follicular activation in conjunction with increased basal PI3K signaling. Additionally, ovaries from obese mice were more sensitive to DMBA-induced ovotoxicity, potentially due to increased bioactivation by higher basal and DMBA-induced EPHX1 levels expression and reduced detoxification due to lack of GSTP1 induction. In chapter 7 we investigate the effects of HS on insulin mediated PI3K and steroidogenic signaling in gilt ovaries. These results suggest that HS alters the expression of ovarian insulin mediated-PI3K signaling pathway members potentially impacting follicle activation and viability. Additionally, hyperthermia may act as an endocrine disrupting environmental exposure altering ovarian steroidogenesis with subsequent malproduction of critical ovarian hormones for fertility and pregnancy maintenance, leading to female infertility. Chapters 2-7 are presented as manuscripts published or submitted to respective journals. Chapter 8 presents the general conclusions from all these studies. In the appendix, some results which were not included in the manuscripts prepared for publication are summarized.
CHAPTER 1: GENERAL INTRODUCTION

The Ovary

The word “ovary” is derived from the Latin word “ovarium” which literally means “egg” or “nut”. Histologically (Figure 1), mammalian ovaries contain follicles at different stages of development and, post-ovulation, the corpus luteum (CL) (110). Individual follicles contain an innermost nucleated oocyte which is surrounded by granulosa cells (GC) and an outer layer of theca cells (TC) (110). The mammalian ovary plays two significant roles; 1) production of oocytes required for perpetuation of the species and 2) generation of hormones mainly progesterone, P4; 17β-estradiol, E2; inhibins and relaxin as well as peptide growth factors, which are critical for ovarian function and development of female secondary sex characteristics (67).

Both the ovary and oocytes differentiate from the somatic lineages of the embryo during embryogenesis (67, 101). Briefly, during fertilization, a zygote is formed by fusion of the female oocyte and the male spermatozoon. The zygote goes through different stages of embryogenesis and once the sixteen-cell blastmeres are formed, cells will undergo differentiation until three lineages; trophectoderm (future placenta), epiblast (future embryo), and primitive endoderm (future yolk sac) are defined (67, 101). After implantation, cells within the epiblast undergo further differentiation to form the precursors of the primordial germ cells (PGCs), the first cells of the future ovary to be defined (67, 101). In due course, the ovary develops and permits the PGCs to differentiate into oocytes, which under coordinated signals from several proteins and
small RNAs, proliferate and enter meiosis 1 where they are eventually arrested in diplotene stage of prophase 1 (67, 101). The meiotically arrested oocytes later become surrounded by pre-granulosa cells and form individual primordial follicles. These primordial follicles comprise the resting pool of oocytes and retain the potential to be activated and recruited into the growing follicle pool during puberty, with the capacity to be ovulated and fertilized to contribute to the next generation (67, 73, 101).

**Ovarian folliculogenesis**

In mammals, ovarian folliculogenesis consist of three major stages namely, formation of the primordial follicles; recruitment into the growing pool to form primary, secondary and tertiary follicles; and lastly ovulation and subsequent formation of the CL (73, 74, 101, 164). Before ovarian follicles are formed, oocytes are present within germ cell clusters called germ cell cysts or nests (101). During primordial follicle formation, the germ cell cluster undergoes breakdown and the surviving oocytes are individually surrounded with a single layer of flattened squamous-shaped pre-granulosa cells. This marks the first stage of folliculogenesis taking 1-2 days in mice and the latter half of fetal development in humans (67, 100). These follicles are believed to be dormant, showing little to no biological activity, however, they retain the potential throughout their lifetime to be activated and recruited into the growing follicle pool from puberty to menopause under well-coordinated signaling cascades that continue to be discovered (67, 74, 100). Principally, the primordial follicles transition to the primary follicle
histologically characterized by a morphological change in the pre-granulosa cells from a squamous to cuboidal structure. During the primary stage, the oocyte genome is activated to allow gene transcription and follicles at this stage develop rudimentary receptors for the follicle stimulating hormone (FSH), but remain incapable of responding to gonadotropins until the antral stage (73). Also at this follicle development stage, the zona pellucida forms around the oocyte, separating it from the surrounding layer of GC (74). The primary follicles further transition to secondary follicles which contain an oocyte surrounded by multiple layers of stratified GC. At the secondary stage, another layer of somatic cells called the TC are recruited to the follicular structure (67, 72, 198). Thus at this stage, histologically the follicles are characterized by oocytes surrounded by multiple layers of GC and TC. In humans from initial recruitment to the secondary stage may take several months (245). The follicles continue to mature forming a fluid-filled cavity called the antrum. Follicles that acquire this structure are now referred to as antral or graafian follicles (245). The GC and TC continue to undergo mitosis in conjunction with increased antrum. At the tertiary follicular stage, the TC expresses luteinizing hormone (LH) receptors for androgen production and subsequent aromatization to estrogen by the GC. Also at this stage the majority of the follicles that began growth almost 9 – 12 months earlier have died (101), thus only a subset of activated follicles are eventually selected for dominance and ovulation (164).

Although the ovary contains a number of follicles at different stages of development, in mammalian species, the number of primordial follicles is finite and
becomes the only oocyte reservoir available to female mammals throughout their reproductive lifespan (101). On average, the number of primordial follicles is about seven million follicles at or immediately after birth (245). However, this pool reduces drastically to as low as approximately <1 million follicles by the time a human female reaches puberty (101, 245). This number continues to decrease until menopause, a physiological condition where the female is incapable of experiencing the menstrual cycle, due to her ovary being devoid of follicles (101, 245). Therefore the rate at which this precious pool of primordial follicles is lost determines the rate and time at which females approach menopause. The process by which follicles die is believed to be through atresia (101, 123), which is a form of hormonal controlled programmed cell death (apoptosis) (111). Although atresia occurs at all stages of folliculogenesis, it is believed that the majority of follicles become atretic at the antral stage (100), such that only a few of them undergo gonadotropin stimulation to reach the preovulatory stage (101). It is also important to note that germ cell cluster breakdown, primordial follicle formation and subsequent recruitment of primordial follicles to the growing follicular pool, remain the least understood steps of folliculogenesis. However, some key regulators of these initial stages of follicle development such as insulin, kit ligand (KITLG) and the phosphatidylinositol 3-kinase (PI3K) pathway have been identified to be involved (67, 164, 210) and this research area continues to determine novel regulators of these processes. Despite the many unanswered questions during this crucial period, the concept of ovarian cross talk between oocytes and somatic cells is apparent from the formation of primordial follicles onward (67, 69, 163).
Steroid hormone production in the ovary

Ovarian steroidogenesis is accomplished by two families of enzymes, the cytochrome P450-heme containing proteins and the hydroxysteroid dehydrogenases (3, 88). LH released from the anterior pituitary will bind to LH receptors on ovarian TC to trigger signaling cascades that will increase the expression of genes encoding enzymes required to covert cholesterol to androgens (Testosterone and Androstenedione). In response, FSH is released from the anterior lobe of the pituitary and binds to the FSH receptor localized on the ovarian GC with a subsequent increase in expression of genes encoding enzymes necessary for aromatization of TC-derived androgens to estrogens (17β-estradiol and estrone). In TC, cholesterol is either synthesized de novo (159) or derived from lipoproteins through low density lipoprotein receptors (87). Once in the cytoplasm, cholesterol is then acted upon by steroidogenic acute regulatory (StAR) protein localized in the outer membrane of the mitochondria (140, 168, 169). This protein is also believed to be the rate-limiting enzyme in steroid hormone production due to its key role in shuttling cholesterol from the outer to the inner mitochondrial membrane, an event which facilitates the cleavage of cholesterol into pregnenolone (140, 169). Cytochrome P450, Family 11, Subfamily A, Polypeptide 1 (Cyp11a1), previously known as cholesterol side chain cleavage, is a 56 kDa protein located in the inner mitochondrial matrix that is required for the conversion of cholesterol to pregnenolone (88, 226, 227). Pregnenolone exits the mitochondria to the endoplasmic reticulum where under the influence of Hydroxyl-Delta-5-Steroid Dehydrogenase, 3 Beta- and Steroid Delta-Isomerase 2 (HSD3B2) in humans or Hsd3b1 in mice, commonly known as 3β-
hydroxysteroid dehydrogenase is converted to progesterone (3, 136). Cytochrome P450, Family 17, Subfamily A. Polypeptide 1 (Cyp17a1), also localized to the endoplasmic reticulum, catalyzes the conversion of progesterone to androstenedione (7, 56, 88, 120, 170, 195, 227, 244). Androstenedione can diffuse to the GC prior to its conversion to testosterone or in the presence of hydroxysteroid 17-β dehydrogenase 1 (HSD17B1) in humans or hydroxysteroid 11-β dehydrogenase 2 in mice (Hsd11b2), androstenedione is first converted to testosterone which enters the GC for further processing. In the GC, testosterone is aromatized to estrogens by Cytochrome P450, Family 19, Subfamily A. Polypeptide 1 (Cyp19a1) also known as aromatase (88, 120, 136).

At puberty, the reproductive cycles of mammalian species are tightly regulated by a system of positive and negative feedback of reproductive hormones secreted and released from the hypothalamus (gonadotrophin-releasing hormone, GnRH) (64, 167), the pituitary (FSH, LH, oxytocin and prolactin) (64, 242), the ovaries (P4, E2, inhibins and relaxin) (120, 202) and the uterus (prostaglandin F2α)(9, 229). Briefly, FSH secreted by the pituitary promotes further granulosa cell proliferation and survival. Ovulation of the dominant follicle occurs in response to a rise in LH, due to a positive surge in ovarian E2. After ovulation, the remaining GC and TC undergo terminal differentiation to form the CL which produces P4. Unlike humans, rodents and pigs ovulate over eight oocytes in one estrus period, however, like humans, the ovulation rate and quality of the ovulating follicles depends on the process of folliculogenesis in the earlier period (229). It is therefore important to keep a balance between folliculogenesis and steroidogenesis.
for optimal fertility since any disruption in these processes will likely compromise ovulation rate, subsequent conception rates and adversely impact embryonic viability, thus leading to impaired fecundity.

**Impact of ovotoxicant exposure on ovarian function**

Human environmental exposure is widespread and if these chemicals are not detoxified to lesser toxic products, various target organs/tissues suffer damage leading to several health complications including reproductive and developmental impairments (62, 63, 77, 138, 233, 249). For instance, relative to non-smokers, women who are cigarette smokers have a greater risk of early onset of menopause and suffer infertility (92, 121, 230). Postmenopausal females have a great risk for developing gynecological cancers, osteoporosis and cardiovascular disease (105-107). Although the liver is the primary site for xenobiotic metabolism, the ovary’s capacity to metabolize xenobiotics without the liver’s contribution is well documented (14, 19, 24, 28, 115, 125, 160, 173, 186, 207). Therefore, in addition to its fundamental functions of oocyte and steroid hormone production, the mammalian ovary plays another critical role of xenobiotic metabolism (24, 56, 105). In animal models, xenobiotic exposures have been demonstrated to target the ovary and destroy the primordial follicles as well as other follicle types, leading to premature ovarian failure, infertility and other health impairments (23, 24, 33, 105, 127).
Xenobiotic biotransformation enzymes

The ovary responds to the environmental exposure through increased expression of genes encoding xenobiotic biotransformation enzymes such as cytochrome P450 isoform 2E1 (CYP 2E1) (44, 126, 206), microsomal epoxide hydrolase (EPHX1) (45, 115), glutathione S-transferase Pi (GSTP1) (25, 127) and glutathione S-transferase Mu 1 (GSTM1) (27) whose expression and activity levels are altered during xenobiotic exposure. These enzymes are primarily involved in the detoxification of xenobiotic compounds (31, 94, 96-99, 103, 130), however, their expression may also lead to bioactivation of some environmental compounds (23, 24, 44, 94, 106, 109, 206), leading to increased toxicity.

GSTP1 and GSTM1 are members of the glutathione S-transferases (GST) superfamily of enzymes (190). The GST protein family is involved in phase II metabolism of xenobiotic compounds by converting a variety of electrophilic and hydrophobic compounds into more soluble, easily excretable compounds through catalysing their conjunction with glutathione (GSH) (222). These enzymes have long been known as part of the cell defense against several endogenous and xenobiotic toxic compounds (94). However, recently these enzymes are now known to be involved in a number of other cellular processes other than detoxification. They are mediators of signaling processes involved in cell proliferation and death (134) and are thought to be involved in intracellular transport and isomerization of steroid hormones (95, 134, 141). These enzymes have also been implicated in the pathophysiology of a range of human
diseases including cancer (78, 83), diabetes (254) and inflammatory diseases (78, 94). In recent years, GSTP1’s and GSTM1’s involvement in the development of chemotherapeutic-drug resistance as well as poor prognosis of certain types of malignancies is well documented (235, 239). Moreover, GSTP1 and GSTM1 are also known for their ability to negatively regulate pro-apoptotic pathways leading to reduced programmed cell death which could explain their implications in poor prognosis of certain types of malignancies (78, 94, 127, 134, 236, 239). For instance, GSTP1 inhibits the phosphorylation of C-Jun N-Terminal Kinase 1 (JNK1) and p38 pro-apoptotic proteins of the Mitogen-Activated Protein Kinase (MAPK) signaling (127, 134, 235, 236, 239), modulating apoptotic pathways. Similarly, GSTM1 interacts with Apoptosis Signal-regulating Kinase 1 (ASK1), repressing its induction of the pro-apoptotic p38 and JNK activities on the MAPK signaling (26, 78, 94, 239). The above literature does not only highlight the multiple roles of the GST enzymes, but it also raises concerns about the potential pleiotropic consequences that may face the ovary if alterations in their expression occur.

Like the GSTs, the xenobiotic biotransformation enzymes, EPHX1 and CYP2E1 are involved in other biological processes other than their known detoxification roles. In rodent models, exposure to the environmental carcinogen (57, 108, 124, 139, 219) and ovotoxicant (24, 33, 115, 125), 7,12-dimethylbenz[a]anthracene (DMBA), has been demonstrated to target and destroy all follicle stages leading to accelerated premature ovarian senescence (23, 24, 115, 162, 246). DMBA is a polycyclic aromatic hydrocarbon
present in organic compounds including coal, cigarettes, and petroleum. Therefore human exposure is widespread and it is mainly through inhalation of smoke from burning of such organic substances (115, 207). This could partially explain why some female cigarette smokers undergo early onset of menopause and suffer infertility compared to female non-smokers of the same age (92, 121, 230). Both the carcinogenic and ovotoxic potency of DMBA are believed to mediated through its metabolite DMBA-3,4-diol-1,2-epoxide which is synthesized by hepatic cytochrome P450 isoforms 1B1 (CYP1B1) and 1A1 (CYP1A1) (115, 160, 186, 207) in conjunction with microsomal epoxide hydrolase (EPHX1) (172, 173) enzymes. Importantly, DMBA bioactivation has been demonstrated to occur in the mammalian ovary even without hepatic contributions (14, 19, 24, 115, 173, 186, 207), underscoring the ovary’s capacity to metabolize environment chemicals.

Another industrial chemical of reproductive importance that has been widely studied is the occupational chemical 4-Vinylcyclohexene (VCH). VCH exposure results from the manufacture of rubber tires, flame retardants, insecticides, plasticizers and antioxidants (72, 109). Like DMBA, VCH requires bioactivation to VCH diepoxide (VCD) its ovotoxic metabolite (126, 206). This processes is accomplished by cytochrome P450 isoform 2E1 (CYP 2E1) together with CYP450 enzymes (126, 206). In rodent models, VCD exposure leads to loss of ovarian primordial and primary follicles (109), compromising animal fertility. Interestingly, EPHX1 detoxifies VCD to a lesser toxic tetrol metabolite (28, 72). Thus, depending on the chemical substrate, the
action of EPHX1 can protect or damage ovarian follicles. This data strengthens the need for ovarian uninterrupted capacity to metabolize and detoxify such compounds in order to protect the female germ cell and ensure sound ovarian function.

The phosphatidylinositol-3 kinase signaling pathway

The lipid kinase, phosphatidylinositol 3 kinase (PI3K) is one of the central signaling pathways that integrate the major functions of the ovary (1, 67). PI3K activation by several growth factors and hormones including insulin and KITLG (46, 209), results in signaling events which regulate a number of biological processes including cell proliferation, survival, death, metabolism and regulation of gene expression (1, 30, 46, 104, 156). Most of the PI3K signaling cascades are mediated through the serine/threonine protein kinase B (AKT). In mammals, AKT has three isoforms, alpha (AKT1), beta (AKT2) and gamma (AKT3), which all contain a pleckstrin homolog domain, a central kinase domain with a threonine 308 phosphorylation site and a regulatory serine 473 phosphorylation site (30, 70). Once PI3K is activated, AKT is recruited to the cell membrane where it becomes phosphorylated (pAKT) at both of these sites and activated (30, 46, 70). Following AKT activation, pAKT translocates to the cell nucleus where it can regulate gene expression of many targets including the functional forkhead box transcription factors (FOXO), FOXO1, FOXO3 and FOXO4 (30, 60, 131, 156, 178). These FOXOs can be involved in unique or divergent cellular responses depending on the tissue and physiological state of
the tissues involved (104). For instance in mammalian oocytes, FOXO3 leads to transcription of molecules involved in apoptosis stress responses and cell cycle arrest (40, 240).

As previously mentioned, the mechanisms underlying the initial primordial follicle activation and recruitment to the growing pool are still elusive. Available evidences however, suggest that, growth factors such as insulin and KITLG and their downstream PI3K signaling pathway are key regulators of this process (67, 142). These studies have demonstrated that ovarian PI3K/AKT/FOXO3 signaling pathway is involved in follicular activation (143, 209), recruitment, survival (2, 210), and development (1, 37, 50, 122, 210) as well as ovarian steroidogenesis (2, 53, 119). In rodent models, Akt⁻/⁻ mice have reduced primordial follicle viability (37), FOXO3OE mice show no activation of primordial follicle pool (144), whereas FOXO3⁻/⁻ mice display global primordial follicle activation by postnatal day 14, leading to oocyte death, early depletion of functional ovarian follicles and secondary infertility (50). This pathway has also been implicated in ovarian carcinoma (1, 34, 37, 50, 119, 125, 143, 208, 210), underscoring the importance of PI3K/AKT/FOXO3 signaling pathway in female reproduction.

In addition to regulation of folliculogenesis, PI3K signaling is also involved in regulation of xenobiotic biotransformation gene expression (25, 28). Inhibition of PI3K increased mRNA and protein abundance of EPHX1, GSTP1, GSTM1 as well as induced
elevations in the mRNA and protein encoding transcription factors known to be involved
in regulation of xenobiotic biotransformation genes, the Arylhydrocarbon receptor (Ahr)
and Nuclear factor erythroid-related factor 2 (Nrf2).

The aryl hydrocarbon receptor (Ahr) is a member of the basic helix-loop-helix
(bHLH) DNA binding protein family (184), located in the cytoplasm in a protein
complex with HSP90 under non-stimulated conditions. Upon activation, the
AHR:HSP90 complex dissociates, and AHR can translocate to the nucleus to dimerize
with the Ahr nuclear translocator (ARNT) and bind to the xenobiotic response element
(XRE) in target gene promoters (90). Using alpha-naphthoflavone (ANF; an
arylhydrocarbon receptor antagonist), AHR’s involvement during DMBA ovotoxicity
has been determined. Co-treatment of ovaries with ANF prevented DMBA-induced
follicle destruction, supporting an involvement of AHR in DMBA bioactivation (25,
224), through activation of CYP1B1 (90).

NRF2 is a cap’n’collar basic-region leucine zipper protein that can bind to the
antioxidant response element (ARE) in target genes. NRF2 protein action is maintained
in an inactive state through interaction with the Kelch-like ECH-associated protein 1
(Keap1), which results in proteasomal degradation of NRF2 (152, 165). When exposure
to a xenobiotic occurs, KEAP1 can be inactivated resulting in NRF2 protein release and
nuclear translocation where gene expression of target genes is activated (152). NRF2
knockout mice have increased susceptibility to DMBA-induced skin (112), and
mammary (15) tumors. NRF2 activates the Gstp gene promoter (116) and interestingly, only 3-20% of hepatic GST expression is observed in NRF2 deficient mice relative to their wild-type littermates (52). Taken together, NRF2 is likely involved in DMBA detoxification, potentially through the action of GSTP.

**Insulin signaling**

Insulin is a pleiotropic hormone, regulating an array of biological processes including, but not limited to, glucose and lipid metabolism (215), xenobiotic metabolism (130) and reproductive function (12, 203, 204). Insulin’s actions are mediated through its receptors and receptor’s substrate (IRS) proteins. Insulin receptor (INSR) is a heterodimer comprised of two alpha (INSRα) and beta (INSRβ) subunits and there are four (IRS 1-4) insulin receptor substrate proteins (129, 234). Binding of insulin to the alpha subunits activates the INSR tyrosine kinase in the beta subunits, with subsequent auto phosphorylation and recruitment of different substrate adaptor (IRS 1-4) proteins. Tyrosine phosphorylated IRS then displays binding sites for numerous signaling partners including the PI3K/AKT signaling pathway (81, 84, 176, 232). Defects in IRS, more specifically IRS1 and IRS2, have been implicated in female infertility and rodent models with such defects have been found to display both ovarian and hypothalamic dysfunction (39, 54, 203). Insulin has been demonstrated to induce primordial follicle transition primary follicle stage as well as further follicle development in cultured human ovarian cortex (147), canine (221), fetal hamster cultured ovaries (253), bovine cortical pieces
as rodents (128, 179). Taken together, these studies suggest a role for insulin signaling in ovarian folliculogenesis.

The proto-tirosine kinase receptor (KIT) and its associated ligand, KITLG, are respectively encoded by the Kit and Kitlg genes (66, 73). Kit is majorly expressed in oocytes (102, 157, 158), whereas Kitlg is predominantly expressed in the theca and granulosa cells (66, 73, 117). Binding of KITLG to its receptor induces signaling cascades that regulate a number of ovarian process, notably folliculogenesis (66, 76, 188, 189, 191, 192, 252) and steroidogenesis (174). Although gonadotropins are critical for later stages of folliculogenesis, initial follicle activation is believed to be independent of their influence (68, 200). Studies in this area have demonstrated that KITLG is one of the essential molecules for initial follicular activation. In a number of studies, KITLG has been demonstrated to stimulate follicle activation in rodents (192, 252), human ovarian follicles (48) and bovine ovarian cortical pieces (76). Previous literature also indicates that KITLG’s actions are not limited to initial follicle activation but as well as regulation of multiples stages of follicle development (66, 113, 188, 191, 213). To date, the mechanism(s) behind KITLG’s action on follicle activation and successive follicle development are not fully known, nevertheless, available evidence suggest that binding of KITLG to its cognate receptor induces phosphorylation of the regulatory subunit of PI3K leading to AKT activation and subsequent FOXO3 under expression (132, 142, 209). Despite their paucity, studies in rats (128, 179), mice (93), and bovine (73, 75, 76) have implicated insulin in KITLG-induced follicle activation and subsequent
development. Overall, these studies accentuate the relevance of insulin-KITLG-KIT-PI3K-AKT-FOXO3 signaling pathway in ovarian folliculogenesis.

**Obesity**

Obesity and overweight are the most prevalent health burdens of the modern society claiming about 2.8 million lives of adults globally every year (71). Obesity is defined as a medical condition in which a person abnormally accumulates body fat, usually 20% or more over his/her ideal body weight (71). Both obesity and overweight are majorly classified based on body mass index (BMI) which is a measure of one’s body weight (kg) relative to their height (m2). Based on this criterion, an individual is overweight if his/her BMI is $25.0 \leq 29.9$. Similarly, an individual with a BMI $\geq 30$ is classified as obese. In the past three decades, the world has seen a rapid rise in the incidence of overweight and obesity which in 2008 was estimated to be 35% and 12% respectively among adults of 20 years and over (231). According to this study (231), much of this increase is seen in the developing countries (904 million people) which have been long afflicted by hunger than developed countries (577 million people), making obesity a global epidemic that requires urgent intervention. Obesity and overweight is also escalating in children and young adolescents, aged 5-19 years (49, 59, 61, 137, 153), predisposing them to diseases such as type 2 diabetes and cardiovascular diseases which were previously considered adult diseases.
Obesity is associated with a number of health problems including diabetes (51, 71, 89, 155), cardiovascular diseases (8, 114, 149, 155), cancer (41, 42, 133, 211) and reproductive impairments (133, 154). Among females, the common reproductive complications associated with obesity are early menarche (225), irregular menstrual cycles and polycystic ovarian syndrome (11, 65), poor quality oocytes (35, 154), impaired fecundity (17, 35, 65), increased risk for gynecological cancers (133), intrauterine fetal death and stillbirth (16, 18, 135, 180), gestational diabetes (16, 133, 135) as well as increased risk for birth defects in offspring (16, 49, 135, 150, 180).

Although the mechanism(s) underlying these complications are still less well understood, available evidence indicate that obesity is associated with metabolic perturbations including hyperinsulinemia (214), hyperleptinemia (145, 171) which could induce alterations in a number of pathways including those critical for folliculogenesis, steroidogenesis, ovulation, implantation and embryonic development. Additionally obesity is associated with elevated lipopolysaccharide (LPS) level (43, 241). In females, higher levels of LPS have been implicated in induction of preterm labor and premature embryonic loss (5, 6, 251). LPS has also been reported to compromise steroidogenic signaling specifically through reduced expression of the P4 and LH receptors (4, 5), required for relaying signaling cascades for the hormones critical for maintenance of pregnancy and ovulation, respectively.
**Obesity-induced inflammation**

Obesity is also associated with macrophage infiltrations and chronic inflammation (212). Macrophages and their derived products can regulate ovarian function (228), specifically, ovarian cellular proliferation, apoptosis (20), ovarian steroidogenesis and ovulation (166, 243, 250), and are therefore essential accessory cells for optimal fertility (38, 55, 181). Derangements in inflammatory signals have been implicated in the development of infertility and other obesity-associated adverse reproductive health outcomes (29, 49, 177, 183, 193, 194, 201, 218). For example, aggravated activation of inflammatory cytokines has been associated with detrimental deposition of excess lipid in extra-ovarian tissues (58, 85, 86), generation of reactive oxygen species and oxidative stress, scenarios both of which could have detrimental consequences for human fertility (118). In addition, several studies have reported that macrophages and/or macrophage-derived product deregulation are implicated in a number of ovarian pathologies (175) including polycystic ovary syndrome (80, 151, 205), endometriosis (47) and premature ovarian failure (250). Importantly, obesity is associated with hypertrophic expansion of intra-abdominal adipose tissue which could be potentially impact ovarian physiology due to the proximity of this tissue and the ovary. Adipose tissue is associated with microphage infiltration and inflammation which could impact ovarian physiology. Despite this realization, little is known about how changes that obesity induces in this tissue could impact ovarian tissue and function.
Hyperthermia

Like obesity, heat stress (HS) is associated with elevated plasma insulin and LPS (13, 182, 196, 197, 220, 248), metabolic perturbations that may have direct effects on reproductive performance (91). In production animals including cows, sheep, chicken, and pigs, HS can compromise the animals’ productive and reproductive efficiency (13, 79, 82). Heat stressed animals normally display phenotypes including decreased spermatogenesis (91, 247), impaired folliculogenesis (21, 146), steroidogenesis (22, 146, 216, 217), dysregulated estrous cycles (187, 223), reduced conception rate (32, 91, 146), higher frequency of embryonic death (161, 237), and impaired fertility (32, 36, 146, 148, 199). Moreover, these animals, specifically sows, experience a prolonged period of seasonal infertility for which the effects last from July to December, perhaps due to compromised conception rates and increased embryonic lethality induced by HS, particularly during summer hot months (185, 238). Although these deleterious phenotypes have been long appreciated, the underlying causative mechanism(s) remain largely unknown. Available evidences have implicated photoperiodism as a potential causative factor (10), however such a phenomenon does not fully account for the occurrences of same phenotypes during elevated ambient temperatures in areas with equal days and nights (32). While such scenarios do not rule out the involvement of photoperiodism, they are suggestive that other players are causative in seasonal infertility (10). Since insulin levels are elevated during HS and insulin influences ovarian physiology, investigating how heat stress-induced hyperinsulinemia may be responsible for seasonal infertility and poor sow performance during the summer months is critical.
Summary

The literature reviewed so far indicates that ideal reproduction requires a balance between folliculogenesis and steroidogenesis in addition to proper ovarian inflammatory signaling and ovotoxicant chemical metabolism. Alterations in these processes are likely to alter ovarian function and consequently lead to impaired fecundity and other associated reproductive pathologies. The literature also reveal that the reproductive disorders-induced during central metabolic states like during obesity and HS are phenotypes that could be related to the derangements in molecular pathways controlling ovarian folliculogenesis and steroidogenesis in addition to inflammatory and chemical metabolism processes. Although this correlation is provoking, little is known of how changes to central metabolism could impact such ovarian processes. This dissertation research therefore focused on investigating impacts of metabolic perturbations on ovarian folliculogenesis, steroidogenesis and xenobiotic biotransformation. We hypothesized that hyperinsulinemia induced during central metabolic perturbations, alters ovarian insulin-mediated PI3K signaling, negatively impacting ovarian folliculogenesis, steroidogenesis and xenobiotic biotransformation. To test this hypothesis, mRNA and/or protein expression profiles of insulin, PI3K, steroidogenic, inflammatory and chemical metabolism members were quantified using qRT-PCR, Western blot or immunohistochemistry techniques using three models: 1) high fat diet (HFD)-induced obesity, 2) a transgenic mouse model of progressive obesity and 3) a porcine model of hyperinsulinemia.
References


57. Crist KA, Zhang Z, You M, Gunning WT, Conran PB, Steele VE, and Lubet RA. Characterization of rat ovarian adenocarcinomas developed in response to direct


108. Hoyer PB, Davis JR, Bedrnicek JB, Marion SL, Christian PJ, Barton JK, and Brewer MA. Ovarian neoplasm development by 7,12-dimethylbenz[a]anthracene


152. Ma Q, Kinneer K, Bi YY, Chan JY, and KanYW. Induction of murine NAD(P)H: quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-p-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction. Biochemical Journal 377: 205-213, 2004.


Figure 1. Model of the mammalian ovary. The dynamic ovarian structures are follicles and corpora lutea. The most immature form of follicle growth is termed the primordial stage, composed of an oocyte surrounded by a single layer of squamous shaped pre-granulosa cells. Once activated, the primordial follicle develops into a primary follicle, an oocyte still surrounded by a single layer of cuboidal granulosa cells. The follicle progresses to the secondary stage in which granulosa cells proliferate to form a multicellular layer surrounding the oocyte. At this stage, the follicle also obtains a layer of theca interna cells which encompass the granulosa cell layer. The theca interna and granulosa cells co-operate to produce 17β-estradiol (E2), the major ovarian estrogen. As follicular development continues, a fluid-filled antral cavity forms at which point the follicle is termed antral pre-ovulatory. The majority of follicles do not progress to ovulation and die by a process of atresia (apoptosis). Following ovulation, the oocyte is extruded from the follicle and ovary and enters the oviduct, surrounded by several layers of granulosa cells known as the corona radiata. Within the ovary residual follicular cells undergo a process known as luteinization to form the mature corpus luteum (CL) which is required for progesterone (P4) production. Should pregnancy occur, the CL will be maintained through an endocrine signal from the blastocyst known as the maternal recognition of pregnancy (human chorionic gonadotrophin in humans, E2 in pigs). In a non-pregnant female, the corpus luteum regresses due to lack of this signal and the ovarian cycle resumes (from Keating, A.F. and Hoyer, P.B. 2009. Mechanisms of reproductive toxicity. In: Drug metabolism in pharmaceuticals; Concepts and Applications. Chapter 24: 697-734.), with copyright permission.
CHAPTER 2: HIGH FAT DIET INDUCED OBESITY ALTERS OVARIAN PHOSPHATIDYLINOSITOL-3 KINASE SIGNALING GENE EXPRESSION

A paper published in Reproductive Toxicology 42 (2013) 68-77


Contribution Statement:
I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Ross, J.W. provided expertise on microRNA quantification; Perfield, J.W. provided ovarian tissue from high fat diet fed female mice; Keating, A.F. designed experiments, aided in data interpretation and edited the manuscript.

Abstract

Insulin regulates ovarian phosphatidylinositol-3-kinase (PI3K) signaling, important for primordial follicle viability and growth activation. This study investigated diet-induced obesity impacts on: 1) insulin receptor (Insr) and insulin receptor substrate 1 (Irs1); 2) PI3K components (Kit ligand (Kitlg), kit (c-Kit), protein kinase B alpha (Akt1) and forkhead transcription factor subfamily 3 (Foxo3a)); 3) xenobiotic biotransformation (microsomal epoxide hydrolase (Ephx1), Cytochrome P450 isoform 2E1 (Cyp2e1), Glutathione S-transferase (Gst) isoforms mu (Gstm) and pi (Gstp)) and 4) microRNA’s 184, 205, 103 and 21 gene expression. INSR, GSTM and GSTP protein levels were also measured. Obese mouse ovaries had decreased Irs1, Foxo3a, Cyp2e1, MiR-103, and MiR-21 but increased Kitlg, Akt1, and miR-184 levels relative to lean littermates. These results support that diet-induced obesity potentially impairs ovarian function through aberrant gene expression.
Introduction

Globally, the prevalence of overweight and obesity is increasing, predisposing female to health hazards including diabetes, cardiovascular disease, cancer and compromised reproductive capacity [1-4]. 1.5 billion adults are overweight of whom 200 million men and 300 million women are obese. Further, approximately 43 million children (age 5-19) are overweight [5-10]. About 65% of the world’s population live in countries where being overweight is more responsible for morbidity than underweight, and currently about 2.8 million adults globally die every year as a result of being overweight or obese [1]. Formerly considered a problem of developed countries, this epidemic is now dramatically on the rise in low- and middle-income countries which have had a long incidence of food insecurity, now squaring them up for a binary affliction of disease. While toiling with infectious disease and under-nutrition, they are also paradoxically experiencing a rapid upsurge in non-communicable disease risk factors including obesity and overweight [11-13].

The mammalian ovary is the female gonad responsible for gamete production, as well as the female sex steroids, 17β-estradiol and progesterone. The ovary contains a finite number of primordial follicles at the time of birth, which serve as the follicular pool. Once depleted, primordial follicles cannot be replaced [14-22]. Though this theory has been to a certain extent challenged in recent years [23-25], it remains that the number of primordial follicles declines over the female lifetime. This is because the
process of follicular growth is an irreversible process; once follicles are recruited from the resting pool into the growing pool they will undergo cell death if not selected for further growth to ovulation [26-33]. Unlike the cyclic recruitment of follicles to ovulation, initial follicular activation is independent of the pituitary gonadotropins [17, 18, 34, 35]. The intrinsic ovarian mechanism(s) triggering individual follicle activation are not yet fully known, however, several lines of evidence have identified potential candidate factors including phosphatidylinositol-3 kinase (PI3K). An overall balance among dormancy, activation and death of primordial follicles is believed to be the determining factor which decides the female reproductive lifespan [29, 31, 36-38]. Any external factor that could accelerate follicle activation could hasten the rate at which the ovary become devoid of the follicular pool, thus threatening the reproductive potential of the female.

Obesity has been demonstrated to have detrimental effects on female reproductive function. Obese women have an increased likelihood to display signs of polycystic ovarian syndrome (PCOS), ovulation defects, reduced fecundity and poor quality oocytes [4, 39, 40]. There is also an association between obesity and an increased risk of birth defects, premature and still-births, and gestational diabetes [4, 41], however, the molecular mechanisms involved are still lacking. Obesity is a contributing factor for development of type 2 diabetics, characterized by elevated blood glucose and impaired insulin signaling [42]. Diet-induced obesity with concomitant diabetes is a stimulus that provokes alterations in insulin level and its subsequent sensitivity on its target tissues. It
has been long postulated that obesity not only compromises insulin sensitivity in classical tissues like muscle and liver but also in other insulin responsive tissues like the ovary [42-44]. This theory has recently been challenged in a diet-induced obesity model, where ovaries from female mice fed on 60% kcal of fat for 12 weeks maintained insulin sensitivity, despite that other classical tissue like muscle and liver became insulin resistant [45].

The ovary possesses both insulin and insulin-like growth factor 1 (IGF-1) receptors which can be directly stimulated by their hormone ligands. Insulin not only controls critical energy functions such as glucose and lipid metabolism but also plays a crucial role in reproductive function. Insulin receptor (INSR) is a heterodimer comprised of two alpha subunits and two beta subunits [46, 47]. Insulin binds to the alpha subunits which activates the insulin receptor tyrosine kinase in the beta subunits, with subsequent auto phosphorylation and recruitment of different substrate adaptors such as the Insulin receptor substrate (IRS 1-4) family of proteins. Tyrosine phosphorylated IRS then display binding sites for numerous signaling partners including the PI3K/Protein kinase B (PKB/AKT) signaling pathway. Defects in IRS, more specifically IRS1 and IRS2, have been implicated in female infertility and rodent models with such defects have been found to display both ovarian and hypothalamic dysfunction [48-50].

Several lines of evidence have identified the importance of PI3K signaling in ovarian function [51]. Once PI3K is activated, AKT is recruited to the cell membrane
where it becomes phosphorylated (pAKT), and mediates many downstream events [52-54]. PI3K/AKT has been implicated in regulating follicular activation [36, 38] recruitment, survival [29, 31], and development throughout the female reproductive lifespan [31, 51, 55-57]. PI3K also plays a role in proliferation and differentiation of granulosa cells (GC) in response to gonadotropins [29, 58] and it has been associated with ovarian carcinoma [31, 37, 38, 51, 55, 57-60]. In rodent models, Akt/-/- mice were found to have reduced primordial follicle viability [57]. AKT has the ability to phosphorylate and inactivate several targets including forkhead transcription factors (FoxO). FoxO3 is critical for early stages of follicular growth [55] and it has been reported to trigger apoptosis through either up-regulation of genes necessary for cell death or down-regulation of anti-apoptotic genes [61-64]. FOXO3/-/- mice had global primordial follicle activation by postnatal day 14, leading to oocyte death, early depletion of functional ovarian follicles and secondary infertility [55] while in FOXO3OE mice, no activation of primordial follicle pool was observed [65].

Insulin-mediated PI3K activation has been also suggested to regulate xenobiotic biotransformation genes [66]. Inhibition of ovarian PI3K increases both mRNA and protein levels of microsomal epoxide hydrolase (Ephx1)[67] Glutathione S-transferase Pi (Gstpi) [68] and Glutathione S-transferase Mu 1 (Gstmu) [69] suggesting that these enzymes could be downstream members of the PI3K signaling pathway. GSTP and GSTM are members of the GST protein family involved in phase II metabolism of xenobiotic compounds by converting a variety of electrophilic and hydrophobic
compounds into more soluble, easily excretable compounds through catalyzing their conjunction with glutathione (GSH) [70]. EPHX1 and Cytochrome P450 isoform 2E1 (CYP 2E1) ensure the rapid detoxification of epoxides generated during the oxidative metabolism of xenobiotics, thus providing cellular protection against free radical and carcinogenic compounds [66, 71-73]. Any alteration in expression patterns of genes that encode for ovarian chemical biotranformation enzymes can pose a risk for the onset of ovarian dysfunction. This is because exposure to a number of chemical classes can destroy follicles of all types threatening the reproductive potential of exposed females through accelerated premature ovarian insufficiency, premature ovarian failure (menopause) and other associated health problems [22, 67, 74-77].

Several genes that are components of the PI3K pathway also are regulated by MicroRNAs (miR’s) [78-80]. MiR’s are small (19 – 25 bp) non-coding RNA that can positively or negatively regulate gene expression [81-85]. It is known that miR-21 inhibits phosphatase and tensin homolog (PTEN), an antagonist of PI3K [86, 87]. In vivo loss of miR-21 has been reported to increase ovarian apoptosis and as well compromise ovulation rates in rodent models [86, 88]. MiR-184 is believed to play a critical role in development as well as a mediator of apoptosis [79]. Up regulation of miR-184 can interfere with AKT action, repressing PI3K action [78, 85]. Also, miR-103 has been implicated in insulin sensitivity [89]. Thus, miR’s may mediate the response to insulin signaling, through the PI3K pathway.
In summary, obesity results in reproductive dysfunction and an increase in negative consequences for offspring, and insulin signaling is impaired in obesity. Additionally, insulin can activate PI3K signaling, which is critical for controlling the rate of activation of primordial follicles, and is an upstream regulator of xenobiotic metabolism gene expression. There remains a dearth of knowledge regarding whether obesity can influence ovarian xenobiotic metabolism, thus we hypothesized that obesity caused by a high fat diet would alter ovarian PI3 kinase signaling with subsequent effects on genes encoding xenobiotic metabolism enzymes in female mice.

**Materials and Methods**

**Reagents**

2-β-mercaptoethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulfate, glycerol, N’,N’,N’,N’-Tetramethyl-ethylenediamine (TEMED), Tris base, Tris HCl, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (Vitamin C), phosphatase inhibitor, protease inhibitor and transferrin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Hanks’ Balanced Salt Solution (without CaCl2, MgCl2, or MgSO4), DAPI nuclear stain and superscript III one-step RT-PCR System were obtained from Invitrogen Co. (Carlsbad, CA). miRNeasy Mini Kit, miScript Reverse Transcription Kit, miScript SYBR Green PCR Kit, RNeasy Mini kit, QIAshredder kit, RNeasy MinElute kit, TaqMan® microRNA Reverse Transcription Kit and Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Pierce
Biotechnology (Rockford, IL). Custom designed primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University. Ponceau S was purchased from Fisher Scientific (Waltham, MA, USA). ECL plus chemiluminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK). Anti-GSTP and anti-GSTM antibodies were purchased from Millipore (Temecula, CA, USA). Anti-pAKTSer473 and β-Actin antibodies were from Cell Signaling Technology and anti-insulin receptor (INSR) antibody was purchased from Abcam (Cambridge, MA, USA). Secondary antibodies were obtained from EMD Millipore (Billerica, MA).

**Animal and diets**

Ovarian tissues were obtained from a study at the University of Missouri. The experimental protocols were approved and performed in accordance with the guidelines of the University of Missouri Institutional Animal Care and Use committee as previously described [90]. Briefly, twelve 6 weeks old C57Bl/6J female mice were randomized into two groups (n = 6 per group). The control group was fed a standard chow mice diet (Purina 5001; 4.5g/100g fat) while the treatment group was fed a high-fat diet (HFD; Research Diets D12492; 35g/100g fat) for approximately 7 months. The animals were housed at constant room temperature, 12h light:12h darkness cycle, diet and water were provided ad libitum. Body weight for the control and HFD mice were 24.8g ± 0.45 and 46.5g ± 2.0, respectively. Gonadal adipose tissue weight was 0.44g ± 0.05 and 3.74g ± 0.35 in the control and HFD mice, respectively [90]. After 7 months glucose tolerance testing confirmed that HFD-fed mice had elevated blood glucose, relative to their
control-diet fed littermates [90]. There was no impact of HFD on plasma estradiol [90]. The HFD mice are subsequently referred to as obese, and their non-HFD fed littermates are referred to as lean.

**Tissue collection**

Animals were euthanized by CO2 asphyxiation. Ovaries were removed, trimmed of excess fat, weighed and snap frozen for gene expression analysis or fixed in 4% paraformaldehyde for histological analysis.

**RNA Isolation**

Total ovarian RNA was isolated using Qiagen RNeasy® Mini Kit (n = 6 per dietary treatment). Briefly, ovaries were lysed and homogenized using a hand held homogenizer. The homogenate was then applied to a QIAshredder column placed in a collection tube and centrifuged at 16100 RCF for 2 minutes at room temperature. The flow through was then applied to an RNeasy Mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter and concentrated using an RNeasy MinElute kit. RNA was eluted using 14 µl of RNase-free water and quantified using an ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE).
First Strand cDNA synthesis and quantitative Real-Time quantitative polymerase chain reaction (qRT-PCR)

Total RNA (0.5 µg) was reverse transcribed to cDNA using Invitrogen™ Superscript III Reverse Transcriptase according to the manufacturer’s protocol. Diluted cDNA (2 µl; 1:20 dilution) were amplified on an Eppendorf Mastercycler using Quantitect™ SYBR Green PCR kit and primers specific for mouse Actb, Insr, Irs1, Kit Ligand (Kitlg), c-Kit, Akt1, Foxo3a, Cyp2e1, Ephx1, Gstp, and Gstm (See table 1 for primer sequences). The PCR cycling program consisted of a 15 min hold at 95 oC and 40 cycles of: denaturing for 15 at 95 oC, annealing for 15s at 58 oC and extension at 72 oC for 20s. Product melt conditions were determined using a temperature gradient from 72 oC to 99 oC with a 1 oC increase at each step. Three replicates of each sample (n = 6 per dietary treatment) were included. Statistical analysis was performed on the cycle numbers at which each sample reached a threshold level. The relative mRNA expression for each of the above genes was normalized using mouse Actb as a housekeeping gene and relative fold change calculated using the 2-ΔΔCT method. The results are presented as mean fold change ± standard error relative to the control group.

mirScript miRNA PCR array

Purification of miRNA-enriched fractions was performed using a miRNeasy Mini Kit according to manufacturer’s protocol (n = 3 per dietary treatment). MiRNA-enriched fractions (250 ng) in a reverse-transcription reaction of 20 µl were converted to cDNA using miScrip Reverse Transcription Kit. cDNA was then diluted by adding 200
µl of RNase-free water, from which, 100 µl was used as template cDNA for real-time PCR quantification of multiple microRNAs using miScrip SYBR Green PCR Kit. Data analysis was performed using the web-based miScript miRNA PCR Array Data Analysis package. TaqMan® microRNA Reverse Transcription Kit was used to validate the expression of miR-21. The amount of target miRNA was normalized using RNU43 as a housekeeping miRNA.

Histological analysis of ovarian tissue

One ovary from each animal was fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned (5 µM) at the Iowa State University Veterinary Medicine Histopathology laboratory. Sections were mounted (3-4 per animal), and one section was stained with hematoxylin and eosin (H&E). Digital images were acquired with a Leica DMI300B Fluorescent Microscope.

Protein Isolation and Immunoblot analysis

Total ovarian protein was isolated and immunoblots performed as previously described [75]. Briefly, ovaries were homogenized in ~ 200 µl of iced-cold extraction buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP40 and 1% Protease inhibitor, followed by centrifugation for 30 minutes at 9300 RCF at 4 oC. Protein concentration was determined using a standard BCA protocol. Emission absorbance values were detected with a λ = 560 nm excitation on a Synergy™ HT Multi-Detection Microplate Reader using KC4™ software (Bio-TekR Instruments Inc. Winooski, VT).
Total protein (20 µg) was separated using 10% SDS-PAGE and electro-transfer of proteins from the gel to a nitrocellulose membrane was performed for 60 minutes at 100 V. The membranes were stained with Ponceau S to visualize the amount of total protein transferred in each lane. To reduce non-specific binding, membranes were pre-incubated overnight on a rocker at 4°C in a blocking buffer (5% non-fat dry milk, 5 M NaCl, 20 mM Tris-HCl, 0.15% Tween-20, pH 8). Membranes were probed using specific primary antibodies; Rabbit Anti-INSRα (1:500); Rabbit anti-GSTP (1:200); and mouse Anti-ACTB (1:2000) diluted in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TTBS) overnight at 4°C. Following washing for three times (10 min each) in TTBS, membranes were incubated at room temperature for 1 hour with HRP-conjugated suitable secondary antibodies (1:2000) against the primary antibodies. Membranes were washed three times in TTBS followed by a single wash in Tris-Buffered Saline (TBS). Autoradiograms were visualized on X-ray films in a dark room following 5 min incubation of membranes in ECLplus chemiluminescence detecting reagent. Densitometry of the appropriate band was quantified using Image J software (NCBI). Equal loading was confirmed by Ponceau S staining of membranes and protein expression was normalized to ACTB densitometry values.

**Immunofloourescence staining**

Two slides per animal were deparaffinized in citrol buffer and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1M, pH 6.1). Sections were then blocked in
5% BSA for 1 h at room temperature. Sections were incubated with a primary antibody directed against pAKTSer473 (1:100) overnight at 4°C. After washing in 1% PBS, sections were incubated with a goat anti-rabbit secondary antibody conjugated to fluorescein for 1 h. Slides were counterstained with DAPI nuclear stain for 5 min. Images were taken using a Leica fluorescent microscope. Analysis of pAKTSer473 protein level in small oocytes (pre-antral follicles), large oocytes (antral follicles) and theca cells of antral follicles was performed using ImageJ software (NCBI). Mean densitometry value was divided by the cellular area measured.

**Statistical analysis**

Statistical analysis was performed using the unpaired t-test function of GraphPad Prism 5.5 software with a statistical significance level set at P < 0.05. P < 0.1 was considered a trend for a difference between treatments.

**Results**

**Effect of obesity on ovarian size and weight**

There was no impact of HFD on ovarian weight (P ≥ 0.05) ovarian weight (Figure 1A) yet the ovaries appeared visually smaller relative to their lean littermates (Figure 1B and 1C).
Effect of obesity on ovarian insulin signaling members

Obesity decreased mRNA level of the gene encoding the Irs1 (P < 0.05) with a trend for a decrease in Insr (P = 0.08; Figure 2A). Additionally there was a strong trend (P = 0.06) for decreased INSR protein levels in ovaries of obese female mice compared to lean mice (Figure 2B and 2C).

Effect of obesity on ovarian PI3K signaling

Relative to lean ovaries, obesity increased the mRNA levels of Kitlg by 0.6-fold (P < 0.05) and there was a trend for increased mRNA level of the KITLG receptor, c-Kit of 0.4-fold (P = 0.07; Figure 3). Obesity also induced a 1.1-fold increase (P < 0.05) in mRNA levels of Akt1, with a concomitant 0.6-fold decrease (P < 0.05) in mRNA levels of Foxo3a relative to lean ovaries (Figure 3). pAKTSer473 protein was localized to the oocyte of pre-antral and antral follicles and also to the theca cells (Figure 4A,B). Obese females had a strong trend (P = 0.056) for decreased pAKTSer473 protein in the oocytes of pre-antral follicles (Figure 4C), with no impact observed in the antral follicle oocytes (Figure 4D). Interestingly, there was a trend (P = 0.09) for increased pAKTSer473 protein in theca cells from obese females (Figure 4E).

Effect of obesity on ovarian expressed xenobiotic metabolism genes

There was decreased mRNA level for the gene encoding Cyp2e1 (P < 0.05). In addition a trend (P < 0.1) for increased Ephx1 and decreased Gstp1 and Gstm1 mRNA
levels was observed (Figure 5A). No impact of obesity on GSTM protein was observed, however, there was a trend for decreased GSTP protein level (P < 0.1).

**Effect of obesity on ovarian expression of miR-103, miR-21, miR-184 and miR-205**

Ovaries from obese mice had decreased (P < 0.05) miR-21, and miR-103 (P < 0.05), with a strong trend (P = 0.06) for increased miR-205. In contrast, obesity up-regulated (P < 0.05) miR-184 levels (Figure 6A). The obesity-induced decrease in miR-21 was validated using qRT-PCR (P < 0.05; Figure 6B).

**Discussion**

Several studies have highlighted a strong correlation between obesity, infertility and adverse reproductive health outcomes, however, the underlying mechanisms remain unclear [91-95], thus comprehension of the mechanisms by which obesity affects ovarian function is of significant relevance. We hypothesized that obesity could alter factors regulating both primordial follicular activation and xenobiotic biotransformation. The females in this study were approximately 8.5 months of age, and did not differ in levels of plasma estradiol [90].

Previous studies in humans and rat models have reported that hyperinsulinemia down-regulates ovarian Insr expression [96, 97]. Similar studies in insulin-resistant and hyperinsulinemic mice demonstrated altered duration of estrous cycles as well as aberrant distribution and morphology of ovarian follicles [42, 49, 98]. The metabolic
effects of insulin mainly involve tissue-specific actions which result in changes in gene expression, protein phosphorylation and function of the INSR and its downstream adaptor proteins, IRS1-4 [99]. Phosphorylation of IRSs lead to activation of downstream mediators including the PI3K/AKT serine/threonine kinase [50]. There are several pathological effects associated with dysregulated PI3K pathway in the ovary. For instance, over activation of PI3K in oocytes has been associated with premature ovarian failure (POF), while, on the other hand, over activation of the pathway in granulosa cells is highly correlated with defects in follicle cyclic recruitment and ovulation and carcinogenesis derived from ovarian surface epithelium [29-31, 37, 58, 100].

In order to determine the molecular effects of obesity on ovarian function, we first investigated the effect of obesity on expression of genes encoding the insulin signaling members, INSR and IRS1, both of which are critical for the response to insulin. Obesity decreased the mRNA level of Irs1 concomitant with a trend for a reduction in total ovarian Insr mRNA and protein expression. The effect of obesity on expression of insulin signaling members in other tissues is well reported but most ovarian studies have reported inconsistent results. For instance consistent with our findings, reductions in expression of INSR and IRS1 during obesity [101-103] or hyperinsulinemia [96, 97] have been reported, while in contrast, increased phosphorylation of both IRS1 and IRS2 with obesity have been also observed in other cell types [45, 96, 104-107]. Additionally, other studies have observed no effect of obesity on INSR [3, 42] or IRS1 [3, 108, 109]. Taken together, these results implicate
the effect of obesity on insulin signaling members to be a complex mechanism that may be species-, tissue- and/or time-specific.

Following these observations we postulated the insulin-mediated PI3K/AKT pathway could be compromised. To our surprise our results demonstrated that reduction in mRNA encoding insulin signaling factors did not inactivate the PI3K/AKT pathway in ovaries of obese mice. Obese mice had increased Akt1 mRNA levels with a concomitant decrease in Foxo3a mRNA compared to lean mice. Several other studies show that insulin could have a tissue-isoform and species specific differential effect on Akt isoforms [53, 110]. In obese rodent models, insulin-stimulated AKT1 activity was reported to decrease in muscle and adipose tissue but increased in liver, in contrast, AKT2 activity was decreased in muscle and liver but increased in adipose tissue [53]. Thus, our data indicate that ovarian AKT1 responds in a similar fashion to hepatic tissue in the face of changing insulin levels. Whether these changes are direct interactions between the IRS or involve other signaling mediators is difficult to surmise from the current data.

Since obesity mildly reduced expression of Insr and Irs1 in mice ovaries while the PI3K signaling pathway is markedly up-regulated compared to lean mice in our study, we asked what other insulin-independent pathways involved in activation of the ovarian PI3K/AKT pathway were altered in the obese mouse ovary. Stem cell growth factor receptor (c-KIT), a receptor protein tyrosine kinase is expressed by the oocyte
and its ligand, Kit Ligand (KITLG; also called Stem cell factor or Steel factor), is expressed by granulosa cells [113]. Several lines of evidence have indicated that KITLG/c-KIT signaling is essential for oocyte viability and survival and that this pro-survival pathway also regulates follicle recruitment [114-116]. Once c-KIT is bound by KITLG the PI3K pathway is activated [36-38, 55, 56, 59]. Based on those previous reports and our data we asked if HFD-induced obesity affected the ovarian mRNA level of genes encoding c-KIT and KITLG. Our data shows that obesity increased Kitlg mRNA levels with a trend for increased mRNA encoding its receptor, c-Kit. To the best of our knowledge this is the first study to demonstrate that HFD-induced obesity can alter the expression of the c-Kit ligand in murine ovaries. In oocytes of cultured neonatal mouse and rat ovaries, it has been demonstrated that KITLG leads to phosphorylation of FOXO3a through the action of pAKT [36]. Based on the results presented herein hyperactivation of KITLG/c-KIT-PI3K/AKT could be a potential mechanism underlying obesity related infertility problems. It is known that hyperstimulation of primordial follicles into the growing follicular pool leads to their eventual destruction [55, 57, 59]. Over-activation of the KITLG/c-KIT dependent PI3K/AKT pathway could imply that in obese female mice, there is over stimulation of primordial follicles into the growing follicular pool; a scenario that could accelerate the rate at which mice become infertile, although it should be noted that follicle numbers were not classified in our study. Interestingly, we also noted a trend for increased theca cell pAKTSer473, and it is known that the interaction between KITLG and c-KIT has a regulatory role in steroidogenesis in rat granulosa cells [117].
Several studies have demonstrated that insulin and other growth factors regulate xenobiotic chemical metabolizing enzyme gene expression, including cytochromes p450, GSTs and EPHX1 through several kinases including PI3K/AKT signaling pathway [66, 118, 119]. The ovary has the capacity to metabolize ovotoxic compounds [60, 67, 68, 75, 76, 120-122], however to date, there is a dearth of literature examining potential effects of obesity on expression of xenobiotic metabolizing enzymes in the ovary. Since diet-induced obesity can alter insulin levels and/or sensitivity, we hypothesized that obesity may alter the ovarian expression of ovotoxicant metabolizing enzymes genes. Previous studies have demonstrated that diabetes is strongly associated with increased hepatic Cyp2e1 [123] and on the other hand decreased Ephx1 [124]. Also, Cyp2e1 mRNA expression is decreased in a dose dependent manner by insulin in rat cultured hepatocytes [123, 125, 126]. PI3K inhibitors such as wortmannin and LY294002 were shown to reverse the insulin-mediated down-regulation of Cyp2e1 mRNA levels [127]. Furthermore, in cultured neonatal ovaries, inhibition of PI3K signaling using LY294002 resulted in increased mRNA and protein levels of Ephx1, Gstp and Gstm [67-69]. In the present study decreased mRNA encoding Cyp2e1 was observed in ovaries of obese mice compared to the lean mice. In contrast to Cyp2e1, there was a trend for increased Ephx1 mRNA level. This differential effect of a physiological paradigm on ovarian Cyp2e1 and Ephx1 expression has been previously reported [76, 128]. Studies on the GSTs have reported both increased and decreased gene expression during diabetes [66]. In rat cultured hepatocytes, neither insulin nor
glucagon affected the mRNA levels of Gstm while, on the other hand, glucagon reduced Gstp mRNA levels [129]. In the current study there was a trend for decreased Gstp mRNA and protein level in the obese ovary. These results suggest that obese females may have altered xenobiotic metabolism and therefore if such females are exposed to ovotoxicants, the rate at which they would approach premature ovarian insufficiency and failure would be accelerated. Further still, these results suggest that obese females may have increased risks from exposure to carcinogens and teratogens which could also potentially explain the increased rates of miscarriage and increased offspring birth defects in obese mothers.

PI3K signaling can be regulated through posttranscriptional gene silencing by the action of miRs [78, 80, 89, 130]. In order to evaluate if obesity had any effect on miR’s that could at least partially explain the effects observed on PI3K signaling, levels of miR-103, miR-21, miR-184 and miR-205 were measured. It was found that the ovaries from obese mice had decreased miR-21 and miR-103; however, miR-184 levels were increased. Recent reports have indicated that miR-21 expression is important for regulation of apoptosis [131, 132]. Decreased expression of miR-21 expression has been reported to increase cell apoptosis in a variety of cell culture systems including the granulosa cells from mouse pre-ovulatory follicles both in vivo and in vitro [86]. In addition, miR-21 has been identified as promoting follicular cell survival during ovulation and miR-21 inhibition also has been reported to reduce ovulatory rates [86]. Although many different cell types undergo apoptosis in response to inhibition of miR-
21 action, the miR-21 targets implicated vary widely for different cells and the mechanism by which miR-21 suppresses apoptosis in GCs remain to be identified [133-135].

miR-184 has been shown to act as a physiological suppressor of general secretory activity of progesterone and estradiol [135, 136]. Additionally, miR-184 is believed to play a critical role in development as well as being a mediator of apoptosis. Up-regulation of miR-184 has been reported to interfere with the ability of miR-205 to lead to repression of AKT signaling [78]. Though in this study we observed an elevated level of miR-184 with a trend for a decrease in miR-205 level, there was an up-regulation of Akt1 mRNA levels together with a subsequent decrease in Foxo3a mRNA levels thus indicating that Akt1 activation is not being compromised by miR-205 action. Several studies from both human and rodent models have reported that miR-103 is up-regulated during obesity [89, 137, 138]. It has been further suggested that silencing miR-103 improves insulin sensitivity in adipocytes mainly through increased caveolin-1 expression, which in turn leads to stabilization of the INSR and thus enhancing insulin signaling [89]. In contrast to the above reports miR-103 expression has also been found to be down-regulated in the mouse model of genetic insulin resistance and obesity (ob/ob mice) [139]. This is consistent with our findings, where obesity decreased miR-103 levels in mice ovaries. These results further confirm miRNA cell and tissue specific actions [140].
Conclusions

In summary, the data presented herein, though preliminary in nature, demonstrate perturbations to ovarian PI3K signaling caused by obesity in females. Under basal conditions, AKT1 is positively regulated by both KITLG and IRS1. In addition, AKT1 is positively regulated by miR21 and negatively regulated by miR184. Our data collectively indicates that obesity decreases IRS1 levels but increases KITLG which can then result in increased AKT1 levels. Although miR21 is decreased, which should result in decreased AKT1, we propose that increased levels of miR184 may counteract decreased miR21 to result in AKT1 activation (summarized in Figure 7). One consequence of obesity-induced altered PI3K includes changes in the downstream components Akt1 and Foxo3a that may alter the rate of primordial follicle activation, leading to fertility problems. Another result of altered PI3K observed is that expression of major xenobiotic biotransformation enzymes that are important in the ovarian response to ovotoxicant exposure, are changed. Thus, obesity may have negative consequences for follicle activation and oocyte viability, as well as altering how the ovary responds to chemical exposures. All of these scenarios can lead to impairment of ovarian function, and may at least partially explain why reproduction is compromised in obese females.

Acknowledgements

The project described was supported by the National Institutes of Environmental Health Sciences [R00ES016818] to A.F.K. The content is solely the responsibility of
the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
References


Table 1. Sequences of primers used

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insr-for</td>
<td>ATGGGCTTCGGGAGAGGAT</td>
</tr>
<tr>
<td>Insr-rev</td>
<td>GGATGTCCATACCAGGGGCAC</td>
</tr>
<tr>
<td>Irs1-for</td>
<td>GGATGTCCATACCAGGGGCAC</td>
</tr>
<tr>
<td>Irs1-rev</td>
<td>CAGCCCCGCTTTGTAGATGTTG</td>
</tr>
<tr>
<td>Cyp2e1-for</td>
<td>CCAAGGTCTTTAAACCAAGTGGGC</td>
</tr>
<tr>
<td>Cyp2e1-rev</td>
<td>CTTCATGTGGGTCCATTATTGA</td>
</tr>
<tr>
<td>c-kit-for</td>
<td>GCCACGTCTCAGCCATCTG</td>
</tr>
<tr>
<td>c-kit-rev</td>
<td>GTCGGGATCAATGCACGTCA</td>
</tr>
<tr>
<td>Kitlg-for</td>
<td>GAATCTCCGAAGGGCGCAAGA</td>
</tr>
<tr>
<td>Kitlg-rev</td>
<td>GCTGCAACAGGGGTGAACAT</td>
</tr>
<tr>
<td>Akt1-for</td>
<td>CCACCTGTCTCTAGGGGTCCA</td>
</tr>
<tr>
<td>Akt1-rev</td>
<td>CATGGGACACAGCAACAAAC</td>
</tr>
<tr>
<td>Gstp-for</td>
<td>CCAAGTTTGAGGATGGAGA</td>
</tr>
<tr>
<td>Gstp-rev</td>
<td>CAGGGCCCTTCAGTGACAT</td>
</tr>
<tr>
<td>Gstm-for</td>
<td>GAGGGATCCGGTGCAGACAT</td>
</tr>
<tr>
<td>Gstm-rev</td>
<td>ACTTGGGCTCAAACATACG</td>
</tr>
<tr>
<td>Foxo3a-for</td>
<td>GGTACCAGGCTGAAGGATCA</td>
</tr>
<tr>
<td>Foxo3a-rev</td>
<td>CAGTCTCTGTGGGGTTAGGG</td>
</tr>
</tbody>
</table>
Figure 1. Obesity does not alter ovarian weight in mice. Six weeks old C57Bl/6J female mice (n = 6 per treatment group) were fed either a standard chow mice diet or a high-fat diet for approximately 7 months and were euthanized by CO2 asphyxiation. Ovaries were removed, trimmed of excess fat and weighed. (A) Ovarian weight; (B) lean and (C) obese ovarian sections stained with hematoxylin and eosin (H&E).
Figure 2. Obesity decreases ovarian Irs1 mRNA levels in mice. Ovaries were removed from lean and obese mice (n = 6 per treatment group). (A) RNA was isolated and Insr and Irs1 mRNA levels were evaluated by quantitative RT-PCR. Values represent fold-change relative to a control value of 1 ± SE, normalized to Actb. (B) Total protein was isolated and Western blotting performed to measure INSR level. Values represent fold-change relative to a control value of 1 ± SE, normalized to ACTB. Different from control, *P < 0.05. (C) Representative Western blot for INSR and ACTB; Lean = L, Obese = O.
Figure 3. Obesity enhances ovarian KITLG/KIT-PI3K/AKT1 signaling pathway in mice. Ovaries were removed from lean and obese mice (n = 6 per treatment group). RNA was isolated and Kitlg, cKit, Akt1 and Foxo3a mRNA levels were evaluated by quantitative RT-PCR. Values represent fold-change relative to a control value of 1 ± SE, normalized to Actb. Different from control, *P < 0.05.
Figure 4. Altered pAKTSer473 levels are observed in the ovaries of obese mice. Ovaries were removed from (A) lean and (B) obese mice, serially sectioned and mounted onto slides (n = 6 per treatment group). Immunofluorescence staining to detect pAKTSer473 was performed, with counterstaining to detect the nucleus. Image J software was used to quantify levels of pAKTSer473 in (C) small (pre-antral; indicated by arrowhead) and (D) large oocytes (antral; indicated by broken arrow) and (E) theca cells (indicated by unbroken arrow).
Figure 5. Obesity down-regulates ovarian Cyp2e1 mRNA levels. Ovaries were removed from lean and obese mice and RNA isolated (n = 6 per treatment group). (A) Cyp2e1, Ephx1, Gstp and Gstm mRNA levels were measured by quantitative RT-PCR. Values represent fold-change relative to a control value of 1 ± SE, normalized to Actb. Different from control, *P < 0.05. (B) Western blotting was performed to determine any impact of obesity on GSTP. (C) Representative Western blot for GSTP and ACTB; Lean = L, Obese = O.
Figure 6. Obesity alters levels of ovarian miR. Ovaries were removed from lean and obese mice and RNA isolated (n = 3 per treatment group). (A) Enriched miR fractions were analyzed using a miR array. (B) qRT-PCR was performed on miR-21 to confirm the array data. Values represent fold-change relative to a control value of 1 ± SE, normalized to RAU43. Different from control, *P < 0.05.
Figure 7. Proposed model of obesity effects on AKT1 signaling. Under basal conditions, AKT1 is positively regulated by both KITLG and IRS1. In addition, AKT1 is positively regulated by miR21 and negatively regulated by miR184. Our data indicates that obesity decreases IRS1 levels but increases KITLG leading to increased AKT1 levels. The increased levels of miR184 may counteract the decreased miR21 in AKT1 activation. AKT regulates xenobiotic metabolism, primordial follicle activation and viability as well as ovarian steroidogenesis. Thin arrows indicate positive regulation, negative regulation is indicated by the broken arrow. Block arrows indicate the impact of obesity.
CHAPTER 3: DIET-INDUCED OBESITY ALTERS IMMUNE CELL INFILTRATION AND EXPRESSION OF INFLAMMATORY CYTOKINE GENES IN MOUSE OVARIAN AND PERI-OVARIAN ADIPOSE DEPOT TISSUES.


**Contribution Statement:**
I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Ortinau L.C. and Perfield, J.W. provided ovarian tissue from high fat diet fed female mice; Keating, A.F. designed experiments, aided in data interpretation and edited the manuscript.

**SUMMARY**

Dysregulation of immune cells and/or altered inflammatory signaling have been implicated with reproductive dysfunction. Physiological changes leading to perturbations in the profile of immune cells and/or pro-inflammatory cytokines in, or around, female reproductive tissue could potentially have profound effects on ovarian function. Obesity is associated with chronic low-grade inflammation due, in part, to increased immune cell infiltration and inflammation in visceral adipose depots. This study investigated the impact of diet-induced obesity on immune cell infiltration and inflammation in peri-ovarian adipose tissue and mRNA expression of key inflammatory markers and Micro-RNAs (miR) in ovarian tissue. Six week old female C57Bl/6J mice were fed a standard chow or high-fat diet (HFD; 60% kcal fat) for approximately 7 months at which time peri-ovarian adipose tissue and ovarian tissues were collected for analysis. Histological analysis of obese peri-ovarian adipose tissue, revealed increased \( P < 0.05 \) adipocyte
size and the presence of crown-like structures, the morphological presentation of infiltrating immune cells in adipose tissue, along with increases ($P < 0.05$) in the mRNA levels of markers of T-cells, activated macrophages, inflammatory cytokines and chemokines. Ovarian mRNA levels of $Il1b$, $Il6$, $Tnfa$, $p55$, $p75$, $Ccl2$, $Ikbkb$ and $Rela$ were increased by obesity ($P < 0.05$), with a strong trend ($P = 0.06$) for an increase in $Nos2$ and RELA protein. Additionally, ovarian $miR125b$ and $miR143$ levels were decreased ($P = 0.1$). These data demonstrate that diet-induced obesity elevates expression of inflammatory mediator genes in both the ovary and surrounding adipose depot, potentially negatively affecting ovarian function.
INTRODUCTION

The mammalian ovary is a heterogeneous tissue, containing follicles at different developmental stages (Hirshfield 1991). Regulation of the ovarian cycle is primarily dependent on proper interaction between ovarian steroids and the pituitary gonadotropins, however, it is suggested that macrophages can also regulate ovarian cellular proliferation, apoptosis (Benyo and Pate 1992), inflammation and steroidogenesis (Wu et al. 2004), and are therefore essential accessory cells for optimal fertility (Brännström et al. 1994; Cohen et al. 1999; Norman and Brannstrom 1994). Macrophages and/or macrophage-derived product deregulation are implicated in a number of ovarian pathologies (Moller 2000) including polycystic ovary syndrome (Gazvani et al. 2000; Ma et al. 2010; Qiao and Feng 2011), endometriosis (Carlberg et al. 2000) and premature ovarian failure (Wu et al. 2004). Physiological changes which could lead to perturbations in the profile of immune cells and/or pro-inflammatory cytokines in, or in close proximity to, the female reproductive tract could potentially have profound effects on ovarian function.

Obesity is an independent risk factor for a range of metabolic complications including reproductive disorders (Malnick and Knobler 2006; Rachoń and Teede 2010; Raj and Kumar 2010; Teede et al. 2010). The exact mechanism(s) by which obesity affects ovarian function remain poorly understood. Low-grade systemic inflammation has been implicated in the development of infertility and other obesity-associated adverse reproductive health outcomes (Blencowe et al. 2012; Carmichael et al. 2010;
Inflammation originating from intra-abdominal adipose tissue contributes significantly to systemic inflammation and is due in large part to the pro-inflammatory actions of bone marrow-derived immune cells that infiltrate obese adipose tissue and selectively localize to dead adipocytes forming a crown-like structure (CLS) (Cinti et al. 2005; Strissel et al. 2007; Weisberg et al. 2003a). CLS’s are predominantly found in intra-abdominal fat depots, whereas subcutaneous adipose tissue appears to be protected from inflammatory increases (Strissel et al. 2007); however, these depot-specific differences in the distribution of immune cells and inflammatory factors have yet to be investigated in peri-ovarian adipose tissue.

Chronic inflammation has also been reported to alter MicroRNA (miRNA) levels. Aggravated activation of inflammatory cytokines has been associated with detrimental deposition of excess lipid in extra-ovarian tissues (Cummings and Schwartz 2003; Guilherme et al. 2008; Gustafson et al. 2009), generation of reactive oxygen species and oxidative stress scenarios both of which could have detrimental consequences for human fertility (Jabbour et al. 2009). In addition, several studies have reported that a variety of cytokines can regulate ovarian function (Adashi 1990; Andreani et al. 1991; Salmassi et al. 2001; Spangelo et al. 1995). Interleukin-6 (Il6) is a pleiotropic cytokine with diverse roles including regulation of ovarian steroid production and ovulation (Mikuni 1995; Van der Hoek et al. 1998). Nitric oxide synthase 2 (Nos2) is an oxidative stress marker
involved in a number of biological processes including insulin signal transduction, fertility, embryogenesis, and fetal development (Agarwal et al. 2005a; Agarwal et al. 2005b; Agarwal et al. 2006; Osborn et al. 2002; Rosselli et al. 1998). Tumor necrosis factor-alpha (Tnfa) is a pleiotropic cytokine that acts on multiple tissues via two distinct membrane receptors (a 55-kDa isoform (p55) and a 75-kDa isoform (p75)) that activate both unique and synergistic responses (Crespo et al. 2010; Erickson et al. 1994; Sakumoto and Okuda 2004; Schreyer et al. 1998; Tartaglia et al. 1991). In the ovary, TNFa has been reported to regulate cell differentiation, proliferation and apoptosis (Amsterdam et al. 2003a; Amsterdam et al. 2003b; Roby and Terranova 1990), inflammation (Roby et al. 1990) as well as steroid production (Amsterdam et al. 2003a). The nuclear factor kappa B (RelA) transcription factor is a heterodimer protein composed of two subunits; p50 and p65, which can be induced by TNFa (Dhanalakshmi et al., 2002) and which can activate inflammatory components such as IL6 (Albertini et al., 2012). RELA is maintained in an inactive state in the cytoplasm through interaction with members of the IkB kinase (IKK), until IKK phosphorylation results in ubiquitination of IKK and subsequent release of RELA by the action of CHUK and/or IKBKB (Mercurio et al., 1999).

In recent years, miRNAs have been shown to regulate the activity of key cellular processes, including insulin release in pancreatic β cells, adipocyte differentiation (Williams and Mitchell 2012; Xie et al. 2009), and insulin sensitivity (Trajkovski et al. 2011) and are therefore thought to contribute to the pathologies linked with exacerbated inflammatory cytokines. For instance, miR125b negatively regulates Tnfa mRNA
(Huang et al. 2012) whereas increased Tnfa itself down-regulates miR143 (Xie et al. 2009).

In this study, we examined the impact of high fat diet (HFD)-induced obesity on peri-ovarian adipose tissue morphology and mRNA expression of immune cell markers and inflammation therein. Relative mRNA expression of key inflammatory mediators and miRNAs were also investigated in ovarian tissue. We hypothesized that HFD-induced obesity results in elevated immune cell infiltration and expression of inflammatory cytokine genes in these two tissues and contribute to obesity-induced reproductive dysfunction.

RESULTS

**Obesity increases adipocyte size and relative gene expression of markers of immune cells and inflammation in the peri-ovarian fat pad.**

Consistent with reported changes in the epididymal adipose tissue of obese male mice (Strissel et al. 2007; Weisberg et al. 2003b), the peri-ovarian adipose tissue of obese female mice was characterized by increased adipocyte size and elevations in markers of immune cells and pro-inflammatory cytokines. Average adipocyte size within obese peri-ovarian adipose tissue was more than twice that of the lean adipose tissue (Figure 1A). A frequency distribution graph of adipocyte size shows that this increase was associated with a reduction in the proportion of small adipocytes and a distribution pattern that was more Gaussian in appearance (Figure 1B). An increase in
adipocyte size is typically associated with an increased presence of immune cells in adipose tissue. CLS’s are clusters of immune cells that localize to dead adipocytes in adipose tissue and are the predominant source of inflammatory cytokines in obese adipose tissue (Cinti et al. 2005; Lumeng et al. 2007a; Lumeng et al. 2007b; Nguyen et al. 2007; Strissel et al. 2007). The frequency of CLS in obese peri-ovarian adipose tissue was increased dramatically as compared to lean peri-ovarian adipose tissue (Figure 2). This increase in CLS abundance was associated with an increase in the relative gene expression of T cell (Cd3, Cd4 and Cd8), macrophage (F4/80) and dendritic cell (Itgax) markers in obese peri-ovarian adipose tissue (Figure 3A). Consistent with an increased infiltration of immune cells, gene expression of pro-inflammatory cytokines (Il1b, Il6, Tnfa) and chemokines (Ccl2 and Ccl5) were also increased in obese peri-ovarian adipose tissue (Figure 3B).

**Obesity increases mRNA levels of genes encoding inflammatory cytokines and chemokines in ovarian tissue**

In a similar manner to the peri-ovarian fat depot, obesity increased ovarian mRNA encoding Il1b, Il6, Tnfa, the TNFa receptor genes p55 and p75, and the chemokine Ccl2 ($P < 0.05$; Figure 4A). Also, a tendency for increased (0.6-fold increase; $P = 0.06$) Nos2 mRNA expression was observed in ovaries from obese females compared to their lean littermates (Figure 4). Furthermore, obesity increased mRNA levels of RelA, a downstream target of TNFa signaling, and increased Ikbkb (Figure 4). No impact of obesity on Chuk was observed. Further, in agreement with the changes
observed in the TNFa receptor genes, RELA tended to be increased (25% increase; \( P = 0.051 \)) in the obese ovaries, relative to those from lean females (Figure 5).

**Effect of obesity on ovarian levels of miR125b and miR143**

Since *Tnfa* mRNA was increased we next examined the effect of obesity on a microRNA known to negatively regulate *Tnfa* expression, miR125b (Huang et al., 2012). Also, we determined if a microRNA that is regulated by TNFa, miR143 (Xie et al. 2009), was altered by obesity. There was a trend for decreased (\( P = 0.1 \)) levels of both miR125b (74% decrease) and miR143 (96% decrease) in ovaries from obese females compared to lean littermates (Figure 7).

**DISCUSSION**

Obesity, activation of pro-inflammatory macrophages and compromised reproductive function are associated pathologies (Ferrante 2007; Hotamisligil 2006; Wellen and Hotamisligil 2003). In a number of obesity models, local and systemic markers of inflammation and oxidative stress are increased coincident with an increased presence of immune cells (Hofmann et al. 1994; Hotamisligil et al. 1995; Hotamisligil et al. 1993; Kern 1997; Kern et al. 1995; Nickelson et al. 2012; Saghizadeh et al. 1996). However, there is currently limited information regarding the effects of obesity on the inflammatory state of the peri-ovarian adipose tissue and the ovary it surrounds. Understanding potential changes in these tissues is important as exacerbated activation of these inflammatory pathways in the ovary could alter ovarian physiology (Carlberg et
In the present study we therefore determined the impact of HFD-induced obesity on the expression profiles of select pro-inflammatory cytokines in the murine peri-ovarian fat pad and ovarian tissue.

Obese female mice had increased adipocyte size in the peri-ovarian fat pad compared to the lean littermates. Additionally, there was an increased presence of immune cells in peri-ovarian adipose tissue as evidenced by an increase in the number of CLS and the mRNA expression of immune cell markers. Consistent with increased cell size and immune cell infiltration, relative mRNA expression of inflammatory markers were also elevated in peri-ovarian adipose tissue. These data are the first, as far as the Authors are aware, to demonstrate that hypertrophic expansion of obese peri-ovarian adipose tissue is associated with CLS formation and inflammation in obese female mice. While the observed changes are consistent with reported changes in other intra-abdominal adipose tissue depots of obese male and female mice (Strissel 2007 and Nickelson et al. 2012), this confirmation is important given the proximity of the adipose tissue to the ovary and the ability of estrogen to regulate adipose tissue expansion and inflammation (Heine et al. 2000; Stubbins et al. 2012). Although humans and mice differ in the deposition of fat around the ovary, inflammatory cytokines produced in intra-abdominal adipose tissue in humans could potentially impact the ovary due to the proximity of both tissues.

Although Il6 mRNA levels were unchanged with obesity in peri-ovarian adipose tissue, there were elevated Il6 mRNA levels in the ovaries of obese mice compared to
lean mice. Elevated levels of IL6 have been reported in patients with ovarian hyperstimulation syndrome (Buyalos et al. 1992; Loret de Mola et al. 1996a; Loret de Mola et al. 1996b), epithelial ovarian cancer (Lane et al. 2011) as well as other forms of ovarian cancer (Costanzo et al. 2005; Nilsson et al. 2007). IL6, together with TNFα and IL1β, has also been reported to be part of the cytokine network implicated in ovarian cancer (Anglesio et al. 2011; Coward et al. 2011; Kulbe et al. 2007). Additionally, IL6 activation has been associated with enhanced tumor cell activation (Chou et al. 2005), survival, and increased resistance to chemotherapy (Duan et al. 2006). Thus, aberrant expression of Il6 caused by HFD-induced obesity as observed in our study may have negative consequences for ovarian function.

In addition to elevated Tnfa in the peri-ovarian fat depot, obese mice had elevated ovarian mRNA levels of Tnfa and both TNFα receptors as compared with lean mice. These findings are in agreement with other studies which reported obesity-induced increased Tnfa mRNA or protein expression in human adipose and muscle tissues (Hotamisligil et al. 1995; Kern et al. 1995; Saghizadeh et al. 1996), rat adipose tissue (Hotamisligil et al. 1993) and mouse muscle and adipose tissue (Hofmann et al. 1994). Whether the temporal pattern of Tnfa induction differs between the tissues measured remains unclear from the current study. Further, RELA protein level was increased in the obese ovaries, relative to lean controls, confirming that the TNFα pathway was activated by obesity, and the Ikbkb gene was increased in obese ovaries but not the Chuk, supporting that Ikbkb is involved in RELA activation in obese ovaries.
Aggravated oxidative stress in the ovary could adversely affect ovarian function (Agarwal et al. 2005b; Hefler and Gregg 2002; Pierce et al. 2004; Szczepańska et al. 2003) and hence compromise the female reproductive potential. We observed a strong trend for increased $\text{Nos2}$ mRNA in association with elevated $\text{Tnfa}$ mRNA levels in ovaries of obese mice compared to their lean littermates, potentially indicating increased macrophage infiltration to the ovary. These data are in agreement with previous studies which showed that $\text{Nos2}$ expression is elevated in both genetic and diet-induced models of obesity and is activated in response to pro-inflammatory cytokines including TNFa (Agarwal et al. 2005b; Lee et al. 2000; Perreault and Marette 2001; Rosselli et al. 1998).

Given their roles in regulation of the activity of key cellular processes; including insulin release in pancreatic $\beta$ cells, differentiation of adipocytes (Williams and Mitchell 2012; Xie et al. 2009), and insulin sensitivity (Trajkovski et al. 2011), miRNA’s may contribute to the pathologies associated with exacerbated inflammatory cytokines (Huang et al. 2012; Sonkoly and Pivarcsi 2009). The observed elevation of $\text{Tnfa}$ expression in the obese ovary led us to query if obesity modulated the expression of miR125b; which is known to negatively regulate $\text{Tnfa}$ mRNA (Huang et al. 2012), and miR143 whose expression is suppressed by elevated TNFa (Xie et al. 2009). Decreased circulating miR125b associated with increased fat mass has been reported in morbidly obese men (Ortega et al. 2013). Also, miR143 is reported to be critical for formation of the primordial follicle pool in utero (Zhang et al. 2013), raising concerns about the
impact of obesity on the neonatal ovary. While our data cannot confirm a direct involvement of miR125b and miR143 with the TNFα pathway, taken together with the mRNA and protein changes observed, these data support that miR125b and miR143 may be involved in physiological alterations that occur in ovarian tissue during obesity.

It is worth noting that a thorough evaluation of the reproductive status of lean and obese females was not performed in this study however plasma estradiol concentrations were not different between the age-matched groups of mice (Nickelson et al. 2012). Whole ovary samples were used to determine if broad changes in the inflammatory profile of the organ were occurring during obesity, and our lack of controlling for cycle stage may certainly have introduced additional variation to the ovarian data resulting in an underestimation of the significance of the data reported. However, future studies that control for stage of estrous cycle would shed light on the impact of changing hormone profiles on the ovarian inflammatory profile during obesity.

In summary, HFD-induced obesity resulted in increased adipocyte size, immune cell infiltration and increased mRNA expression of inflammatory markers in peri-ovarian adipose tissue. Changes in peri-ovarian adipose tissue were associated with increased mRNA levels of pro-inflammatory cytokines, TNFα, RELA, Il6 and the oxidative stress marker, Nos2 in the ovaries of obese mice. While the current study established that obesity is associated with inflammatory changes in the ovary, future studies will be required to address the cellular origin and cause-effect relationship
between observed changes in peri-ovarian adipose tissue and the ovary and the implications of these changes on ovarian function. Improved understanding of the mechanisms that are involved in the onset and progression of obesity-associated changes in the ovary will be of value in order to develop improved strategies to mitigate reproductive dysfunction in obese females.

**MATERIALS AND METHODS**

**Reagents**

2-β-mercaptoethanol was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Hanks' Balanced Salt Solution (without CaCl2, MgCl2, or MgSO4) and superscript III one-step RT-PCR System were obtained from Invitrogen Co. (Carlsbad, CA). miRNeasy Mini Kit, miScrip Reverse Transcription Kit, miScrip SYBR Green PCR Kit, RNeasy Mini kit, QIAshredder kit, RNeasy MinElute kit, TaqMan® microRNA Reverse Transcription Kit and Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). RELA primary antibody was purchased from Cell signaling (Danvers, MA). Custom designed primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University.

**Animal and diets**

Ovarian tissues were obtained from a study at the University of Missouri. The experimental protocols were approved and performed in accordance with the guidelines of the University of Missouri Institutional Animal Care and Use committee as previously
described (Nickelson et al. 2012). Briefly, 6 weeks old C57Bl/6J female mice were randomized into two groups. The control group (lean) was fed a standard chow mice diet (Purina 5001; 4.5g/100g fat) while the treatment group (obese) was fed a high-fat diet (Research Diets D12492; 35g/100g fat) for approximately 7 months. The animals were housed at constant room temperature, 12h light:12h darkness cycle, diet and water were provided ad libitum. Body weights were measured weekly and a glucose tolerance testing confirmed that obese mice were less glucose tolerant than their lean littermates (Nickelson et al. 2012).

**Tissue collection**

Animals were euthanized by CO$_2$ asphyxiation followed by exsanguination via cardiac puncture. Ovaries and the surrounding peri-ovarian adipose tissue were removed and separated from each other. Both the ovaries and peri-ovarian adipose tissue were snap frozen for gene expression analysis or fixed in 4% paraformaldehyde for histological analysis.

**RNA Isolation**

Total RNA was isolated from ovaries using the Qiagen RNeasy® Mini Kit and from peri-ovarian adipose tissue using the Qiagen RNeasy® Lipid Mini Kit. Briefly, tissues were lysed and homogenized using a hand held homogenizer. The homogenate was then applied to a QIAshredder column placed in a collection tube and centrifuged at 16100 RCF for 2 minutes at room temperature. The flow through was then applied to an
RNeasy Mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter and concentrated using an RNeasy MinElute kit. RNA was eluted using RNase-free water and quantified using an ND-1000 Spectrophotometer ($\lambda = 260/280$ nm; NanoDrop technologies, Inc., Wilmington, DE).

**First Strand cDNA synthesis and quantitative Real-Time quantitative polymerase chain reaction (qRT-PCR)**

0.5µg of total RNA was reverse transcribed to cDNA using Invitrogen™ Superscript III Reverse Transcriptase according to the manufacturer’s protocol. 2 µl of diluted cDNA (1:25) were amplified on an Eppendorf Mastercycler using Quantitect™ SYBR Green PCR kit and primers specific for mouse Actb, Rps3, Itgax, Cd3, Cd4, Cd8, Emr1, Il6, Tnfa, Nos2, Il1B, Mcp1 and Ccl5. The PCR cycling program consisted of a 15 min hold at 95 °C and 40 cycles of: denaturing for 15 at 95 °C, annealing for 15s at 58 °C and extension at 72 °C for 20s. Product melt conditions were determined using a temperature gradient from 72 °C to 99 °C with a 1 °C increase at each step. Three replicates of each sample (n = 6) were included. The relative mRNA expression for each of the above genes was normalized using mouse housekeeping genes Actb (ovary) and Rps3 (peri-ovarian adipose tissue) and relative fold change calculated using the $2^{-\Delta\Delta CT}$ method. The results are presented as mean fold change ± standard error relative to the lean control group.
mirScript miRNA PCR array

Purification of miRNA-enriched fractions was performed using a miRNeasy Mini Kit according to manufacturer’s protocol. 250 ng of miRNA-enriched fractions in a reverse-transcription reaction of 20 µl were converted to cDNA using miScrip Reverse Transcription Kit. cDNA was then diluted by adding 200 µl of RNase-free water, from which, 100 µl was used as template cDNA for real-time PCR quantification of multiple microRNAs using miScrip SYBR Green PCR Kit. Data analysis was performed using the web-based miScript miRNA PCR Array Data Analysis package.

Protein Isolation and Immunoblot analysis

Total ovarian protein was isolated and immunoblots performed as follows. Briefly, ovaries were homogenized in ~ 200 µl of iced-cold extraction buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP40 and 1% Protease inhibitor, followed by centrifugation for 30 minutes at 9300 RCF at 4 °C. Protein concentration was determined using a standard BCA protocol. Emission absorbance values were detected with a λ = 560 nm excitation on a Synergy™ HT Multi-Detection Microplate Reader using KC4™ software (Bio-Tek Instruments Inc. Winooski, VT). Total protein (20 µg) was separated using 10% SDS-PAGE and electro-transfer of proteins from the gel to a nitrocellulose membrane was performed for 60 minutes at 100 V. The membranes were stained with Ponceau S to visualize the amount of total protein transferred in each lane. To reduce non-specific binding, membranes were pre-incubated overnight on a rocker at 4°C in a blocking buffer (5% non-fat dry milk, 5 M NaCl, 20 mM Tris-HCl, 0.15%
Tween-20, pH 8). Membranes were probed using a specific primary antibody against RELA (1:500 dilution) diluted in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TTBS) overnight at 4 °C. Following washing for three times (10 min each) in TTBS, membranes were incubated at room temperature for 1 hour with HRP-conjugated suitable secondary antibodies (1:2000) against the primary antibodies. Membranes were washed three times in TTBS followed by a single wash in Tris-Buffered Saline (TBS). Autoradiograms were visualized on X-ray films in a dark room following 5 min incubation of membranes in ECLplus chemiluminescence detecting reagent. Densitometry of the appropriate band was quantified using Image J software (NCBI). Equal loading was confirmed by Ponceau S staining of membranes and protein expression was normalized to ACTB densitometry values.

**Histological analysis of peri-ovarian adipose tissue**

A portion of the peri-ovarian adipose tissue was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Digital images were acquired with an Olympus BX51 light microscope using an Olympus DP70 camera. For each animal, images of three distinct regions within two or three sequential histologic sections were used to determine frequency of crown-like structures (CLSs) and cell size. All CLS and adipocytes within an image were counted and the percentage of CLS was calculated as (number CLS/number total adipocytes) x 100. Adipocyte size (volume) was determined using the cross-sectional area obtained from perimeter tracings using Image J software (Sun Microsystems, Santa Clara, CA, USA). Frequency
distribution for adipocyte size was calculated as (number of cells within a specific size range/total adipocytes) x 100.

**Statistical analysis**

Statistical analysis was performed using the unpaired t-test function of GraphPad Prism 5.5 software with a statistical significance level set at $P < 0.05$. A trend for a biological change was considered if the $P$-value was below 0.1.

**ACKNOWLEDGEMENTS**

The project described was supported by the National Institutes of Environmental Health Sciences [R00ES016818] to A.F.K. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
REFERENCES


Loret de Mola JR, Flores JP, Baumgardner GP, Goldfarb JM, Gindlesperger V, Friedlander MA. 1996b. Elevated interleukin-6 levels in the ovarian hyperstimulation


Table 1. Primer Sequences

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnfa</td>
<td>CCAGACCCCTCACACTAGATCA</td>
<td>CACTTGGTGTTTGGCTACGAC</td>
</tr>
<tr>
<td>p55</td>
<td>AGCCACACCCACAAACCTTAG</td>
<td>ATCTCCCTGCCCTGCCACTCAAG</td>
</tr>
<tr>
<td>p75</td>
<td>ATGCTAGGCAAACGCAATC</td>
<td>GGAACCTGCTGGGGATTC</td>
</tr>
<tr>
<td>Ikbkb</td>
<td>AGGCTGTCTGCTTTGGACTC</td>
<td>CATCGCCATCACTACGACAT</td>
</tr>
<tr>
<td>Chuk</td>
<td>TTAGGGAACTGCTACGTT</td>
<td>GCACCATCGCTGCCTGTTT</td>
</tr>
<tr>
<td>Rela</td>
<td>GCTACCTGTGAGTTCTCCAT</td>
<td>TAGGTCTTTTCGGCTTCTC</td>
</tr>
<tr>
<td>Il1b</td>
<td>TCACAGCAGCACATCAACAA</td>
<td>TGTCCTCATCTGGGAAGGTC</td>
</tr>
<tr>
<td>Il6</td>
<td>CAGAGATACAAAGATGCAATC</td>
<td>GGAACTGCTGCTTTGGAGTC</td>
</tr>
<tr>
<td>Nos2</td>
<td>AATCTTGGAGCGAGTTTGGTGG</td>
<td>CAGGAAGTGGAGGGCTTGG</td>
</tr>
<tr>
<td>Cd3</td>
<td>TGGTCATGTTCGAGGGCA</td>
<td>CCTCGGTGTGGCCACTT</td>
</tr>
<tr>
<td>Cd4</td>
<td>CAAGGGCTAAACAGAGATGG</td>
<td>CACTTGGCAGAAGGACAG</td>
</tr>
<tr>
<td>Cd8</td>
<td>ACGGGCATTGCTCCCTTCTT</td>
<td>ACAGGAGCAAGCTGACTG</td>
</tr>
<tr>
<td>Emr1</td>
<td>CTTGGCTATGGGCTCCAGTC</td>
<td>GCAAGGAGCAGAGCTTATGTG</td>
</tr>
<tr>
<td>Ccl2</td>
<td>ACTGAGCCAGCCTGCTCCCTTCCCT</td>
<td>TCCCTGTGGGGTGTCAGCAGAC</td>
</tr>
<tr>
<td>Ccl5</td>
<td>CAGCAAGCAAGTGCTCACAATCTT</td>
<td>TTCTTGAACCCACTTCCCTCCTG</td>
</tr>
<tr>
<td>Itgax</td>
<td>CTGGATAGCCCTTCTTCTTCCTG</td>
<td>GCACACTTGGTCGAACTG</td>
</tr>
</tbody>
</table>
Figure 1. Obesity increases adipocyte size in the peri-ovarian fat pad in mice. The peri-ovarian fat pad was removed from lean or obese C57Bl/6J female mice. H&E stains of lean and obese peri-ovarian adipose tissue were used to determine average adipocyte area (A) and the overall size distribution of adipocytes (B) from both lean and obese peri-ovarian adipose tissue. Data are reported as mean ± SE; n = 6 per group; *P < 0.05.
Figure 2. Obesity increases the presence of crown-like structures in peri-ovarian adipose tissue. Representative H&E stains of peri-ovarian adipose tissue from lean (A) and obese (B) female mice. Sections were used to quantify the presence of crown-like structures (C). Arrows identify CLSs in the adipose tissue; scalebar = 100 µm. Data are reported as mean ± SE; n = 6 per group. *P < 0.05.
Figure 3. Obesity increases mRNA expression of immune cell and pro-inflammatory markers in peri-ovarian adipose tissue. The peri-ovarian adipose tissue was removed from lean or obese C57Bl/6J female mice. Total RNA was isolated and qRT-PCR performed. Relative mRNA expression of markers for immune cell infiltration (A) and inflammation (B) were determined. Data are reported as mean ± SE; n = 6 per group; Values represent fold-change relative to a control value of 1 ± SE, normalized to Rps3, *P < 0.05.
Figure 4. **Obesity up-regulates pro-inflammatory cytokine mRNA expression in ovarian tissue.** Ovaries were removed from lean or obese C57Bl/6J female mice. Total RNA was isolated, followed by quantification of *Il1b*, *Il6*, *Nos2*, *Tnfa*, TNFα receptors *p75* and *p55*, *Ccl2*, *Chuk*, *Ikbkb* and *RelA* mRNA levels by quantitative RT-PCR. Data are reported as mean ± SE; n = 6 per group; Values represent fold-change relative to a control value of 1 ± SE, normalized to *Actb*, *P* < 0.05.
Figure 5. Obesity increases the downstream target of TNFα, RELA. Ovaries were removed from lean (L) or obese (O) C57Bl/6J female mice. Total protein was isolated, followed by quantification of RELA by (A) Western blotting. (B) Data are reported as densitometric mean, normalized to ACTB ± SE; n = 4 per group; Difference from control are indicated by *P < 0.05.
Figure 6. Obesity down-regulates levels of miR125b and miR143 associated with inflammatory cytokine signaling in the ovary. Ovaries were removed from lean or obese C57Bl/6J female mice. Enriched miR fractions were analyzed using a miR array. Data are reported as mean; n = 6 per group; Values represent fold-change relative to a control value of 1; †P ≤ 0.1.
CHAPTER 4: PROGRESSIVE OBESITY ALTERS OVARIAN PRO-INFLAMMATORY AND STEROIDOGENIC SIGNALING

A paper to be submitted to *American Journal of Physiology* - *Endocrinology and Metabolism*

Nteeba, J., Ganesan, S., Keating, A.F.

**Contribution Statement:**
I performed the animal studies, all analyses on ovarian tissue, designed the experiments, interpreted data and wrote the paper. Ganesan, S. aided in glucose tolerance testing and tissue collection; Keating, A.F. designed experiments, aided in data interpretation and edited the manuscript.

**Abstract**

Diet-induced obesity induces immune cell infiltration and inflammation in peri-ovarian adipose tissue and mRNA expression of inflammatory markers in ovarian tissue. Whether these changes are associated with the onset or progression of obesity remains unknown. In the present study, qRT-PCR and Western blot techniques were utilized to compare ovarian mRNA and protein expression of ovarian immune cell and inflammation markers, along with NF-κB and steroidogenic pathway members in normal wild type non-agouti (a/a; lean) and lethal yellow mice (KK.CG-A^yJ; obese) at 6, 12, 18 or 24 weeks of age. Our data revealed that, beginning at 12 weeks of age, NFκB inflammatory signaling members were elevated \((P < 0.05)\) in obese females. Interestingly obesity had contrasting effects on steroidogenic members. Obesity decreased \((P < 0.05)\) STAR protein at 12, 18 and 24 weeks of age. CYP11A1 and CYP19A1 proteins were increased \((P < 0.05)\) at 12 weeks but decreased \((P < 0.05)\) at 18
and 24 weeks. Interestingly, CYP19A1 was increased in obese mouse ovaries at 6 weeks of age, potentially indicating early puberty onset. These data demonstrate that obesity alters expression of ovarian inflammatory and steroidogenic pathway genes in ways which could adversely affect ovarian function.
Introduction

The mammalian ovary is essential for production of oocytes and the steroid hormones, 17β-estradiol (E2) and progesterone (P4). E2 and P4 are essential for ovarian function and development of female secondary sex characteristics (45, 65, 91, 107, 125). Circulating lipoproteins (54) and de novo biosynthesis (83) provide the steroid hormone precursor, cholesterol, to the theca (TC) and granulosa (GC) cells under the influence of luteinizing hormone (LH) and follicle stimulating hormone (FSH), respectively (65, 91, 105, 107). Steroidogenic acute regulatory protein (STAR), mediates the transfer of cholesterol from the outer to the inner mitochondrial membranes of the TC or GC, where cytochrome P450 side chain cleavage (CYP11A1) catalyzes its conversion to pregnenolone (55, 77, 89, 90, 118). Pregnenolone then exits the mitochondrion matrix to the cytoplasm where 3-beta-hydroxysteroid dehydrogenase (3β-HSD) converts it to P4 (5, 76). The GC lack critical enzymes (17α-Hydroxylase and C17,20-lyase) for androgen production, thus, P4 diffuses to the TC where conversion to androstenedione under the action of 17α-Hydroxylase and C17,20-lyase enzymes occurs (13, 55, 65, 119, 125). Likewise, the TC lack CYP19A1 (aromatase), required for conversation of androgens to estrogen. Therefore, androstenedione will diffuse into the GC where CYP19A1 catalyzes its’ conversion to estrone and subsequently E2 by the action of 17β-hydroxysteroid dehydrogenase (17β-HSD). Alternatively, androstenedione can be converted to testosterone by 17β-HSD which diffuses to the GC to be aromatized to E2 by CYP19A1 (55, 65, 76).
Obesity is a complex metabolic disorder associated with reproductive impairments (99, 102, 104, 109, 123). The deleterious effects of obesity on reproduction including polycystic ovarian syndrome (PCOS), poor oocyte quality, abridged fertility (21, 109) and increased risk of birth defects and still-birth (17, 29, 53, 111) have been long appreciated, however, the underlying molecular mechanisms involved are unclear. Obesity is linked with low grade systematic inflammation which is implicated in infertility (93, 99, 103). Additionally, obesity alters ovarian steroid hormone synthesis and metabolism (104) potentially by modifying the expression and activity of steroidogenic enzymes. Macrophages and inflammatory pathways can serve as modulators of ovarian function (1, 2, 9, 19, 24, 37, 64, 95, 128). The chemoattractants interleukin 8 (IL-8) and chemokine (C-C motif) ligand 2 (CCL2), are major inflammatory response mediators, and are involved in ovarian follicular development, steroidogenesis, ovulation and atresia (19). Furthermore, interleukin 6 (IL-6), through its ability to modulate cyclic AMP (cAMP) (19), potentially also regulates ovarian steroid production. Similarly, tumor necrosis factor and interleukin 1 (IL-1) can stimulate ovarian P4 production (19). Additionally, the pro-inflammatory cytokines TNFα and interleukins have been implicated in a number of ovarian pathologies (64, 82, 88) including polycystic ovarian syndrome (108), ovarian hyperstimulation (79, 80), ovarian cancer (8, 33, 72, 74), endometriosis (28) and impaired steroid production (9, 11, 128). Therefore it is possible that derangements in ovarian proinflammatory cytokine cues could compromise ovarian function and consequently lead to suboptimal fertility.
The lethal yellow mouse has a deletion mutation in the normal wild type non-agouti (a/a) background which results in ectopic expression of agouti in various tissues including the hypothalamus and adipose tissue (30, 44, 71, 87). Hypothalamic agouti overexpression interferes with the alpha-melanocyte-stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART) by inhibiting melanocyte stimulating hormone (MSH) receptor (20, 81), leading to hyperphagia (71, 81). Hence this serves as a useful model for understanding the effects of progressive obesity on ovarian function. The main objective of the present study was to determine the effects of obesity onset and progression on expression of ovarian pro-inflammatory and steroidogenesis pathway members in mice. To achieve this objective, changes in gene expression between the normal wild type non-agouti -a/a (lean) and lethal yellow KK.CG-Ay/J (obese), mice at 6, 12, 18 and 24 weeks of age were compared using qRT-PCR and Western blotting techniques.

Materials and Methods

Reagents

Custom designed primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University. D-Glucose, 2-β-mercaptoethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulfate, glycerol, N,N',N',N'-Tetramethylethylenediamine (TEMED), Tris base, Tris HCl, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (Vitamin C), phosphatase inhibitor, protease inhibitor and transferrin were purchased from Sigma-Aldrich Inc. (St. Louis,
MO, USA). Bayer Contour Blood Glucose meter and glucose strips (Bayer Ag, Leverkusen, Germany). RNALater was obtained from Ambion Inc (Austin, TX, USA). Hanks’ Balanced Salt Solution (without CaCl₂, MgCl₂, or MgSO₄) and superscript III one-step RT-PCR System were obtained from Invitrogen Co. (Carlsbad, CA, USA). RNaseasy Mini kit, QIAshredder kit, RNaseasy MinElute kit and Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA, USA). Ponceau S was purchased from Fisher Scientific (Waltham, MA, USA). Goat anti-rabbit and rabbit anti-mouse secondary antibodies along with anti-STAR, anti-CYP11A1, anti-CYP19A1, anti-pIkBαSer32/36, anti-pIKKa/βSer176/180, anti-pNFκBp65Ser536 primary antibodies and SignalFire™ ECL Reagent were from Cell Signaling Technology® (Danvers, MA, USA). The anti-3β-HSD antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Donkey anti-goat secondary antibody was purchased from Pierce Biotechnology (Rockford, IL, USA). Restore™ PLUS Western Blot Stripping Buffer was purchased from Thermo SCIENTIFIC (Rockford, IL, USA).

Animal procedures

All experimental protocols and procedures were approved by the Iowa State University Animal Care Committee (IACUC). At 4 weeks of age, female normal wild type non-agouti (a/a; designated lean; n = 20) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 20) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and housed at the animal facility at Iowa State University under controlled room temperature (21-22°C) and lighting (12h light:12h darkness cycle). All
animals had *ad libitum* access to feed and water. Body weight was measured once weekly. Glucose tolerance testing followed by euthanasia and ovary collection was performed at 6, 12, 18 or 24 weeks of age (n = 5 per group).

*Estrous cyclicity monitoring*

To determine any changes in the estrous cycle, fresh, wet unstained vaginal smears were examined daily using a Leica DMI300B Fluorescent Microscope for 14 consecutive days prior to the end of each time point (at 12, 18 and 24 weeks). Classification of estrous cycle stages was based on the presence or absence of leucocytes, nucleated epithelial, and cornified epithelial cells as previously described in (26). Briefly, a stage was classified as proestrus if the vaginal smears were characterized with mostly small, round nucleated epithelial cells, some cornified epithelial cells and none to a few leucocytes. Estrus was when vaginal smears were characterized by the presence of numerous large cornified cells with degenerate nuclei. During metestrus vaginal smears were primarily characterized by the presence of many leucocytes and a few cornified cells. In diestrus, vaginal smears were largely populated by many polymorphonuclear leucocytes and few nucleated epithelial cells.

*Glucose tolerance testing*

At the end of each experimental time point (6, 12, 18 and 24 weeks of age; n = 4 per treatment per time point), a glucose tolerance test was performed. Mice were weighed and placed in separate cages with access to water but not feed for 16 hours
Glucose solution (0.25 g/ml; 2.5 g of D-glucose in 10 ml milliQ water) was prepared the day before the test and left to stand at room temperature overnight. Blood from the lateral saphenous vein was collected to determine fasting blood glucose (at time designated 0 min) followed by intraperitoneal injection (1g/kg body weight) of glucose solution. Blood from the lateral saphenous vein was used to measure blood glucose concentration at 30, 60, 90, and 120 min after the injection using a hand held glucometer.

**Tissue collection**

At the end of each experimental time point (6, 12, 18 and 24 weeks of age; n = 4 per treatment per time point), mice were euthanized by CO\textsubscript{2} asphyxiation. Ovaries were collected, trimmed of excess fat and weighed. One ovary was fixed in 4% paraformaldehyde for histology and follicle counts while the other ovary was stored in RNALater at -80\(^\circ\)C for RNA and protein expression studies.

**RNA isolation**

Total RNA was isolated from one half of each sample ovary using the Qiagen RNeasy\textsuperscript{®} Mini Kit (n = 4 per treatment per time point) according to the manufacturer’s procedure. Briefly, tissues were lysed and homogenized using a hand held homogenizer. The homogenate was then applied to a QIAshredder column placed in a collection tube and centrifuged at 10000 rpm for 2 minutes at room temperature. The flow through was then applied to an RNeasy Mini column, allowing RNA to bind to the filter cartridge.
Following washing, RNA was eluted from the filter using RNase-free water and concentrated using an RNeasy MinElute kit. The concentration was determined using an ND-1000 Spectrophotometer ($\lambda = 260/280$ nm; NanoDrop technologies, Inc., Wilmington, DE).

*First strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)*

Equal amount (0.5µg) of total RNA was reverse transcribed to cDNA using Invitrogen™ Superscript III Reverse Transcriptase according to the manufacturer’s protocol. Two microliters of diluted cDNA (1:25) were amplified on an Eppendorf Mastercycler using Quantitect™ SYBR Green PCR kit and primers specific for mouse *Tnfa*, *Tnfr1(p55)*, *Tnfr2(p75)*, *Il1b*, *Il6*, *Nos2*, *Cd3e*, *Cd4*, *Cd8b*, *Emr1*, *Ccl2*, *Ccl5*, *Chuk*, *Ikbkb*, *Rela* (for sequences see (97)), *Gapdh*, *Il1rn*, *Il10*, *Ikbkg*, *Nfkb1*, *Star*, *Cyp11a1*, *Cyp19a1*, *Erα*, and *Erβ* (for sequences see Table 1). The PCR cycling program consisted of a 15 min hold at 95 °C and 40 cycles of: denaturing for 15 at 95 °C, annealing for 15s at 58 °C and extension at 72 °C for 20s. Product melt conditions were determined using a temperature gradient from 72 °C to 99 °C with a 1 °C increase at each step. Three replicates of each sample (n = 4) were included. The relative mRNA expression for each of the above genes was normalized using the housekeeping gene *Gapdh* and relative fold change calculated using the $2^{-\Delta\Delta CT}$ method. The results are presented as mean fold change ± standard error relative to the lean matched control group.
Protein isolation and Western blot analysis

Total ovarian protein was isolated and Western blotting performed as previously described (97). Briefly, ovaries were homogenized in 300 µl of ice-cold extraction buffer followed by two rounds of centrifugation each for 15 minutes at 9300 RCF at 4 °C. Protein concentration was determined using a standard BCA protocol and absorbance values were detected with a λ = 560 nm excitation on a Synergy™ HT Multi-Detection Microplate Reader using KC4™ software (Bio-Tek® Instruments Inc. Winooski, VT). Total protein (25 µg) was separated using 10-12% SDS-PAGE and electro-transfer of proteins from the gel to a nitrocellulose membrane was performed for 1.2hrs at 100 V. The membranes were stained with Ponceau S to visualize the amount of total protein transferred in each lane. To reduce non-specific binding, membranes were pre-incubated for 2 hours on a rocker at room temperature in a blocking buffer containing 5% non-fat dry milk, 5 M NaCl, 20 mM Tris-HCl, 0.15% Tween-20, pH 8. Membranes were probed using a specific primary antibody against anti-STAR (1:1000), anti-CYP11A1 (1:1000), anti-3β-HSD (1:500), anti-Aromatase (1:500), anti-pIkBαSer32/36 (1:500), anti-pIKKα/βSer176/180 (1:250), anti-pNFκBp65Ser536 (1:100) diluted in 5% BSA in Tris-buffered saline with Tween-20 (TTBS) overnight at 4 °C. Following washing for three times (5 min each) in TTBS, membranes were incubated at room temperature for 1 hour with HRP-conjugated suitable secondary antibodies (1:10,000) against the primary antibodies. Membranes were washed three times in TTBS followed by a single wash in Tris-Buffered Saline (TBS). Autoradiograms were visualized on X-ray films in a dark room following 5-10 min incubation of membranes with 1X SignalFire™ ECL reagent.
Densitometry of the appropriate sized bands was measured using Carestream molecular imaging software version 5.0 (Carestream Health Inc., Rochester, NY) which eliminates background noise. Values were normalized to Ponceau S staining.

Histology and follicle counting

Ovaries were fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and processed at the Iowa State University Veterinary Medicine Histopathology laboratory. Each ovary was dehydrated and embedded in paraffin blocks. The entire ovary was serially sectioned (5 μM) and every 6th section was mounted, and stained with hematoxylin and eosin (H & E). The number of healthy follicles in every 6th section was determined using direct counts on all consecutive 5 μm sections in both lean and obese female mice using a Leica DMI300B Fluorescent Microscope. A follicle was considered healthy if it contained a distinct oocyte nucleus. A follicle was classified as a primordial follicle if its nucleated oocyte was surrounded by a partial or complete layer of squamous granulosa cells. A primary follicle contained a single layer of cuboidal granulosa cells surrounding its oocyte. A follicle whose nucleated oocyte was surrounded by multiple layers of granulosa cells was classified as a secondary follicle. A follicle was classified as a pre-ovulatory follicle if its nucleated oocyte was surrounded with at least two layers of granulosa cells and a fluid-filled antrum.
Statistical analysis

Statistical analysis was performed using the unpaired t-test function of GraphPad Prism 5.5 software with a statistical significance level set at $P < 0.05$.

Results

Increased body weight and temporal increased fasting blood glucose in the lethal yellow mice relative to their controls

There was no difference in body weight between control and lethal yellow mice at 6 weeks. From 12 weeks of age onward, a progressive increase ($P < 0.05$) in body weight was observed in the lethal yellow mice compared to their controls (Table 2), thus from this point onwards the controls are designated as lean and the lethal yellow mice are designated as obese. Increased ($P < 0.05$) fasting blood glucose was observed in obese compared to lean females from 12 weeks onward (Table 2). In addition, glucose tolerance testing revealed that relative to lean mice, obese mice had reduced clearance of glucose following two hours of a glucose bolus challenge at 12, 18, and 24 weeks (Data unpublished). There was no impact of obesity on ovarian weight across the time points of this study (Table 2).

Progressive obesity alters estrus and diestrus phases without altering overall length of the estrous cycle

To evaluate the impact of progressive obesity on estrous cyclicity, fresh, wet unstained vaginal smears obtained daily for 14 consecutive days prior to the end of each
time point (12, 18 and 24 weeks) from both lean and obese mice were examined (Table 3). There was no impact of obesity on proestrus, metestrus, or the overall length of the estrous cycle; however, at both 18 and 24 weeks, obese mice displayed a decrease ($P < 0.05$) in the length of the estrus phase and an increase ($P < 0.01$) in diestrus phase length relative to their lean counterparts (Table 3).

**Obesity decreases the number of healthy primordial and primary follicles in female mice**

The impact of obesity advancement on the number of ovarian healthy follicle populations was determined (Figure 1). Ovaries from obese mice had decreased ($P < 0.05$) numbers of healthy primordial (Figure 1A) and primary (Figure 1B) follicles at 12, 18, and 24 weeks of age as compared to ovaries from their lean matched controls. Although there was no impact of obesity on secondary follicles across time points, at 18 weeks, there was a tendency ($P = 0.07$) for an increased number of healthy secondary follicles in ovaries of obese mice relative to their lean counterparts (Figure 1C). Interestingly, obesity increased ($P < 0.05$) the number of antral follicles at 12 and 24 weeks but not at 18 weeks (Figure 1D). To rule out any impact of genotype on follicle number, ovaries examined at 6 weeks of age, when there is no difference in body weight, and no difference in any follicle type number was observed between the two strains of mice (Figure 1A-D). Interestingly, the numbers of primordial and small primary follicles were decreased in lean mice with aging (Figure 1A and B).
**Obesity alters mRNA expression of ovarian steroidogenic members in mice**

The impact of continuing obesity on mRNA expression of genes encoding enzymes involved in ovarian steroidogenesis (Figure 2) was examined. At 6 and 24 weeks, there was no difference in Star mRNA levels between ovaries from lean and obese mice, however at both 12 ($P < 0.0001$) and 18 ($P < 0.05$) weeks, obesity reduced ovarian Star mRNA abundance (Figure 2A). There was no impact of obesity on ovarian Cyp11a1 mRNA levels at any time point tested in this study (Figure 2B). We observed a decrease in Cyp19a1 mRNA levels in ovaries obtained from obese mice relative to their matched controls across all time points (Figure 2C). We further investigated the impact of progressive obesity on the two estrogen receptors (Figure 1D & E). At 6 and 12 but not at 18 or 24 weeks of age, ovaries from obese mice had increased ($P < 0.05$) Erα mRNA levels compared to their respective lean controls (Figure 2D). Furthermore, progressive obesity increased ($P \leq 0.01$) Erβ mRNA expression from 12 weeks onward (Figure 2E).

**Progressive obesity alters protein expression of ovarian steroidogenic members in mice**

No difference in STAR protein expression between the two groups was observed at 6 weeks of age, however, ovaries from obese mice had decreased STAR protein levels compared to ovaries from their matched lean controls at 12 ($P < 0.01$), 18 ($P < 0.05$), and 24 ($P < 0.01$) weeks of age (Figure 3A). While there was no difference in CYP11A1 protein expression between the two groups at 6 or 18 weeks, a decrease ($P < 0.01$) in
CYP11A1 protein levels was observed at 24 weeks of age in ovaries from obese females (Figure 3B). In contrast, at 12 weeks of age, ovaries from obese mice had increased \((P < 0.001)\) CYP11A1 protein levels compared to those from lean mice (Figure 3B). We did not find any differences between lean and obese females in 3β-HSD protein levels for all time points tested during this study (Figure 3C). Interestingly, at 6 weeks of age increased \((P < 0.05)\) ovarian CYP19A1 protein levels were observed in the obese relative to the lean group (Figure 3D). Although there was no impact of obesity on CYP19A1 protein level at 12 weeks; there was a tendency \((P < 0.1)\) for decreased CYP19A1 protein expression in the obese mice compared to their lean counterparts at 18 and 24 weeks (Figure 3D).

**Impact of progressive obesity on relative mRNA expression of ovarian immune cells, inflammation and NF-κB pathway members in mice**

The expression profiles of immune cell infiltration and inflammatory marker genes were investigated over the temporal pattern of obesity onset and advancement (Table 4). The mRNA level of \(Tnfa, p55, p75, Cd3e, Cd8eb1, F4-80, Ikbkb, Ikbkg\) and \(RelA\) were not impacted by obesity (Table 4). However, the mRNA levels of \(Nos2\), a marker for oxidative stress was increased at 6 \((P < 0.01)\), 18 \((P < 0.01)\), and 24 \((P < 0.001)\) weeks of age (Table 4). Additionally, the mRNA expression of the anti-inflammatory gene, \(Il10\), was increased \((P < 0.05)\) with obesity at both 18 and 24 weeks (Table 4). Further still, the mRNA expression of \(Il1b\) was decreased at 12 and 24 \((P < 0.05)\) weeks, but increased at 18 \((P < 0.01)\) weeks with obesity. Ovaries from obese mice
also showed a marked decrease in Il1ra mRNA at 12 (P < 0.0001), 18 (P < 0.0001), and 24 (P < 0.01) weeks compared to ovaries from lean matched mice. Cd3 mRNA was increased (P < 0.05) by obesity only at 24 weeks. At 12 weeks, obesity decreased (P < 0.01) Cd4 mRNA but at 18 weeks, Cd4 mRNA was slightly increased (P < 0.05) with obesity. Although Mcp1 (P < 0.001) and Ccl5 (P < 0.01) mRNA levels were decreased by obesity at 12 weeks, at 24 weeks only Mcp1 was decreased (P < 0.001) by obesity. Of the NF-κB pathway members tested in this study, only Ikaka (P < 0.001) and Nfkb1 (P < 0.05) mRNA levels were decreased by obesity at 18 weeks (Table 4).

**Progressive obesity increases ovarian protein expression of NF-κB pathway members in mice**

There was no impact of progressive obesity on phosphorylated IKKα/β$^{\text{Ser176/180}}$ protein levels across time points, (Figure 4A). However, phosphorylated IκB$^{\text{Ser32/36}}$ protein level was decreased (P < 0.01) at 6 weeks, but increased at 12 (P < 0.01) and 24 (P < 0.05) weeks in obese females relative to their lean littermates (Figure 4B). Similarly, ovarian pNFκBp65$^{\text{Ser536}}$ was reduced by obesity at 6 weeks (P < 0.05), but increased (P < 0.01) from 12 weeks onwards (Figure 4C).

**Discussion**

Obesity is associated with impaired fertility (21, 36, 47, 48, 130). Although the reasons for this association are not fully known, available evidence suggest that obese females have higher incidences of poor oocyte quality (27, 40, 66), irregular menstrual
cycles (31, 42, 56), anovulation (35), and increased miscarriage rates (15, 49, 67), phenotypes suggestive of derangements in ovarian folliculogenesis and steroidogenesis. Additionally, obesity is associated with low grade inflammation which if not properly regulated can lessen reproductive function (50, 61, 127). The main objective of this study was to determine the impact of obesity onset and progression on the expression profiles of immune cell and inflammation markers as well as the NF-κB and steroidogenic pathway members in murine ovaries. The lethal yellow mouse, a model of progressive obesity was utilized to compare gene expression beginning at 6 weeks of age when there is no reported difference in body weight between genotypes (129), which we confirmed in this study.

Beginning at 12 weeks of age, a progressive increase in body weight among the lethal yellow mice compared to their a/a matched controls was observed. Increased fasting blood glucose was also evident as body weight increased from 12 weeks onwards. Glucose tolerance testing revealed that relative to their age matched controls, obese mice had reduced clearance of glucose following a glucose bolus challenge at 12, 18, and 24 weeks (Data not shown). While we did not measure blood insulin levels in these animals, previous data showed that the lethal yellow mice have increased circulating insulin and leptin levels compared to the age-matched lean controls from 12 weeks onwards (129), matching our phenotypic data. Thus, elevated fasting blood glucose, insulin levels (129) and reduced glucose clearance rate indicate that these mice have compromised insulin sensitivity.
Obesity impacted ovarian cyclicity by decreasing the length of time spent in estrus with a concomitant increase in the length of the diestrus phase. The impact of obesity on the estrous cycle has been previously reported in rats (7, 14). In agreement with our results, HFD-fed obese rats exhibited longer diestrus but shorter estrus stages compared to chow fed lean animals (7). Physiologically, the ovarian steroids predominantly associated with estrus and diestrus phases are E2 and P4, respectively (84). Therefore, shorter estrus stages would imply lower E2 levels while longer diestrus stages would imply higher P4 levels in obese females. In premenopausal females, an inverse relationship between E2 and body mass index has also been reported (51, 110, 121), and higher P4 levels have also been observed in obese rodent models (7). Taken together these data suggest that alterations in the estrous cycle during obesity could impact ovulation and subsequently compromise the reproductive capacity of obese females.

In order to separate the effects of obesity from the Ay/J genotype, we included a 6 week old group in which we observed no differences in body weight from the a/a genotype mice. Importantly, also at this time point, there was no difference in the number of all follicular stages. However, despite a lack of any impact of progressive obesity on ovarian weight, fewer primordial and primary follicles and increased numbers of tertiary follicle subtypes was present as body mass increased. These data are in agreement with a previous study from our group in which 20 week old obese mice which
were found to have reduced primordial and small primary, but increased numbers of secondary and antral follicles (96). Furthermore, Sprague-Dawley rats fed a HFD for 18 weeks demonstrated a decreased number of primordial follicles but increased number of developing and atretic follicles (126). This difference in follicle populations could indicate increased activation of follicles from the primordial follicle pool. This scenario is possible because obesity is associated with elevated insulin levels, obese females have higher levels of insulin in their follicular fluid (112), and insulin administration promoted primordial to primary transition in neonatal rat ovaries (70).

Several studies have reported an inverse relation between steroid hormone level and primordial follicle activation (22, 23, 32, 69). Estradiol levels are lower in HFD-induced obese rats (14) and obese females (51, 110, 121) and reduced E2 levels stimulated primordial follicle activation from the resting into the growing follicular pool (22, 23). In mice (22, 32) and in neonatal rats (69), administration of E2 or P4 was associated with impaired primordial follicle assembly, and decreased numbers of both primordial and primary follicles. Further still, increased activation of ovarian phosphatidylinositol-3 kinase (PI3K) signaling (45, 78, 101), which we have previously reported to be altered during HFD-induced obesity (98), is involved in early stages of folliculogenesis. Therefore it is possible that the observed low numbers of primordial follicles and higher numbers of growing follicles observed in ovaries from obese relative to lean mice is indicative that obesity triggers changes in the intrinsic ovarian signals responsible for initiation of follicle activation and recruitment.
Ovarian steroidogenesis is mediated by FSH and LH through the action of several enzymes including STAR, CYP11A1, 3β-HSD, and CYP19A1. In the testes, during PI3K activation, insulin inhibited cAMP-induced STAR, CYP11A1 and 3β-HSD gene expression (6). In the present study, we found that obesity has differential effects of the expression profiles of genes involved in ovarian steroidogenesis. With the exception of CYP19A1 protein expression, which was increased at 6 weeks of age in the lethal yellow mice, potentially indicating early onset of puberty, there was no impact of genotype (6 weeks of age) on levels of ovarian steroidogenic members (mRNA or protein). Progressive obesity decreased both Star mRNA and protein levels indicating that the rate limiting step in steroidogenesis could be compromised during an obese state. Additionally, despite its increase in the early stages of obesity (at 12 weeks), CYP11A1 protein expression was markedly downregulated with progressive obesity, reaching a 50% reduction by 24 weeks of age. Erα mRNA levels were increased at 6 and 12 in obese females, while Erβ mRNA levels were elevated from 12 onwards due to the obese phenotype, suggesting a temporal pattern of E2 receptor response to increased body mass. 3β-HSD protein levels were unchanged, but Cyp19a1 mRNA and protein were both negatively impacted by obesity. Taken together, these results indicate that obesity may alter ovarian steroidogenesis contributing to the reproductive disorders experienced by obese females. Our data also reveal a temporal effect of obesity on ovarian steroidogenic genes underscoring the importance of considering the timepoint at which data is collected when interpreting obesity-associated ovarian impacts.
Ovarian steroids may vary depending on energetic conditions of the cell (46) and inflammatory changes (34) both of which are impacted by obesity. Elevated TNFα and IL-6 levels have been reported in the follicular fluid of obese women (73), while obesity-induced increased Tnfa expression has been observed in human adipose tissue (62, 68), muscle (116), rat adipose tissue (63), mouse muscle and adipose tissues (60). IL-6 through its ability to modulate cAMP, can regulate ovarian steroid production (19) and ovulation (88, 124). TNFα and Il-1 can also stimulate ovarian P4 production (9, 19), regulate ovarian cell differentiation, proliferation and apoptosis (9, 10, 113) as well as inflammation (114). Derangements in these inflammatory pathways have been linked to various reproductive disorders (1, 11, 64, 117, 120). For example, elevated levels of IL-6 have been associated with ovarian hyperstimulation syndrome (25, 79, 80), tumor cell activation (33), and chemotherapeutic resistance (43). Furthermore, IL-6, in conjunction with TNFα and IL-1β, has been associated with ovarian neoplasia (12, 38, 39, 72, 74, 94).

Similar to previously reported elevations in Nitric oxide synthase 2 (Nos2) mRNA in animal models of obesity (4, 75, 106, 115), we observed a temporal increase in Nos2 mRNA in ovaries of obese mice compared to their lean littermates. Nos2 is a marker for oxidative stress, dysregulation of which has been linked to impaired ovulation (58), ovarian cancer (59), endometriosis (122), and infertility (3, 100, 115). Similarly, ovarian mRNA expression of the anti-inflammatory gene, Il10, was increased
by obesity. In contrast, obesity decreased Il1ra mRNA, a modulator of the pro-inflammatory actions of IL-1. Interestingly, although Il1b mRNA was decreased at 12 and 24 weeks, it was subsequently increased at 18 weeks with obesity. Also, although Mcp1 and Ccl5 mRNA levels were both decreased by obesity, only the decrease in Mcp1 was maintained at the time points studied. MCP1 is involved in ovarian follicular development, steroidogenesis, ovulation and atresia (19). Interpreted locally, these mRNA results would suggest a downward trend in expression profiles of both immune cell infiltration and inflammatory markers. Taken together these results indicate that progressive obesity alters inflammatory members and that these changes may have negative consequences for ovarian function.

The mammalian NF-κB family, is made of five proteins; RelA (p65), RelB, c-Rel, NF-κB1 and NF-κB2 carrying a Rel-homology domain (18). Currently, there are two known activation pathways for the NF-κB activity; the canonic pathway which is activated by proinflammatory cytokines including TNFα and other TNF cytokines (18). Before activation, the inactive NF-κB complex is localized in the cytoplasm bound to IκB proteins such as IκBα, IκBβ, and IκBγ, which block its nuclear localization sequence function of the Rel-homology domain (18). During the canonic activation pathway, proinflammatory cytokines, work through different TNF receptors (TNFR) and Toll-like receptor-interleukin-1 receptor (IL-1R) superfamilies to activate the IκB kinases (IKKs) (18). Like the IκB complex, the IKK complex is mainly made up of two catalytic subunits, IKKα and IKKβ and a regulatory subunit IKKγ. Activated IKK
proteins can catalyze the phosphorylation of IκB proteins mainly at the serine 32 and 36 sites of IκBα, leading to proteasome degradation of the IκB complex and subsequent release and translocation of the NF-κB proteins to the nucleus (18, 57). Once in the nucleus, the NF-κB proteins will bind and activate transcription of several genes particularly those involved in immune and inflammatory cascades (18, 57).

Although progressive obesity did not impact the mRNA expression profiles of TNFα-NF-κB pathway members as previously observed during HFD-induced obesity (97), we found that obesity advancement increased abundance of protein expression of NF-κB pathway member proteins, pIκBSer32/36 and pNFκB p65Ser536 indicating increased activation of NF-κB signaling pathway in ovaries of obese mice. Surprisingly, before the onset of obesity (at 6 weeks of age), ovaries from the lethal yellow mice displayed a marked decrease in pIκBSer32/36 and pNFκB p65Ser536 proteins compared to non-agouti (a/a) mice, potentially indicating that the Ay/J genotype has some culpability – it is more likely, however, that even though no differences in body weight are detectable at this age point, that changes in central metabolism have been activated, this is strengthened by the fact that the lethal yellow mice displayed increased CYP19A1, a marker that puberty onset is impending at an earlier time than the control genotype.

NF-κB pathway is activated in response to inflammatory responses (18, 85, 86), therefore its sustained activation in ovaries from obese mice is indicative of aggravated inflammatory signaling, which in turn is associated with a number of ovarian pathologies
including polycystic ovary syndrome (52, 108), endometriosis (28, 41) and premature ovarian failure (128). Additionally, since inflammatory signals can influence generation of reactive oxygen species and oxidative stress (64), ovarian steroid production and ovulation (9, 10, 88, 124, 128) as well as cell differentiation, proliferation and apoptosis (9, 10, 16, 113), deregulation of inflammatory signals in the ovary may adversely impact tissue function and pose dire consequences for female fertility.

In conclusion, our results indicate that progressive obesity alters the expression of inflammatory and steroidogenesis signaling pathway members in ways which could alter optimal ovarian function.

Grants: The project described was supported by award number R00ES016818 to AFK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
References


Table 1. Primer sequences used in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward Sequence: 5’-3’</th>
<th>Reverse Sequence: 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>GTG GAC CTC ATG GCC TAC AT</td>
<td>GGA TGG AAT TGT GAG GGA GA</td>
</tr>
<tr>
<td>H110</td>
<td>GCT CAT TGC TGG GTA CTT ACA A</td>
<td>CCA GAC TTG GCA CAA GAC AGG</td>
</tr>
<tr>
<td>Ikbkg</td>
<td>ATC GAT TTC TCC CCT GTG AA</td>
<td>TGT CAA ATT CAT TCA TGG CCT</td>
</tr>
<tr>
<td>Nfkb1</td>
<td>AAA GTT GGC TGC CAT GAG TC</td>
<td>GAA AGG AGT GGT GAG CTT G</td>
</tr>
<tr>
<td>Star</td>
<td>ATG TTC CTC GCT ACG TTC AAG</td>
<td>CCC AGT GCT CTC CAG TTG AG</td>
</tr>
<tr>
<td>Cyp11a1</td>
<td>AGG TCC TTC AAT GAG ATC CCT T</td>
<td>TCC CTG TAA ATG GGG CCA TAC</td>
</tr>
<tr>
<td>Cyp19a1</td>
<td>ATG TTC TTG GAA ATG CTG AAC CC</td>
<td>AGG ACC TGG TAT TGA AGA CGA G</td>
</tr>
<tr>
<td>Erα</td>
<td>AAT TCT GAC AAT CGA CGC CAG</td>
<td>GTG CTT CAA CAT TCT CCC TCC TC</td>
</tr>
<tr>
<td>Erβ</td>
<td>CTG TGC CTC TTC TCA CAA GGA</td>
<td>TGC TCC AAG GGT AGG ATG GAC</td>
</tr>
</tbody>
</table>
Body weights were measured once per week starting at 6 weeks of age across the entire experimental period. At each time point (6, 12, 18 or 24 weeks of age; n = 4 per treatment per time point), fasting blood glucose was determined using the hand held glucometer from blood collected from the lateral saphenous vein of mice fasted for 16 hours (overnight). To determine the impact of progressive obesity, mice were euthanized at the end of each experimental time point; ovaries were collected, trimmed of excess fat and weighed.

Table 2. Progressive obesity effects on fasting blood glucose and ovarian weight.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>6 weeks</th>
<th>12 weeks</th>
<th>18 weeks</th>
<th>24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean</td>
<td>Obese</td>
<td>Lean</td>
<td>Obese</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25 ± 1.043</td>
<td>26.2 ± 0.629</td>
<td>32.40 ± 1.238</td>
<td>42.20 ± 0.503*</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mM)</td>
<td>129.2 ± 23.66</td>
<td>134 ± 13.61</td>
<td>90 ± 8.651</td>
<td>142.2 ± 19.289*</td>
</tr>
<tr>
<td>Ovary weight (g)</td>
<td>0.0096 ± 0.001</td>
<td>0.0095 ± 0.001</td>
<td>0.0127 ± 0.002</td>
<td>0.0154 ± 0.002</td>
</tr>
</tbody>
</table>
Table 3. Impact of progressive obesity on estrous cyclicity. Fresh, wet unstained vaginal smears were obtained once a day (8 – 9 AM) for 14 consecutive days prior to the end of 12, 18, and 24 weeks from both lean and obese mice. The stage of estrous cycle was determined based on presence or absence of leucocytes, cornified epithelial, and nucleated epithelial cells as detailed in the materials and methods section.

<table>
<thead>
<tr>
<th>Estrus Cycle (days)</th>
<th>12wks</th>
<th>18wks</th>
<th>24wks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean</td>
<td>Obese</td>
<td>Lean</td>
</tr>
<tr>
<td>Proestrus</td>
<td>1.00 ± 0.245</td>
<td>0.800 ± 0.374</td>
<td>1.50 ± 0.250</td>
</tr>
<tr>
<td>Estrus</td>
<td>1.400 ± 0.200</td>
<td>1.600 ± 0.316</td>
<td>1.750 ± 0.408</td>
</tr>
<tr>
<td>Metestrus</td>
<td>0.800 ± 0.375</td>
<td>1.000 ± 0.315</td>
<td>1.000 ± 0.250</td>
</tr>
<tr>
<td>Diestrus</td>
<td>1.800 ± 0.400</td>
<td>2.00 ± 0.274</td>
<td>1.50 ± 0.250</td>
</tr>
</tbody>
</table>
Table 4. Impact of progressive obesity on mRNA level of markers of immune cells, inflammation and NF-κB pathway members. Ovaries were removed from lean or obese female mice at the end of each experimental time point (6, 12, 18, and 24 weeks of age). Total RNA was isolated, reverse transcribed to cDNA and relative mRNA of target genes quantified by qRT-PCR using specific primer sets as detailed in the materials and methods section. After normalization to Gapdh the relative fold change was calculated using the 2-ΔΔCT method. The results are presented as mean fold change ± standard error relative to the lean matched control value of 1 ± SE. * indicates different from control at P < 0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>6 weeks</th>
<th>12 weeks</th>
<th>18 weeks</th>
<th>24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean</td>
<td>Obese</td>
<td>Lean</td>
<td>Obese</td>
</tr>
<tr>
<td>tfnp</td>
<td>1.00±0.09</td>
<td>0.80±0.035</td>
<td>1.00±0.145</td>
<td>0.74±0.057</td>
</tr>
<tr>
<td>p53</td>
<td>1.00±0.067</td>
<td>1.13±0.058</td>
<td>1.00±0.121</td>
<td>0.95±0.09</td>
</tr>
<tr>
<td>p75</td>
<td>1.00±0.014</td>
<td>1.16±0.087</td>
<td>1.00±0.134</td>
<td>0.95±0.093</td>
</tr>
<tr>
<td>il6</td>
<td>1.00±0.122</td>
<td>1.15±0.094</td>
<td>1.00±0.169</td>
<td>0.66±0.114</td>
</tr>
<tr>
<td>il10</td>
<td>1.00±0.288</td>
<td>1.78±0.405</td>
<td>0.01±0.095</td>
<td>0.78±0.314</td>
</tr>
<tr>
<td>il12</td>
<td>1.00±0.146</td>
<td>1.32±0.521</td>
<td>1.00±0.155</td>
<td>0.50±0.090*</td>
</tr>
<tr>
<td>ii10</td>
<td>1.00±0.292</td>
<td>0.31±0.068</td>
<td>1.00±0.078</td>
<td>0.33±0.031*</td>
</tr>
<tr>
<td>ccl2</td>
<td>1.00±0.054</td>
<td>1.50±0.122*</td>
<td>1.00±0.117</td>
<td>1.34±0.139</td>
</tr>
<tr>
<td>ccf1</td>
<td>1.00±0.067</td>
<td>1.37±0.339</td>
<td>1.00±0.162</td>
<td>0.85±0.080</td>
</tr>
<tr>
<td>ccf2</td>
<td>1.00±0.075</td>
<td>0.72±0.114</td>
<td>1.00±0.075</td>
<td>0.47±0.061*</td>
</tr>
<tr>
<td>ccf3</td>
<td>1.00±0.397</td>
<td>1.39±0.462</td>
<td>1.00±0.135</td>
<td>0.50±0.062</td>
</tr>
<tr>
<td>ccf4</td>
<td>1.00±0.326</td>
<td>1.01±0.203</td>
<td>1.00±0.139</td>
<td>0.91±0.129</td>
</tr>
<tr>
<td>ccf5</td>
<td>1.00±0.146</td>
<td>0.45±0.197</td>
<td>1.00±0.103</td>
<td>0.38±0.054*</td>
</tr>
<tr>
<td>ccf6</td>
<td>1.00±0.255</td>
<td>0.61±0.180</td>
<td>1.00±0.092</td>
<td>0.59±0.083*</td>
</tr>
<tr>
<td>ccf7</td>
<td>1.00±0.705</td>
<td>2.51±0.752</td>
<td>1.00±0.125</td>
<td>0.92±0.085</td>
</tr>
<tr>
<td>ccf8</td>
<td>1.00±0.227</td>
<td>0.35±0.063</td>
<td>1.00±0.134</td>
<td>0.98±0.106</td>
</tr>
<tr>
<td>ccf9</td>
<td>1.00±0.314</td>
<td>0.61±0.164</td>
<td>1.00±0.106</td>
<td>0.73±0.127</td>
</tr>
<tr>
<td>ccf10</td>
<td>1.00±0.267</td>
<td>0.71±0.065</td>
<td>1.00±0.190</td>
<td>0.39±0.077</td>
</tr>
<tr>
<td>ccf11</td>
<td>1.00±0.076</td>
<td>0.96±0.491</td>
<td>1.00±0.126</td>
<td>0.86±0.086</td>
</tr>
</tbody>
</table>
Figure 1: Effect of obesity on follicle number. At 6, 12, 18 or 24 weeks of age, mice were euthanized and ovaries collected. One ovary was fixed in 4% paraformaldehyde, complete serial sections were mounted and healthy follicles was classified and counted in both lean and obese (Figure 1A-D); (A) healthy primordial follicle number; (B) healthy primary follicle number; (C) healthy secondary follicle number; and (D) healthy antral follicle number. Bars represent means ± SEM. * indicate significant ($P < 0.05; n = 4$) difference from respective lean group.
Figure 2. Obesity alters mRNA expression of ovarian steroidogenic members in mice. At the end of each experimental time point (6, 12, 18, and 24 weeks of age) ovaries were removed from lean or obese mice, total RNA isolated (n = 4 per treatment per time point) and reverse transcribed to cDNA. mRNA level of Star, Cyp11a1, Cyp19a1, Erα, and Erβ were quantified by quantitative RT-PCR. After normalization to Gapdh relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method. The results are presented as mean fold change ± standard error relative to the lean matched control value of 1 ± SE. * indicates different from control at $P < 0.05$. † indicate $P < 0.1$. 
Figure 3. Obesity impacts protein abundance of ovarian steroidogenic members. At the end of each experimental time point (6, 12, 18, and 24 weeks of age), ovaries were removed from lean (L) or obese (O) female mice and total ovarian protein isolated (n = 3-4 per treatment group per time point). Western blotting was performed to measure (A) STAR, (B) CYP11A1, (C) 3β-HSD, and (D) CYP19A1 protein levels. Densitometry of the appropriate bands was performed using Carestream molecular imaging software and normalized to Ponceau S staining prior to statistical analysis. Bars represent means ± SEM and * indicate significant difference from respective lean group at $P < 0.05$. 
Figure 4. Ovarian NF-κB pathway member protein level are affected by obesity. At each time point (6, 12, 18, and 24 weeks of age), total ovarian protein was isolated from both lean (L) and obese (O) female mice. Western blotting was performed to quantify (A) pIκKα/βSer176/180, (B) pIκBαSer32/36, and (C) pNFκB p65Ser536 protein levels. Densitometry of the appropriate bands was performed using Carestream molecular imaging software and normalized to Ponceau S staining prior to statistical analysis. Bars represent means ± SEM and * indicate significant difference from respective lean group at $P < 0.05$. 

---

**A**

**B**

**C**
CHAPTER 5: OVARIAN XENOBIOTIC BIOTRANSFORMATION ALONG WITH INSULIN AND PHOSPHATIDYLINOSITOL-3 KINASE SIGNALING PATHWAYS ARE ALTERED DUE TO OBESITY

A paper to be submitted to *American Journal of Physiology - Endocrinology and Metabolism*

Nteeba, J., Ganesan, S., Keating, A.F.

**Contribution Statement:**
I performed the animal studies, all analyses on ovarian tissue, designed the experiments, interpreted data and wrote the paper. Ganesan, S. aided in glucose tolerance testing and tissue collection; Keating, A.F. designed experiments, aided in data interpretation and edited the manuscript.

**Abstract**

Mechanisms underlying obesity-associated reproductive impairment are ill-defined, however, the presence of hyperinsulinemia as well as other metabolic perturbations are well recognized. Insulin activates phosphatidylinositol 3-kinase (PI3K) signaling, which plays roles in ovarian folliculogenesis, steroidogenesis as well as xenobiotic metabolism. The impact of progressive obesity on expression profiles of ovarian genes involved in insulin-mediated PI3K activation and signaling along with xenobiotic biotransformation, insulin receptor (*Insr*), insulin receptor substrate 1 (*Irs1*), 2 (*Irs2*), and 3 (*Irs3*); kit ligand (*Kitlg*), stem cell growth factor receptor (*Kit*), protein kinase B (AKT) alpha (*Akt1*), beta (*Akt2*), forkhead transcription factor (FOXO) subfamily 1 (*Foxo1*), and subfamily 3 (*Foxo3a*), microsomal epoxide hydrolase (*Ephx1*), cytochrome P450 family 2, subfamily E, polypeptide 1 (*Cyp2e1*), glutathione S-
transferase (GST) class Pi (Gstp1) and class mu 1 (Gstm1) were determined in normal wild type non-agouti (a/a; lean) and lethal yellow mice (KK.CG-A^yJ; obese) at 6, 12, 18 or 24 weeks of age. At 6 weeks, ovaries from obese mice had increased ($P < 0.05$) Insr and Irs3 but decreased ($P < 0.05$) Kitlg, Foxo1 and Cyp2e1 mRNA levels. Interestingly, starting at 12 weeks, a temporal increase ($P < 0.05$) in Kitlg and Kit mRNA, pIRS1$^{\text{Ser302}}$, pAKT$^{\text{Thr308}}$, EPHX1, and GSTP1 protein level was observed due to obesity. In contrast, reduced Cyp2e1 mRNA and CYP2E1 protein occurred with progressive obesity. Taken together progressive obesity affected PI3K signaling as well as ovarian xenobiotic metabolism which could participate in obesity-associated reproductive disorders.
Introduction

The mammalian ovary is a female gonad responsible for production of oocytes and steroid hormones (28, 54, 95). At birth, a female is born with a finite number of primordial follicles, arrested in the diplotene stage of prophase 1 (35, 50). Once primordial follicles are depleted, the female enters into ovarian senescence. Primordial follicles are composed of a nucleated oocyte surrounded by squamous granulosa cells (GC), which become cuboidal and proliferate as the follicle matures toward ovulation. Pre-ovulatory follicles are the major source of steroids and under the influence of gonadotropins, a selected cohort of dominant antral follicles become capable of ovulation. Following ovulation, the GC and theca cells (TC) luteinize to form the corpus luteum (CL) which produces progesterone, essential for implantation and pregnancy maintenance. Adverse reproductive outcomes including anovulation, impaired fecundity and premature ovarian failure can result from unregulated folliculogenesis.

In the absence of ovarian function, females have a greater risk for development of gynecological cancers, osteoporosis and cardiovascular disease (54-56). A number of chemical exposures can result in follicle depletion leading to early ovarian senescence (5, 6, 13, 54, 65). In addition, DNA damage (101) and altered folliculogenesis (63) due to ovotoxicant exposure have been reported. The ovary has the capacity to respond to such exposures through increased biotransformation gene expression, including cytochrome P450 isoform 2E1 (CYP 2E1) (19, 64, 104), microsomal epoxide hydrolase (EPHX1) (20, 58), glutathione S-transferase Pi (GSTP1) (7, 65) and glutathione S-
transferase Mu 1 (GSTM1) (8) whose expression and activity levels are altered during xenobiotic exposure. These enzymes are principally involved in the detoxification of xenobiotic compounds (12, 46, 48, 53, 68), however, their expression may also lead to bioactivation of some environmental compounds (5, 6, 19, 55, 57, 104).

The adverse effects of obesity on reproductive health and fecundity have been documented, and include reduced conception and implantation (17, 73), impaired fecundity (29, 73, 103, 132), increased infertility (17, 108), and an increase in offspring birth defects (10, 27, 34). The phosphatidylinositol-3 kinase (PI3K) signaling pathway plays a pivotal role in regulating ovarian folliculogenesis (61, 86, 105, 107) and steroidogenesis (24, 78, 85, 133). Additionally, the PI3K pathway regulates ovarian genes encoding xenobiotic metabolism enzymes (1, 9, 62, 63). We (94) and others (2, 84, 123, 128), have found that obesity alters insulin mediated signaling pathways, including PI3K. Altered expression of genes involved in these pathways could sabotage proper ovarian function and consequently lead to impaired fertility. Despite a strong positive correlation between obesity and impairments in reproductive capacity, the underlying mechanisms have not been clearly defined. To understand the onset and duration of these changes during obesity, we designed a temporal study using the agouti lethal yellow (KK.Cg-Ay/J) mice, an excellent model for progressive human obesity (22, 72, 129). The impact of obesity onset and progression on expression of ovarian genes encoding PI3K pathway members and enzymes involved in ovarian xenobiotic biotransformation were investigated.
Materials and Methods

Reagents

D-Glucose, 2-β-mercaptopethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulfate, glycerol, N′,N′,N′,N′-Tetramethyl-ethylenediamine (TEMED), Tris base, Tris HCl, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (Vitamin C), phosphatase inhibitor, protease inhibitor and transferrin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Custom designed primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University. Hanks' Balanced Salt Solution (without CaCl₂, MgCl₂, or MgSO₄) and superscript III one-step RT-PCR System were purchased from Invitrogen Co. (Carlsbad, CA, USA). RNAlater was obtained from Ambion Inc (Austin, TX, USA). RNeasy Mini kit, QIAshredder kit, RNeasy MinElute kit and Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). Ponceau S was purchased from Fisher Scientific (Waltham, MA, USA). Restore™ PLUS Western Blot Stripping Buffer was purchased from Thermo Scientific (Rockford, IL, USA). SignalFire™ ECL Reagent, goat anti-rabbit secondary antibody, anti-pAKT<sup>Thr308</sup> and anti-pIRS1<sup>Ser302</sup> antibodies were from Cell Signaling Technology® (Danvers, MA, USA). Anti-FOXO3 and anti-GSTP1 antibodies were purchased from Millipore (Temecula, CA, USA). Donkey anti-goat secondary antibody was purchased from Pierce Biotechnology (Rockford, IL). Anti-CYP2E1 antibody was purchased from Abcam (Cambridge, MA, USA). Anti-mEH (EPHX1) antibody was purchased from Detroit R&D, Inc. (Detroit, MI, USA).
**Animal procedures and tissue collection**

All experimental animal protocols used in this study were approved by the Iowa State University Animal Care Committee (IACUC). Four week old female normal wild type non-agouti (a/a; designated lean; n = 20) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 20) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The mice were housed at the animal facility at Iowa State University under identical conditions of room temperature (21-22°C), lighting (12h light:12h darkness cycle) and ad libitum access to feed and water until 6, 12, 18 or 24 weeks of age. Phenotypically, the lethal yellow mice had elevated body weight, and fasting glucose levels from 12 weeks onwards (Nteeba et al., under review). Estrous cyclicity was also impacted by shorter time spent in estrus and longer time in diestrous. In addition, primordial and small primary follicle numbers were decreased, while secondary and antral follicle numbers were increased in number with progressive obesity (Nteeba et al., under review). At the end of each experimental time point, mice were euthanized by CO₂ asphyxiation. Ovaries were collected, cleaned off excess fat, and stored in RNAlater at -80°C for RNA and protein analyses.

**RNA isolation, first strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was isolated from both lean and obese mice using Qiagen RNeasy® Mini Kit (at 6, 12, 18 and 24 weeks, n = 3-4 per group per time point) per the manufacturer’s protocol. Briefly, ovaries were lysed and homogenized using a hand-held
homogenizer followed by applying the homogenate to a QIAshredder column with subsequent centrifugation at 16100 RCF for 2 minutes at room temperature. The resulting supernatant was applied to an RNeasy Mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter and concentrated using an RNeasy MinElute Kit according to the manufacturer’s protocol. The total RNA was eluted using 14 µl of RNase-free water and concentration determined using an ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE). For cDNA synthesis, RNA (0.5µg) was reverse-transcribed using Invitrogen™ Superscript III Reverse Transcriptase according to the manufacturer’s protocol. cDNA (2 µL; 1:25 dilution) were amplified on an Eppendorf Mastercycler using a Quantitect™ SYBR Green PCR kit and primers specific for mouse *Gapdh, Insr, Irs1, Kitlg, cKit, Akt1, Gstm1, Gstp1, Ephx1 and Cyp2e1* (see sequences in (94)), *Irs2, Irs3, Akt2, Foxo1 and Foxo3a* (for sequences see Table 1). The PCR cycling program consisted of a 15 min hold at 95 °C and 40 cycles of: denaturing for 15 at 95 °C, annealing for 15s at 58 °C and extension at 72 °C for 20s. Product melt conditions were determined using a temperature gradient from 72 °C to 99 °C with a 1 °C increase at each step. Three replicates of each sample (n = 3- 4) were included. The relative mRNA expression for each gene was normalized using the housekeeping gene *Gapdh* and relative fold change calculated using the $2^{-\Delta\Delta CT}$ method. The results are presented as mean fold change ± standard error relative to the lean matched control group.
Protein isolation and Western blot analysis

At each time point (6, 12, 18 or 24 weeks of age, n = 3-4 per group per time point), total ovarian protein was isolated and Western blotting performed as previously described (93) with the following modifications. Ovaries were homogenized in 300 µl of ice-cold tissue lysis buffer and protein concentration quantified using a standard BCA protocol on a 96-well assay plate. Equal total protein (20 µg) was separated on a 10-12% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Ponceau S staining was performed to visualize and confirm equal amount of protein loading and transfer. Following blocking for 2 hours at room temperature, membranes were probed with specific primary antibodies [Rabbit Anti-GSTP1 (1:200), Goat anti-EPHX1 (1:500), Rabbit anti-pIRS1Ser302 (1:200), Rabbit anti-pAKTThr308 (1:250), Rabbit anti-FOXO3 (1:250), Rabbit anti-CYP2E1 (1:200)] in 5% BSA in TTBS for 24-48 h at 4°C. HRP-conjugated secondary antibodies (1:10000 – 1:20000) were added for 1h at room temperature, and then membrane-bound HRP were washed three consecutive times for 5 minutes each time in TTBS. Autoradiograms were visualized on X-ray films following 10 min incubation of membranes with 1X SignalFire™ ECL reagent. Densitometry of the appropriate sized bands was measured using Carestream molecular imaging software version 5.0 (Carestream Health Inc., Rochester, NY) which eliminates background noise. Densitometric values of appropriate target proteins were normalized to Ponceau S staining prior to statistical analysis.
Statistical Analysis

Statistical analyses were performed using the unpaired t-test function of GraphPad Prism 5.5 software with a statistical significance level set at \( P < 0.05 \). Bars represent means ± SEM.

Results

Obesity temporally increases ovarian mRNA expression of insulin signaling members

Since obese mice displayed reduced glucose clearance rate in addition to having higher fasting blood glucose levels (Nteeba et al., under review), we sought to determine the impact of progressive obesity on ovarian mRNA expression of insulin signaling members (Figure 1, n = 4 per group per time point). At 6 weeks of age, ovaries from the obese mice had increased \( Insr \) (Figure 1A, \( P < 0.05 \)) and \( Irs3 \) (Figure 1D, \( P < 0.01 \)) but showed no difference in either \( Irs1 \) or \( Irs2 \) mRNA levels relative to ovaries from the lean mice. At 12 weeks, only \( Irs1 \) mRNA was increased (Figure 1B, \( P < 0.001 \)) with obesity but other members were not impacted by obesity (Figure 1). Similarly, ovaries from obese mice displayed increased \( Irs1 \) (Figure 1B, \( P < 0.001 \)) and \( Irs3 \) (Figure 1D, \( P < 0.05 \)) without significant effect on \( Insr \) (Figure 1A) and \( Irs2 \) (Figure 1C) mRNA levels at 18 weeks of age. In the 24 week old group, obesity increased ovarian expression of \( Insr \) (Figure 1A, \( P = 0.05 \)), \( Irs1 \) (Figure 1B, \( P < 0.0001 \)), \( Irs2 \) (Figure 1C, \( P < 0.01 \)), and \( Irs3 \) (Figure 1D, \( P < 0.01 \)).
Progressive obesity increases ovarian phosphorylated insulin receptor substrate 1 protein

Following the impact of obesity on ovarian mRNA levels of insulin signaling members, we investigated protein levels of pIRS1_{Ser302} (Figure 2). Although there was no difference in phosphorylation of IRS1 protein at the serine302 site at 6 weeks, relative to lean matched controls, ovaries from obese mice showed a tendency for increased pIRS1_{Ser302} protein levels at 12 weeks (P = 0.07), and increased protein at 18 (P < 0.05) and 24 (P < 0.01) weeks (Figure 2).

Ovarian Kitlg/cKit-P13K pathway members are elevated during obesity

At 6 weeks, ovaries from the obese group had lower (P < 0.01) Kitlg (Figure 3A) and Foxo1 (Figure 3E) but not cKit, Akt1, Akt2 or Foxo3a mRNA levels than ovaries from lean females (Figure 3). At 12 weeks, there was a marked increase in ovarian Kitlg (Figure 1A, P < 0.05), cKit (Figure 1B, P < 0.05), and Foxo1 (Figure 1E, P < 0.0001) in obese compared to lean females, but there was no impact of obesity on Akt1 (Figure 3C), Akt2 (Figure 3D) or Foxo3a (Figure 3F) mRNA expression. Likewise, at 18 weeks, obesity did not affect ovarian Akt1 (Figure 3C), Akt2 (Figure 3D) and Foxo1 (Figure 3E) mRNA levels, but increased Kitlg (Figure 1A, P < 0.001), cKit (Figure 1B, P < 0.0001), and Foxo3a (Figure 1E, P < 0.01) mRNA levels. There was increased (P < 0.01) Kitlg, cKit, Akt1, Akt2, Foxo1 and Foxo3a mRNA levels in ovaries from obese compared to lean mice at 24 weeks (Figure 3A-F).
**Ovarian phosphorylated AKT is increased by obesity without impacting FOXO3 protein levels**

There was no differences in ovarian $\text{pAKT}^{\text{Thr308}}$ protein levels between lean and obese females at 6 and 12 weeks, but obesity increased ($P < 0.01$) $\text{pAKT}^{\text{Thr308}}$ protein expression at 18 and 24 weeks (Figure 4A, $n = 4$ per group per time point). Total ovarian FOXO3A protein levels did not differ between lean and obese groups at any time points (Figure 4B).

**Progressive obesity alters mRNA expression of ovarian xenobiotic metabolism genes**

Despite lack of a genotype effect on $\text{Gstm1}$, $\text{Gstp1}$ and $\text{Ephx1}$ mRNA levels at 6 weeks (92), obesity increased ($P < 0.001$) ovarian $\text{Gstm1}$ (Figure 5A) and $\text{Ephx1}$ (Figure 5C) mRNA expression at 12, 18 and 24 weeks. On the contrary, ovarian $\text{Cyp2e1}$ mRNA expression was lower ($P < 0.001$) in the obese group at all of the time points investigated (Figure 5D). $\text{Gstp1}$ mRNA levels were decreased ($P < 0.05$) by obesity at 12 weeks, however, at 24 weeks there was a tendency ($P = 0.06$) for increased ovarian $\text{Gstp1}$ in obese versus lean females (Figure 5B). At 18 weeks there was no difference between lean and obese females in ovarian $\text{Gstp1}$ mRNA levels (Figure 5B).

**Abundance of ovarian xenobiotic metabolism proteins is impacted by increased body mass**

At 6 weeks, there was no difference in ovarian EPHX1, CYPE1 and GSTP1 protein levels between the two groups of mice (Figure 6). In a similar manner to the
mRNA results, ovaries from obese mice had increased ovarian EPHX1 protein levels relative to their lean counterparts at 12 ($P < 0.05$), 18 ($P < 0.01$), and 24 ($P < 0.0001$) weeks (Figure 6A). Likewise, CYP2E1 protein expression was reduced in ovaries from obese females compared to their lean littermates at 12 ($P < 0.01$), 18 ($P < 0.05$) and 24 ($P < 0.01$) weeks (Figure 6B). Relative to their matched lean controls, obese females displayed a higher ($P < 0.05$) expression of ovarian GSTP1 at 18 and 24 weeks (Figure 6C).

**Discussion**

Obese mothers have increased risk for miscarriage (96), poor oocyte quality (102), and birth defects in their offspring (96). In a recent study, we discovered that ovaries from obese females may have increased susceptibility to environmental exposures due to increased levels of EPHX1 and altered GST levels (92). Although the mechanism(s) remain unclear, we propose that ovarian biotransformation capacity may be altered during obesity, leading to increased susceptibility to ovotoxicant effects. We also hypothesize that these impacts may be mediated through altered insulin-regulated PI3K signaling, thus we chose a model of progressive obesity in which to determine impacts of obesity onset and establishment on these endpoints.

Many rodent models of obesity have mutations in the satiety hormone leptin (ob/ob) (134) or its receptor (db/db) (114). While these mutations result in rapid onset of
obesity, mutations of this kind which result in complete dysregulation of body weight control are rare in the human population (14, 22). The lethal yellow mouse has a deletion mutation in the normal wild type non-agouti (a/a) background which results in ectopic expression of agouti (22, 33, 72, 87). Hypothalamic agouti overexpression inhibits the melanocyte stimulating hormone (MSH) receptor (81) leading to hyperphagia due to hindering of the inhibitory effects of feeding imparted by alpha-melanocyte-stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART) (16, 81). Subsequently, hyperphagia coupled with reduced energy expenditure results in the development of progressive obesity (72, 81). Starting at 12 weeks of age, these mice are hyperinsulinemic (129), hyperleptinemic (15), and display insulin (72) and leptin (44) resistance. Additionally, premature reproductive failure is a common phenotype of the lethal yellow mouse (15, 41, 51, 92). Elevated insulin levels have been reported in both serum and follicular fluids of obese females (109, 121). However, studies investigating whether the ovary retains insulin sensitivity during hyperinsulinemia are contradictory (2, 18, 94, 128).

We found that progressive obesity was associated with a temporal pattern of increased *Irs1*, *Irs2* and *Irs3* mRNA as well as pIRS1<sup>Ser302</sup> protein levels, perhaps indicating that ovarian insulin signaling pathway retains activity in ovaries from obese mice, supporting our previous data with both high fat diet (HFD) fed mice (94) and the lethal yellow mouse (92). The IRS1 contains several tyrosine/serine phosphorylation sites that serve as docking sites for numerous downstream mediators of insulin’s growth-
promoting and metabolic functions (39, 43, 90, 113). Although most serine phosphorylations of IRS lead to insulin signaling attenuation (43), phosphorylation of IRS1 at serine 302 in rodents, which corresponds to serine 307 of the human IRS1 (30, 135), is associated with insulin stimulation and it is believed to have a positive action in subsequent IRS1 tyrosine phosphorylations (30, 39, 135). In several studies, inhibition of pIRS1 Ser302 by glucose starvation or deprivation of short term amino acid correlates with decreased IRS1 tyrosine phosphorylation and subsequent reduced insulin activity (39, 43, 124). Also increased pIRS1 Ser302 has been observed during peripheral insulin resistance in the skeletal muscle of both rodents (49) and humans (110, 125). Whether the observed temporal increase in ovarian pIRS1 Ser302 protein levels during progressive obesity in this study represents positive or negative feedback to insulin stimulation is hard to discern.

In cultured human ovarian cortex, insulin alone or in combination with IGF-I and IGF-II increased the percentage of primordial follicles transitioning to the primary stage (80). In canines (111) and fetal hamster cultured ovaries (131), elevated insulin concentrations promoted preantral follicular growth and viability. Insulin promoted the transition from primordial to primary follicle stage in rat ovaries (66) and bovine cortical pieces (36). Moreover, insulin had an additive effect with KITLG on increasing follicle activation in cultured neonatal rat ovaries (66, 91). These studies suggest a functional role for insulin signaling in ovarian folliculogenesis regulation. Insulin acting through its receptor and the receptor substrate proteins can activate the PI3K-AKT dependent
signaling pathway (70). AKT activation involves two phosphorylations, one at threonine 308 (Thr308) normally by phosphoinositide dependent kinase (PDPK1) and the other at serine 473 (Ser473) by mammalian target of rapamycin complex 2 (mTORC2) (11, 118, 122). In mouse liver (31) or skeletal muscle (79) lacking IRS1 or IRS2, AKT Thr308 phosphorylation (pAKTThr308) is undetectable; however, AKT phosphorylation at Ser473 (pAKTSer473) is retained (25), thus pAKTSer473 appears not to be a suitable indicator of IRS-mediated PI3K-AKT signaling (26, 122). We demonstrate a temporal pattern of increased pAKTThr308 with obesity progression indicating increased PI3K-AKT activation in obese females. FOXO3 is a downstream AKT target that is negatively regulated upon AKT activation (3, 11, 61, 106). We noted a trend for a 60% and 70% reduction in this protein in ovaries from obese mice at 12 and 24 weeks, respectively, suggestive of increased activation of insulin-PI3K/AKT signaling pathway during progressive obesity. Hyperactivation of the PI3K-AKT-FOXO3 signaling pathway, has been shown to increase the rate of primordial follicles activation and recruitment into the growing pool with eventual death of most of those follicles, leading to accelerated ovarian senescence (23, 60, 76, 77, 107).

In addition to insulin, PI3K-AKT-FOXO3a can be activated by Kit ligand, KITLG, through its protein-tyrosine kinase receptor, cKIT (71, 75, 106). cKit is expressed in the oocyte (52, 82, 83), whereas Kitlg originates from the GC and TC (32, 36, 59). In the ovary, KITLG binding to cKIT induces phosphorylation of the regulatory subunit of PI3K and subsequent activation of AKT1 (71, 75, 106). We previously found
that HFD-induced obesity increased Kitlg concomitant with increased Akt1 and decreased Foxo3a mRNA expression in murine ovaries (94). In the present study we also observed a temporal pattern of increased ovarian Kit and Kitlg mRNA concomitant with increased pAKT\textsuperscript{Thr308} protein in obese mice relative to their lean littermates. The ovarian KITLG-cKIT pathway plays a pivotal role in regulation of ovarian folliculogenesis (32, 37, 97-100, 130) and steroidogenesis (88). In human ovarian follicles, blocking the KIT receptor using an anti-KIT antibody, ACK2 induced ovarian follicular atresia (21). KITLG supplementation promoted the transition of primordial to the primary follicle stage in bovine ovarian cortical pieces (37), and stimulated follicle activation in rodents (100, 130). Taken together, our data support that activation of insulin-KITLG-KIT-AKT1 signaling pathway is induced by obesity, and this could be a potential mechanism underlying obesity-induced over-activation of primordial follicles into the growing follicular pool previously noted in both this mouse model (92) and rats (123).

In primary cultured rat hepatocytes, insulin administration decreased Cyp2e1 mRNA expression in a dose dependent manner, and this was reversible by either addition of glucagon or PI3K inhibitors (Wortmannin or LY294002) (126, 127). Increased Cyp2e1 mRNA and/or CYP2E1 protein abundance have been reported during diabetes (4, 40, 112), glucagon treatment (127) and starvation (40, 68); physiological states that are normally associated with decreased insulin activity. Interestingly, EPHX1 expression pattern is opposite to CYP2E1 during these physiological states. Insulin
treatment or re-feeding increases Ephxl mRNA, EPHX1 protein expression and enzyme activity (68, 69, 117), however, diabetes (68, 117), glucagon (69) or starvation (68, 117) or PI3K inhibition (69), decreases Ephxl expression. Similar to the liver, opposing expression patterns of ovarian EPHX1 and CYP2E1 expression have been previously reported (64, 94). In the present study, similar to the mRNA results, there was a temporal increase in ovarian EPHX1 with a concomitant decrease in CYP2E1 protein levels in ovaries from obese females compared to their age matched lean counterparts.

In a similar manner to EPHX1 and CYP2E1, the expression and activities of GST enzymes can be mediated through PI3K/AKT signaling (7, 38, 67). In male rats, addition of insulin reversed decreased hepatic GST activity that had been induced by diabetes (117). Also, re-feeding (associated with insulin stimulation), restored starvation-induced reduced liver GST enzyme activities (117). We recently observed increased ovarian levels of Gstm1 mRNA, GSTM1 and GSTP1 proteins due to an obese phenotype (92). In the data reported herein, we observed a temporal increase in ovarian Gstm1 mRNA and GSTP1 protein expression with progressive obesity. GST’s are critical for detoxification of a number of xenobiotic compounds (7, 47, 74). In addition, ovarian GSTP1 (Bhattacharya et al., 2012) and GSTM1 (Bhattacharya and Keating, 2012) negatively regulate pro-apoptotic proteins, thus their increased level in obesity may reflect a reduction in apoptosis during obesity. Both of these functions are in agreement with GSTP1 and GSTM1 being implicated in susceptibility toward and poor prognosis from various forms of gynaecological tumours (38, 42, 45) and development.
of anti-cancer drug resistance (89, 115, 116, 119, 120). Therefore increased abundance of ovarian GSTM1 and GSTP1 during obesity could be part of the underlying mechanisms behind obesity-induced reproductive disorders in obese females.

Taken together, our data demonstrates that progressive obesity increased mRNA and/or protein of ovarian signaling pathways that regulate folliculogenesis as well as ovotoxicant metabolism. These findings are of concern since dysregulated activation of follicles into the growing pool will eventually result in their depletion, accelerating entry into ovarian senescence. In addition, altered ovarian capacity to biotransform chemicals could pose a threat to both folliculogenesis and stability of the germ line.

**Grants:** The project described was supported by award number R00ES016818 to AFK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
References


85. McDonald CA, Millena AC, Reddy S, Finlay S, Vizcarra J, Khan SA, and Davis JS. Follicle-Stimulating Hormone-Induced Aromatase in Immature Rat Sertoli Cells Requires an Active Phosphatidylinositol 3-Kinase Pathway and Is Inhibited via the Mitogen-Activated Protein Kinase Signaling Pathway. *Molecular Endocrinology* 20: 608-618, 2006.


Table 1: Primer Sequences used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irs2</td>
<td>GAA GCG GCT AAG TCT CAT GG</td>
<td>GAC GGT GGT GGT AGA GGA AA</td>
</tr>
<tr>
<td>Irs3</td>
<td>TCG GCT CAC CGT TTC CTT G</td>
<td>TCG CTC TCG TAG CAC TCC A</td>
</tr>
<tr>
<td>Akt2</td>
<td>TGG ACC ACA GTC ATC GAG AG</td>
<td>CTT GTA ATC CAT GGC GTC CT</td>
</tr>
<tr>
<td>Foxo1</td>
<td>GAG TGG ATG GTG AAG AGC GT</td>
<td>TGC TGT GAA GGG ACA GAT TG</td>
</tr>
<tr>
<td>Foxo3a</td>
<td>CTG GGG GAA CCT GTC CTA TG</td>
<td>TCA TTC TGA ACG CGC ATG AAG</td>
</tr>
</tbody>
</table>
Figure 1: Obesity temporally increases ovarian mRNA expression of insulin signaling members. Total ovarian RNA was isolated from lean or obese mice at 6, 12, 18 or 24 weeks (n = 4, per group per time point), reverse transcribed and qRT-PCR performed to quantify mRNA levels of (A) *Insr*, (B) *Irs1*, (C) *Irs2*, and (D) *Irs3*. Target gene mRNA expression values were normalized to *Gapdh* as a housekeeping gene. Results are presented as relative fold-change means ± SEM. * indicates significant difference from age matched lean females at *P* < 0.05.
Figure 2: Progressive obesity increases ovarian phosphorylated insulin receptor substrate 1 protein. Total ovarian protein was isolated from lean (L) and obese (O) mice at 6, 12, 18, or 12 weeks (n = 3-4 per group per time point). Protein expression of pIRS1 (Ser302) was determined by Western blotting, followed by densitometric quantification of the appropriate protein band using Carestream molecular imaging software. Bars represent means ± SEM in arbitrary units. * indicates significant difference from age matched lean females at $P < 0.05$. 
Figure 3: Ovarian Kitlg/cKit-PI3K pathway members are elevated during obesity. Total ovarian RNA was isolated from lean or obese mice at 6, 12, 18 or 24 weeks (n = 4, per group per time point), reverse transcribed and qRT-PCR performed to quantify the mRNA levels of (A) Kitlg, (B) Kit, (C) Akt1 (D) Akt2 (E) Foxo1 and (G) Foxo3a. Target gene mRNA expression values were normalized to Gapdh as a housekeeping gene. Results are presented as relative fold-change means ± SEM. * indicates significant difference from age matched lean females at P < 0.05.
Figure 4: Ovarian phosphorylated AKT is increased by obesity without impacting FOXO3 protein levels. Total ovarian protein was isolated from lean (L) and obese (O) mice at 6, 12, 18, or 12 weeks (n = 3-4 per group per time point). Protein expression was determined by Western blotting, followed by densitometric quantification of the appropriate protein bands using Carestream molecular imaging software. (A) pAKT<sub>Thr308</sub> and (B) FOXO3 protein levels. Bars represent means ± SEM in arbitrary units. * indicates significant difference from age matched lean females at P < 0.05.
Figure 5: Progressive obesity alters mRNA expression of ovarian xenobiotic metabolism genes. Total ovarian RNA was isolated from lean or obese mice at 6, 12, 18 or 24 weeks (n = 4, per group per time point), reverse transcribed and qRT-PCR performed to quantify the mRNA levels of (A) Gstm1, (B) Gstp1, (C) Ephx1, and (D) Cyp2e1. Target gene mRNA expression values were normalized to Gapdh as a housekeeping gene. Results are presented as relative fold-change means ± SEM. * indicates significant difference from age matched lean females at $P < 0.05$. 
Figure 6: Abundance of ovarian xenobiotic metabolism proteins is impacted by increased body mass. Total ovarian protein was isolated from lean (L) and obese (O) mice at 6, 12, 18, or 12 weeks (n = 3-4 per group per time point). Protein expression was determined by Western blotting, followed by densitometric quantification of the appropriate protein band using Carestream molecular imaging software. (A) GSTP1, (B) EPHX1, and (C) CYP2E1 protein levels. Bars represent means ± SEM in arbitrary units. * indicates significant difference from age matched lean females at $P < 0.05$. 
CHAPTER 6: IMPACT OF OBESITY ON OVOTOXICITY INDUCED BY 7,12-DIMETHYLBENZ[A]ANTHRACENE IN MICE

A paper accepted by Biology of Reproduction

Nteeba, J., Ganesan, S., Keating, A.F.

Contribution Statement:
I performed the animal studies, all analyses on ovarian tissue, designed the experiments, interpreted data and wrote the paper. Ganesan, S. aided in glucose tolerance testing and tissue collection; Keating, A.F. designed experiments, aided in data interpretation and edited the manuscript.

Summary sentence: Ovaries from obese mice have altered capacity for chemical biotransformation and increased DMBA-induced ovotoxicity.

Abstract

Insulin, elevated during obesity, regulates xenobiotic biotransformation enzymes, potentially through phosphatidylinositol 3-kinase (PI3K) signaling, in extra-ovarian tissues. PI3K regulates oocyte viability, follicular activation and ovarian chemical biotransformation. 7,12-dimethylbenz[a]anthracene (DMBA), a carcinogen and ovotoxicant, destroys all stage follicles, leading to premature ovarian failure. Obesity has been reported to promote DMBA-induced tumors, but it remains unknown whether obesity impacts ovarian xenobiotic metabolism. Therefore, we investigated ovarian expression of xenobiotic metabolism genes (microsomal epoxide hydrolase (Ephx1), Glutathione S-transferase (GST) class Pi (Gstpi) and class mu 1 (Gstm1)) and PI3K
signaling members (protein kinase B (AKT) alpha (Akt1), beta (Akt2), and the forkhead transcription factor subfamily 3 (Foxo3)) in lean and obese female mice after DMBA exposure (1 mg/Kg; intraperitoneal injection for 14 days). Relative to lean, obese mice had decreased ($P < 0.05$) healthy primordial and primary follicle numbers but increased ($P < 0.05$) secondary and pre-ovulatory follicles numbers. Obesity increased ($P < 0.05$) Akt1, Akt2, Gstm1, Ephx1 mRNA and pAKT$^{Ser473/Thr308}$, GSTM1, GSTP1 and EPHX1 protein levels. DMBA decreased ($P < 0.05$) ovarian weight in lean and obese mice, however, obese DMBA-treated females had a greater reduction ($P < 0.05$) in ovarian weight. In both lean and obese mice, DMBA decreased ($P < 0.05$) all stage healthy follicle numbers, increased Gstp1, Ephx1 mRNA and GSTM1, GSTP1 and EPHX1 protein levels and decreased Akt1, Akt2 mRNA, pAKT$^{Ser473}$ or pAKT$^{Thr308}$, FOXO3 and pFOXO3$^{Ser253}$ protein expression. There was an additive effect between obesity and DMBA exposure for increased Gstm1, Ephx1 mRNA, and GSTM1 and EPHX1 protein expression.
Introduction

Obesity is positively correlated with a number of health hazards some of which, including diabetes [1-5], cardiovascular disease [6-9] and cancer [10-14], are the leading causes of preventable death. The prevalence of obesity and obesity-related health complications, such as type 2 diabetes, which were previously considered “adult diseases”, are now also rising dramatically in children [8, 15, 16] leading to premature death and infertility. In recent years, a strong association between increased body mass index with enhanced incidence in reproductive health impairments has been reported [12, 17-20]. Obese women have an increased likelihood to display signs of polycystic ovarian syndrome (PCOS), reduced fecundity and poor quality oocytes [19, 21]. There has also been a strong link between obesity and an increased risk of birth defects, premature and still-birth [22-28] and gestational diabetes [29, 30]. Although, there is strong association between obesity and compromised reproductive health, the molecular mechanisms involved remain ill-defined. Additionally, despite the alarming prevalence of obesity and obesity associated maladies, little is known of how this epidemic may influence ovarian xenobiotic metabolism.

Ovaries are important for supplying the germ cell necessary for perpetuation of species and production of hormones essential for female growth and development [31-33]. At birth, females are born with a limited number of primordial follicles, which once depleted, cannot be replenished [33-35]. It is accepted that the process of folliculogenesis is an irreversible process; once follicles are recruited from the resting
pool to the growing pool they will undergo atresia if not selected for further growth to ovulation [36-38]. Unlike the cyclic maturation of follicles to ovulation, initial primordial follicular activation has been identified to be regulated, independent of the pituitary gonadotropins, largely by local ovarian factors including the phosphatidylinositol 3-kinase (PI3K) pathway [38-41]. A balance between dormancy, activation and atresia of primordial follicles is critical for the female reproductive lifespan [37, 38, 42]. Any environmental factor(s) that could accelerate the rate of primordial follicle activation and the process of atresia, would greatly threaten the reproductive potential of the female.

Obesity can alter insulin sensitivity in a number of target tissues including muscle, liver, adipose tissue and the ovary [2, 43-46]. Insulin hormone binds to its receptor resulting in auto-phosphorylation and recruitment of the insulin receptor substrate proteins (Irs) [47-49], which in turn regulate numerous downstream insulin-mediated signaling pathways including PI3K signaling [2, 43]. PI3K signaling events are largely mediated through Protein kinase B (AKT), a subfamily comprising of three mammalian isoforms Akt1, Akt2 and Akt3 [50]. Upon PI3K activation, it recruits AKT to the membrane where AKT is phosphorylated (pAKT). pAKT dissociates from the membrane and shuttles to the cell nucleus where it has the ability to phosphorylate and inactivate several targets including forkhead transcription factor subfamily 3 (FOXO3).
A number of environmental chemicals can target the ovary and destroy the primordial follicles as well as other follicle types, leading to premature ovarian failure, infertility and other health impairments [31, 32, 51-53], making ovarian xenobiotic metabolism critical for protection of the female germ cell. The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA), is an environmental carcinogen [54-58] as well as ovotoxicant [51, 52, 59, 60]. Human exposure to DMBA is mainly through smoke or fumes from burning of organic substances such as coal, car exhaust and cigarette smoke [59, 61]. Relative to non-smokers, women who are cigarette smokers undergo early onset of menopause and suffer infertility [62-64]. Studies from animal models have demonstrated that DMBA exposure can destroy follicles of all types resulting in accelerated premature ovarian insufficiency and other reproductive complications [32, 51, 59, 65, 66]. In the liver, the parent compound DMBA is bioactivated to its more toxic metabolite, DMBA-3,4-diol-1,2-epoxide by cytochrome P450 isoforms 1B1 (CYP1B1) and 1A1 (CYP1A1) [59, 61, 67, 68] in conjunction with microsomal epoxide hydrolase (EPHX1) [69, 70]. Several studies have also demonstrated the ovary’s capacity to metabolize DMBA to its more ovotoxic metabolite, DMBA-3,4-diol-1,2-epoxide via the action of EPHX1 [51, 59, 61, 68, 70-72].

Insulin can regulate the expression xenobiotic biotransformation genes products such as the CYP’s, Glutathione S-transferases (GST) and EPHX1 through PI3K/AKT signaling [73, 74]. Inhibition of PI3K has also been reported to alter the expression of EPHX1, GSTP1 and GSTM1 genes in postnatal day 4 cultured rat ovaries [75-77].
GSTP1 and GSTM1 are members of the GST superfamily of proteins involved in phase II metabolism of xenobiotic compounds. These enzymes function in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione [78]. Though the effects of obesity on DMBA-induced ovotoxicity have not been previously explored, regulation of EPHX1 by insulin in non-ovarian tissues has been reported [73, 74]. Since obesity can alter insulin action to its target tissues including the ovary, we hypothesized that obesity-induced increased insulin could increase PI3K signaling and alter xenobiotic gene expression leading to accelerated DMBA-induced ovotoxicity.

Materials and Methods

Reagents

7,12-dimethylbenz[a]anthracene (>98% DMBA), 2-[β-mercaptoethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulfate, glycerol, N’N’,N’,N’-Tetramethyl-ethylenediamine (TEMED), Tris base, Tris HCl, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (Vitamin C), phosphatase inhibitor, protease inhibitor and transferrin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). RNAlater was obtained from Ambion Inc (Austin, TX). Hanks’ Balanced Salt Solution (without CaCl$_2$, MgCl$_2$, or MgSO$_4$) and superscript III one-step RT-PCR System were obtained from Invitrogen Co. (Carlsbad, CA). RNeasy Mini kit, QIAshredder kit, RNeasy MinElute kit and QuantitectTM SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). Custom designed primers were obtained
from the DNA facility of the Office of Biotechnology at Iowa State University. Ponceau S was purchased from Fisher Scientific (Waltham, MA, USA). SignalFire™ ECL Reagent and Anti-pAKT<sup>Ser473</sup> antibody were from Cell Signaling Technology® (Danvers, MA, USA). Anti-FOXO3, anti-pFOXO3<sup>Ser253</sup>, anti-alpha Tubulin (TUBA), anti-GSTP1 and anti-GSTM1 antibodies were purchased from Millipore (Temecula, CA, USA). Anti-pAKT<sup>Thr308</sup> antibody was purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit and donkey anti-goat secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL). Restore™ PLUS Western Blot Stripping Buffer was purchased from Thermo SCIENTIFIC (Rockford, IL, USA).

**Animals**

Four week old female wild type normal non-agouti (a/a; designated lean; n = 15) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 15) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and housed at the animal facility at Iowa State University. All experimental protocols and procedures were approved by the Iowa State University Animal Care Committee (IACUC). Animals were maintained under controlled lighting (12 hr light/12 hr dark) and temperature (21-22°C) conditions. Food and water were provided *ad libitum*. At 6 weeks of age, non-agouti and agouti lethal yellow mice (n = 5/genotype) were sacrificed. At 18 weeks of age, glucose tolerance testing confirmed that obese mice were less glucose tolerant than their lean littermates and had higher systemic basal glucose level. Both lean and obese mice (n = 10/genotype), were intraperitoneally (i.p.) dosed with sesame oil or DMBA (> 98%;
1mg/kg/day) for 14 days. This dose was chosen based on destruction of approximately 50% of primary and secondary follicles, with a higher loss of primordial follicles [52].

**Tissue collection**

Mice were euthanized at 6 weeks of age or 3 days after the end of dosing (approximately 20 weeks of age) during the pro-estrus stage of cyclicity and body weight was recorded. Ovaries were collected, trimmed of excess fat and weighed. One ovary was fixed in 4% paraformaldehyde for histological analysis while the other ovary was stored in RNAlater at -80°C for RNA and protein expression studies.

**Histology and Follicle Counting**

The histology work was performed at the Iowa State University Veterinary Medicine Histopathology laboratory. Briefly, one ovary from each animal was fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, dehydrated, embedded in paraffin blocks, serially sectioned (5 µM) and every 6th section (4-6 sections/slide) was mounted (15–20 slides per animal), and stained with hematoxylin and eosin (H & E). Digital images were acquired with a Leica DMI300B Fluorescent Microscope. Numbers of healthy follicles (oocyte-containing follicles showing a distinct oocyte nucleus) were classified and counted in every 12th section according to the procedures as previously described [79, 80]. Briefly, primordial follicles contained an oocyte surrounded with a single layer of squamous-shaped granulosa cells, primary follicles contained an oocyte surrounded by a single layer of cuboidal-shaped granulosa cells, secondary follicles
contained an oocyte surrounded by multiple layers of granulosa cells and antral follicles contained an oocyte surrounded by at least two layers of granulosa cells and a fluid-filled antral space.

RNA Isolation

Total RNA was isolated using Qiagen RNeasy® Mini Kit (n = 3 ovaries per treatment) according to the manufacturer’s protocol. Briefly, ovaries were lysed and homogenized using a hand-held homogenizer followed by applying the homogenate to a QIAshredder column with subsequent centrifugation at 16100 RCF for 2 minutes at room temperature. The resulting supernatant was applied to an RNeasy Mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter and concentrated using an RNeasy MinElute Kit according to the manufacturer’s protocol. The final total concentrated RNA was eluted using 14 µl of RNase-free water and RNA concentration determined using an ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE).

First strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA (0.5 µg) was reverse transcribed into cDNA utilizing the Invitrogen™ Superscript III Reverse Transcriptase as detailed in the manufacturer’s procedures. Two microliters of diluted cDNA (1:25) was amplified on an Eppendorf Mastercycler using a Quantitect™ SYBR Green PCR kit and primers specific for mouse
*Gapdh, Ephx1, Gstp1, Gstm1, Akt1* (sequences as indicated in Nteeba et al [81]) and *Akt2* (forward primer: 5′- TGG ACC ACA GTC ATC GAG AG-3′; reverse primer: 5′-CTT GTA ATC CAT GGC GTC CT-3′). The PCR cycling program consisted of a 15 min hold at 95°C and 45 cycles of: denaturing at 95°C for 15 sec, annealing at 58°C for 15 sec, and extension at 72°C for 20 sec at which point data was acquired. Product melt conditions were determined using a temperature gradient from 72°C to 99°C with a 1°C increase at each step. Three replicates of each sample (n = 3 wells per treatment) were included. Statistical analysis was performed on the normalized ∆CT for each sample. There was no difference in ovarian *Gapdh* mRNA level between sesame control and DMBA-treated ovaries in lean or obese mice. Therefore, for each sample, relative mRNA expression of each of the above genes was normalized using *Gapdh* as a housekeeping gene and relative fold change calculated using the $2^{-\Delta\Delta CT}$ method. The results are presented as mean fold change ± standard error relative to the sesame control group.

**Protein Isolation and Western Blot Analysis**

Total ovarian protein was isolated and immunoblots performed according to the procedure of Nteeba et al. [81]. Briefly, ovaries (n = 3 per treatment) were homogenized in 300 µl of ice-cold tissue lysis buffer and protein quantified using a standard BCA protocol on a 96-well assay plate. Total protein (15 µg) was separated on a 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Following blocking, membranes were incubated with specific primary antibodies [Rabbit Anti-GSTP1
Rabbit Anti-GSTM1 (1:200), Goat Anti-EPHX1 (1:500), Rabbit Anti-pAKTSer473 (1:500), Rabbit Anti-pAKTThr308 (1:500), Rabbit Anti-FOXO3 (1:500), Rabbit Anti-pFOXO3Ser253 (1:1000)] in 5% BSA in TTBS for 15-20 h at 4°C. HRP-conjugated secondary antibodies (1:2000 – 1:5000) were added for 1h at room temperature, and then membrane-bound HRP were washed three times for 5 minutes in TTBS. Autoradiograms were visualized on X-ray films in a dark room following 7 min incubation of membranes with 1X SignalFire™ ECL reagent. Equal protein loading was confirmed by Ponceau S staining of nitrocellulose membranes prior to antibody incubation. Additionally, blots were stripped and probed with an anti-TUBA or anti-GAPDH antibody. Densitometry of the appropriate sized bands was measured using Carestream molecular imaging software version 5.0 (Carestream Health Inc., Rochester, NY) which eliminates background noise. Values were normalized to the appropriate loading control (TUBA, GAPDH, or Ponceau S staining).

Statistical Analysis

Statistical analyses were performed using either the Two-Way ANOVA followed by Bonferroni multiple comparisons to assess interaction between strain and drug or the One-Way ANOVA followed by Tukey’s multiple pairwise comparison function of GraphPad Prism 5.5 software with a statistical significance level set at \( P < 0.05 \). Bars represent means ± SEM. Different letters indicate significant difference from respective pairs; \( n = 3 \) per treatment.
Results

*Obesity decreases the number of healthy primordial follicles in murine ovaries*

We determined the effect of DMBA exposure on ovarian weight and the number of healthy follicles in lean and obese mice (Fig. 1). There was no effect of DMBA on body weight (Fig. 1A); however, compared to sesame oil, DMBA decreased ovarian weight in both lean ($P < 0.05$) and obese ($P < 0.001$) treated groups (Fig. 1B). There was no difference between ovarian weight of lean and obese sesame treated groups; however, DMBA treatment further reduced ($P < 0.05$) ovarian weight in the obese compared to lean mice (Fig. 1B). In both lean and obese females, DMBA treatment significantly decreased ($P < 0.0001$) the number of healthy follicles compared to sesame-treated females (Fig. 1C-1F). Interestingly, ovaries from obese mice had both decreased ($P < 0.0001$) number of healthy primordial (Fig. 1C) and primary (Fig. 1D) follicles but increased ($P < 0.001$) secondary (Fig. 1E) and pre-ovulatory (Fig. 1F) follicles compared to ovaries from lean sesame-treated mice. No impact of obesity or DMBA on corpora lutea numbers were observed.

*DMBA and obesity have an additive effect on ovarian Ephx1 and Gstm1 gene expression*

Relative to lean counterparts, ovaries from obese mice had increased ovarian Gstm1 (Fig. 2A; $P < 0.01$) and Ephx1 (Fig. 2C; $P < 0.05$) mRNA levels. However, obesity did not affect Gstp1 (Fig. 2B) mRNA levels in murine ovaries. In lean ovaries,
DMBA did not alter \textit{Gstm1} mRNA expression. \textit{Gstp1} and \textit{Ephx1} mRNA levels were increased ($P < 0.05$) in ovaries of both lean and obese treated groups compared to those of their respective control groups (Fig. 2A – 2C). Compared to lean DMBA-treated females, ovaries from obese DMBA-treated females displayed increased ($P < 0.01$) \textit{Gstm1} and a strong tendency for increased ($P = 0.06$) \textit{Ephx1} but no difference in \textit{Gstp1} mRNA levels (Fig. 2A – 2C). Lack of any impact of genotype on \textit{Gstm1}, \textit{Gstp1} or \textit{Ephx1} mRNA level was confirmed in ovaries from 6 week old mice (pre-obese) (Fig. 2D).

\textit{DMBA increases ovarian GSTM1, GSTP1, and EPHX1 protein levels in obese but not in lean mice}

Ovaries from obese mice had increased ($P < 0.05$) basal ovarian GSTM1 protein levels relative to their lean counterparts (Fig. 3A). DMBA exposure did not impact GSTM1 protein level in lean ovaries however there was increased ($P < 0.05$) GSTM1 protein levels in ovaries of obese treated groups compared to their respective control groups (Fig. 3A). Relative to lean counterparts, ovaries from obese mice had increased basal ovarian GSTP1 ($P < 0.001$) protein levels (Fig. 3B). Interestingly, DMBA increased ($P < 0.05$) GSTP1 protein level in ovaries of lean mice, but decreased ($P < 0.05$) ovarian GSTP1 protein level in obese treated groups compared to those of their respective control groups (Fig. 3B). Similar to mRNA results, ovaries from obese mice had increased ($P < 0.05$) basal ovarian EPHX1 protein levels relative to their lean counterparts (Fig. 3C). DMBA increased ($P < 0.05$) EPHX1 protein levels in ovaries of
both lean and obese treated groups compared to their respective control groups (Fig. 3C). Relative to lean DMBA treated mouse ovaries; obesity resulted in a greater increase ($P < 0.01$) in EPHX1 protein levels, relative to the obese sesame oil-treated mouse ovaries (Fig. 3C).

**Obesity and DMBA have opposing effects on ovarian Akt1/2 mRNA expression**

Ovaries from obese females displayed higher ($P < 0.05$) Akt1 (Fig. 4A) and Akt2 (Fig. 4B) mRNA levels than lean females; however, DMBA exposure significantly reduced ovarian Akt1/2 mRNA levels in both lean ($P < 0.001$) and obese ($P < 0.05$) treated groups (Fig. 4). Akt1 and Akt2 mRNA levels were not found to differ between genotype in ovaries from 6 week old mice (pre-obese) (Fig. 4C).

**Obesity increases ovarian phosphorylated AKT without affecting phosphorylated FOXO3 protein levels**

DMBA decreased ($P < 0.05$) ovarian pAKT$^{\text{Ser473}}$ protein in lean mice, while obesity increased ($P < 0.05$) basal pAKT$^{\text{Ser473}}$ levels and the reduction observed in the lean mice was absent from ovaries of obese mice. In lean mice, there was no impact of DMBA on pAKT$^{\text{Thr308}}$ protein, however obesity increased ($P < 0.05$) basal ovarian pAKT$^{\text{Thr308}}$ protein levels (Fig. 5). In contrast to lean ovaries, those from obese mice had a decrease in pAKT$^{\text{Thr308}}$ when exposed to DMBA. Lean and obese females did not differ in FOXO3 protein level, and although there was a visible reduction in DMBA-
treated lean mice, this was not significant due to variation. pFOXO3^{Ser253} protein expression was reduced by DMBA in both lean and obese ovaries, and there was a lower level basally ($P < 0.05$) of pFOXO3^{Ser253} due to obesity.

**Discussion**

Obesity is associated with elevated blood glucose and insulin levels, altering insulin’s action on a number of organs including the ovary [2, 43-46]. Recently, it has been demonstrated that the ovary maintains insulin sensitivity during obesity even though other classical tissues including the liver, muscle and adipose tissue develop insulin resistance [43, 81]. Insulin has been previously shown to regulate hepatic xenobiotic metabolism [73, 74, 82, 83] and in the ovary insulin can activate the PI3K/AKT signaling, a pathway that has been demonstrated to play critical roles in metabolism and reproduction [84]. Previous work in rodent models has shown the involvement of PI3K signaling in insulin-mediated hepatic regulation of xenobiotic metabolism [83, 85]. Recently, the involvement of PI3K signaling in ovarian metabolism of chemicals including DMBA has been demonstrated [60, 75, 86]. Since obesity is associated with elevated plasma insulin levels, we hypothesized that obesity-induced increased insulin could increase PI3K signaling and alter xenobiotic gene expression leading to accelerated DMBA-induced ovotoxicity. To test this hypothesis, we used the lethal yellow mouse, an excellent model for human obesity [87], to investigate whether obese females have increased susceptibility to DMBA-induced ovotoxicity. We designed our experiments on the impact of obesity on chemical metabolism when mice
were 18 weeks of age because elevated insulin has been shown in this mouse model at this timepoint [88]. In addition, we confirmed that the obese mice had elevated basal glucose and had a compromised response to a glucose tolerance test (data not shown) and that there was no impact of the lethal yellow genotype on follicle numbers at 6 weeks of age (our unpublished data). Further, to eliminate any impact of genotype outside of the obese phenotype, we characterized gene expression in 6 week old mice; at this point there is no difference in body weight and the lethal yellow mice are considered pre-diabetic.

Relative to sesame oil, DMBA treatment did not impact body weight; however, compared to sesame oil, DMBA decreased ovarian weight in both lean and obese treated groups. Interestingly, we observed lower than expected primordial follicle numbers in ovaries of both lean and obese sesame oil treated animals, which could be due to their being 20 weeks of age, and potentially this is a time when follicle numbers are naturally beginning to decline. As expected, DMBA treatment significantly decreased the number of healthy follicles of all types in both lean and obese treated females compared to sesame-treated females. The observed decrease in ovarian weight and the number of healthy follicles in DMBA-treated lean and obese females could be as a result of increased follicle loss induced by DMBA exposure as previously reported [59, 61]. Surprisingly, though we saw no difference between ovarian weight between lean and obese sesame oil-treated groups; there was a significant reduction in ovarian weight of obese females treated with DMBA compared to their lean DMBA-treated littermates,
indicating that the obese ovaries suffered greater levels of DMBA-induced ovotoxicity, outside of follicle depletion. This data supports that the ovaries from obese, DMBA-treated mice had greater levels of ovotoxicity than their lean counterparts that received the same DMBA exposure. It was surprising that larger numbers of growing follicles did not impact ovarian weight, it has previously been shown that ovaries from mice lacking the Arylhydrocarbon receptor had lower numbers of all stage follicles without any difference in ovarian weight [89]. Despite lack of ovarian weight differences between lean and obese mice, ovaries from obese mice had both decreased number of healthy primordial and primary follicles but increased secondary and pre-ovulatory follicles compared to ovaries from lean sesame-treated mice. This difference in follicle populations could indicate increased activation of follicles from the primordial follicle pool. Insulin has been shown to activate primordial to primary transition in neonatal rat ovaries [90]. Additionally, high fat diet fed rats have been recently demonstrated to have increased number of growing and reduced numbers of primordial follicles [91]. Several studies have also reported an inverse relation between BMI and estradiol levels in premenopausal women [92-94]. Decreased estradiol levels have been associated with increased activation of primordial follicle in mice [95]. So it is possible that obesity could trigger changes in the intrinsic ovarian signals responsible for initiation of follicle activation and recruitment.

DMBA has been demonstrated to have both carcinogenic and ovotoxic properties in animal models [54, 59, 61]. These two properties are mediated through DMBA’s
metabolite 3,4-diol-1,2-epoxide which is formed during DMBA metabolism facilitated by CYP1B1, EPHX1 and CYP1B1/1A1 enzymes [59, 96-99]. Although the liver is the primary site for xenobiotic metabolism, the ovary has been demonstrated to have the capacity to metabolize xenobiotic compounds [60, 32, 67, 68, 75, 76] including DMBA in absence of hepatic contributions [59, 96-99]. Several studies have confirmed that EPHX1 [59, 61, 98] required for DMBA bioactivation, is expressed at high levels in the ovary and is increased following DMBA exposure. Although obesity has been reported to promote DMBA-induced tumors [100], there is a dearth of literature examining potential effects of obesity on ovarian xenobiotic metabolism including DMBA.

Consistent with previous studies, ovaries from obese mice had increased ovarian Ephx1 mRNA [81] and EPHX1 protein [59, 61] levels relative to their lean counterparts. While DMBA treatment increased Ephx1 mRNA and EPHX1 protein levels in ovaries of both lean and obese treated groups compared to their respective control groups, ovaries from obese DMBA-treated females displayed a greater increase in Ephx1 mRNA and EPHX1 protein expression compared to lean DMBA-treated females. To the best of our knowledge, this is the first study to report a DMBA-obesity synergistic effect for increased ovarian Ephx1 mRNA and EPHX1 protein expression in mice. In extra-ovarian tissues, insulin has been demonstrated to increase hepatic EPHX1 expression [73, 74, 83] while glucagon to inhibit EPHX1 expression [83]. In rats, conditions of insulin deficiency induced by either Type 1 diabetes or starvation were associated with decreased EPHX1 enzyme activity yet on the other hand, addition of insulin or re-
feeding restored EPHX1 activity [74]. Taken together, these data demonstrate that insulin plays a role in induction of EPHX1 expression and activity. Since obesity is associated with elevated levels of insulin, it is more likely that one of the mechanisms by which obesity accelerates ovarian EPHX1 expression is through hyperactivation of insulin-mediated EPHX1 induction signaling pathways such as the PI3K pathway. In cultured primary rat hepatocytes, administration of insulin increased EPHX1 mRNA and protein expression in a time- and concentration-dependent manner [83], conversely, inhibition of PI3K by Wortmannin and LY294002 or the mTOR inhibitor, rapamycin, modulated the insulin-induced increase in EPHX1 [83], supporting the involvement of PI3K signaling in insulin-induced hepatic regulation of EPHX1. Furthermore, Ki and Kim [85] confirmed the involvement of PI3K signaling in induction of EPHX1 through C/EBP transcription factors. With recent reports demonstrating the involvement of PI3K signaling in ovarian metabolism of DMBA [60, 75, 86], it is highly probable that the observed DMBA-obesity synergistic effect on ovarian Ephx1 mRNA and EPHX1 protein expression is mediated through insulin/PI3K signaling pathway.

Just like EPHX1, regulation of GSTM1 and GSTP1 by insulin in non-ovarian tissues has been reported [73, 74, 101]. Insulin administration is known to increase GST gene expression through the PI3K/AKT/mTOR pathway while glucagon decreases such gene expression [101]. In alloxan- and streptozotocin-diabetic male Fischer-344 rats, diabetes decreased the activities of rat liver soluble GST’s, yet application of insulin to alloxan-diabetic individuals approximately restored the initial enzyme activities [74].
Similarly, starvation of Fischer-344 rats, resulted in reduced activities of GST enzymes, however, re-feeding restored their initial activities [74]. Taken together, these results suggest that insulin also regulates the hepatic activities of GST enzymes. Although in the current study we did not measure the activities of these enzymes, we observed increased \textit{Gstm1} mRNA, GSTM1 and GSTP1 protein levels in ovaries from obese sesame oil-treated females compared to lean mice, mimicking the trend observed during normal insulin activity. Taken together, these results could support the notion that despite insulin resistance in other tissues, the ovary maintains insulin sensitivity.

DMBA-induced increases in \textit{Gstp1} mRNA and GSTP1 protein levels in PND4 cultured rat ovaries have been reported [76], however the effect of DMBA on ovarian expressed GSTM1 has not been previously reported. In the present study, DMBA treatment had contrasting effects on GSTP1 and GSTM1 in both lean and obese treated mice. Interestingly, DMBA increased ovarian \textit{Gstm1} mRNA and GSTM1 protein levels in obese but not in lean mice yet on the other hand DMBA increased ovarian \textit{Gstpl} mRNA in both lean and obese females and GSTP1 protein levels in only lean but decreased GSTP1 protein expression in obese females. Furthermore, though there was no difference in ovarian \textit{Gstpl} mRNA and GSTP1 protein levels between lean DMBA- and obese DMBA-treated females, relative to lean DMBA-treated females; ovaries from obese females exhibited a greater increase in both \textit{Gstm1} mRNA and GSTP1 protein expression, following DMBA treatment. Given the divergent roles played by GST enzymes, depending on the physiological conditions and type of cells involved, changes
in expression of genes encoding for these enzymes could be beneficial or have detrimental consequences for the cells. Although best known for their detoxification role in metabolism [53, 76, 102-105], GST enzymes have also been implicated in cell signaling, intracellular transport and isomerization of steroid hormones [105-107] as well as development of chemotherapeutic-drug resistance [104, 105, 108-111] and a variety of diseases including cancer, diabetes and inflammatory diseases [101, 104, 110, 112-114]. In particular, overexpression of GSTP1 has been associated with inactivation of cigarette smoke carcinogens [105] including detoxification of DMBA-induced toxicity [53, 76, 102], and development of drug resistance [105, 108, 110, 111, 114, 115], susceptibility to and poor prognosis of several cancers including breast, cervical and ovarian cancer [112]. This is could be due to its ability to selectively inhibit pro-apoptotic p38 and C-Jun N-Terminal Kinase 1 (JNK1) portions of the Mitogen-Activated Protein Kinase (MAPK) signaling cascades [53, 107, 108, 110, 114]. Like GSTP1, there is a growing body of evidence supporting the role of GSTM1 in regulating apoptotic pathways through direct protein-protein interactions, with Apoptosis Signal-regulating Kinase 1 (ASK1), which is upstream of JNK. GSTM1 sequesters ASK1 through complex formation and subsequent prevention of its induction of the pro-apoptotic p38 and JNK portions of the MAPK signaling cascade [101, 104, 108, 116]. It is important to note that increased expression of GSTs in obese females and during DMBA treatment in both lean and obese females did not avert DMBA-induced follicle loss. This would imply that the role of overexpressed GSTM1 and GSP1 could be not
principally involved in detoxification but rather be involved in other cellular processes that are yet to be determined.

Insulin regulates xenobiotic metabolism through PI3K/AKT pathway [83, 85, 101]. Previous studies have also implicated PI3K/AKT/mTOR pathway in DMBA-induced ovotoxicity [86]. In the current study, obesity and DMBA had opposing effects on PI3K/AKT signaling pathway members’ gene expression. Ovaries from sesame oil-treated obese females displayed higher Akt1 and Akt2 mRNA levels concomitant with upregulated pAKT$^{\text{Ser473/Thr308}}$ protein levels, without affecting total FOXO3, but reduced pFOXO3$^{\text{Ser253}}$ protein expression compared to their lean counterparts. Surprisingly, DMBA treatment significantly reduced ovarian Akt1/2 mRNA levels in both lean and obese treated groups, mirrored by decreased pAKT$^{\text{Ser473}}$ protein in lean but not obese ovaries. A decrease in pAKT$^{\text{Thr308}}$ protein expression was observed in the ovaries from obese females. Interestingly, though pFOXO3$^{\text{Ser253}}$ was decreased in both lean and obese DMBA-treated mice, FOXO3 protein expression was not impacted by either obesity or DMBA. These alterations to the PI3K pathway may be at least partially responsible for the altered dynamics of follicle activation observed in the obese mouse ovary.

In conclusion, the ovary contains a finite number of primordial follicles, which once depleted, cannot be replaced; therefore ovarian xenobiotic metabolism of chemical compounds like DMBA which can deplete the primordial follicle pool is critical for protection of the female germ cell. Since insulin regulates hepatic expressed xenobiotic
metabolism enzymes and obesity alters insulin sensitivity in a number of target tissues, understanding how obesity might influence the ovary’s capacity to metabolize chemicals is critical. Our data is in agreement with previous studies which demonstrated that insulin induces the hepatic activities of EPHX1 and GST enzymes [73, 74, 101]. We have shown that obesity increased mRNA and protein levels of pAKT, EPHX1, GSTM1 and GSTP1 compared to lean mice, mimicking the trend observed during insulin administration or re-feeding. Therefore, our data support the notion that despite insulin resistance in other tissues, the ovary seems to maintain insulin sensitivity and that obesity-induced increased insulin could increase PI3K signaling and alter xenobiotic gene expression leading to accelerated DMBA-induced ovotoxicity.

Grants: The project described was supported by award number R00ES016818 to AFK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
References


18. Barber TM, Franks S. The link between polycystic ovary syndrome and both Type 1 and Type 2 diabetes mellitus: what do we know today? Womens Health (Lond Engl) 2012; 8:147-154.


227


70. Miyata M, Motoki K, Tamura E, Furukawa M, Gonzalez FJ, Yamazoe Y. Relative importance of maternal and embryonic microsomal epoxide hydrolase in


86. Sobinoff AP, Mahony M, Nixon B, Roman SD, McLaughlin EA. Understanding the Villain: DMBA-Induced Preantral Ovotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling. Toxicological Sciences 2011; 123:563-575.


92. Freeman EW, Sammel MD, Lin H, Gracia CR. Obesity and reproductive hormone levels in the transition to menopause. Menopause 2010; 17:718-726.


Figure 1: Effect of DMBA on ovarian weight and follicle numbers in lean and obese mice. At 18 weeks of age lean and obese mice were intraperitoneally (i.p) dosed with sesame oil or DMBA (95%; 1mg/kg) for 14 days. Mice were euthanized 3 days after the end of dosing; body weight measured (A), ovaries collected, trimmed of excess fat and weighed (B). One ovary was fixed in 4% paraformaldehyde and complete serial sections were prepared and subjected to histological examination for the number of healthy follicles in both lean and obese treated groups (C-F); (C) healthy primordial follicle number; (D) healthy primary follicle number; (E) healthy secondary follicle number; (F) healthy pre-ovulatory follicle number. Bars represent mean follicle number per ovary ± SEM. Different letters indicate significant ($P < 0.05$; $n = 3$) difference from respective pairs.
Figure 2: Effects of DMBA on ovarian metabolism gene expression in lean and obese mice 18 weeks old lean and obese female mice were treated with sesame oil or DMBA (95%; 1mg/kg; i.p) for 14 days. Three days after dosing, ovaries were collected, trimmed of excess fat and total ovarian RNA isolated. Using quantitative (q) RT-PCR, relative mRNA levels of (A) *Gstm1*, (B) *Gstp1* and (C) *Ephx1* were evaluated after normalization to the housekeeping gene, *Gapdh*. (D) Ovarian *Gstm1*, *Gstp1*, *Ephx1* mRNA levels in 6 week old lean and obese mice. Values represent relative fold-change means ± SEM. Different letters indicate significant difference from respective pairs, $P < 0.05$; n = 3.
Figure 3: Effect of DMBA on ovarian GSTM1 protein in lean and obese mice. 18 weeks old lean and obese female mice were treated with sesame oil or DMBA (95%; 1mg/kg; i.p) for 14 days. Three days after dosing, ovaries were collected, trimmed of excess fat and total ovarian protein was isolated (n = 3 per treatment group) from lean control, (LC), lean DMBA (LD), obese Control (OC) and obese DMBA (OD). (A) GSTM1, (B) GSTP1 or (C) EPHX1 proteins were quantified by Western blotting, followed by densitometric quantification of the protein band using Carestream molecular imaging software version 5.0. Bars represent means ± SEM. Different letters indicate significant difference from respective pairs; P < 0.05; n = 3.
Figure 4: Effects of DMBA on ovarian Akt1/2 expression in lean and obese mice. At 18 weeks of age lean and obese mice were intraperitoneally dosed with sesame oil or DMBA (95%; 1mg/kg) for 14 days. Ovaries were collected 3 days after the end of dosing, trimmed of excess fat, and total ovarian RNA isolated (n = 3 per treatment group). Relative mRNA expressions of (A) Akt1 and (B) Akt2 were normalized using Gapdh as a housekeeping gene and quantified using qRT-PCR. (C) Ovarian Akt1 and Akt2, mRNA levels in 6 week old lean and obese mice. Bars represent means ± SEM. Different letters indicate significant difference from respective pairs, p < 0.05; n = 3.
Figure 5: Effect of DMBA on ovarian PI3K proteins in lean and obese mice. 18 weeks old lean and obese female mice were treated with sesame oil or DMBA (95%; 1mg/kg; i.p) for 14 days. Three days after dosing, ovaries were collected, trimmed of excess fat and total ovarian protein was isolated (n = 3 per treatment group) from lean control, (LC), lean DMBA (LD), obese Control (OC) and obese DMBA (OD). Western blotting was performed to measure (A) pAKT<sup>Ser473</sup>, (B) pAKT<sup>Thr308</sup>, (C) FOXO3, and (D) pFOXO3<sup>Ser253</sup> levels. Densitometry of the appropriate bands was performed using Carestream molecular imaging software version 5.0. Bars represent means ± SEM. Different letters indicate significant difference from respective pairs; P < 0.05; n = 3.
CHAPTER 7: HEAT STRESS ALTERS OVARIAN INSULIN MEDIATED
PHOSPHATIDYLINOSITOL-3 KINASE AND STEROIDOGENIC
SIGNALING IN GILT OVARS

A paper to be submitted to American Journal of Physiology - Endocrinology and Metabolism


Contribution Statement:
I performed all analyses on ovarian tissue, designed the ovarian experiments, interpreted data and wrote the paper; Sanz-Fernandez, M.V. performed the animal studies from which ovarian tissues were obtained; Rhoads, R.P., Ross, J.W. and Baumgard, L.H. are the investigators on the grant that funded the animal experiments; Keating, A.F. designed the ovarian experiments, aided in data interpretation and edited the manuscript.

Abstract
Heat Stress (HS) compromises a variety of reproductive functions in several mammalian species. Inexplicably, HS animals are frequently hyperinsulinemic despite marked hyperthermia-induced hypophagia. Our objectives were to determine the effects of HS on insulin signaling and key steroidogenic synthetic machinery components in the pig ovary. Gilts (35±4 kg) were exposed to constant thermal neutral (TN; 20°C; 35-50% humidity) or HS conditions (35°C; 20-35% humidity; n = 3) for either 7 (n = 3) or 35 d (n = 3). After 7d, HS increased (P < 0.05) ovarian mRNA abundance of the insulin receptor (IR), insulin receptor substrate 1 (IRS1), protein kinase B subunit 1 (AKT1), low density lipoprotein receptor (LDLR), luteinizing hormone receptor (LHR), and aromatase (CYP19a). After 35d, HS increased IR, IRS1, AKT1, LDLR, LHR, CYP19a,
steroidogenic acute regulatory protein (STAR), and forkhead box O3 (FOXO3) ovarian mRNA levels. In addition, after 35d HS increased ovarian phosphorylated ISR1 (pIRS1), phosphorylated AKT (pAKT), STAR and CYP19a protein levels. Immunostaining analysis revealed similar localization of IR and pAKT1 in the oocyte cytoplasm and cytoplasmic membrane of all stage follicles, and in theca and granulosa cells. Taken together, these results demonstrate that HS alters ovarian insulin mediated-PI3K signaling pathway members which likely impacts follicle activation and viability.

In summary, environmentally-induced HS is an endocrine disruptor that alters ovarian physiology potentially resulting in malproduction of ovarian hormones essential for fertility and pregnancy maintenance.
Introduction

Ambient temperatures above an animals’ thermoneutral zone can cause heat stress (HS) that has detrimental effects on animal welfare and productivity (27). In a number of livestock species HS is also associated with a variety of female reproductive issues including dysregulated estrous cyclicity (39, 51, 52), reduced conception rate (14, 28), reduced embryonic survival (58), and higher frequency of stillbirths (28, 58). From an agricultural perspective, HS-induced sub-optimal productivity and reproductive performance limits the production of high quality protein for human consumption. Consequently, HS jeopardizes global food security, and this is especially true for developing countries (8).

Several studies have demonstrated that heat-stressed animals have elevated basal circulating insulin concentrations (10, 38, 41, 61) and an increased insulin response to a glucose tolerance test (11). Energetically, this is paradoxical as HS causes a well-conserved decrease in nutrient intake and is generally considered a hypercatabolic condition (9). Reasons for elevated blood insulin (a potent anabolic signal) during HS are not yet fully understood, but likely include insulin’s key role in activating and up-regulating heat shock proteins (32). Though traditionally known for regulating blood glucose homeostasis, insulin is a pleiotropic hormone, and it also plays a pivotal role in influencing normal follicle development (43, 45) through its ability to stimulate ovarian steroidogenesis (7). In insulin responsive cells, insulin binds to its extracellular receptor (IR) resulting in autophosphorylation and recruitment of the insulin receptor substrate
(IRS) proteins (16, 43, 57), which in turn regulate numerous downstream insulin-mediated signaling pathways including the phosphatidylinositol-3 kinase (PI3K) pathway (2, 65). In humans, polycystic ovarian syndrome (PCOS) is associated with impaired reproduction, and these females are typically hyperinsulinemic and have hypersecretion of luteinizing hormone (LH) (3-5). Elevated circulating LH increases 17β-estradiol (E2), decreases oocyte maturation and fertilization, impairs embryo quality, increases embryo loss and decreases overall fecundity (29). Additionally, both insulin and LH regulate ovarian E2 synthesis, thus altered insulin and/or LH action could profoundly affect E2 physiology and consequently reduce follicle recruitment from the primordial pool through a negative feedback system (24). Therefore we hypothesize that there may be a link between HS-induced seasonal infertility and aberrant insulin signaling.

Proper insulin-mediated PI3K signaling is not only critical for regulating primordial follicle activation (PFA) and recruitment (22, 30, 47, 49), but is also necessary for follicle maturation (15, 33), oocyte viability (49) and ovarian steroidogenesis (18, 68). Most downstream PI3K signaling events are governed through serine-threonine protein kinase B (AKT), a subfamily comprised of three mammalian isoforms (Akt1, Akt2 and Akt3; (23)). Upon PI3K activation, AKT is phosphorylated and translocated to the cell nucleus where it can regulate several targets including forkhead box O (FOXO 1-4) transcription factors which are critical for follicle activation and maturation (17) as well as apoptosis (21, 26, 54, 66). In rodent models, Akr1c mice
have impaired fertility due to reduced primordial follicle viability (15), while, Foxo3−/− mice experience secondary infertility as a result of global PFA and their subsequent depletion (17). Interestingly, in oocyte specific FOXO3 overexpressing mice, unlike Foxo3−/− mice, PFA is prevented (34). Therefore, a proper balance in the insulin mediated PI3K/AKT/FOXO3 signaling pathway is crucial for female reproduction.

Understanding the molecular mechanism(s) by which HS compromises reproductive performance is a prerequisite for developing strategies and therapeutics to mitigate HS-induced suboptimal fertility. Since insulin influences ovarian PI3K signaling, we hypothesized that heat-induced hyperinsulinemia and aberrant insulin signaling may be a contributing factor to seasonal infertility during the warm summer months.

**Materials and Methods**

*Animals and Tissue Collection*

Tissues analyzed in this manuscript were obtained as a subset from previous experiments. Details regarding experimental design, animal handling, environmental conditions and results have been previously described (40, 53). In brief, crossbred prepubertal gilts (35±4 kg) were housed in constant climate controlled rooms in individual pens with *ad libitum* feed intake. Gilts were exposed to thermal neutral (TN) conditions (20°C; 35-50% humidity; n = 3) or HS conditions (35°C; 20-35% humidity; n = 3) for 7 or 35 days. To eliminate the effects of dissimilar feed intake, an additional
group of gilts (n = 3) were pair-fed to the HS treatment but remained in thermal neutral conditions for 7d. All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Gilts were euthanized by captive bolt penetration, one ovary was snap frozen in liquid nitrogen and stored at -80°C and the other ovary was fixed in 4% paraformaldehyde.

**RNA isolation and real-time polymerase chain reaction (RT-PCR)**

Frozen ovaries were ground in liquid nitrogen using a mortar and pestle. The resulting powder was divided into two halves; one half was used for RNA isolation while the other half for protein purification. Total RNA was isolated using an RNeasy Mini kit and concentrated using an RNeasy MinElute kit according to the manufacturers’ protocols. RNA was eluted using 14 µL of RNase-free water and RNA concentration determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE). Prior to RT-PCR, total RNA was reverse transcribed into cDNA utilizing the Superscript III One-Step RT-PCR system. Two microliters of diluted cDNA (1:50) was amplified using gene-specific primers (see Table 1) on an Eppendorf Mastercycler using a Quantitect™ SYBR Green PCR kit. The cycling program consisted of a 15 min hold at 95°C and 45 cycles of: denaturing at 95°C for 15 sec, annealing at 58°C for 15 sec, and extension at 72°C for 20 sec at which point data will be acquired. Following amplification, a melting curve analysis was conducted. Since there was no difference in ovarian glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level across treatments, the relative mRNA expression of each target
gene measured in the current study was normalized using *GAPDH* as a housekeeping gene. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method and the results are presented as mean fold change ± standard error relative to the TN control group.

*Protein Isolation and Western Blot Analysis*

Using tissue lysis buffer, samples were homogenized and the ovarian protein homogenates were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min at 4°C. Supernatant was collected and protein concentration determined using a standard BCA protocol on a 96-well assay plate during which emission absorbance values were detected with a $\lambda=540$ nm excitation on a Synergy™ HT Multi-Detection Microplate Reader using KC4™ software (Bio-Tek® Instruments Inc., Winooski, VT). Samples were then stored at -80°C until further use. Using 10% SDS-PAGE, 50 µg of protein homogenates were separated and subsequently transferred to nitrocellulose membranes. Briefly, membranes were blocked for 1-4 h with shaking at 4°C in 5% milk in Tris-buffered saline (TBS) with Tween-20 (TTBS). Membranes were incubated with primary antibody in 5% milk in TTBS for 1 h at 4°C. Equal protein loading was confirmed by Ponceau S staining of nitrocellulose membranes prior to antibody incubation. Membranes were washed with TTBS three times for 10 min. HRP-conjugated secondary antibody was added for 1 h at room temperature. Membranes were again washed in TTBS, followed by a single wash for 10 min in TBS. Western blots were detected using chemiluminescence (ECL plus reagent) and exposed to X-ray film. Densitometry of the appropriate sized bands was measured using Carestream molecular
imaging software version 5.0 (Carestream Health Inc., Rochester, NY) which eliminates background noise. Proteins of interest were normalized to Ponceau S measurement.

**Immunofluorescence staining and microscopy analysis**

Immunofluorescence staining was performed as previously described (47). Briefly, following euthanasia, ovaries were fixed in 4% paraformaldehyde for 24 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned, and every 6th section mounted. Three slides per ovary were deparaffinized and incubated with primary antibody specific to either IRα or pAKT^{Ser473} (1:100) at 4°C overnight. Secondary antibodies conjugated to fluorescein were applied for 1 h, followed by counterstaining with DAPI nuclear stain for 7 min. Immunofluorescence was visualized on a Leica DMI300B fluorescent microscope at λ = 461 and 665 nm for DAPI (blue) and fluorescein conjugated (green), respectively.

**Statistical Analysis**

All data were statistically analyzed using GraphPad Prism software. Comparison of two treatments was performed using t-test; comparison of more than two treatments was performed by ANOVA. A $P$-value $<$ 0.05 was considered significantly different.
Results

The porcine ovary is highly enriched with insulin receptors

Although the ovary possesses both insulin (IR) and insulin-like growth factor 1 (IGF-1) receptors (9, 54, 56), localization of IR in porcine ovaries was confirmed by immunofluorescence staining to detect the alpha subunits of IR (green color in Figure 1). It is evident that the oocyte is highly enriched in the alpha subunit of the IR (IRα) protein (Figure 1). The IRα was located in the oocyte cytoplasm and cytoplasmic membrane, and staining was also evident in the theca and granulosa cells.

The mRNA of ovarian IR and IRS1 genes are upregulated during HS

Using qRT-PCR primers the effect of HS on mRNA encoding the IR and IRS1 were determined. After 7d, HS markedly increased ($P < 0.001$) ovarian IR compared to TN controls (Figure 2A). Although not to the same extent, IR mRNA abundance was also increased in the PF gilts compared to the TN controls (Figure 2A). The HS-induced increased ovarian IR response was maintained ($P < 0.01$) after 35d (Figure 3B). In contrast to IR mRNA, the IRS1 mRNA abundance on 7d was only increased in the HS ovaries as the PF and TN controls had similar levels of expression (Figure 2A). Although the magnitude of increase was less, the IRS1 mRNA level remained increased after 35d in the HS compared to TN controls (Figure 2B).
**HS increases ovarian phosphorylated IRS1 in the ovary**

Concomitant with HS-induced ovarian IR and IRS1 mRNA levels, 7d ovarian phosphorylation of the Tyrosine 632 residue on the IRS1 protein was increased \((P < 0.05)\) in HS relative to the TN gilt ovaries (Figure 3A) while PF pIRS1 abundance was intermittent and not statistically different from either TN or HS groups. The increase in ovarian pIRS1 was maintained for 35d of HS compared to the TN controls (Figure 3B).

**AKT1 and FOXO3 mRNA is increased during HS**

Following increased activation of insulin signaling pathway members during HS, whether the downstream insulin mediated PI3K members, AKT1 and FOXO3, are also impacted by HS was determined. Ovarian AKT1 mRNA was increased \((P < 0.05)\) after 7d of HS (Figure 4A), compared to PF and TN controls and remained increased after 35d of HS relative to TN controls (Figure 4B). Interestingly, it is noteworthy that although there was no difference \((P > 0.05)\) in FOXO3 mRNA between ovaries obtained from TN and HS gilts, reduced \((P < 0.05)\) ovarian FOXO3 mRNA levels were observed after 7d of PF (Figure 4A). However, after 35d and in a similar pattern to AKT1, ovarian FOXO3 mRNA expression was increased \((P < 0.05)\) in HS gilts relative to their TN counterparts (Figure 4B).

**HS increases ovarian AKT1 phosphorylation**

Similar to the localization pattern of IR, immunofluorescence staining revealed that pAKT1 protein is highly expressed in the porcine oocyte, in addition to being
localized to the theca and granulosa cells (Figure 5A, B). Western blotting to quantify the impact of HS on phosphorylated and activated AKT1 (pAKT1) protein demonstrated that ovaries from HS gilts, had increased ($P < 0.05$) pAKT1 protein levels compared to TN gilts after 35d of thermal treatment (Figure 5C).

**Expression of genes involved in ovarian steroid hormone production are affected by HS**

Since PI3K signaling was impacted by HS, and it is known that the insulin mediated-PI3K pathway can regulate ovarian steroidogenesis (1, 41, 55), we determined the impact of HS on mRNA expression of the genes involved in ovarian steroid production (Figure 6). Our data indicates that compared to TN and PF controls, mRNA levels of LDLR, LHR and CYP19a were elevated ($P < 0.05$) after 7d (Figure 6A) and that this HS-induced increase in mRNA abundance of these genes was maintained for 35d (Figure 6B). Interestingly, STAR was not impacted by 7d of HS at the mRNA level (Figure 6A), but was increased after 35d of HS. In addition, after 35d of HS (Figure 6B), all other members of the steroidogenic pathway measured were increased.

**Ovarian STAR and CYP19 are elevated during HS**

Since the increase in STAR and CYP19a mRNA were unexpected, protein levels of these two critically important enzymes were quantified during HS. Relative to TN gilts, ovaries from HS gilts had increased ($P < 0.05$) STAR protein following 35d of thermal treatment (Figure 7). It is important to note that the seeming lack of STAR in
the TN gilts was not surprising, since they are pre-pubertal females. Compared to TN and PF females, ovarian CYP19a protein was elevated ($P < 0.05$) by HS after 7d (Figure 8A) and this HS-induced increase was maintained for 35 d (Figure 8B). There was no difference ($P > 0.05$) in CYP19a protein levels between PF and TN gilt ovaries (Figure 8A).

Discussion

Insulin plays a pivotal role in controlling carbohydrate, protein and lipid metabolism as well as influencing normal reproductive function (31, 43, 45). Physiological conditions characterized by hyperinsulinemia, including obesity and PCOS, are often associated with reduced fecundity. Hyperinsulinemia has been reported in a number of environmentally-induced HS models, including pigs, therefore the HS gilt was utilized as a model of hyperinsulinemia to explore impacts of elevated insulin on ovarian function. Since gilts in this experiment were in a prepubertal physiological state, this model presented an opportunity to determine ovarian impacts of environmentally-induced hyperthermia in the absence of gonadotrophin influence.

Upon insulin binding, the IR undergoes conformation changes with subsequent auto-phosphorylation of IRβ subunits which in turn relay kinase cascades leading to phosphorylation of IRS proteins at various tyrosine-serine sites. Phosphorylated IRSs provide binding sites for the activation of various insulin mediated downstream effectors including PI3K. We report increased ovarian $IR$ mRNA levels during acute (7d) and
chronic (35d) HS. Interestingly, ovarian IR mRNA levels were also elevated in ovaries from PF gilts suggesting that restricted feeding and presumably decreased nutrient delivery to the ovary increases the initial capacity for insulin signaling. Concomitant with increased IR mRNA expression, ovaries from HS gilts demonstrated increased IRS1 mRNA and pIRS1 protein levels during acute and chronic HS, confirming that the insulin-mediated signaling pathway was activated. These results suggest that the ovary not only is responsive to insulin but that insulin signaling is upregulated during HS. This is also in agreement with previous work in rat models of diet-induced obesity, where in the face of elevated circulating insulin, the ovary maintained insulin sensitivity (65), and in our previous work using high fat diet-induced obesity in mice (Nteeba, et al., 2013). In rodent models, defects in IRS1 and IRS2 cause ovarian and hypothalamic dysfunction leading to impaired female fertility (16, 19, 43). Since our model is independent of hypothalamic input, it is likely that the observed HS effects on IRS1 are ovarian specific. Despite the upregulation of the IR, the PF pigs had decreased IRS1:pIRS1 protein compared to the HS pigs and this suggests a reduced capacity of intracellular insulin signaling. This differential effect between the HS and PF pIRS1 also demonstrates that HS directly (independent of reduced nutrient intake) alters insulin signaling. The increased ovarian insulin action is similar to how non-lethal HS ameliorates systemic proxies of insulin insensitivity in diabetic rodents (Kokura et al., 2007, 2010) or rodents fed high fat diets (Gupte et al., 2009). Further, it is also similar to reports indicating thermal therapy (saunas and hot baths) improves insulin sensitivity in humans (McCarty et al., 2009). One potential mechanism by which heat offers protection from insulin
resistance is by upregulating HSP72; which inhibits the activation of stress kinases c-Jun N-terminal kinase (JNK) and inhibitor of kappa B kinase β (IKKβ; Kondo et al., et al., 2011; Simar et al., 2012), enzymes involved in insulin resistance.

To determine if the downstream pathways mediated by insulin’s binding to the IR are altered during HS, we investigated mRNA encoding genes involved in PI3K signaling. Both acute and chronic HS increased the expression of both AKT1 and FOXO3. Interestingly, it is noteworthy that during reduced feed intake in the PF group, FOXO3 is reduced, thus the impact of inadequate nutrient intake during HS did not impact FOXO3 as would be energetically expected. Previous studies have demonstrated that FOXO3 plays a pivotal role in maintaining the resting primordial follicular pool (17) in addition to influencing cell apoptosis (21, 26, 54, 66). Therefore, increased FOXO3 during HS could be associated with impaired PFA similar to that observed in FOXO3OE mice (34). Such a scenario could negatively impact reproduction and result in a “barren” ovary since a lack of growing follicles progressing towards ovulation would result in a reduced number of dominant follicles required for E2 production. In addition, increased FOXO3 could indicate increased apoptosis, which is important in the ovary since 99% of oocytes are eliminated through pro-apoptotic pathways, potentially as a mechanism to ensure ovulation of oocytes that have the greatest reproductive potential. Thus, apoptosis prevention is not necessarily beneficial for the ovary and alterations to this process may result in ovulating an imperfect oocyte. Similar to the localization pattern of the IR, pAKT1 (33, 34, 48, 49) is highly expressed in the porcine oocyte (in addition to being
localized to the theca and granulosa cells) and is increased by HS. We hypothesize that increased AKT activation could be an ovarian attempt to sustain oocyte viability, and also could increase steroid production by the dominant follicles. Since the ovulation rate and quality of the ovulating follicles depend on the process of folliculogenesis in the earlier period (55), these data indicate that HS could directly (not associated with decreased feed intake) alter the ovarian mechanisms that regulate oocyte quality, viability and growth activation - all of which are potentially negative events for reproductive success.

Similar to humans, porcine reproductive cycles are tightly regulated by a system of positive and negative feedback of reproductive hormones secreted and released from the hypothalamus (gonadotrophin-releasing hormone), the pituitary (follicle-stimulating hormone, luteinizing hormone, oxytocin and prolactin), the ovaries (progesterone, 17β-estradiol, inhibins and relaxin) and the uterus (prostaglandin F2α) (55). While the major physiological functions of E2 in ovulation and regulation of follicular recruitment are well documented, it is also important to recognize that E2 is necessary for estrus display and maternal recognition of pregnancy in the pig, thus altered insulin signaling impacting E2 production could contribute to compromised fertility and increase the potential for early embryonic death (24, 29). Unlike humans, pigs can ovulate more than 15 oocytes during estrus, however, like humans, the ovulation rate and follicle quality depends on the preceding folliculogenesis period (55). Thus, any disruption in folliculogenesis could compromise ovulation and subsequent conception, while impaired
steroidogenesis could reduce conception rate and cause embryonic death. Additionally, unlike cyclic follicle recruitment which is controlled by gonadotropins, initial PFA is gonadotropin independent and it is thought to be tightly regulated by the PI3K signaling pathway (17, 22, 36, 42).

Since both insulin (7, 69) and PI3K (18, 68) signaling pathways can influence ovarian steroidogenesis, we next examined the impact of HS on expression of genes that enable steroid production. Our data revealed that mRNA levels of \textit{LDLR}, \textit{LHR}, \textit{STAR} and \textit{CYP19a} were elevated during both HS stages, though the increase in \textit{STAR} was delayed. HS also increased \textit{STAR} and \textit{CYP19a} protein levels, relative to PF and TN conditions indicating the effects of HS are direct and not mediated by restricted feeding. Normally, \textit{STAR} is upregulated in steroid producing cells in response to conditions that induce acute steroid biosynthesis. Under such circumstances, \textit{STAR} increases cholesterol translocation from the outer to the inner mitochondrial membranes, the rate-limiting step in steroid production (56). Therefore increased \textit{STAR} expression during HS could be locally interpreted as an increased steroid production capacity, however, we suspect that HS compromises steroid biosynthesis and therefore \textit{STAR} is being activated to compensate for the steroid production needs of ovarian cells. Additionally, increased \textit{LHR} during HS could indicate that the ovary is attempting to upregulate the amount of LH receptors necessary to ensure ovulation. Increased \textit{CYP19a} could also locally imply that HS potentially alters the amount of circulating E2 since the pathway that produces E2 is increased, resulting in compromised fertility and estrus derangement. However, we
hypothesize that E2 levels are likely decreased and that the increase in the production pathway is a counteractive effort by the ovary to curtail dwindling levels of this critical reproductive hormone. The impact of HS on reproductive hormone levels have been previously noted (6, 12, 13, 20, 28). In dairy cows, blood LH levels have been reported to either increase (50), decrease (35, 63) or stay unchanged (20, 25) during HS. Similarly, plasma P4 concentrations have been reported to increase (20, 59, 60), decrease (20, 67) or be unaltered (51, 62) with HS. Furthermore, HS has been associated with reduced blood E2 concentrations (20, 62, 64) in dairy cows. Reasons for the inconsistencies within the literature are not clear but likely include differences in 1) species, 2) length and magnitude of the heat load, and 3) the incorporation of a pair-feeding model. Regardless, these data suggest that HS alters gonadotropins and ovarian steroid production, in ways that are likely detrimental to reproduction.

Taken together, our data indicates that the utilization of a hyperinsulinemic prepubertal porcine model has benefits for understanding ovarian physiological alterations in the absence of hypothalamic-pituitary input and that HS could be thought of as an endocrine disrupting environmental exposure that can negatively affect female reproduction. Further, our data clearly demonstrate that the negative effects of HS on ovarian insulin signaling and steroid synthesis are the direct effects of environmental hyperthermia and not mediated by reduced nutrient intake.
Grants: This work was supported by the Iowa Pork Producers Association grant to L.H.B., J.W.R. and A.F.K. and by the Agriculture and Food Research Initiative competitive grant no 2001-67003-3007 from the USDA National Institute of Food and Agriculture.
References


### Table 1: Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence (5′ – 3′)</th>
<th>Reverse Primer Sequence (5′ – 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACCCAGAAGACTGTGGATGG</td>
<td>AAGCAGGGATGATGTTCTGG</td>
</tr>
<tr>
<td>LDLR</td>
<td>GAGTTGGCTTTTGCTCTGCT</td>
<td>GGGTTTTGGTGTAATGAATGG</td>
</tr>
<tr>
<td>LHR</td>
<td>CATGGCAACCGATCTCTTCTCT</td>
<td>CGGAATGCCCTTTTGAAAT</td>
</tr>
<tr>
<td>STAR</td>
<td>TTGGAAGAGACGGGATGGAAG</td>
<td>CCCACATTCCCTGCTATTGCT</td>
</tr>
<tr>
<td>CYP11α</td>
<td>AGGCCAATGTTACCGAGATG</td>
<td>ATTGCAGCATCTTGCTTTGTG</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>GACACACCTCCCAAAAGCTA</td>
<td>TGTAGGAGACGTTGAAACACG</td>
</tr>
<tr>
<td>CYP19α</td>
<td>GGAGCTTGGGTTAATGGAT</td>
<td>GGGAAGGATGCTCTTTGATGB</td>
</tr>
<tr>
<td>IRβ</td>
<td>AGAGATTTGCTGATGGGATGGB</td>
<td>AAAGGACCACATGTCGGAAAAG</td>
</tr>
<tr>
<td>IRS1</td>
<td>GTGCGACAGAGCATCATTGT</td>
<td>GCCTGTGTTTAAGCCTCTGC</td>
</tr>
<tr>
<td>AKT1</td>
<td>ATCGTGTGGCAGGATGTTGTA</td>
<td>CTGGCCGAGTGGAGAGAAGAATGB</td>
</tr>
<tr>
<td>FOXO3</td>
<td>TCAGCCAGTCTATGCAAACC</td>
<td>CCATGTACATTCACAGCTC</td>
</tr>
</tbody>
</table>
Figure 1: Effects of 7 days of HS (35°C) on IR protein levels in ovaries from pre-pubertal gilts. Immunofluorescence staining was performed on ovarian sections from gilts after 7d of HS or TN conditions. Thin arrow indicates small primary or primordial follicles; arrowhead indicates oocyte in secondary follicle, block arrow indicates oocyte in antral follicle. Green staining indicates IR protein; Blue staining represents DNA.
Figure 2: Impact of HS on ovarian IR or IRS1 mRNA. qRT-PCR was performed on ovarian tissues from gilts after (A) 7d or (A) 35d of TN, PF or HS conditions. Different letters indicate statistical difference between treatments; $P < 0.05$. * indicates $P < 0.05$, different from TN.
Figure 3: **HS-induced effects on ovarian pIRS<sup>Tyr632</sup> protein.** Western blotting was performed on ovarian tissues from gilts after (A) 7d or (A) 35d of TN, PF or HS conditions. Different letters indicate statistical difference between treatments; $P < 0.05$. * indicates $P < 0.05$, different from TN.
Figure 4: Impact of HS on ovarian AKT1 or FOXO3 mRNA. qRT-PCR was performed on ovarian tissues from gilts after (A) 7d or (A) 35d of TN, PF or HS conditions. Different letters indicate statistical difference between treatments; \( P < 0.05 \). * indicates \( P < 0.05 \), different from TN.
Figure 5: Effect of HS on ovarian pAKT1 protein localization and level. (A, B) Immunofluorescence staining was performed on ovarian sections from gilts after 7d of HS or TN conditions. Thin arrow indicates small primary or primordial follicles; arrowhead indicates oocyte in secondary follicle. Green staining indicates pAKT1 protein; Blue staining represents DNA. (C) Western blotting was performed on ovarian tissues from gilts after 35d of TN or HS conditions. * indicates $P < 0.05$, different from TN.
Figure 6: Impact of HS on ovarian steroidogenic gene mRNA. qRT-PCR was performed on ovarian tissues from gilts after (A) 7d or (A) 35d of TN, PF or HS conditions. Different letters indicate statistical difference between treatments; $P < 0.05$. * indicates $P < 0.05$, different from TN.
Figure 7: HS-induced effects of ovarian STAR protein. Western blotting was performed on ovarian tissues from gilts after 35d of TN or HS conditions. * indicates $P < 0.05$, different from TN.
**Figure 8: Effect of HS on ovarian CYP19a protein.** Western blotting was performed on ovarian tissues from gilts after (A) 7 or (B) 35d of TN, PF or HS conditions. Different letters indicate statistical difference between treatments; $P < 0.05$. * indicates $P < 0.05$, different from TN.
CHAPTER 8: GENERAL CONCLUSION

The ovary performs two major roles; 1) production of oocytes required for perpetuation of species and 2) synthesis of steroid hormones required for cyclicity and secondary sexual development. Optimal reproduction is achieved when there is a balance between folliculogenesis and steroidogenesis in addition to proper ovotoxicant chemical metabolism to ensure protection of the ovarian follicular pool and developing embryo from potential damage. Perturbations in these processes are likely to alter ovarian function and consequently lead to impaired fecundity and other associated reproductive pathologies. This dissertation research focused on investigating impacts of metabolic perturbations on ovarian folliculogenesis, steroidogenesis and xenobiotic biotransformation. Specifically, mRNA and/or protein expression profiles of insulin, PI3K, steroidogenic, inflammatory and chemical metabolism members were quantified using qRT-PCR, Western blot or immunohistochemistry techniques using three models: 1) high fat diet (HFD)-induced obesity, 2) a transgenic mouse model of progressive obesity and 3) a porcine model of hyperinsulinemia.

Determination of suitable mouse model

Although HFD-fed mice develop obesity, they require a long period of dietary treatment (~7 months). Additionally, although the mice develop diabetes associated with obesity as a result of overnutrition (80), there is no frank hyperglycaemia present in genetically normal animals and hence they are not the ideal model in which to mimic the obese state in humans. This prompted us to use the lethal yellow mouse which develops
progressive obesity starting at 8 weeks of age (41, 47). By 12 weeks, these mice also display progressive hyperinsulinemia (93), hyperleptinemia (7), along with insulin (41) and leptin (32) resistance. Since they lack a satiety signal due to hypothalamic agouti expression, they eat a normal diet until overweight - these characteristics make the lethal yellow mouse an excellent model for human obesity.

**Impact of changes in central metabolism on ovarian signaling pathways**

1) **Insulin signaling**

Proper insulin signaling is essential for ovarian folliculogenesis, steroidogenesis and metabolism. Events that induce dysregulation of insulin signaling cascades in the reproductive axis result in a number of reproductive impairments. In the two models of obesity, increased body weight was associated with increased fasting blood glucose, accompanied by reduced glucose clearance. These observations were suggestive of reduced insulin activity and therefore we hypothesized that during obesity, ovarian insulin signaling is impaired. Our data revealed that during high fat diet (HFD)-induced obesity, there was a trend for decreased ovarian Insr, Irs1 mRNA and total INSR protein expression, locally meaning compromised insulin signaling. However, progressive obesity was associated with a temporal increase in Irs1, Irs2 and Irs3 mRNA as well as pIRS1\textsuperscript{Ser302} protein levels, perhaps indicating that the ovarian insulin signaling pathway is active in ovaries from obese mice. Previously reported effects of obesity on expression of insulin signaling members in a number of tissues are not conclusive. For instance consistent with our HFD results, reductions in expression of insulin receptor numbers
during obesity has been observed in human muscle (75) and adipose tissue (19). In contrast and similar to our progressive obesity data, increased phosphorylation of both IRS1 and IRS2 with obesity also have been observed in the rodent reproductive axis (11, 89). Similarly, insulin induced-PI3K activation is maintained in the liver despite its downregulation in the muscle of diabetic rodent models, HFD-fed obese mice and obese monkeys (25). Interestingly, other studies have observed no effect of HFD-induced obesity on INSR (3, 26) or IRS1 (26, 38, 40). Taken together, these results indicate the complexity of obesity effects on insulin signaling members which may be species-, tissue- and/or time-specific. Despite the disharmony in available data, it is known that follicular fluid contains insulin and that insulin levels are elevated in both serum and follicular fluids of obese females compared to lean controls (71, 84). Therefore, our obesity data suggest that perturbation in insulin signaling is one of the underlying mechanisms for obesity-associated reproductive disorders.

In a similar manner to progressive obesity, ovarian *INSR, IRS1* mRNA and pIRS1 protein levels were increased during acute and chronic heat stress, suggesting that the ovary was responsive to HS-induced hyperinsulinemia. Interestingly, ovarian *INSR* mRNA levels were also elevated in ovaries from PF gilts confirming ovarian sensitivity to changing blood glucose levels due to underfeeding. Since these were pre-pubertal gilts, it is likely that the HS effects on IRS1 are independent of hypothalamic input and therefore can be considered ovarian specific. Rodent models, with defects in IRS1 and IRS2 experience ovarian and hypothalamic dysfunction concomitant with impaired
female fertility (13, 20, 63). In a number of in vitro studies, insulin administration increases the percentage of primordial follicles transitioning to the primary stage in cultured human ovarian cortex (46), rat ovaries (37, 52) and bovine cortical pieces (28). In canines (74) and fetal hamster cultured ovaries (97), elevated insulin concentrations promoted pre-antral follicular growth and viability, underscoring the importance of insulin signaling in regulating different stages of ovarian folliculogenesis. Overall, our data in conjunction with the above literature suggest that aberrant insulin signaling induced by changes in central metabolism is likely one of the mechanisms underlying reproductive disorders associated with both obesity and HS. Future studies to decipher the molecular pathways mediating hyperinsulinemia-induced alterations to ovarian physiology will advance our understanding and pave ways for therapeutic intervention. Additionally, although serum insulin levels are elevated during HS, we don’t know insulin levels in follicular fluid during HS.

2) PI3K signaling

PI3K signaling is a central pathway that integrates the reproductive and metabolic functions of the mammalian ovary (1). Alterations in expression of proteins involved in this pathway have been previously reported to undermine reproductive potential (1, 45, 67, 68). Both insulin and KITLG through interaction with their cognate receptors can activate the PI3K-AKT dependent pathway in a number of tissues including the ovary (39, 43, 69). For the first time, we demonstrated that obesity increases ovarian mRNA levels of Kitlg and its receptor concomitant with increased
Akt1 and decreased Foxo3a mRNA expression (56), and that in mice, this increase follows a temporal pattern with progressive obesity (Nteeba, et al. under review). It is interesting that unlike the inconsistency in changes induced by HFD-induced and progressive obesity models in insulin signaling members, alterations to ovarian Kitlg and cKit were consistent in both models of obesity used in our studies. KITLG-cKIT signaling is a pro-survival pathway that is essential for optimal ovarian folliculogenesis (43, 61, 94) and steroidogenesis (50, 60). Blocking of KIT receptor using an anti-KIT antibody, ACK2 in human ovarian follicles was demonstrated to induce ovarian follicular atresia (17). Moreover, like insulin, KITLG supplementation in culture media has been demonstrated to promote the transition of primordial to the primary follicle stage in bovine ovarian cortical pieces (29) and rodents (61, 94).

Importantly, both insulin and KITLG signaling seems to impact similar ovarian pathways (PI3K-AKT) and lead to similar changes in ovarian folliculogenesis (increased follicle activation). It is therefore not surprising that previous studies using cultured neonatal rat ovaries (37, 52), found that insulin and KITLG have an additive effect for increased primordial follicle activation. Therefore, in addition to aberrant insulin signaling, hyperactivation of KITLG-cKIT signaling could be another pathway underlying obesity-induced reproductive impairments. Although our studies revealed that members in these two signaling pathways are altered during obesity, future studies that could separate the effects of insulin from those of KITLG during obesity will advance our understanding.
We have also demonstrated that during obesity pAKT\textsuperscript{Ser473} and/or pAKT\textsuperscript{Thr308} are increased in theca cells (56) and total ovarian homogenates (54), indicating increased ovarian PI3K-AKT activation in obese females. Although total ovarian FOXO3A protein was not significantly impacted during our studies (54), there was a trend for reduction in total FOXO3A protein expression (Nteeba, et al. under review) and a significant under expression of pFOXO3\textsuperscript{Ser253} (54) during obesity. Whether these changes are direct interactions between the IRS or involve other signaling mediators is difficult to surmise from the current data. However, hyperactivation of PI3K-AKT signaling pathway, could adversely impact ovarian function through accelerating the rate of primordial follicles activation and recruitment in to the growing pool with eventual death of most of those follicles, leading to accelerated premature ovarian failure (18, 36, 44, 45, 70). Therefore the upregulation of insulin-KITLG-cKIT-AKT1 signaling pathway by obesity as observed in our studies could be a potential mechanism underlying obesity-induced over-activation of primordial follicles into the growing follicular pool previously noted in rodent model (54, 86).

In gilts, mRNA expression of AKT1, FOXO3 and pAKT1 protein levels were elevated during acute and chronic HS, indicating that PI3K-AKT1 dependent signaling pathway is activated by HS, likely due to increased circulating insulin. FOXO3 plays important roles in apoptosis (24, 31, 78, 92), cell cycle arrest (14, 83) and is believed to regulate the rate of initial primordial follicle activation (5, 8, 18). In rodent models, FOXO3-deficient mice display global primordial follicle activation while oocyte specific
FOXO3-overexpression primordial follicle activation is prevented (45). Although each of these FOXO3 alterations had contrasting effects on the primordial follicular pool, they all resulted in impaired fertility, highlighting the importance of proper FOXO3 expression in ovarian physiology. In the cycling sow, increased FOXO3 expression could mean impaired primordial follicle activation, a scenario which could halt folliculogenesis leading to reduced number of dominant follicles required for E2 production and subsequently compromise reproductive potential. Additionally, if increased FOXO3 is channeled towards increased apoptosis, this could be important in ensuring that imperfect oocytes are eliminated before ovulation and thus minimizing the risk of passing on damaged germline. Although, we did not determine FOXO3 protein levels in these samples due to challenges in finding appropriate antibodies against porcine FOXO3, we think that the observed HS-induced increased FOXO3 is directed towards impaired folliculogenesis and less apoptotic pathways. This is likely true since HS animals have compromised fecundity, and recently FOXO3 knockdown in pre-pubertal pigs was associated with increased primordial oocyte activation (51). Future studies should also investigate the impact of HS on KITLG-cKIT signaling members, since this pathway is critical for porcine oocyte survival.

3) Folliculogenesis

Although no impact of obesity on ovarian weight was observed, ovaries from obese mice had fewer primordial and primary follicles with increased larger/tertiary follicle subtypes (54). This difference in follicle populations could indicate increased
activation of follicles from the primordial follicle pool, which could lead to accelerated premature ovarian insufficiency. This scenario is also supported by upregulated ovarian insulin-KITLG-KIT-PI3K signaling. Hyperactivation of these signaling molecules is associated with increased activation of primordial follicles to the growing follicles. Additionally, our results show that the steroidogenic pathway leading to E2 production is altered by progressive obesity and hyperthermia both of which are associated with hyperinsulinemia. In a number of studies, reduced E2 levels have been reported to stimulate primordial follicle activation from the resting to the growing pool (9, 10). Although we did not count follicles in HS gilts, these data support the observed low numbers of primordial follicles and higher numbers of growing follicles observed in ovaries from obese relative to lean mice. Taken together, these results are indicative that changes to central metabolism, may trigger changes in the intrinsic ovarian signals responsible for initiation of follicle activation and recruitment, adversely impacting female fecundity.

4) Steroidogenic members

Progressive obesity impacted ovarian cyclicity by decreasing the length of time spent in estrus with a concomitant increase in the length of the diestrous phase. Physiologically, ovarian E2 is predominantly associated with estrus and P4 with diestrous phases (48). Therefore, shorter length in the estrus stage would imply lower E2 levels while longer diestrous stages would imply more sustained P4 levels in obese females. E2 is required for ovulation, whereas P4 is required for implantation along with
establishment and maintenance of pregnancy. In premenopausal women, a higher P4/E2 ratio during follicular phase is associated with anovulation and/or premature luteinization (95), leading to compromised fertility. Interestingly, although genes involved in ovarian steroidogenesis were upregulated during early stages of obesity, at later stages of obesity establishment and consistent with the cyclicity data these genes were downregulated. Thus, this data implies that the pathways for production of both P4 and E2 are compromised with progressive obesity, which could impact ovulation and subsequently compromise the reproductive capacity of obese females. Furthermore, these results underscore the importance of considering the time point at which data is collected when interpreting obesity associated ovarian impacts. Similar to early stages of obesity, ovarian steroidogenic members were elevated during HS in pre-pubertal gilts, which could alter the amount of circulating E2 and P4, resulting in estrus derangement and impaired fertility. Taken together these data suggest that changes to central metabolism lead to alterations in the estrous cycle and signaling cascades necessary for production of ovarian steroidogenic hormones, potentially negatively impacting oocyte competency, ovulation, conception rate and embryonic survival, thereby compromising female reproductive capacity.

5) **Inflammatory members**

Inflammatory pathways are involved in several ovarian processes to aid optimal fertility (12, 22, 53). For example they are involved in ovarian cellular proliferation and apoptosis (6) as well as steroidogenesis and ovulation (49, 85, 88). Despite their
beneficial effects, alterations in inflammatory pathways could negatively impact ovarian function. During HFD-induced obesity, obese female mice had increased adipocyte size, immune cell infiltration and the mRNA expression of immune cell and inflammatory markers in the peri-ovarian fat pad compared to the lean littermates (55). This was the first study to demonstrate that hypertrophic expansion of obese peri-ovarian adipose tissue is associated with crown-like structure formation and inflammation in obese female mice. Although humans and mice differ in the deposition of fat around the ovary, inflammatory cytokines produced in intra-abdominal adipose tissue in humans could potentially impact ovarian physiology due to the proximity of both tissues. Additionally, changes in peri-ovarian adipose tissue were associated with increased mRNA levels of pro-inflammatory cytokines, TNFα, RELA, Il6 and the oxidative stress marker, Nos2 in the ovaries of obese mice (55). Similarly, progressive obesity was also associated with increased TNFα-NF-κB dependent pathway indicating increased activation of inflammatory pathways in ovaries of obese mice. Dysregulation in ovarian Nos2 has been linked to impaired ovulation (33), ovarian cancer (34), endometriosis (81), and infertility (2, 58, 72). Aggravated inflammation in the ovary is associated with polycystic ovary syndrome (30, 65), endometriosis (15, 23) and premature ovarian failure (88). Therefore obesity-induced changes in inflammatory signals can adversely impact ovarian function and pose dire consequences for female fertility.
6) Xenobiotic metabolism members

The ovary contains a finite number of primordial follicles, which once depleted, cannot be replaced; therefore ovarian xenobiotic metabolism of chemical compounds like DMBA which can deplete the primordial follicle pool is critical for protection of the female germ cell. Since insulin regulates hepatic expressed xenobiotic metabolism enzymes and obesity alters insulin sensitivity in a number of target tissues, understanding how obesity might influence the ovary’s capacity to metabolize chemicals is critical. Our data demonstrated that ovarian expressed mRNA and/or protein levels of GSTM1, GSTP1 and EPHX1 were upregulated but CYP2E1 was downregulated during both HFD-induced (56) and progressive obesity (Nteeba, et al. under review). Furthermore and for the first time, our results demonstrated that obesity accelerated DMBA-induced ovotoxicity (54). Therefore, our results not only show that ovaries from obese female mice have altered xenobiotic metabolism, these results are also suggestive that if similar changes are occurring in humans, those females would have increased susceptibility to environmental exposures including carcinogens and teratogens which could also potentially explain the increased rates of miscarriage (59, 66) and increased offspring birth defects in obese mothers (59) in addition to poor oocyte quality (64). Although, these enzymes are principally involved in the detoxification of xenobiotic compounds, their action can also lead to bioactivation of some environmental compounds, leading to increased toxicity. The ovary therefore requires the capacity to metabolize and detoxify these compounds in order to protect the female germ cell and ensure sound ovarian function.
7) MicroRNAs

MicroRNAs are known posttranscriptional regulators of a number of biological processes including insulin signaling (27, 82, 87), PI3K signaling (57, 82, 91, 96), steroidogenesis (76, 77), adipocytes differentiation (90) and inflammation (35, 79, 90). During HFD-induced obesity, it was found that the ovaries from obese mice had decreased miR-21, miR-103, miR125b and miR143, however, miR-184 levels were increased (55, 56). Given the fact that these microRNAs regulate many targets, their dysregulation in the ovary during obesity could have wide implications. For instance in rodent models, downregulation of miR-21 (21) and upregulation of miR-184 (76, 77), negatively impacted ovarian steroidogenesis leading to reduced ovulatory rates. Additionally, loss or inhibition of miR-21 (16, 42) and increased miR-184 concomitant with reduced miR-205 (4, 96) expression negatively regulates PI3K-AKT signaling, potentially impacting PI3K-mediated death/survival functions and ovarian folliculogenesis. However, our data revealed that despite elevated miR-184 concomitant with decreased miR-205 and miR-21 levels, PI3K-AKT signaling was upregulated, suggesting that either the actions of these microRNAs were not sufficient to modulate PI3K-AKT actions or their changes are directed towards other ovarian processes including steroidogenesis and apoptosis (16).

Our data also revealed that ovaries from obese mice displayed a 74% and 96% decrease in miR-125b and miR-143 levels, respectively, supporting increased Tnfa transcription activity. Downregulation of miR-125b (35) and miR-143 (90) is associated
with increased \textit{Tnfa} mRNA expression which is supported by increased inflammatory signaling pathway members observed during HFD-induced (55) and progressive obesity (Nteeba et al. under review). Besides its role in inflammation, \textit{miR-143} is critical for \textit{in utero} primordial follicle development, raising concerns about the impact of obesity on the neonatal ovary. Lastly, although the impact of obesity on \textit{miR-103} levels in extra-ovarian tissues is inconsistent with increased (62, 73, 82) and decreased (90) expression being reported, it has been suggested that silencing \textit{miR-103} improves insulin sensitivity in adipocytes (82). Therefore observed low \textit{miR-103} level in ovaries from obese mice is in agreement with increased insulin signaling during progressive obesity.

\textbf{Future areas to address}

Although these studies have resulted in fascinating novel findings, it is important to note that these results have also generated interesting questions for future considerations. For instance our data demonstrated that obesity is associated with lower number of primordial and primary follicles but increased number of growing follicle subtypes, perhaps indicating increased follicle activation and recruitment. While these changes were accompanied by upregulation of insulin-KITLG-KIT-AKT dependent pathway, which notion could support the observed changes in follicle numbers, it was not possible from our data to clearly determine which molecule is the major regulator and whether other factors are involved in these changes. Therefore further studies will be needed to clearly define the mechanism(s) underlying obesity-induced changes in follicle subpopulations.
During HFD-induced obesity, changes in the peri-ovarian pad associated with changes in ovarian tissue were observed, however we could not answer from our studies how much of the changes we see are due to adipose tissue contributions.

From our data it is apparent that during obesity, both KITLG-cKIT and insulin signaling members are upregulated however, the relationship between insulin and KITLG signaling remains unclear at the moment.

Obesity and HS are both associated with a number of additional metabolic perturbations including hyperinsulinemia, hyperleptinemia and/or LPS which could all influence ovarian physiology. From our studies it was not possible to decipher which of these alterations is chiefly responsible for the molecular changes we saw in ovaries from obese mice and HS pigs.

We did not measure the steroid hormones at each time point. These measurements would have allowed us to see how ovarian steroids change over time from the onset through progression of obesity.

During HS study, we used pre-pubertal gilts, future studies need to explore if these changes are similar in cycling sows. Additionally, future studies need to explore how HS impacts steroid hormone concentrations in both the follicular fluid and serum and determine which concentrations correlate with ovarian changes observed during HS.
Overall summary

The data presented herein, demonstrates that ovarian insulin-KITLG-KIT-AKT signaling pathway is active and upregulated during central metabolic alterations. Perturbations to ovarian insulin-KITLG-KIT-AKT signaling pathway are likely to impact 1) follicle activation, oocyte viability and recruitment, 2) steroid hormone biosynthesis, and 3) xenobiotic biotransformation, potentially accelerating susceptibility to chemical exposure. All of these scenarios can lead to impairment of ovarian function, and may at least partially explain why female fecundity is compromised during altered metabolic states. Future studies will be required to address the cellular origin and cause-effect relationship between the observed changes in the ovary and the implications of these changes on ovarian function. This is critical because although the central focus for this dissertation has been on insulin, elevated circulating leptin and LPS present in both obesity and HS could potentially also affect ovarian physiology. Further understanding of the mechanisms that are involved in the onset and progression of the central metabolic-associated changes in the ovary will be of value in order to develop improved strategies to mitigate reproductive dysfunction experienced by both obese females and HS animals.
References


57. **Näär AM.** MiRs with a sweet tooth. *Cell Metab* 14: 149-150, 2011.


APPENDIX

Figure 1. Impact of genotype on glucose clearance in mice. A glucose tolerance test (GTT) was performed at 6 weeks, by administration of bolus glucose (1 mg/kg), followed by measurement of blood glucose levels over time (30, 60, 90 and 120 min). There was no difference ($P > 0.05$) in fasting blood glucose levels and glucose clearance between the lean (CT) and obese (LY) mice. $n = 5$ mice per treatment.
Figure 2. Obesity decreased glucose clearance rate in mice at 12 weeks of age. A glucose tolerance test (GTT) was performed at 12 weeks, by administration of bolus glucose (1 mg/kg), followed by measurement of blood glucose levels over time (30, 60, 90 and 120 min). Obese (LY) mice had increased ($P < 0.05$) fasting blood glucose levels relative to their lean (control) littermates in addition to reduced ($P < 0.0001$) glucose clearance, indicating that obese mice were becoming insulin insensitive. $n = 5$ mice per treatment.
Figure 3. Obesity decreased glucose clearance rate in mice at 18 weeks of age. A glucose tolerance test (GTT) was performed at 18 weeks, by administration of bolus glucose (1 mg/kg), followed by measurement of blood glucose levels over time (30, 60, 90 and 120 min). Obese (LY) mice had increased ($P < 0.01$) fasting blood glucose levels relative to their lean (control) littermates in addition to reduced ($P < 0.0001$) glucose clearance, indicating that insulin sensitivity is being compromised in obese. $n = 4$ mice per treatment.
Figure 4. Obesity decreased glucose clearance rate in mice at 24 weeks of age. A glucose tolerance test (GTT) was performed at 24 weeks, by administration of bolus glucose (1 mg/kg), followed by measurement of blood glucose levels over time (30, 60, 90 and 120 min). Obese (LY) mice had increased ($P < 0.05$) fasting blood glucose levels relative to their lean (control) littermates in addition to reduced ($P < 0.0001$) glucose clearance, indicating that obese mice were becoming less insulin sensitive. $n = 5$ mice per treatment.
Figure 5. Impact of obesity on ovarian energy utilization gene expression in mice. At 18 weeks, we determined the impact of obesity on glucose metabolism using PCR arrays. Obesity increased genes involved in gluconeogenesis (Fbp2, G6pc and Fbp1) while decreasing those in glycosis (Pklr and Gck). Interestingly, some members of the TCA cycle were increased (Sdha, Sdhb and Suclg2) while others being decreased (Acly and Mdhl1b). Similar effects of obesity were observed on gene expression partner of the pentose phosphate pathway members where Rpe was increased but Rapia tended to decrease with obesity. Glycolysis, the TCA cycle and the pentose phosphate pathways, breakdown glucose into metabolites necessary for energy production, while Gluconeogenesis stores excess energy as glucose. These data suggest that the ovary is responsive to changes in energy intake.
Figure 6. Impact of HS on porcine ovarian energy utilization gene expression. Using the 7 day time point group, we investigated the impact of HS on glucose metabolism using PCR arrays. Both PF and HS increased genes involved in gluconeogenesis. The genes involved in glycolysis showed a general trend of reduction by PF but an increased trend in HS; demonstrating that thermal stress itself may impact energy requirements by the ovary. The genes in the TCA cycle were regulated in the same manner by PF and HS, with the exception of ACO1, however, the pentose phosphate pathway was reduced by HS. Genes in the regulation of glucose metabolism group were increased by HS compared to TN and PF. These data are not useful only in the context of HS, but also provide valuable information regarding the ovarian response to nutrient deprivation.