Phase III biotransformation enzyme involvement in the ovarian response to ovotoxic environmental exposures

by

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DEDICATION

I give all glory to God for the strength and knowledge He has graced upon my life. I dedicate this thesis to my beloved and forever-cherished mother, Rachel Ann Thomas, who has supported every endeavor and made sacrifices for this very moment to be achieved. You are my eternal sunshine and I will keep shining for you; live in peace my angel. I would also like to dedicate this thesis to my immediate family, Roy L. Thomas, Sam Thomas, LaToya Thomas, Keisha Norfleet, and Lexie Norfleet! Without the support and prayers through the good moments and the rough patches this would not be possible. I love you all so much. To my precious nephews, Jacovie and Titan, let this be a representation that regardless of where you may start in life, with hard work, faith, and extraordinary people supporting you, ANYTHING is POSSIBLE! I love you both so much and hope that one day when you are in a similar position that you may look back at this and find inspiration. I also dedicate this to my life-long friends, the five lanes, who have supported me through every failure and victory, we did it again! Lastly, I dedicate this thesis to all the hard working graduate students who endures the challenges of science for betterment of mankind.

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ABBREVIATIONS

PI3K: Phosphotidylinositol-3 Kinase
FSH: Follicle Stimulating Hormone
LH: Luteinizing Hormone
STAR: Steroid Acute Regulatory Protein
CYP450: Cytochrome P450
GnRH: Gonadotropin-Releasing Hormone
HS: Heat Stress
PM: Phosphoramide Mustard
CPA: Cyclophosphoramide Mustard
CEZ: Chloroethylziridine
PND4: Postnatal day 4
PGCs: Primordial germ cells
pf: Post fertilization
ZP: Zona pellucida
IGF-1: Insulin-like growth factor
CL: Corpus luteum
PgF2α: Prostaglandin F2alpha
LDL: Low density lipoproteins
3βHSD: 3β-hydroxysteroid dehydrogenase
DHEA: Dehydroepiandrosterone
17βHSD: 17β-hydroxysteroid dehydrogenase
GC: Granulosa cells
TC: Theca cells
HPG: Hypothalamic-pituitary-gonadal
POF: Premature ovarian failure
XBE: Xenobiotic biotransformation enzymes
EH: Epoxide hydrolases
GST: Glutathione *S*-transferase
ABC: ATP-binding cassette
AHR: Arylhydrocarbon receptor
NRF2: Nuclear factor erythroid related factor 2
XRE: Xenobiotic response element
KITLG: Kit ligand

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ABSTRACT

The mammalian ovary is comprised of follicles at various stages of growth and is responsible for gamete and steroid hormone production. Conservation of ovarian function is vital for female reproductive and general health, and disruption can lead to infertility and/or ovarian senescence. Stressors affect the ovary by inflicting damage to the primordial oocyte and/or inducing follicular activation thereby depleting the follicular reserve and disrupting biological pathways or defense mechanisms necessary for maintaining proper ovarian function. Cyclophosphamide (CPA) is a chemotherapy drug that biotransforms into an ovotoxic metabolite phosphoramide mustard (PM) via hepatic biotransformation. Our previous findings demonstrated that PM (60 μM) exposure depleted primordial follicles as well as all follicle types in cultured postnatal day (PND) 4 rat ovaries. With the advancement of science and medicine, there has been a decline in mortality rates of females who receive chemotherapy. The viability and health of the ovary post-chemotherapy treatment is of major concern for female cancer survivors due to the harsh effects of anti-neoplastic agents. An ovarian defensive mechanism would challenge the adverse effects of PM-induced ovotoxicity. We hypothesized that the ovarian defense response to PM-induced damage involves both the ABCB1 and ABCC1 proteins to excrete PM from the ovary and that their regulation involved the PI3K pathway. ABCB1 and ABCC1 are involved phase III chemical biotransformation, having enzymatic activity that requires ATP derived energy for the transportation of endogenous and exogenous compounds. Briefly, PND4 rat ovaries were cultured in PM (60 μM), LY294002 (20 μM), or vehicle control (1% DMSO) followed by protein isolation and western blotting. Adult

female mice (15 wks) were intraperitoneally (i.p) dosed once with sesame oil (SO) or PM (95%; 25 mg/kg), euthanized 3 days post dosing followed by removal of ovaries and fixation in paraformaldehyde (4%). Ovaries were sectioned and mounted on slides followed by immunohistochemistry (IHC) performance to localize ABCC1 and ABCB1 proteins. PM exposure decreased ABCB1 protein abundance in PND4 cultured rat ovaries, however, no impact was observed in ABCC1 in either PND4 or cultured ovarian rat granulosa cell models. Localization of ABCB1 and ABCC1 in PND4 ovaries was observed in the oocyte cytoplasm post PM exposure. ABCB1 was localized in granulosa cells, interstitial tissue, and the oocyte of adult mice ovaries, and there was striking ABCB1 oocyte peri-cytoplasmic and peri-nuclear immunofluorescence staining. ABCC1 was localized in the nucleus of oocyte in PND4 ovaries, but confined to theca cells, oocyte cytoplasm, and interstitial tissue of adult mouse ovaries. Additionally, pre-ovulatory follicles expressed ABCC1 in the oocyte peri-cytoplasmic membrane in control animals, but this staining pattern was absent in PM-exposed mice. In regards to PI3K, inhibition resulted in down regulation of both ABCB1 and ABCC1, however, PI3K activation via kit ligand treatment had no effect on ABCB1 or ABCC1.

Heat stress has a negative impact on agriculture livestock production. Our previous studies suggested that HS alters ovarian steroidogenic signaling impacting the productivity of reproduction in swine (Nteeba et al., 2015). This thesis investigated whether SULT1E1 and ABCC1 were involved in metabolism and excretion of 17β-estradiol synthesis during HS. It was hypothesized that HS alters SULT1E1 and ABCC1 impacting 17β-estradiol. Briefly, pre-pubertal gilts were exposed to thermoneutral (TN; 20°C) or heat stress (HS; 35°C) conditions for 5, 7, or 35 d with an additional group of thermoneutral pair-fed

(PFTN) gilts included to eliminate bias of feed intake followed by protein isolation, RNA isolation, western blotting, and qRT-PCR. Additionally, post-pubertal gilts were synchronized in their follicular phase with Matrix feeding and exposed to TN (20.3°C) or HS (26-32°C) conditions for 5 d followed by protein isolation, RNA isolation, western blotting, and qRT-PCR. We found that chronic HS (35 d) of pre-pubertal gilts increased both ABCC1 and SULT1E1 protein abundance without having an effect at the transcriptional level. HS during the follicular phase in post-pubertal estrous synchronized gilts resulted in reduction of *ABCC1* mRNA levels and increased levels of SULT1E1 protein, but no impact on ABCC1 protein was observed. The data generated from these studies suggest that PI3K plays a critical role in ovarian phase III biotransformation enzymes providing evidence that the ovary has the ability to conduct phase III drug metabolism and that SULT1E1 and ABCC1 are active in the ovary and may alter 17β-estradiol metabolism during HS.

ORGANIZATION OF THE THESIS

The main focus of this thesis regards PM-induced ovotoxicity and HS-induced steroidogenic disruption impacting ovarian function followed by defense mechanisms along with identification of ovarian proteins affected. It is organized in four separate chapters including a table of contents section. Chapter 1 details general background information on ovarian physiology, folliculogenesis, steroidogenesis, hypothalamic-pituitary-gonadal axis, ovotoxicity, heat stress, biotransformation enzymatic activity, and role of PI3K pathway. Chapter 2 consists of specific information on the project in regards to PM toxicity of the ovary and phase III biotransformation defensive mechanisms that are regulated by PI3K pathway signaling in ovarian rat models. Chapter 3 investigates the effects of heat stress on ovarian SULT1E1 and ABCC1 in pre-pubertal and cyclic gilt ovaries. Chapter 4 is a discussion of the literature review, data, and future research ideas from the author's perspective.

CHAPTER 1

GENERAL INTRODUCTION

Ovarian Physiology

The female reproductive system, is comprised of the ovaries, uterus, fallopian tubes, vagina, vulva, and mammary gland, which are collectively vital for the facilitation of gamete development, fertilization, pregnancy maintenance and nourishment of the offspring. The ovary is the major female gonad and has two main functions: to produce 1) the female sex steroid hormones (17β-estradiol (E_2) and progesterone (P_4)) and 2) the female gamete, the oocyte. The ovaries are located on the right and left side of the pelvic body cavity and contain follicles that consist of the oocyte, granulosa and theca cells, dependent on the stage of follicular maturation (**Figure 1.1**) [1].

In humans, oogenesis is a process for the production of oocytes that occurs in the fetal developmental stage prior to birth where the female will produce all the oocytes that will be used during the entire lifespan, comprising the ovarian reserve [2]. This process involves both mitosis and meiosis, where initially oogonia produce oocytes through mitosis [3-7]. Briefly, the oocyte undergoes meiosis but it is arrested during prophase I of the diplotene stage until menarche [3, 8, 9]. The female follicular pool consists of a finite population: the gestational population of oocytes ranges around 14 million and decreases drastically at birth with an estimation of 1.5 million. At puberty onset the number of oocytes further declines to approximately 300,000. Considering that women ovulate only 400-500 oocytes during a lifetime, a fraction of oocytes mature to ovulation. The majority of oocytes die by a process known as atresia. By the time a woman enters menopause there

remain approximately 100 oocytes in the follicular pool [2, 3]. Follicular depletion is irreversible, thus, reproductive senescence ensues, known as menopause in humans [10].

Folliculogenesis

The ovary is a compartmentalized structure that contains follicles at different maturation stages (**Figure 1.1**). These classification of follicles include primordial, primary, secondary, and antral follicle stages. Folliculogenesis is the process where primordial follicles mature to pre-ovulatory follicles via regulation by both paracrine and endocrine factors [11]. Follicles are produced from primordial germ cells (PGCs) that migrate to the genital ridge and settle in the mass of mesoderm on dorsal body wall (indifferent gonad) [12-14]. The somatic cells (granulosa, theca, endothelial, interstitial tissue) of the follicle are descended from the embryonic indifferent gonad, which consists of mesenchyme from the genital ridge [3, 15]. Following invasion of the indifferent gonad, PGCs lose their motile function and undergo rapid proliferation [13, 14, 16]. The PGCs undergo mitosis while the somatic cells also continue to rapidly divide [13]. The PGCs become clustered together and the somatic cells enclose the PGCs by development of histologically distinct cord structures. In the rat and mouse model, ovarian cords are not sharply defined but they are very distinctive in the pig [3]. The ovarian cords are responsible for separating the embryonic ovary into two compartments that are divided by a basement membrane. The embryonic ovary contains an epithelioid compartment containing PGCs and an interstitial compartment without PGCs [17, 18]. Completion of PGC mitotic division results in their transformation to begin prophase of meiosis, however, the oocyte is arrested at the diplotene stage [6]. In humans, oocytes enter meiosis by 2 months post-coitum [19], at day

17.5 post fertilization (pf) in the rat [20], and day 14 pf in the mouse [21]. The somatic cells within the ovarian cords continue to proliferate throughout the embryonic phase and they enclose oocytes into germ cell nests, precursors of primordial follicles.

Primordial follicles consist of an oocyte surrounded by a single layer of flattened granulosa cells and the basal lamina [7] (**Figure 1.2**). Primordial follicles can remain dormant for months or years. The fate of primordial follicles rely on a process known as initial recruitment where intraovarian factors stimulate activation of primordial follicles into the growing follicular pool [11]. This process occurs after primordial follicle formation even prior to pubertal onset [11]. As growth occurs, the flattened granulosa cells resume proliferation and assume a cuboidal morphology, the size of the oocyte increases, and formation of a non-cellular layer between the oocyte and granulosa cells known as the zona pellucida (ZP) occurs [22-24], forming a primary follicle (**Figure 1.2**). In primary follicles, the granulosa cells and oocyte makes contact by transmission of microvilli through the ZP forming gap junctions with the oocyte [3].

Further follicle development results in the formation of the secondary follicle stage where 2-3 layers of granulosa cells are present. Secondary follicles are also referred as pre-antral since they lack an antrum. At this stage, recruitment of an additional theca cell layer occurs [25]. Arterioles attach to the theca cell layer in order to facilitate a self-regulating blood supply [26]. The thecal layer consists of an outer layer, the theca externa, and an inner layer, the theca interna. The theca externa mainly contains nerves, blood vessels, and connective tissue while the theca interna layer is entailed of steroid-producing cells. Theca

cells are thought to be recruited from ovarian stroma by factors that are released from activated follicles [2, 3].

The antral, or Graafian follicle, is the largest follicle that is both LH- and FSH-dependent. It is also a major site for protein and steroid synthesis. At this point, the granulosa cells further proliferate into the corona radiata and cumulus oophorus. The corona radiata is a single layer of granulosa cells adjacent to the ZP that sustain gap junction for intercellular transport of metabolites and nutrients to the oocyte [27]. The multilayer granulosa cells that surround the corona radiata are the cumulus oophorus that are attached to the corona radiata during ovulation and detach into the oviduct afterwards. Mural granulosa cells, which are granulosa cells located next to the basal lamina, contain the most LH receptors in mature follicles. Once the connections with the granulosa cells forming the corona radiata and mural cells are disrupted, it causes a secretion of fluid that forms a cavity known as the antrum. The follicular fluid inside the antrum is a very rich steroid environment and contains thecal blood constituents [28]. Fully mature follicles also house insulin-like growth factor (IGF-1) receptors, gonadotropin-releasing hormone (GnRH) binding sites, and steroidogenic enzymes, which are discussed in a later section [29-35].

Towards the end of the follicular phase, an LH surge causes the pre-ovulatory follicle's cumulus oophorus layer to develop an opening, expelling the oocyte from the follicle in a process known as ovulation [36]. By this point, meiosis I resumes and the first polar body is formed resulting in a haploid oocyte. The primary oocyte is now considered a secondary oocyte and meiosis II is initiated [5-7, 13, 21, 37].

The corpus luteum (CL) is a temporary endocrine structure that is morphologically and biochemically generated from transformed theca and granulosa cells. It synthesizes the production of progesterone (P_4) that is necessary for the maintenance of pregnancy. The CL is developed during the luteal phase of the estrous or menstrual cycle and converts to a blood filled follicular structure, called the corpus hermorrhagicum, immediately following ovulation. In humans and animals with long estrous cycles, such as the pig, the CL forms right after ovulation during the luteal phase of the cycle. Eventually, if fertilization does not occur, the CL undergo luteolysis forming a corpus albicans. In humans, this process is not well investigated, but is hypothesized to be caused by prostaglandin F2alpha ($PgF2\alpha$) [2].

In contrast to initial recruitment, cyclical follicle recruitment after pubertal onset is initiated by an FSH spike that rescues a cohort of antral follicles for ovulation and potential fertilization during each reproductive cycle [11]. Follicle recruitment is associated with slight increase in circulating FSH. For example, rats exhibit a secondary spike of FSH on the day of estrus before the following cohort of ovulatory follicles are recruited [38]. Basal FSH is slightly elevated at the beginning of the follicular phase compared to the later or luteal phases in primates [39, 40]. Early antral follicle size in rats that are considered as recruitable range between 0.2-0.4 mm in diameter, whereas human follicles are larger, being 2-5 mm in diameter [11]. Follicles that are not recruited undergo atresia via a default pathway [11]. It is still unclear to why out of a cohort of 10 dominant follicles in young women only one develops faster than the rest and produces high levels of inhibin and

estrogens. It is suggested that the dominant ovulatory follicle may be more sensitive to FSH due to the higher expression of FSH/LH receptors [41-43]. It is also suggested that E_2 and inhibin secretion from the largest follicle can suppress pituitary FSH release during the mid-follicular phase which is needed for survival in the other growing follicles [44]. Administration of exogenous E_2 in women suppresses follicle development while exogenous administration of gonadotropins increased the growth rate of multiple pre-ovulatory follicles [45-47]. This demonstrates the negative selection of subsidiary follicles as a result of the negative feedback by the dominant follicle through inhibin and E_2 production [11].

The human menstrual cycle is quite variable ranging from 23-35 days with 14 days therein comprising the luteal phase, which is the time between ovulation and menstruation [2]. Unlike humans, in domestic animals including pigs, the first day of the estrous cycle is dictated by the female being in behavioral heat and receptive to the male. In pigs, the estrous cycle is approximately 21 days in length and consists of the follicular phase (pro-estrus and estrus) and the luteal phase (metestrus and diestrus). Pigs are a polytocous species ovulating 15-30 oocytes per cycle [48]. The rodent estrous cycle is 4-6 days and also consists of estrus, proestrus, diestrus, and metestrus stages. Interestingly, primordial follicle formation via germ cell nest breakdown occurs during the fetal life of humans, primates and domestic animals, whereas in rodents it occurs post-parturition [8].

Steroidogenesis

A second major function of the ovary is the synthesis of sex hormones through steroidogenesis. The major products of steroidogenesis are estrogens (E_2 and estrone-3 sulfate) and P_4 [49]. The process is initiated when the ovary obtains cholesterol as either the dietary form of cholesterol bound to low density lipoproteins (LDL) or via *de novo* synthesis from acetyl-CoA as a precursor [50]. Cytochrome P450 (CYP) enzymes mediate each stage of ovarian hormone synthesis where steroidogenic acute regulatory protein (StAR) enables cholesterol transportation from the outer into the inner mitochondrial membrane where it is then converted to pregnenolone via CYP11A1 and P450-side chain cleavage (CYP450scc) enzymes. Pregnenolone is exported to the cytoplasm and undergoes conversion to P_4 by 3β-hydroxysteroid dehydrogenase (3βHSD). Pregnenolone can also be converted to 17α-OH-pregnenolone via CYP17 which is further processed into dehydroepiandrosterone (DHEA), a weak androgen. DHEA is converted into androstenedione by 3βHSD and then P_4 is converted to androstenedione by CYP17. Androstenedione induces the formation of testosterone by 17β-hydroxysteroid dehydrogenase (17βHSD). Both testosterone and androstenedione can be metabolized to E_2 and estrone via aromatase, CYP19a [2, 49, 50].

The steroidogenic process is mediated by granulosa cells (GC) and theca cells (TC) within the ovary through stimulation by FSH and LH. Both GC and TC express CYP11A1 and StAR [51], however, GC do not express CYP17A [52] therefore synthesis of pregnenolone from cholesterol occurs along with subsequent conversion to P_4 in the CL. In the late follicular phase, the preovulatory follicle primarily produces E_2 [49]. The follicle is

dependent on the interaction between the GC and TC and therefore, production of 17β-estradiol is referred to as the two-cell-two gonadotropin theory [34, 53-59]. As the follicle approaches ovulation, the GC increase the synthesis of P4, is required for luteinization initiation [2, 49].

The hypothalamic-pituitary-gonadal (HPG) axis

The hypothalamic-pituitary-gonadal (HPG) axis is a system that consists of a hormonal interaction between the hypothalamus, anterior pituitary gland, and the female gonads and is regulated by both negative and positive feedback mechanisms [60] (**Figure 1.4**). The hypothalamus is located beneath the thalamus and forms a lateral wall and floor of the third ventricle. It encompasses neurosecretory tissue and extends to the anterior pituitary gland where it influences hormone production [61]. It contains three different zones (periventricular, lateral, and medial) and the arcuate nucleus that is important for gonadotropin releasing hormone (GnRH). The secretion of GnRH is vital for stimulation of gonadotrophs in the anterior pituitary [61] that produce the gonadotrophins; FSH and LH. The pituitary gland functions as the master endocrine gland and contains two major components: the anterior and posterior lobes, with the anterior lobe being particularly pertinent to control of the mammalian reproductive cycle [61].

The HPG axis regulates the proliferative (follicular) phase, ovulation, and the secretory (luteal) phase, of the estrous/menstrual cycle. The initiation of the HPG process begins with the onset of menses, the mechanisms of which remain to be fully elucidated. The hypothalamus releases GnRH into the anterior pituitary where it stimulates FSH and LH

production and release. FSH promotes follicular development leading to the production of E_2 from multiple layers of GC. LH signals the TC for the production of androgens where they are transported to the GC to be transformed into E_2 [49]. Elevated E_2 from the dominant follicle(s), positively feedback onto the hypothalamus and pituitary, thereby causing the LH surge that is critical for ovulation [61].

Activin produced by GC amplifies FSH secretion by boosting formation of GnRH receptors [62, 63]. However, activin is inhibited by inhibin and follistatin. Inhibin is a polypeptide that is also secreted from the GC in response to FSH [64] and has the ability to inhibit FSH secretion from the anterior pituitary via negative-feedback regulation [65]. Follistatin is also a peptide that can inhibit FSH secretion and synthesis by requisition of activin [66, 67]. Inhibition of follistatin is promoted by inhibin while activin stimulates follistatin production [66, 67].

The residual GC content that are left behind after ovulation form the P_4-producing CL. P_4 converts the endometrial lining of uterus to the secretory phase for embryo implantation [61]. If a pregnancy occurs, the maternal recognition of pregnancy signal maintains the CL for production of P_4 suppressing the release of GnRH through negative feedback on the hypothalamus [61]. However, if fertilization does not occur, the CL undergoes a luteolysis process forming the corpus albicans which encourages the P_4 decline resulting in endometrium instability signaling a new menstrual cycle [61].

The HPG axis therefore regulates promotion of follicle maturation and ovulation [68] as well as sex steroid hormone (E_2 and P_4) production. E_2 is important for female secondary sex characteristic at puberty and development of mammary glands for lactation while P_4 is critical for implantation of the blastocyst and maintenance of pregnancy.

Ovarian senescence

Approximately 11 percent of women are infertile [69] for which the etiology is unknown but may involve environmental exposures or genetic causes [70]. As mentioned previously, viability and maintenance of the primordial follicular pool is important because once depleted they cannot be replaced [71, 72]. Women typically experience the onset of menopause on average by 52 years of age [10, 73, 74]. Premature ovarian failure (POF) is defined as menopause onset prior to 40 years of age. POF can be induced due to hypoestrogenism and/or elevated abundance of gonadotropins that can lead to exhaustion of the ovarian follicular pool [75]. It is also induced by altered HPG axis function which disrupts the proper synthesis of the sex steroid hormones [76].

Xenobiotic-induced infertility

Ovotoxicants are chemicals which detrimentally affect ovarian function **(Table 1.1).** They can affect primordial or growing follicle viability, folliculogenesis or steroidogenesis [70] and can lead to infertility or POF [73, 77]. The stage of follicle development targeted by an ovotoxicant determines the impact on reproduction. Disruption to antral follicle viability results in short-term deficiency due to the ability for replacement of follicles via recruitment from the finite primordial pool, thus once the exposure is removed, fecundity

is restored [77]. Destruction of primordial follicles represents a more insidious exposure since permanent infertility and POF can be induced which may not be recognized for months or years and for which there is no replacement [70]. Additionally, the life stage of exposure can impact the reproductive outcome: prenatal exposures could affect the PGC population; neonatal exposure could affect puberty onset; reproductive life span exposures can impact fertility, pregnancy maintenance or lactation and post-menopause exposures can increased neoplasm development in reproductive tissues [71] (**Figure 1.3**).

Cyclophosphamide (CPA) is an alkylating agent used to treat a wide range of cancers and autoimmune diseases [78, 79]. CPA can either be administered alone or given in a cocktail formula with other drugs [78]. CPA is a pro-drug that requires hepatic biotransformation by cytochrome P450 (CYP) enzymatic activity for transformation into its anti-neoplastic metabolite, phosphoramide mustard (PM) [80, 81]. Bioactivation of CPA to PM is initiated by CYP isoforms 3A and 2B [82]. As a detrimental side effect of CPA/PM treatments in females, PM induces primordial, small primary, and growing follicle loss [79, 83]. PM can further spontaneously transform into a volatile, cytotoxic compound, chloroethylaziridine (CEZ) [84], which negatively affects ovarian function [79]. Previous research from our group demonstrated that depleted primordial and growing follicles [79]. Exposure to an ovotoxicant that targets and destroys both pre-antral and growing follicles can accelerate ovarian senescence introducing a risk to general female health.

Xenobiotic biotransformation

Xenobiotic biotransformation enzymes (XBE) are involved in bioactivation, detoxification and/or excretion of xenobiotics [85]. Phase I chemical metabolism involves hydrolysis, reduction, and oxidation. Phase I XBE consist primarily of the cytochrome P450 (CYP) enzymatic family [86]. These enzymes are abundant in all tissues and participate in activation or detoxification of chemicals [86]. Epoxide hydrolases (EH) are also phase I biotransformation enzymes and consist of 5 different forms, microsomal EH being an example [86], which catalyzes water addition to arene oxides and alkene epoxides [86, 87].

Phase II XBE action generally enhances excretion in bile and urine by increasing hydrophilicity of the chemical [87]. There are a number of phase II biotransformation processes including sulfonation, glucuronidation, glutathionylation, acetylation, methylation [86]. Glutathione *S*-transferase (GST) isoforms alpha, pi, mu, omega, and theta catalyze glutathione (GSH) xenobiotic conjugation [86, 88]. Gonadotropins regulate GSH synthesis [89] and mice undergoing ovarian senescence had decreased concentrations of GSH [90, 91]. GSH is plentiful in all cells, as an antioxidant composed of glutamic acid, cysteine, and glycine [86]. The ovary has excessive amounts of GSH [92, 93] and ovulated oocytes contain the highest concentration of GSH compared to any cell type [94].

Phase III XBE's generally comprise drug transporters that excrete and transport foreign and endogenous compounds from the cell [87, 95-98]. The ATP-binding cassette (ABC) proteins are a large phase III XBE protein family [87, 97, 99, 100]. ABC proteins have the ability to derive energy from ATP hydrolysis to: 1) drive transmembrane movement of

specific substrates, 2) regulate penetrability of multi-channel complexes, and 3) open or close certain membrane channels [101]. The most studied ABC transporters are the multi-drug resistance protein (ABCB1), also known as p-glycoprotein, and the multi-drug resistance associated protein (ABCC1) [101]. ABCB1 confers multidrug resistance to cancer cells thereby enabling their evasion of anti-neoplastic chemotherapy treatments [102-105]. ABCB1 is approximately 170 kDa in size (Higgins, 1992; Croop, 1993; Gottesman, 2002) and transports positively charged or uncharged hydrophobic compounds that have been made water soluble by phase I and/or II biotransformation [106]. ABCC1 also causes multidrug resistance phenotype in cancer cells [107-113]. ABCC1 is approximately 190 kDa in size and transports organic anions, hydrophobic compounds, sulfates, glucuronates, 17β-glucuronosyl estradiol, bisglucuronosyl bilirubin, and conjugated GSH substrates [101].

Regulation of phase I, II, and III XBE's involve signaling regulated by the arylhydrocarbon receptor (AHR) [114, 115] and nuclear factor erythroid related factor 2 (NRF2) [116]. AHR, a member of the basic helix loop-helix (bHLH) DNA binding proteins [117], is located in the cytoplasm within a complex of heat shock protein (HSP) 90 during homeostatic conditions [118]. However, after activation AHR translocates to the nucleus, where it dimerizes and binds to xenobiotic response element (XRE) of target gene promoters [118]. NRF2 is a member of the "cap 'n' collar basic leucine zipper" (CNC-bZip) proteins where it is to bound to kelch-like ECH-associated protein (KEAP1), a repressor protein, in the cytoplasm until cells are exposed to stressful conditions requiring action [119, 120].

The phosphatidylinositol-3 kinase pathway

Phosphatidylinositol-3 kinase signaling pathway (PI3K) is imperative for viability of primordial follicles [121] as well as their activation into the growing follicle pool [122-126]. In primordial and small primary follicles, PI3K is regulated by kit ligand (KITLG), which is synthesized in the GC [127] and which binds to the oocyte c-kit receptor [128-131] initiating c-kit auto-phosphorylation which in turn activates PI3K [132]. KITLG-cKIT interaction is involved in initiation of oocyte growth during primordial follicle growth activation [133, 134]. PI3K signaling is negatively impacted by exposure to the ovotoxicant 4-vinylcyclohexene diepoxide (VCD) [135, 136] and inhibition of the pathway protected primordial follicles from VCD-induced follicle loss [137]. Exposure to VCD reduced PI3K signaling in primordial and small primary oocytes [135, 136] by decreasing phosphorylation of c-Kit [138] and over expressing pAKT [136] in PND4 cultured rat ovaries. The pathway is also involved in regulation of ovarian XBE's [139, 140]. Both mRNA and protein abundance of the XBE genes, *meh*, *Ahr*, *Nrf2*, *Gstp*, and *Gstm* were increased when PI3K inhibition was induced in cultured rat ovaries [139, 140]. Thus, ovarian PI3K regulates not only primordial follicle activation and growth, but also contributes to phase I, phase II, and phase III xenobiotic biotransformation.

Heat stress and reproduction

Heat stress (HS) is a thermal imbalance between the net amount of energy flowing from an animal to its surrounding environment which can induce several physiological issues [141]. The United States livestock industry endures economic losses due to HS, and seasonal

infertility induced by HS is estimated by our group to exceed $420 million annually (Pollman, Ross, Baumgard, Keating, unpublished data). HS negatively affects female reproduction and is phenotypically observed in swine and other species as irregular estrous cyclicity [142-144], reduced conception rate [145, 146], reduced embryonic survival [147], and higher frequency of stillbirths [146, 147]. The etiologies behind compromised reproductive efficiency due to HS are unknown, and hyperinsulinemia observed during HS [148, 149] could be one causative factor since insulin can activate PI3K signaling. HS has the potential to alter PI3K signaling which is involved in regulation of ovarian steroidogenesis [150], activation of primordial follicles [122-125, 151-153], maturation and viability of the oocyte [121]. A study investigated impacts of HS on ovarian signaling pathways critical for ovarian function in pre-pubertal gilts and demonstrated increased ovarian insulin signaling, PI3K signaling as well as altered mRNA abundance of genes involved in these processes [154]. HS also affected ovarian steroidogenesis and mRNA and protein abundance of *CYP19a1* and *STAR* were observed to be increased [154]. Altered steroid hormone production within the ovary, either by the dominant follicle or CL could lead to a reduction in fertility and impair embryonic viability [154].

Summary

This literature review details basic components of the female reproductive system and illustrates the susceptibility of the ovarian follicular pool that could contribute to infertility and/or POF. The complexity of the ovary demonstrates that there are many processes that contribute to proper ovarian function and efficient reproduction in the female. Folliculogenesis regulates proper maturation of the oocyte and surrounding follicular

structure, and culminates in production of E_2 synthesis which regulates secondary sex characteristics in conjunction with the hypothalamic pituitary gonadal axis. The homeostasis of the ovary can be interrupted by environmental endocrine disrupting chemicals, commonly used anti-neoplastic agents (CPA/PM), and environmentally natural occurrences such as heat stress; all of which can lead to infertility and/or reduced fecundity. Chemical biotransformation enzymatic activities are biological defense methods. The phase III biotransformation enzymes, ABCB1 and ABCC1, derive energy from ATP and transport specific substrates to maintain basal ovarian function. PI3K is an intracellular signaling pathway that regulates folliculogenesis, steroidogenesis and chemical biotransformation genes. In this thesis, we hypothesize that ABCB1 and ABCC1 function are involved in the ovarian mechanistic response to PM exposure, and that they are regulated by the PI3K pathway (**Chapter 2**). We also hypothesize that HS alters SULT1E1 and ABCC1 levels substrate binding thereby impacting E_2 as a response to our previously observed effects of HS on steroidogenic signaling (**Chapter 3**). With a steady increase in numbers of female cancer survivors, the fate of their oocyte viability and reproduction is important to ensure quality life of these women. Additionally, as global temperatures rise, HS has become a major concern and has had a huge impact on livestock production resulting in economic loss. Therefore, understanding negative influences on ovarian function is imperative.

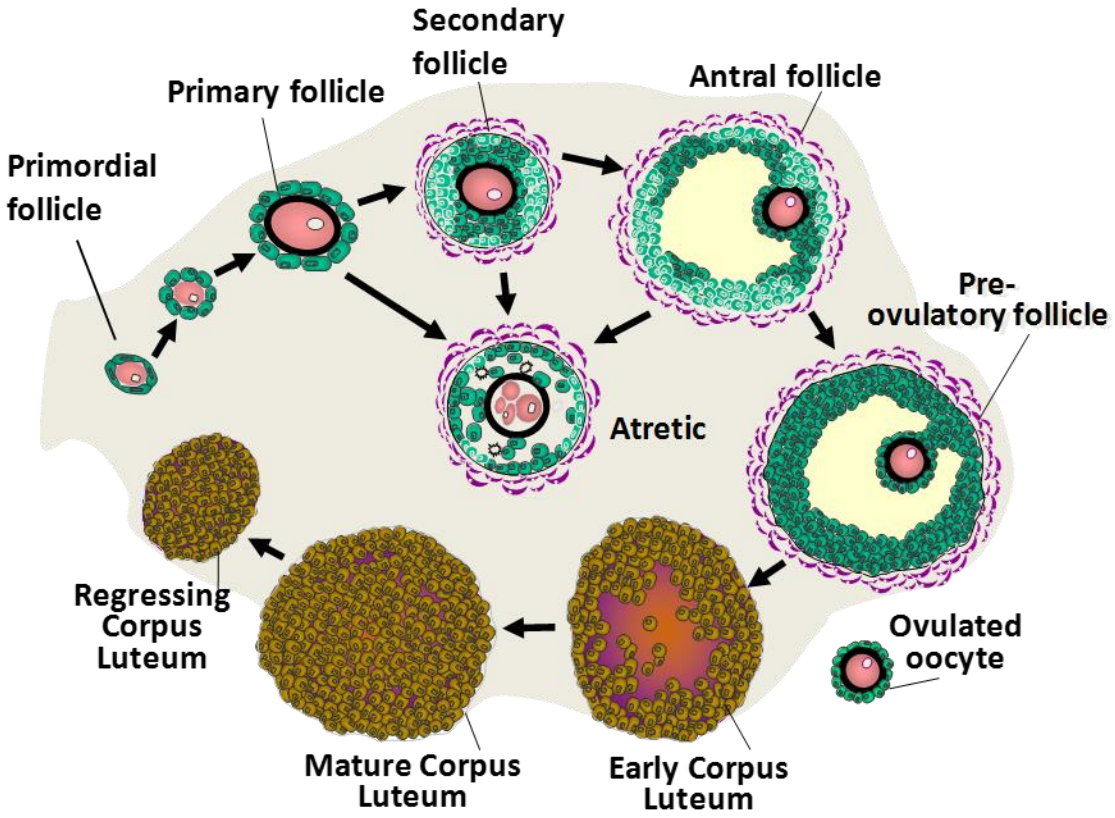

Figure 1.1. Overview of folliculogenesis.
Primordial follicles are signaled to grow and develop through primary, secondary, antral and pre-ovulatory follicular stages towards ovulation. Following ovulation, the remaining tissue differentiates to become the corpus luteum, which undergoes regression if a pregnancy has not been established. The majority (>99%) of follicles die by the natural process of atresia. Adapted from [71] with copyright permission.

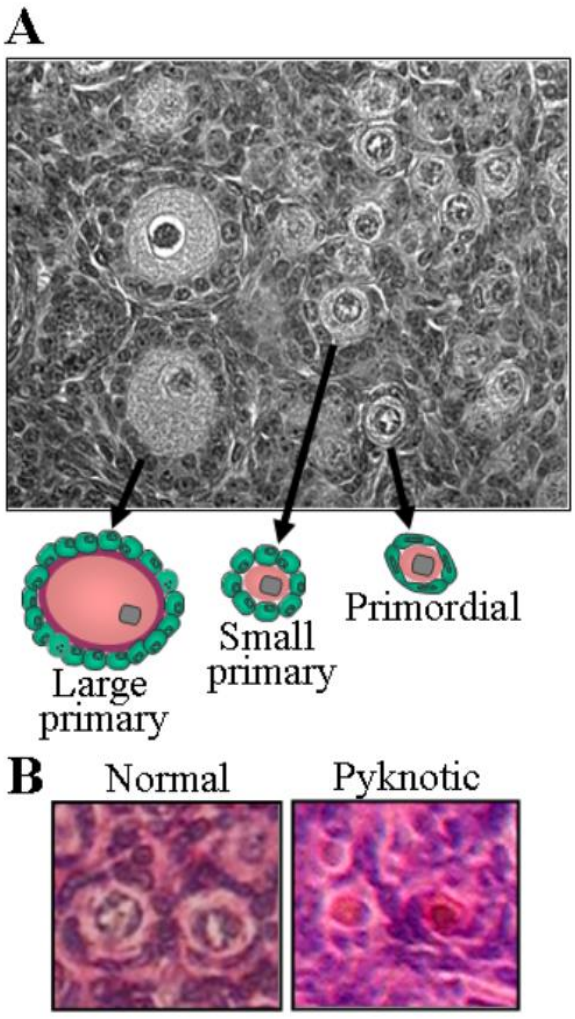

Figure 1.2. Histological morphology of cultured postnatal day (PND) 4 ovaries.
A) Cultured neonatal rodent ovaries contain many primordial follicles, some of which begin developing and growing during the culture period to primary and secondary stages. B) Most follicles appear normal with H&E staining, as shown on the left. Others, predominantly in the center of ovaries or following exposures to toxic substances, have a shrunken, pyknotic (more red or pink) appearance. From P.J. Devine with permission.

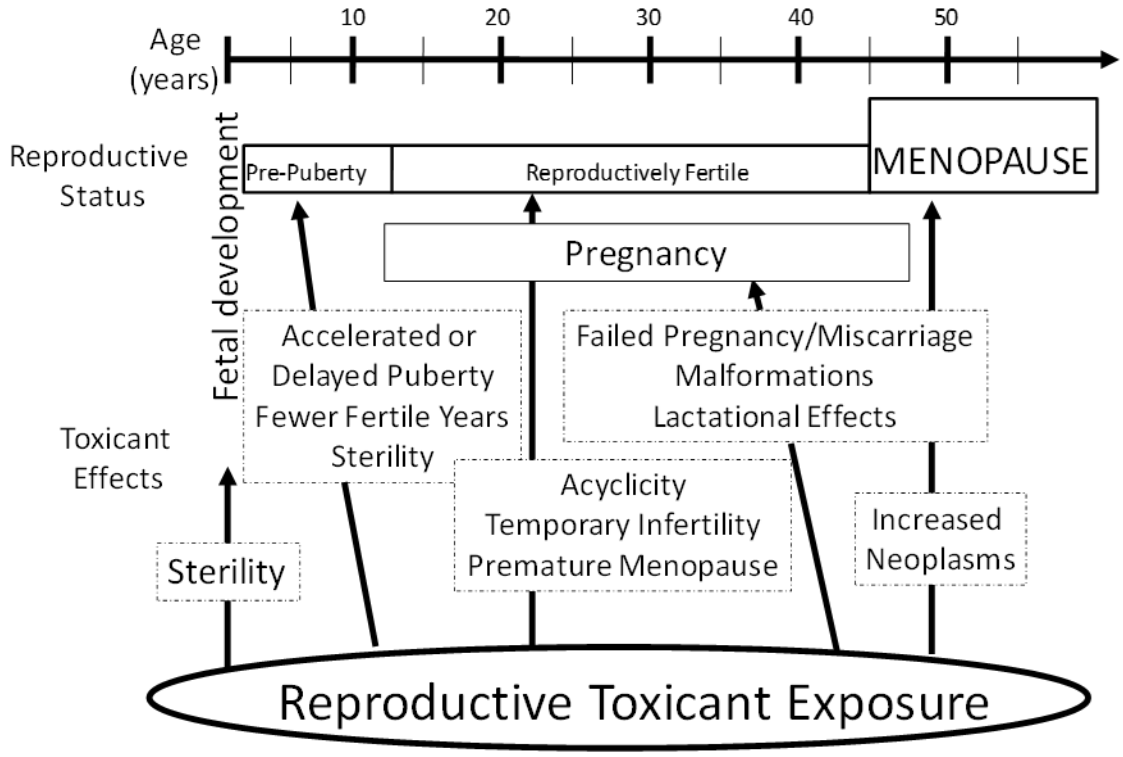

Figure 1.3. Potential age-related effects of reproductive toxicants in females.
The impact(s) of reproductive toxicants are partially dependent upon the reproductive status of the exposed individual. In most cases, direct ovarian toxicity can lead to premature ovarian failure and infertility (menopause). Adapted from [71] with copyright permission.

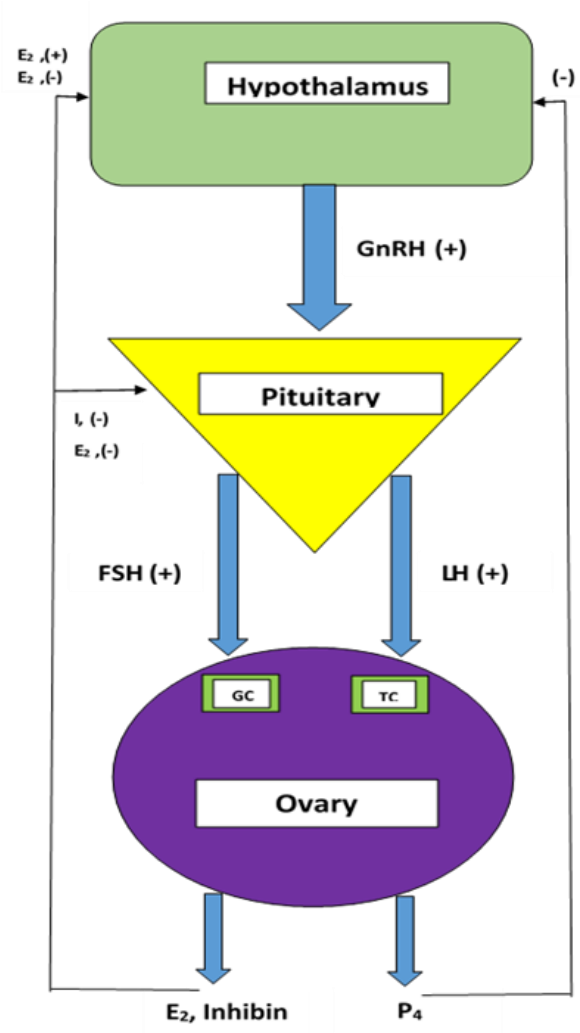

Figure 1.4. The hypothalamic-pituitary-gonadal axis
The primary sex steroid hormones, estrogen (E_2) and progesterone (P_4), are produced due to signaling from the hypothalamus to the anterior pituitary for stimulation of androgen production in granulosa (GC) and theca cells (TC). Briefly, GnRH is produced by the hypothalamus and binds to GnRH receptors in the anterior pituitary to signal the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the endocrine system where they bind respectively to follicular TC and GC receptors. E_2 is produced by developing follicles and generates negative feedback on the hypothalamus and anterior pituitary. GC release inhibin to send negative feedback on anterior pituitary inhibiting FSH production. High levels of E_2 from dominant pre-ovulatory follicles positively feedback to cause the LH surge which is critical for ovulation. Post-ovulation, corpus lutea (CL) form which synthesize and release P_4. P_4 has negative feedback on the hypothalamus for the suppression of GnRH should pregnancy. Absence of implantation causes regression of CL, thereby inhibiting P_4 production and removing the negative feedback of P_4 on the HPG-axis.

Table 1.1. List of some ovotoxicant chemicals and their impact on ovarian function.

Chemical	Small pre-antral follicles	Growing follicles	Hormone Levels
Atrazine	NE	NE	EO
BaP	EO	PE	EO
BPA	EO	NE	NE
Cd	NE	EO	EO
CPA/PM	EO	EO	NE
DEHP	NE	EO	PE
DMBA	EO	EO	EO
Hg	EO	EO	EO
MEHP	NE	EO	EO
MXC	NE	EO	EO
Pb	NE	NE	EO
VCD	EO	NE	NE
VCH	EO	NE	NE

Abbreviations: EO, effect observed; NO, no effect; PE, potential effect.

Chemicals listed: BaP = benzo(a)pyrene; BPA = bisphenol A; Cd = cadium; CPA/PM = cyclophosphamide/phosphoramide mustard; DEHP = di-(2-ethylhexyl) phthalate; DMBA = 9,10-dimethylbenz(a)anthracene; Hg = mercury; MEHP = mono-(2-ethylhexyl)phthalate; MXC = methoxychlor; Pb = lead; VCD = 4-vinylcyclohexene diepoxide; VCH = 4-vinylcyclohexene.

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CHAPTER 2

INVESTIGATION OF PHASE III CHEMICAL BIOTRANSFORMATION ENZYMES ABCC1 AND ABCB1 IN THE OVARIAN RESPONSE TO PHOSPHORAMIDE MUSTARD EXPOSURE AND REGULATION BY PHOSPHATIDYLINOSITOL-3 KINASE.

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Abstract

Phosphoramide mustard (PM), the anti-neoplastic and ovotoxic metabolite of cyclophosphamide causes ovarian DNA adduct formation and depletion of the finite pool of ovarian primordial follicles. Adenosine triphosphate (ATP) binding cassette drug transporters including isoforms B1 (ABCB1) and C1 (ABCC1) contribute to phase III drug metabolism by binding to conjugated xenobiotics contributing to their cellular excretion. We have demonstrated that phosphatidylinositol-3 kinase (PI3K) signaling regulates ovarian phase II drug metabolism enzymes. We had previously reported PM-induced increased mRNA encoding in *Abcb1* and *Abcc1* in an *ex vivo* ovarian culture. Therefore, we hypothesized that ABCB1 and ABCC1 are involved in the ovarian response to PM exposure as a cellular defense mechanism to export the ovotoxicant from the ovary, and that they are regulated by PI3K. Spontaneous immortalized granulosa cells (SIGC) were cultured in media containing vehicle control (DMSO) or media containing PM (6 μM) for 48 h until 80% confluent. Postnatal day 4 (PND4) ovaries were cultured in media containing vehicle control (DMSO) or PM (60 μM) ± LY294002 (20 μM) ± kit ligand (400 ng/ml) for 4 d. Ovaries were also collected from adult mice 3 d post-exposure to saline or PM (25 mg/kg). Western blotting was performed to quantify ovarian abundance of ABCB1 and ABCC1 and their response to PM exposure. Immunofluorescence staining was utilized to determine ABCB1 and ABCC1 protein localization. No impact of PM exposure were observed on ABCC1 protein abundance in either the SIGC or PND4 ovary culture models, but in contrast, ABCB1 protein expression was decreased ($P < 0.10$) in PND4 ovaries exposed to PM. Both ABCB1 and ABCC1 protein abundance were decreased ($P < 0.05$) substantially in ovaries lacking PI3K signaling. Surprisingly, PI3K

activation via kit ligand treatment had no effect on ABCB1 or ABCC1. In PND4 ovaries, both ABCB1 and ABCC1 were localized to the oocyte cytoplasm. ABCB1 was localized in interstitial tissue, granulosa cells and oocyte in adult mouse ovaries. As the oocyte matured towards ovulation, peri-nuclear and peri-cytoplasmic ABCB1 staining was observed. ABCC1 was evident in the oocyte nucleus in PND4 ovaries, but was located in interstitial tissue, theca cells and oocyte cytoplasm in adult ovaries. Pre-ovulatory follicles expressed ABCC1 on the oocyte peri-cytoplasmic membrane in CT-treated adult mice, but this staining pattern was absent in mice exposed to PM. These data indicate a critical role for PI3K in regulation of ovarian phase III drug transporters, as well as demonstrate that the oocyte as well as the vascularized thecal cell layer have the capacity to function in phase III drug metabolism.

Keywords: Ovary, Phosphoramide mustard, Phase III chemical metabolism

Introduction

The mammalian ovaries are the female reproductive organs that are responsible for gamete (oocyte) production and steroid hormone synthesis. The right and left ovary are located on each side of the uterus within the lower region of the abdomen. The ovary is endowed with follicular structures that consist of an oocyte and surrounding granulosa cells in primordial and small primary follicles. As the follicle matures towards ovulation, multiple layers of theca cells are recruited to the follicle and each follicle type serves specific functions based on its maturation stage [1-3]. Production and regulation of the sex steroids 17β-estradiol (E_2), and progesterone (P_4) are vital for follicle maturation and maintenance of pregnancy, respectively [1, 2, 4-7].

Primordial follicle preservation is important because their destruction is irreversible [8-11]. The ovary contains a finite primordial follicular pool and depletion of this follicular reserve can result in infertility and/or premature ovarian failure (POF) [2]. With the advancement of industrial and technological applications, there is concern about the potential harm generated from the by-products of these advancements [12]. Approximately 11% of women suffer from infertility, which may be due to genetically-induced premature ovarian senescence [13, 14], exposure to environmental toxicants, or treatment with chemotherapy agents [8, 15]. Mortality rates have declined in female cancer patients who receive chemotherapy treatments, resulting in a longer life expectancy from the advancement of science and technology. The major concern for survivors is the quality of life and health post-treatment with chemotherapeutic drugs [10, 16-22].

Cyclophosphamide (CPA) is a commonly used anti-neoplastic agent that is bioactivated to phosphoramide mustard (PM) to treat a range of cancers and autoimmune diseases [23, 24]. Bioactivation of CPA to PM requires the hepatic cytochrome P450 (CYP) enzymatic isoforms 3A and 2B [25]. CPA and PM can spontaneously transform into a volatile compound, chloroethylaziridine (CEZ), which has cytotoxic characteristics [26-28], making it a potential threat for those who work with chemotherapy patients or those who are around individuals receiving treatment. We previously demonstrated that both PM and CEZ are ovotoxicants: postnatal day 4 (PND4) cultured rat ovaries exposed to PM (60 μM) had a significant decline in numbers of all follicle types after 4 days of treatment [27]. CEZ also depleted primordial follicles after 4 days in ovaries that did not have direct administration of PM but which were indirectly exposed to CEZ by airborne contact [27].

Phase III chemical metabolism involves drug transporters that excrete and transport both foreign and endogenous compounds [29-33]. ATP-binding cassette (ABC) proteins are one of the largest transporter protein families characterized in all living organisms [34] and 46 ABC transporters have been identified in humans [29, 32, 35]. ABC proteins have the ability to derive energy from ATP hydrolysis for three important functions: 1) to drive transmembrane movement of specific substrates, 2) regulate penetrability of multi-channel complexes, and 3) open or close certain membrane channels [36]. The most studied ABC transporters are the multi-drug resistance protein (MDR1/ABCB1), also known as p-glycoprotein, and the multi-drug resistance associated protein (MRP1/ABCC1) [36]. ABCB1 was the first membrane transporter discovered to play a role in conferring multidrug resistance to cancer cells thereby enabling their evasion of anti-neoplastic

chemotherapy treatment such as CPA [37-40]. ABCB1 is approximately 170 kDa in size (Higgins, 1992; Croop, 1993; Gottesman, 2002) and transports positively charged or uncharged hydrophobic compounds that have been made water soluble by an oxidation process that involves CYP 450 enzymes, conjugation to glutathione (GSH), or exportation before entrance into the cytoplasm [41]. ABCB1 is localized in the plasma membrane of the apical/luminal membrane in polarized cells, endothelial cells of the blood-brain barrier, luminal membrane of renal proximal tubule epithelial cells, as well as hepatocyte biliary cananlicular and brush border membranes [39, 42-44].

Similar to ABCB1, ABCC1 also promotes a multidrug resistance phenotype to cancer cells [45-51]. ABCC1 is approximately 190 kDa in size and transports organic anions, hydrophobic compounds, sulfates, glucuronates, 17β-glucuronosyl estradiol, bisglucuronosyl bilirubin, and conjugated GSH substrates [36]. A major function of ABCC1 is the transport of leukotriene C4 (LTC4) [36]. ABCC1 is typically localized in polarized cells of the basolateral membrane, has ubiquitous expression and is specifically elevated in the testis and lung [29, 36].

We and others have demonstrated that the ovary has the capacity for phase I [52-54] and phase II [55-61] chemical metabolism. We have also discovered that both the xenobiotic biotransformation enzymes transcriptional regulators, arylhydrocarbon receptor (AHR) and nuclear factor erythroid-related factor 2 (NRF2), as well as phase I (microsomal epoxide hydrolase (EPHX1)) and phase II (Glutathione *S*-transferase isoforms pi (GSTP) and mu (GSTM)) enzymes are downstream of the phosphatidylinositol-3 kinase (PI3K)

pathway [55, 57]. Indeed, inhibition of PI3K prevented ovotoxicity induced by 4-vinylcyclohexene diepoxide (VCD) but accelerated that of dimethylbenz[a]anthracene (DMBA) [62], potentially due to increased EPHX1 [57]. Although the basal function of ovarian ABCB1 and ABCC1 have not been well characterized, considering their function in non-ovarian tissues, their necessity in phase III chemical metabolism for intracellular protection of the ovary seems logical. In addition, in light of ABCB1 and ABCC1 involvement in conferring resistance to CPA upon cancer cells, the potential for their involvement in a defense mechanism protecting non-cancerous cells could be beneficial for preservation of the ovary from the cytotoxic effects of CPA and PM. In this study, we hypothesized that ovarian ABCB1 and ABCC1 are involved in the ovarian protective response against PM exposure and PI3K is involved in their regulation.

Materials and Methods

Chemical Reagents

Phosphoramide Mustard (PM) was obtained from the National Cancer Institute (Bethesda, MD). Tris base, 2-β-mercaptoethanol, Glycine, Sodium Dodecyl Sulfate, Sodium Chloride, Tris Hydrochloric Acid, tween-20, Sodium Acetate, Bis- 2-chloroethylamine hydrochloride, Bovine Serum Albumin and Ammonium Persulphate were purchased from Sigma-Aldrich Incorporated (St. Louis, MO). Ponceau S Stain was purchased from Fisher Scientific and Mini-PROTEAN TGX Gels were obtained from Bio-Rad (Philadelphia, PA). RNeasy Mini Elute kit, RNeasy mini kit, Quantitect^{TM} SYBR Green PCR kit and QIA shredder kit were purchased from Qiagen Incorporated (Valencia, CA). EDTA, Pen/Strep, Fetal Bovine Serum, 1X Dulbeco's Modified Eagle Medium and 10X phosphate

buffered saline were purchased from Life Technologies (Grand Island, NY). Corning storage system/vacuum filter and cell culture flasks were obtained from Corning Incorporated (Corning, NY). Clear nail polish was obtained from Electron Microscopy Sciences (Hatfield, PA). X-ray film was purchased from Thermo Scientific (Rockford, IL) and SignalFire ECL western detection reagents were purchased from Cell Signaling Technologies (Beverly, MA). Recombinant mouse kit ligand was obtained from R & D Systems Inc. (Minneapolis, MN). LY294002 was purchased from A.G. Scientific, Inc. (San Diego, CA). Primary antibodies were purchased from Cell Signaling (Beverly, MA) and secondary antibodies were purchased from Southern Biotech (Birmingham, AL).

Rat spontaneously immortalized granulosa cell culture

The rat spontaneously immortalized granulosa cell (SIGC) line was received from Dr. Robert Burghardt at Texas A&M University. SIGC cells were cultured in 25cm^2 flasks in media containing DMEM/F12, 50mg/ml Pen Strep, and 5% FBS media until 80% confluent at 37^{O}C and 5% CO_2. Cells were treated with vehicle control (1% DMSO) or PM (6 µM) for 48 h for protein isolation. This PM concentration and time point was previously demonstrated to cause approximately 30% cell death [63].

Postnatal day 4 (PND4) ovary culture

PND4 female Fisher 344 rat ovaries (n=3; 10 ovaries per pool) were excised, trimmed of excess tissue and placed onto a membrane floating on 250 µl of 1X DMEM nutrient mixture-F-12(Ham) media containing 1 mg/ml Albumax, 1 mg/ml BSA, 5 U/ml penicillin, 50 µg/ml ascorbic acid, and 27.5 µg/ml transferrin. Ovaries were cultured in vehicle

control (1% DMSO) or PM (60 µM) or LY294002 (20 µM) or kit ligand (400 ng/ml) at $37^{O}C$ with 5% CO_2 for 4 d and the media was changed on alternate days. PM treated ovaries were placed in a separate incubator from the control treated ovaries in order to prevent contamination from the ovotoxic volatile compound chloroethylaziridine (CEZ) which is spontaneously liberated from PM [27]. This concentration and time of PM exposure were previously shown to induce follicle loss in the cultured ovarian system [27]. This procedure was approved by the Institutional Animal Care and Use Committee at Iowa State University.

In vivo PM exposure and ovary collection

Adult female mice (15 wks) (n=5) were intraperitoneally (i.p) dosed once with sesame oil (SO) or PM (95%; 25mg/kg). This dose was chosen based on the literature [64, 65]. Mice were euthanized 3 days after the end of dosing in their pro-estrus phase.

Protein isolation and western blotting

Following culture, SIGC cells or PND4 cultured ovaries were processed for total protein isolation by homogenization and placing on ice for 30 min. Samples were centrifuged at 10,000 rpm for 15 min (2X) at 4°C followed by collection of supernatant. Protein concentration was quantified with a ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE). Proteins were separated by SDS-PAGE followed by a 13 minute dry transfer using an iBlot 2 (Life Technologies). Membranes were blocked for 3 h in 5% BSA in phosphate buffered saline containing Tween 20. Membrane was then incubated for 48 h in anti-ABCC1 (1:100 dilution) or anti-ABCB1 (1:100 dilution) at 4°C. Following 3 washes with 1x PBST, membranes were incubated in

a goat anti-rabbit-HRP (1:2000 dilution) secondary antibody for 1 h at room temperature, followed by 3 washes in PBST for 10 minutes each. Membranes were incubated in enhanced chemiluminescence (ECL) reagent for 10 minutes and exposed to X-ray film. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Chemical exposures can impact traditional housekeeping protein abundance (our unpublished data), thus equal protein loading was confirmed by Ponceau S staining of total protein and protein level was normalized to Ponceau S densitometry values. Negative controls used were primary only ABCC1/ABCB1 (1:100), secondary only goat anti-rabbit-HRP (1:2000), and normal goat IgG primary (1:100) with goat anti-rabbit-HRP secondary (1:2000).

Immunofluorescence staining

Ovaries were fixed in paraformaldehyde (4%) for 2 h (PND4 cultured rat ovaries) or 24 h (adult mouse ovaries), transferred to 70% ethanol, embedded in paraffin followed by serial sectioning of 5 μM thick sections and every 10^{th} section mounted onto slides. Slides were deparaffinized in citrisolv 3 times for 8 min each and rehydrated with washes in serial dilutions of 100, 80, 70, and 50 percent ethanol (8 min per wash) followed by one wash with ddH_20. Antigen retrieval was performed by microwaving slides in sodium citrate and citric acid solution (1 M, Ph 6.0) for 10 min. Sections were circled with a PAP pen and washed once in ddH_20 followed by a single wash with 1X TBST for 10 min. Slides were then blocked (5% normal goat serum in TBST) for 1 h at room temperature followed by incubation with a primary antibody directed against ABCC1 (1:25 dilution) or ABCB1 (1:200 dilution) overnight at 4°C. Slides were washed 3X with 1X TBST for 10 min each

followed by incubation in goat anti-rabbit- IgG (H+L) FITC secondary antibody (1:200 ABCC1 and 1:500 ABCB1 dilution) at room temperature followed by 3 washes with 1X TBST. Slides were counterstained with SlowFade Gold antifade reagent with 4-6-diamifino-2-phenylindole (DAPI) and sealed with microscope cover slips with clear nail polish. Slides were allowed to dry for 30 min prior to imaging. Images were captured at 10X and 40X magnification with a Leica fluorescent microscope.

Statistical analysis

Paired 1-tailed t-test comparisons were used to compare control treated and PM exposed cultured ovaries. Unpaired 1-tailed t-test comparisons were used to compare control and PM treated spontaneously immortalized granulosa cell line cell culture. One-way ANOVA analysis to compare ovarian endpoints from control, LY294002, and Kit-Ligand treated cultured ovaries followed by further analysis by Tukey's Multiple Comparison test between all 3 treatment types. Statistical significance was considered if the *P*-value was less than 0.05. A trend towards a biologically significant change was considered at $P < 0.1$.

Results

PM exposure did not impact ABCC1 protein abundance in granulosa cells

ABCC1 was abundant in total protein homogenates from cultured spontaneously immortalized granulosa cell line, but PM exposure did not impact ($P = 0.16$) the abundance of ABCC1 (Figure 2.1).

PM exposure did not alter ABCC1 protein abundance in cultured whole ovaries

Total ovarian protein homogenate contained ABCC1 protein; however, no impact (P = 0.49) of PM exposure on total ovarian ABCC1 protein level was observed (Figure 2.2).

Ovarian ABCB1 is decreased by PM exposure

Total ovarian protein homogenate from cultured neonatal rat ovaries was abundant in ABCB1 protein. Interestingly, the protein abundance of ABCB1 was decreased (P = 0.05) by PM exposure at the time of follicle loss which occurs at day 4 (Figure 2.3).

ABCC1 protein is reduced by inhibition of PI3K signaling

Utilizing protein isolated from ovaries cultured in media containing the PI3K inhibitor, LY294002 (20 μM) or the PI3K activator, Kit Ligand, western blotting demonstrated that in the absence of PI3K, ABCC1 protein was essentially absent (P = 0.03) from the ovary compared to control treatment (Figure 2.4). Interestingly, Kit Ligand treatment, did not impact ABCC1 protein levels and no difference (P = 0.15) from control treated ovaries was observed (Figure 2.4).

ABCB1 protein is reduced by absence of PI3K signaling

Similarly to ABCC1, lack of PI3K signaling in cultured ovaries reduced (P = 0.03) ABCB1 protein abundance, though to a lesser extent than that of ABCC1 (Figure 2.5). Kit Ligand treatment had no impact of ABCB1 protein abundance (P = 0.16) (Figure 2.5).

ABCC1 localization in the ovary and impact of PM exposure

ABCC1 was localized in the nucleus of the oocyte in pre-antral follicles (Figure 2.6A,B) and increased expression observed in growing follicles with CT and PM treatments (Figure 2.6A,B). The adult mouse ovary expressed ABCC1 in the oocyte nucleus, interstitial tissue, and oocyte cytoplasm (Figure 2.6C). Maturing oocytes expressed ABCC1 on their oocyte peri-cytoplasmic and peri-nuclear membrane in the CT treatment mice, but this was ablated by PM treatment treated (Figure 2.6D).

Ovarian localization and impact of PM exposure on ovarian ABCB1

ABCB1 is localized to the oocyte cytoplasm in pre-antral follicles (Figure 2.7A-D). Interestingly, increased PM-induced ABCB1 was observed as follicles matured (Figure 2.7D). In the adult mouse ovary, ABCB1 was localized to the interstitial tissue, to the granulosa cells and the oocyte (Figure 2.7E, F). As the oocyte matured towards ovulation, peri-nuclear and peri-cytoplasmic ABCB1 staining was observed (Figure 2.7G).

Discussion

The primordial follicular pool is vital for female fertility preservation. Disruption of follicular development by exogenous chemical compounds can lead to early senescence of the ovary [8]. Previous studies have shown that CPA induced ovotoxicity in mice and rats and depleted follicles by targeting granulosa cells [66]. We have previously demonstrated that CPA, and its ovotoxic metabolite, PM, induce both primordial and small primary follicle loss. PM is known to spontaneously transform into a cytotoxic volatile compound, CEZ, which also depletes primordial follicles [27]. ABCB1 and ABCC1 were discovered

on the basis of drug resistance to chemotherapy agents in tumors and therefore could encompass potential roles in conservation of the ovary [36]. We hypothesized that phase III chemical metabolism involving ABCB1 and ABCC1 may be an important factor for protection of the ovary during PM exposure and that ABCB1 and ABCC1 are regulated via the PI3K pathway as we have previously observed with Phase I and II chemical biotransformation proteins [55, 57].

ABCB1 regulates a detoxification process by oxidation via CYP 450 enzymes making hydrophobic compounds water soluble [37, 38, 40, 67]. Though CPA is activated in tumors via CYP450 enzymatic activity, its inactivation can be induced by overexpression of CYP450 enzymes [68-74]. Dhaini et al. (2003) demonstrated that increased levels of CYP3A in osteosarcoma tumors of patients resulted in metastasis and poor prognosis confirming that drug efficacy of CPA was altered due to drug metabolizing enzymes [70]. Thus, ABCB1 and ABCC1 could be key factors in understanding potential ovarian protective properties in non-cancer cells induced by CPA and PM toxicity. PM exposure to spontaneous immortalized granulosa cells and cultured whole ovaries did not alter ABCC1 protein expression, but did, however, decrease ABCB1 protein abundance in PND4 rat ovaries. ABCC1 protein abundance may not have been altered by PM exposure because of its substrate specificity for GSH conjugates instead of CYP 450 enzymatic activity, which is vital for CPA's bioactivation [51].

The PI3K pathway promotes primordial follicle recruitment and viability in the ovary [75-78]. PI3K activation is regulated by the binding of kit ligand produced by granulosa cells

to the oocyte receptor c-Kit [79-82]. We have previously demonstrated that PI3K inhibition altered ovotoxicity caused by VCD and DMBA [62, 83]. Inhibition of PI3K completely prevented VCD-induced depletion of primordial follicles but accelerated that induced by DMBA, to such an extent that essentially no primordial follicle oocytes could be identified [62]. We concluded that VCD caused hyperactivation of the primordial follicle pool as an ovotoxicant mechanism [62]. We also attributed these effects to PI3K mediated regulation of EPHX1 protein in the ovary; EPHX1 detoxifies VCD [57] and bioactivates DMBA [83], thus regulation of chemical metabolism enzymes contributes dramatically to resulting ovotoxicity. We therefore interrogated whether ABCB1 and ABCC1 were also regulated via PI3K. Xie et al. (2013) revealed that expression of ABCB1 was down regulated with PI3K inhibition in gastric cancer cells and there was upregulated expression of pro-apoptotic Caspase-3 and Bax [84]. A clinical study by Imai et al., demonstrated that LY294002 promoted inhibition of ABCC1 in human stem cells via competitive inhibition and alteration of protein expression [85]. Cultured PND4 rat ovaries treated with LY294002 resulted in nearly the complete absence of ABCC1 protein levels, but surprisingly, this effect was not impacted by kit ligand treatment. Similar to ABCC1, ABCB1 protein expression significantly decreased with inhibition of PI3K and yet again no impact of kit ligand treatment was observed. Thus, similar to our previous findings that suggest that NRF2, EPHX1, GSTM, GSTP, and AHR are downstream of the PI3K pathway [55, 57], these data support that ABCC1 and ABCB1 are regulated by PI3K. Lack of any effect of kit ligand to overcome the impact of PI3K deficiency on ABCB1 and ABCC1 is an area for further investigation and may yield insight into specific regulators of these phase III drug transporters.

Investigations of porcine and starfish oocytes and somatic cells have demonstrated increased ABCB1 expression concomitant with follicle maturation [86-89]. Further, it was shown that ABCB1 abundance in human and mouse follicles exhibited conflicting results at a transcriptional level compared to the translational level. The study found that transcriptionally, *Abcb1* was increased in Meiosis II (MII) compared to germinal vesicle (GV) phase oocytes, but ABCB1 protein was decreased in MII possibly indicating that MII oocyte ABCB1 expression is stored for later use [86]. Following treatment with PM, we determined that ABCB1 was localized to the oocyte cytoplasm of pre-antral follicles in cultured PND4 rat ovaries and observed increased ABCB1 expression in growing follicles. Adult female mice were treated with PM and ABCB1 was detected in interstitial tissue, granulosa cells, oocyte, and peri-nuclear and peri-cytoplasm structure of the pre-ovulatory follicle. Our findings also suggest that ABCB1 function and expression is expressed in different follicle stages dependent on the developmental stage of the female, since we observed differential locations in postnatal relative to adult ovaries.

ABCC1 was detected in the peri-nuclear membrane of cadmium resistant RH460 lung cancer cells but migrated to the cytoplasmic compartment following cadmium exposure [90]. Our findings demonstrated that ABCC1 was localized in the peri-nuclear region of adult mice pre-ovulatory follicles in control treated mice, but this staining pattern was not obvious in PM exposed ovaries. Redistribution of ABCC1 from the peri-nuclear membrane may have been due to PM-induced follicular damage. ABCC1 was also localized in interstitial tissue, oocyte nucleus and cytoplasm. A different localization

pattern from adult ovaries was observed in neonatal ovaries in which ABCC1 was localized mainly in the oocyte nucleus of and reduced by PM exposure. These data are novel since information regarding ABCC1 expression and function in different follicular developmental stages in the ovary are not well informed and require further investigation.

CPA is often used as a treatment for autoimmune diseases since it induces immunosuppression by inhibiting immune suppressor cells [23]. Tumor cells can elicit an immune response in a mechanism known as immune-editing that renders the cancerous cells non-detectable by the immune system [91]. We noted the appearance of cellular structures in the PM-treated ovary that may represent immune cell infiltration, as their appearance was localized to atretic follicles. Whether the immune system is participating in clearing cellular debris post-PM exposure remains to be confirmed.

In summary, this is the first study investigating ovarian expression of ABCB1 and ABCC1 during PM exposure and we have demonstrated the novel finding that these phase III drug transporters are downstream of PI3K signaling. Considering that depletion of primordial follicles and POF induction result in a longer time frame spent post-menopause, better understanding of proteins involved in the ovarian protective response to ovotoxicants is needed. Also, the importance of understanding the mechanistic pathways and regulation of two key drug transporters, ABCC1 and ABCB1, in response to CPA, which is commonly used for female cancers, could both decrease the mortality rates and improve reproductive function post-treatment. In addition, since ovarian cancer is a “silent” cancer with high chemoresistance and poor prognosis [92], better understanding of the function of ovarian

phase III drug transporters would further our knowledge in this area of female reproductive health.

Chapter 2 Figures

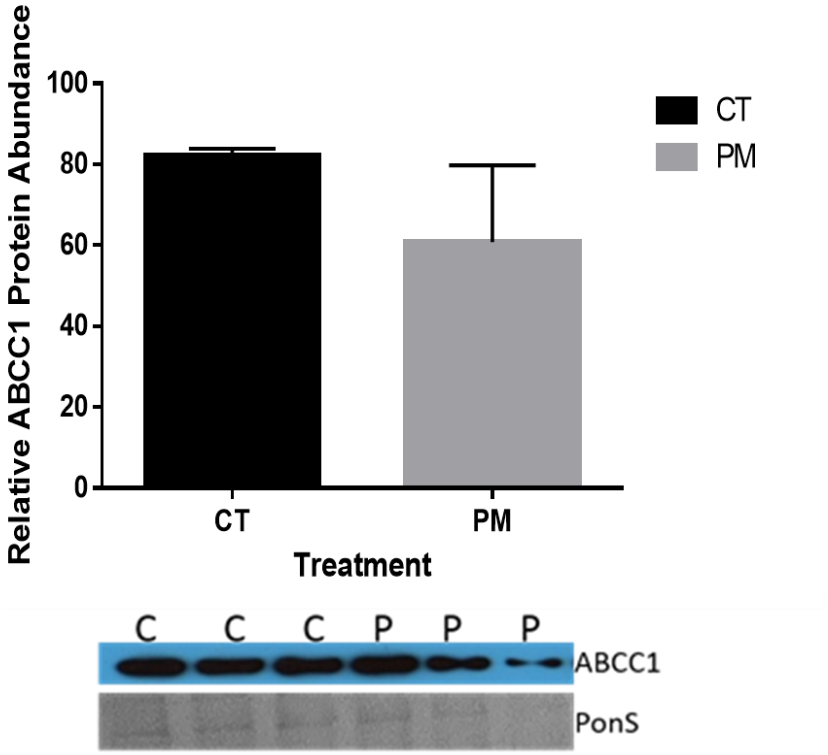

Figure 2.1. Impact of PM exposure on ABCC1 abundance in SIGC. SIGC were cultured in media containing vehicle control (CT; DMSO) or PM (6 µM) for 48 h. Total protein was isolated and ABCC1 protein abundance determined by western blotting. Barchart indicates relative densitometric values for ABCC1 protein in CT (C)- or PM (P)-treated cells; values represent mean ± standard error (n = 3 per treatment); Difference between treatments; $P = 0.16$.

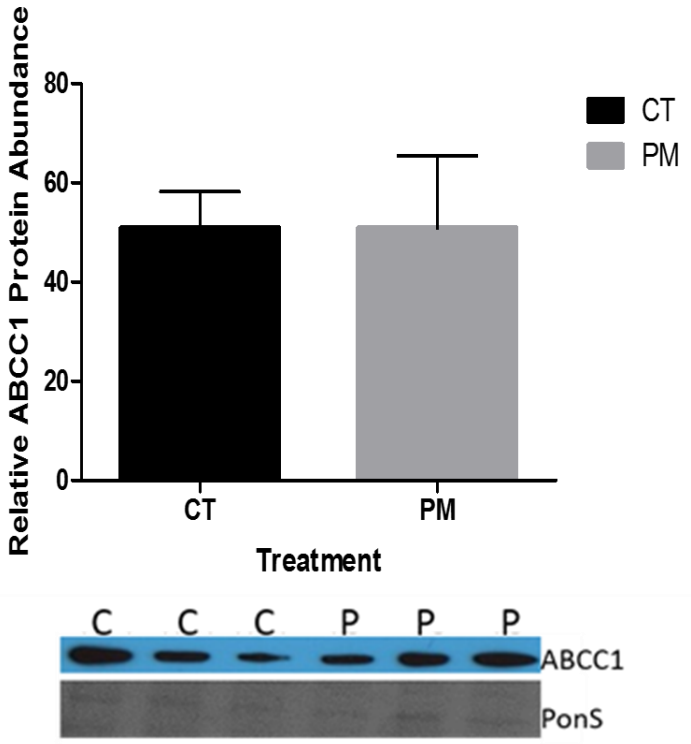

Figure 2.2. Effect of PM treatment on ABCC1 protein abundance in neonatal cultured ovaries. PND4 ovaries were cultured for 4 d in media containing vehicle control (CT; DMSO) or PM (60 µM). Total protein was isolated and western blotting performed to quantify ABCC1 protein abundance. Barchart indicates relative densitometric values for ABCC1 protein in CT (C)- or PM (P)-treated cells; values represent mean ± standard error (n = 3 pools per treatment; 10 ovaries per pool); Difference between treatments; *P* = 0.49.

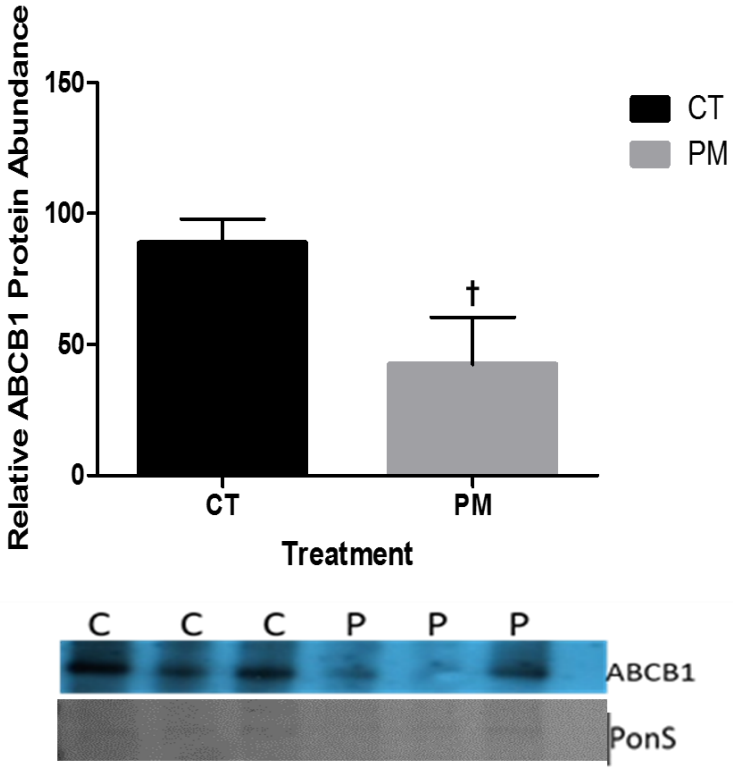

Figure 2.3. Impact of PM exposure on ABCB1 protein in cultured whole ovaries. PND4 ovaries were cultured for 4 d in media containing vehicle control (CT; DMSO) or PM (60 µM). Total protein was isolated and western blotting performed to quantify ABCB1 protein abundance. Barchart indicates relative densitometric values for ABCB1 protein in CT (C)- or PM (P)-treated cells; values represent mean ± standard error (n = 3 pools per treatment; 10 ovaries per pool). † indicates difference between treatments; Difference between treatments; *P* =0.05.

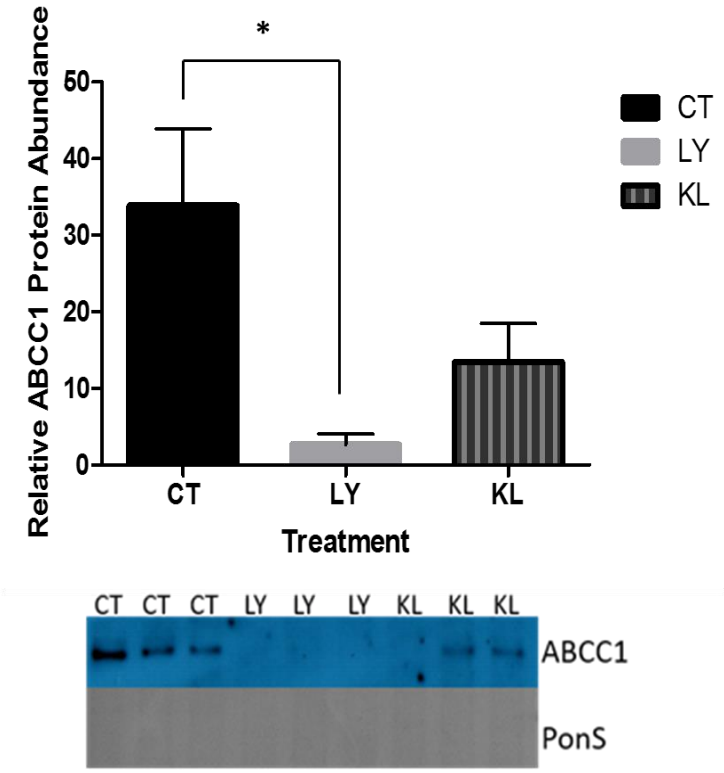

Figure 2.4. Effect of PI3K inhibition on ABCC1 ovarian protein level.
PND4 ovaries were cultured in media containing vehicle control, LY294002 (LY; 20 µM) or kit ligand (KL; 400 ng/ml)) for 4 d. Total protein was isolated and ABCC1 protein abundance determined by western blotting. Barchart indicates relative densitometric values for ABCC1 protein in CT- LY- or KL-treated cells; values represent mean ± standard error (n = 3 pools per treatment; 10 ovaries per pool). * indicates difference between treatments; $P = 0.03$.

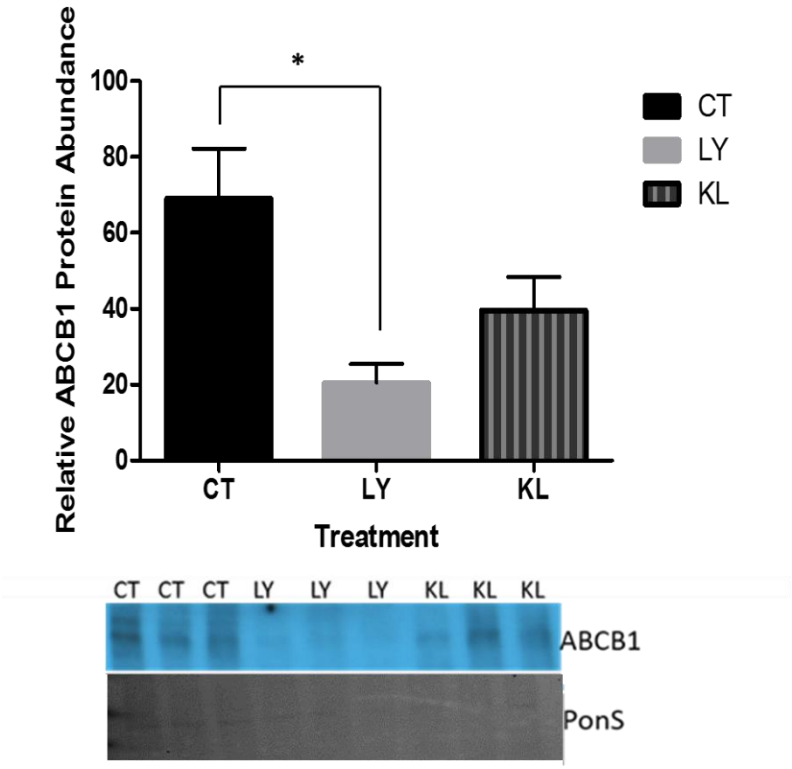

Figure 2.5. ABCB1 protein is reduced by absence of PI3K signaling.
PND4 ovaries were cultured in media containing vehicle control, LY294002 (LY; 20 µM) or kit ligand (KL; 400 ng/ml)) for 4 d. Total protein was isolated and ABCB1 protein abundance determined by western blotting. Barchart indicates relative densitometric values for ABCB1 protein in CT- LY- or KL-treated cells; values represent mean ± standard error (n = 3 pools per treatment; 10 ovaries per pool). * indicates difference between treatments; $P = 0.03$.

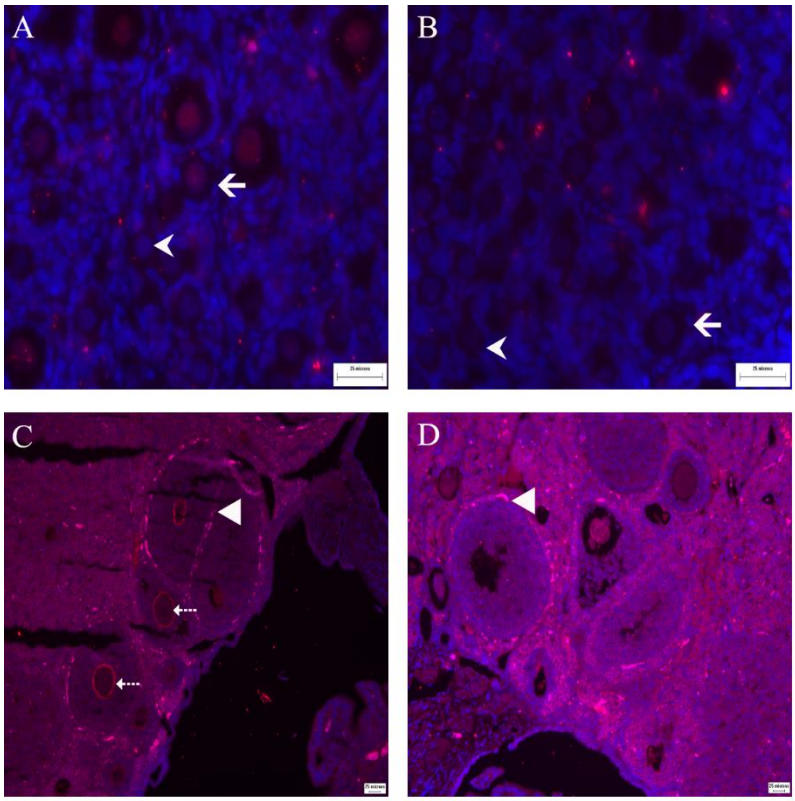

Figure 2.6. Ovarian localization and effect of PM exposure on ovarian ABCC1. Immunofluorescence staining to localize ABCC1 (red staining) was performed on fixed ovarian sections of (A,B) cultured PND4 ovaries treated with media containing vehicle control (A) or PM (60 µM; B) for 4 d. Additionally, ovarian sections from adult mice treated with vehicle control (saline; C)) or PM (25 mg/kg; intraperitoneal injection; D) were stained to localize ABCC1 (red staining). DNA was stained with DAPI (blue staining) to localized cell nuclei. Primordial follicles are indicated with a chevron; arrow = small primary follicles; arrowhead = theca cell layer; broken arrow = pericytoplasmic oocyte membrane. Scalebar = 25 microns.

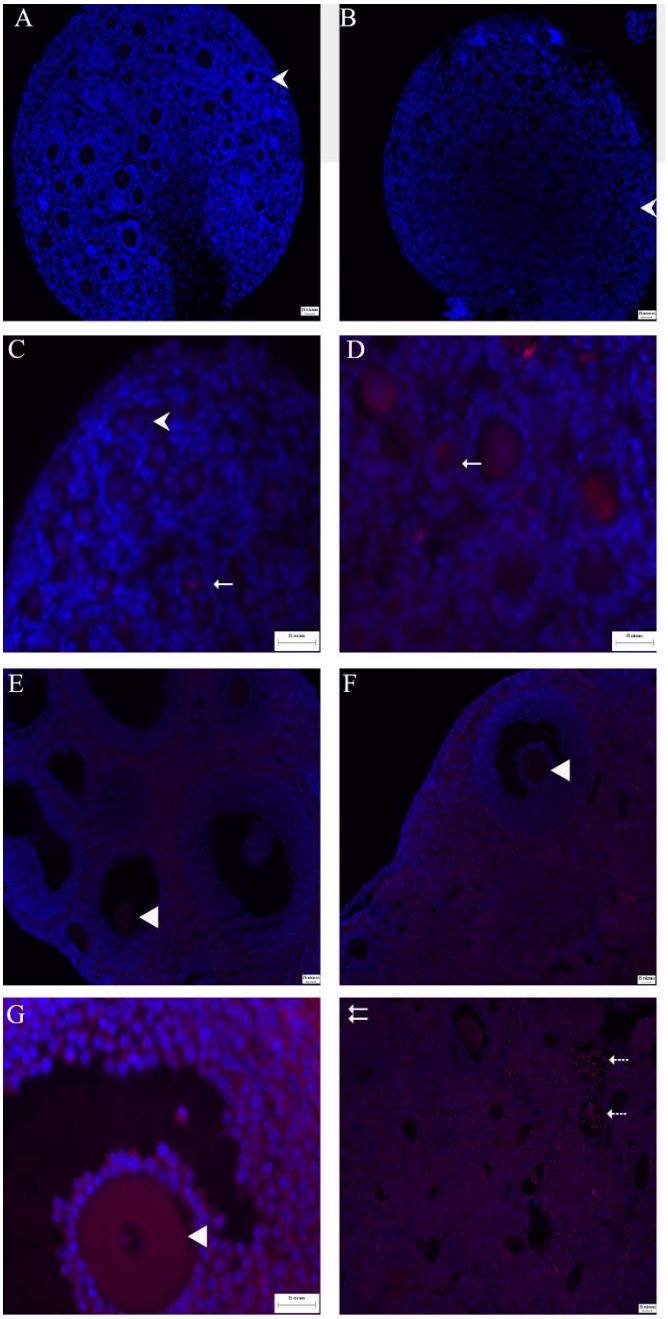

Figure 2.7. Ovarian localization and impact of PM exposure on ovarian ABCB1. Immunofluorescence staining to localize ABCB1 (red staining) was performed on fixed ovarian sections of (A-D) cultured PND4 ovaries treated with media containing vehicle control (A, C) or PM (60 µM; B, D) for 4 d. Additionally, ovarian sections from adult mice treated with vehicle control (saline; E)) or PM (25 mg/kg; intraperitoneal injection; F, G, H) were stained to localize ABCB1 (red staining). DNA was stained with DAPI (blue staining) to localized cell nuclei. Primordial follicles are indicated with a chevron; arrow = small primary follicles; arrowhead = pre-ovulatory oocyte; broken arrow = infiltrating immune cells. Scalebar = 25 microns.

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CHAPTER 3

DETERMINATION OF ALTERED 17B-ESTRADIOL METABOLISM AS A PHYSIOLOGICAL ADAPTATION TO HEAT STRESS

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Contribution Statement: PT performed all molecular analysis in this chapter. AFK was mentor for PT and designed experiments, aided in data interpretation and edited the research chapter. The live phase of the experiments were performed by the research groups of LHB and JWR.

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Abstract

The ovary consists of germ and somatic cells whose major functions are gamete and steroid hormone production, respectively. The anatomy of the porcine and human female reproductive tracts are closely related and both are regulated by the hypothalamic-pituitary-gonadal axis. HS has a major impact on the productivity of livestock agriculture in the US. Our previous studies demonstrated that HS disrupts ovarian steroidogenic signaling potentially altering ovarian function in the heat-stressed pig. The sulfotransferase, SULT1E1, catalyzes 17β-estradiol sulfonation. ABCC1, an ABC cellular transporter, exports and imports GSH, organic anions, and sulfate conjugated substrates, including sulfonated 17β-estradiol. We hypothesized that HS could affect the abundance of both SULT1E1 and ABCC1, thereby impacting ovarian 17β-estradiol and precipitating altered steroidogenic signaling in response. Three models of HS were utilized: pre-pubertal gilts were heat stressed for 7 (n = 5 per treatment) or 35 (n = 3 per treatment) days. Also, post-pubertal gilts were estrus synchronized followed by 5 d of HS during the follicular phase. Chronic HS for 35 d of exposure in pre-pubertal gilts increased ($P < 0.05$) both ABCC1 and SULT1E1 protein expression, without any alteration observed in *ABCC1* mRNA. HS in post-pubertal gilts did not have an impact on *ABCC1* mRNA or protein level, but increased SULT1E1 protein abundance. Our data support that ABCC1 and SULT1E1 are at a high level in the porcine ovary and that both proteins are potentially involved in altering the ovarian level of 17β-estradiol and can be affected by HS.

Keywords: Ovary, Heat Stress, 17β-estradiol, phase III metabolism

Introduction

The general female reproductive system is comprised of oocytes (germ cells) and somatic cells, including granulosa, stromal, and thecal cells [1]. The main functions of the female reproductive tract are for gamete and steroid hormone production to promote follicular development and pregnancy maintenance [2-4]. The anatomy of the porcine reproductive tract is similar to that of humans, except it has a bihorned utuerus that fuses into the uterine body and urogenital opening. Porcine reproductive cycles, which are also similar to humans, are regulated by the negative/positive feedback of the hypothalamic pituitary gonadal axis. The follicular reserve of the female reproductive tract is limited in that, if depleted, it results in early menopause and/or infertility [2]. Regulation of primordial follicle recruitment and activation is managed via the phosphatidylinositol-3 kinase (PI3K) signaling pathway [5-9].

A secondary major function of the ovary is to produce sex hormones and is one of the primary sites for steroidogenesis. Steroidogenesis includes synthesis of the ovarian steroids estrogen and progesterone from the dominant follicles and corpora lutea, respectively. Ovarian hormone synthesis is regulated by cytochrome P450 (CYP) enzymes where up-take of cholesterol occurs in the mitochondria, by the action of steroidogenic acute regulatory protein (StAR). Cholesterol is converted into pregnenolone which diffuses into the cytoplasm and is converted to progesterone (P_4) via the enzyme, 3β-hydroxysteroid dehydrogenase. Alternatively, pregnenolone can be converted to 17α-OH-pregnenolone which is further metabolized into androstenedione and/or testosterone, which both can be irreversibly metabolized to estrone and 17β-estradiol by CYP19a. This

hormone synthesis involves follicular theca and granulosa cell structural compartments, therefore depends on the interaction between thecal and granulosa cells which is now regarded as the two-cell-two gonadotropin theory. The theca cells express luteinizing hormone (LH) receptors which undergo a process for the conversion of cholesterol to androgens, which are then migrated to the granulosa cells where follicle stimulating hormone (FSH) regulates estrone and 17β-estradiol production [10].

Heat stress (HS) is a biological response to high core body temperature that serves as a threat to its homeostatic properties [11-14]. HS is an environmental stressor resulting from high ambient temperatures that impacts agriculture livestock production limiting the manufacturing of high grade quality protein [11]. Pigs have low tolerance for HS since they lack functional sweat glands. HS disrupts the development and function of mammalian reproduction by impairing hormone production secretions, embryo development [15, 16], ovarian folliculogenesis [16, 17], and uterine environment [18, 19]. Studies have reported seasonal infertility, reduced implantation rates, impairment of embryo development, and altered ovarian activity related to HS in gilts and sows [14, 20-22]. Similar findings have been found in boars where total sperm counts were reduced and an increase in percentage of abnormal spermatozoa post-HS [23]. Zeng et al. demonstrated alterations of microfilaments and microtubules in the porcine oocyte during *in vitro* maturation, increased apoptosis, and low developmental proficiency due to HS exposure [15]. Our previous studies demonstrated activation of insulin signaling pathway members during HS exposure which included increased abundance of the PI3K members, *AKT1* and *FOXO3*, at mRNA levels in pre-pubertal gilt ovaries [24]. We also demonstrated that steroid

hormone production genes and proteins were altered by HS. *LDLR*, *LHCGR*, and *CYP19a* mRNA abundance were increased after 7 d of HS and STAR was increased after 35 d of HS. Additionally, CYP19a protein levels increased following 7 d and 35 d of HS [24]. These findings suggest that HS could be associated with early primordial follicle activation, increased apoptosis, and impaired steroidogenesis [24].

ATP-binding cassette (ABC) transporters are involved in the ATP-dependent import or export and a variety of external compounds and endogenous substrates. Multidrug resistance associated protein (MRP1), also known as ABCC1, is part of the ABC transporter family that transports organic anions and glutathione (GSH), glucoronate, and sulfate conjugated substrates [25]. It is expressed in most tissues and localized in polarized cells of the basolateral membrane [25]. Sulfotransferases are known to be involved in phase II drug metabolism [26]. Estrogen sulfotransferase (SULT1E1) is a cytosolic enzyme that catalyzes estrogen sulfonation [26, 27]. In humans, it has a high affinity for catalyzing the sulfoconjugation of estradiol and estrone at the 3-hydroxyl terminal [26]. It is also present in the porcine uterine endometrium and recent studies have demonstrated increased SULT1E1 gene expression observed at day 12 of pregnancy, but persisting at low levels throughout the rest of the gestational term [28]. This enzyme has also been demonstrated to affect tumorigenesis in estrogen-dependent cancers [26]. Free estrogens are biologically active and become inactive when conjugated [29]. Regulation of estrogen levels throughout the estrous cycle are important and rely on the activity of CYP19a, 17β-HSD, and SULT1E1 among others [26]. Cole et al, demonstrated that ABCC1 requires GSH for binding of estrone sulfate [30]. Low activity of SULT1E1 have been reported in the boar

testis [31]. Based upon our previous findings, we hypothesized that HS could affect both SULT1E1 and ABCC1, thereby altering ovarian 17β-estradiol level. Though not previously classified in the porcine ovary, we proposed in this study that SULTE1 enzyme catalyzes sulfonation-mediated inactivation of 17β-estradiol, followed by transport from the ovary by the drug transporter protein ABCC1.

Materials and Methods

Reagents

Tris base, 2-β-mercaptoethanol, Glycine, Sodium Dodecyl Sulfate, Sodium Chloride, Tris Hydrochloric Acid, tween-20, Sodium Acetate, Bis- 2-chloroethylamine hydrochloride, Bovine Serum Albumin and Ammonium Persulphate were purchased from Sigma-Aldrich Incorporated (St. Louis, MO). Ponceau S Stain was purchased from Fisher Scientific and Mini-PROTEAN TGX Gels were obtained from Bio-Rad (Philadelphia, PA). RNeasy Mini Elute kit, RNeasy mini kit, Quantitect^{TM} SYBR Green PCR kit and QIA shredder kit were purchased from Qiagen Incorporated (Valencia, CA). The SULT1E1 H-40 primary antibody was purchased from Santa Cruz (Santa Cruz, CA). Goat anti-Rabbit-HRP secondary antibody was purchased from Southern Biotech (Birmingham, AL). SignalFire ECL western detection reagents purchased from Cell Signaling Technologies (Beverly, MA) and Xray Film was purchased from Thermo Scientific (Rockford, IL). Primary antibody, ABCC1, was purchased from Cell Signaling (Beverly, MA).

Animal Experiments and tissue collection

Pre-pubertal gilt experiment

Tissues analyzed in this manuscript were obtained as a subset from previous experiments with all procedures approved by the Iowa State University Institutional Animal Care and Use Committee. Details regarding experimental design, animal handling and environmental conditions have been previously described [32-34]. In brief, crossbred pre-pubertal gilts (35 ± 4 kg) were housed in constant climate controlled rooms in individual pens with *ad libitum* feed intake. Gilts were exposed to thermoneutral (TN) conditions (20°C; 35-50% humidity; n = 5-6) or HS conditions (35°C; 20-35% humidity; n = 5-6) for 7 (n = 5 per treatment) or 35 (n = 3 per treatment) days. To eliminate the effects of dissimilar feed intake, an additional group of thermoneutral gilts (n = 3) were pair-fed to the HS treatment for 7 d (PFTN). Gilts were euthanized by captive bolt penetration, one ovary was snap frozen in liquid nitrogen and stored at -80^{o}C and the contralateral ovary was fixed in 4% paraformaldehyde.

Post-pubertal gilt experiment

Procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Pubertal gilts (n = 12; 126.02 ± 21.6 kg) were synchronized to the estrus phase through Matrix® feeding 14 days. On the day after Matrix® withdrawal gilts were exposed to TN (n = 6, 20.3°C ± 0.1°C) or HS (n = 6, 26-32°C) conditions for 5 d during the follicular phase. Humidity was recorded during the study period as 33 ± 13% in the TN room and 24 ± 6% in the heat stressed room. Rectal temperature was recorded as 38.68^{o}C in the TN animals and 39.48^{o}C in the HS gilts. All gilts were limit fed (6 lbs per day), thus no

dissimilar feed intake levels were present. Gilts were euthanized by captive bolt penetration, follicular fluid was captured from all dominant follicles and pooled for 17β-estradiol measurement. One ovary was snap frozen in liquid nitrogen and stored at -80^{o}C and the other ovary was fixed in 4% paraformaldehyde.

RNA Isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Ovaries were crushed in liquid nitrogen until in powdered form using a hand mortar and pestle. Total RNA was extracted using an RNeasy Mini kit and RNeasy Mini Elute kit following manufacturer's protocols and eluted in 14 µl RNase–free water. RNA concentration was measured with ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE). Total RNA was reverse transcribed into cDNA with master mix of Superscript III, 1st Strand Buffer, DTT, and RNAse Out® followed by thermocycler incubation at 50^{o}C for 60 min and 70^{o}C for 15 min. cDNA (2 µl; 1:10 dilution) was amplified using *ABCC1* primers on an Eppendorf Mastercycler using a Quantitect SYBR Green PCR kit. Primer design was generated from the NCBI primer blast database (forward primer: 5'-TTTACTTCGCCCTCGTGCTG-3'; Reverse primer: 5'-GGGCAAGGATTAGGGTCGTG-3'). Glyceraldehyde 3-phosphaste dehydrogenase (*GAPDH*) was used as the endogenous control for normalization. The cycling program consisted of a 15 min hold at 95°C and 50 cycles of: denaturing at 95°C for 15 sec, annealing at 60.6°C for 15 sec, and extension at 72°C for 20 sec. Technical controls

included omission of template (cDNA), forward/reverse primers and master mix only, and H_2O. Relative fold change was determined using 2-ΔΔCT method. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method and the results are presented as mean fold difference ± standard error relative to the TN control group.

Protein isolation and western blotting

Total ovarian protein was isolated by powdering frozen ovaries with a mortar and pestle, followed by homogenization in tissue lysis buffer and placing on ice for 30 min. Samples were centrifuged at 10,000 rpm for 15 min (2X) at 4°C followed by collection of supernatant. Protein concentration was quantified with a ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE). Proteins were separated by SDS-PAGE followed by a 13 minute dry transfer using an iBlot 2 (Life Technologies). Membranes were blocked for 3 h in 5% BSA in phosphate buffered saline containing Tween 20. Membrane was then incubated for 24 or 48 h in anti-ABCC1 (1:100 dilution) or SULT1E1 (1:200), respectively, at 4°C. Following 3 washes with 1X PBST, membranes were incubated in goat anti-rabbit-HRP (1:20,000 dilution) secondary antibody for 1 h at room temperature, followed by 3 washes in PBST for 10 minutes each. Membranes were incubated in enhanced chemiluminescence (ECL) reagent for 10 minutes and exposed to X-ray film. Negative controls used were primary only SULT1E1, primary only ABCC1, secondary only goat anti-rabbit-HRP, and normal goat IgG primary/goat anti-rabbit-HRP secondary. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Chemical exposures can impact traditional endogenous control protein abundance

(our unpublished data), thus equal protein loading was confirmed by Ponceau S staining of total protein and protein level was normalized to Ponceau S densitometry values.

Statistical analysis

All data were statistically analyzed using GraphPad Prism software. Comparison of two treatments was performed using unpaired two-tailed t-test; comparison of more than two treatments was performed by one-way ANOVA and Tukey's Multiple Comparisons test for difference between treatments. A P-value < 0.05 was considered significantly different, with $P < 0.1$ considered a trend for a difference.

Results

Ovarian ABCC1 mRNA or protein abundance not impacted by acute HS in the pre-pubertal gilt

ABCC1 mRNA was detectable in the porcine ovary (Figure 3.1). There was no impact of reduced feed intake on ABCC1 mRNA level. In addition, HS did not have any effect on ovarian ABCC1 in the pre-pubertal gilt (Figure 3.1) ($P = 0.44$). ABCC1 protein was also abundant in the porcine pre-pubertal ovary, but neither reduced feed intake nor HS impacted ABCC1 protein level, relative to the TN control gilts (Figure 3.2) ($P = 0.49$).

Chronic HS increases ABCC1 protein abundance in the pre-pubertal gilt ovary

Chronic (35 d) HS did not affect ABCC1 mRNA level in the pre-pubertal gilt ovary ($P = 0.39$) (Figure 3.3), however, ovarian ABCC1 protein abundance was increased ($P = 0.01$) by HS (Figure 3.4).

Ovarian SULT1E1 abundant in the porcine ovary and increased by chronic HS in pre-pubertal gilt ovary

SULT1E1 was detectable in the pre-pubertal gilt ovary albeit at a low level in TN treated gilts. SULT1E1 protein level was increased (P = 0.003) by 35 d of chronic HS (Figure 3.5).

ABCC1 mRNA or protein abundance was not impacted by HS in the presence of circulating gonadotrophins.

The ovary from post-pubertal, estrous synchronized gilts had detectable levels of *ABCC1* mRNA. HS for 5 d during follicular development did not alter (P = 0.09) the abundance of mRNA encoding ABCC1 (Figure 3.6). ABCC1 protein abundance, in correspondence, was not impacted (P = 0.52) by HS in post-pubertal gilts exposed to HS during the follicular phase of the estrous cycle (Figure 3.7).

HS impacts SULT1E1 protein abundance in post-pubertal gilts

Similar to those effects of HS on SULT1E1 protein level observed in the pre-pubertal gilt ovary, there was an impact (P = 0.04) of HS on the abundance of SULT1E1 protein in the estrous synchronized post-pubertal gilt (Figure 3.8).

Discussion

Productivity of commercial livestock agriculture is compromised by high environmental thermal temperatures [11]. HS causes economic losses in US livestock industries by reduction of dry matter intake (DMI) and body weight [11]. In terms of reproduction, HS affects female fertility and fecundity and is culpable for seasonal infertility, a period of reduced reproductive success characterized by an increased wean-to-estrus interval, reduced embryonic development and spontaneous abortion, altered follicular development. Seasonal infertility is estimated by our calculations to cost the US swine industry approximately $420 million annually (Pollman, Ross, Baumgard, Keating, unpublished data). With climate models predicting hotter summer periods in areas with highest swine production, seasonal infertility as a consequence of HS in unlikely to abate in the future.

Our previous findings demonstrated that HS altered ovarian proteins involved in folliculogenesis as well as steroidogenesis. We discovered increased abundance of proteins involved in phosphatidylinositol-3 kinase (PI3K) signaling [24] which is critical for maintenance of follicle viability [35] as well as activation of primordial follicles into the growing follicular pool [5, 36, 37]. We also discovered increased insulin-mediated signaling [24], potentially as a response to hyperinsulinemia due to HS [11]. Ovarian mRNA abundance of genes involved in steroid hormone production were impacted by HS: increased LDLR, LHR, STAR and CYP19A1 were increased by HS – surprising considering that the animals were pre-pubertal. Additionally, both StAR and CYP19A1 protein abundance were increased in HS pre-pubertal gilt ovaries [24]. Considering that 17β-estradiol is increased at the time of estrus in swine, any environmental perturbation

driving increased 17β-estradiol could alter estrus display as well as the LH surge inducing ovulation. In this study, we evaluated whether there are HS-induced changes to the ovarian capacity to regulate 17β-estradiol ovarian concentrations. If so, the increase in steroidogenic protein abundance previously observed could be attributable to increased degradation of 17β-estradiol and may represent a response to altered 17β-estradiol metabolism rather than a direct cause of perturbed ovarian function.

SULT1E1, an enzyme that catalyzes the activity of estrogen sulfonation [26, 27], is present in porcine uterine endometrial tissue. Maintaining an appropriate balance of estrogen within the ovary is regulated by SULT1E1 [38-40]. The role of SULT1E1 in porcine ovarian function has not been characterized, thus the data generated herein is novel and adds to our knowledge of ovarian basal functioning. Appropriate estrogen levels are dependent on SULT1E1 [26]. In humans, SULT1E1 initiates the sulfoconjugation of estradiol and estrone at the 3-hydroxl terminal [26]. Studies have shown the action and abundance of SULT1E1 can impact fetal development [41], tumorigenesis of estrogen-dependent cancers [42], and adipocyte differentiation [43]. A study using human umbilical vein endothelial cells (HUVECs) demonstrated high expression of SULT1E1 therein and that insulin-like growth factor (IGF-1), which is expressed in the ovary and functions as an endocrine/paracrine growth factor, upregulated SULT1E1 in vascular cells [26]. Furbass *et al.*, also demonstrated that SULT1E1 was present in the bovine placentome of the binucleated trophoblast giant cells (TGCs) and uninucleated trophoplast cells (UTCs) [27]. Higher levels of *SULT1E1* mRNA was found in the TGCs compared to the UTCs, however UTCs protein expression was higher than TGCs [27]. This study suggested that UTCs are

the predominant cell type expressing SULT1E in the bovine due to its increased transcriptional levels in a location where free estrogens are sulfonated [27]. SULT1E1 may play a major role in the protection of UTCs against estrogenic stimulation deriving from CYP19A1-expressing TGCs or by building a barrier for prevention of excessive free estrogen into the maternal compartment from the cotyledons in the bovine placentomes [27].

In the *Sult1e1*-deficient mouse ovary, impaired ovulation and cumulus expansion response was observed [44]. This supports the notion that CYP19a and CYP11a1 enzymatic activity potentially induce expression of the SULT1E1 gene in response to the LH surge [45]. 17β-estradiol elevation in the *Sult1e1*-deficient mouse model disrupted the normal endocrine feedback mechanisms therefore potentially altering ovarian cellular functions [45]. Taken together, these studies provide validation of the importance of sulfonation of 17β-estradiol. Chronic HS increased ovarian SULT1E1 protein level in pre-pubertal gilts and also impacted 5d HS estrus synchronized post-pubertal gilts also by increasing SULT1E1 protein levels. These data indicate that ovarian SULT1E1 is involved in 17β-estradiol sulfonation and likely contributes to basal ovarian metabolism of 17β-estradiol. These data also support that HS increased 17β-estradiol metabolism in ovaries of pre-pubertal gilts and post-pubertal gilts. This could indicate that the previously observed increased StAR and CYP19A1 [24] represents a response to increased SULT1E1 during HS or vice versa. Future experiments that could address this would include evaluating the temporal patterns of both increased steroidogenesis and steroid degradation during HS in swine.

ABCC1, a drug transporter that is abundant in many tissues, transports endogenous and exogenous compounds throughout the body [25]. Its major function is to protect cells from damage [40]. ABCC1 is highly expressed in cancerous tissues and has been identified in human cell lung cancer lines that are resistant to anti-neoplastic agents [46]. Sulfonated 17β-estradiol represents a substrate for ABCC1, thus ABCC1 is involved in 17β-estradiol metabolism [47] [26]. ABCC1 is also hypothesized to transport estrone 3-sulfate after catalysis by SULT1E1 because of its hydrophilic properties [48]. In contrast, *Abcc1* knockout-mice exhibited reduced concentrations of 17β-estradiol, androstenedione, and testosterone in the testicles [40]. This study presents the idea that steroid hormone levels in the gonad are involved in negative feedback loops of steroid regulation by possibly compensating the loss of *Abcc1* with upregulating *Cyp17* changing the proportion of 17β-estradiol and estrone 3-sulfate [40]. However, information in regards to regulation of *Abcc1* in concert with *Sult1e1* in the ovary is scant.

We demonstrated that ABCC1 protein is detectable in the porcine ovary in pre-pubertal and post-pubertal gilts. Following 7 d of HS treatment, there were no alterations at the transcriptional or translational levels of ABCC1 and no impact of reduced feed intake on ABCC1 was observed. However, HS for 35 d increased ovarian ABCC1 protein expression, but did not impact *ABCC1* at the mRNA level in pre-pubertal gilts. *ABCC1* mRNA abundance nor ABCC1 protein abundance was impacted by HS in post-pubertal gilts. Our data demonstrate for the first time that ABCC1 is regulated dynamically in the gilt ovary. We also have discovered a response to HS in ABCC1 protein level, though

disparities in this response are dependent on ovarian status as well as on the duration of the HS event.

Consideration of the ovarian status in the porcine models used is necessary to appreciate the biological meaning of our findings. Chronic HS in the post-pubertal gilt increased both ABCC1 and SULT1E1 protein expression, possibly indicating that ABCC1 is transporting the SULT1E1-catalyzed sulfonated 17β-estradiol out of the cell. HS increased SULT1E1 could be indicative of an overproduction of 17β-estradiol or it could have resulted from increased excretion of 17β-estradiol due to the age of the gilts. There were no alterations in *ABCC1* mRNA or ABCC1 protein levels post HS treatment during the follicular phase in post-pubertal gilts, however, SULT1E1 protein levels were increased due to HS exposure. The fact that there were no changes observed in ABCC1 transcriptionally or translationally but an increase in SULT1E1 protein due to HS could be indication that there are high levels of 17β-estradiol present, therefore, sulfonation needed. In addition, though there were no significant changes in *ABCC1* mRNA or protein abundance due to HS, our data demonstrates that mRNA levels decreased while protein levels were increased. Longer treatment exposure to HS could have possibly shown a significant decrease in *ABCC1* mRNA and significant increase in ABCC1 protein which would be required for 17β-estradiol extracellular transport for regulation.

Chapter 3 Figures

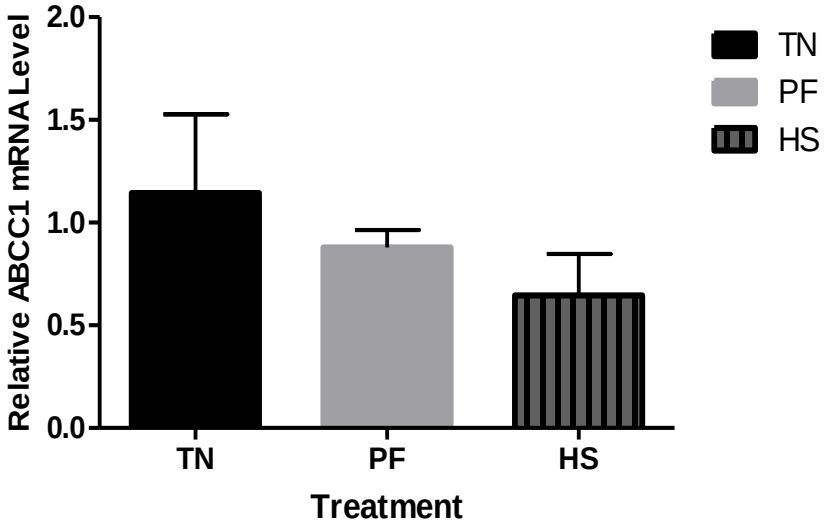

Figure 3.1. Ovarian *ABCC1* mRNA not impacted by acute HS in the pre-pubertal gilt. Quantification of ovarian *ABCC1* mRNA was performed using RT-PCR from total RNA isolated from pre-pubertal gilt ovaries exposed to thermal neutral (TN; 20°C; 35-50% humidity; n = 5-6) or heat stress (HS; 35°C; 20-35% humidity; n = 5-6) conditions for 7 (n = 5 per treatment) days. An additional group of gilts who were pair fed (PF) to those experiencing HS were included to account for dissimilar feed intake. Data represents mean fold-change in mRNA abundance between treatments; $P = 0.44$.

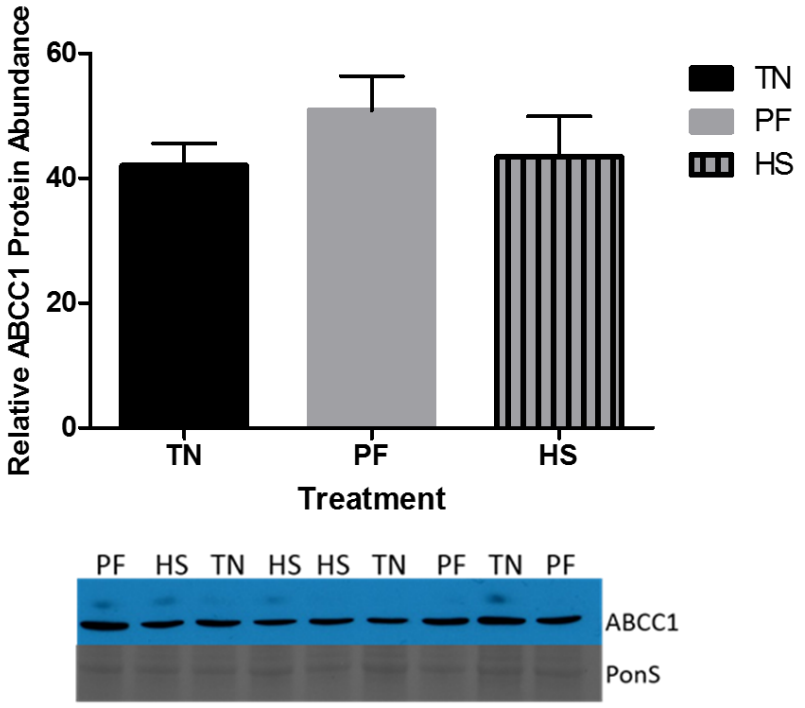

Figure 3.2. ABCC1 protein not impacted by HS in pre-pubertal gilt.
Ovarian ABCC1 protein was quantified by western blotting from protein homogenates isolated from pre-pubertal gilt ovaries exposed to thermal neutral (TN; 20°C; 35-50% humidity; n = 5-6) or heat stress (HS; 35°C; 20-35% humidity; n = 5-6) conditions for 7 (n = 5 per treatment) days. An additional group of gilts who were pair fed (PF) to those experiencing HS were included to account for dissimilar feed intake. Data represents relative protein abundance between treatments. The western blotting image as well as the ponceau S (PonS) total protein staining is provided. Difference between treatments: P = 0.49.

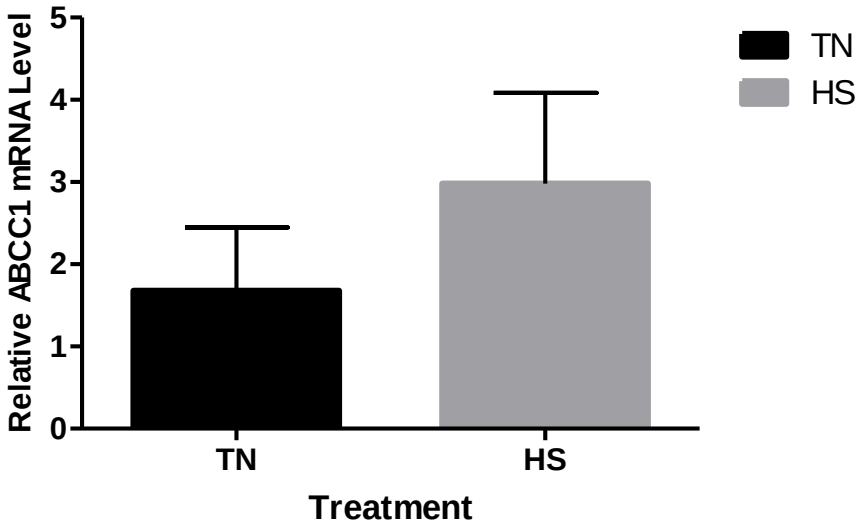

Figure 3.3. Chronic HS does not impact ovarian *ABCC1* mRNA in the pre-pubertal gilt.
Quantification of ovarian *ABCC1* mRNA was performed using RT-PCR from total RNA isolated from pre-pubertal gilt ovaries exposed to thermal neutral (TN; 20°C; 35-50% humidity; n = 5-6) or heat stress (HS; 35°C; 20-35% humidity; n = 5-6) conditions for 35 (n = 3 per treatment) days. Data represents mean fold-change in mRNA abundance between treatments; $P = 0.38$.

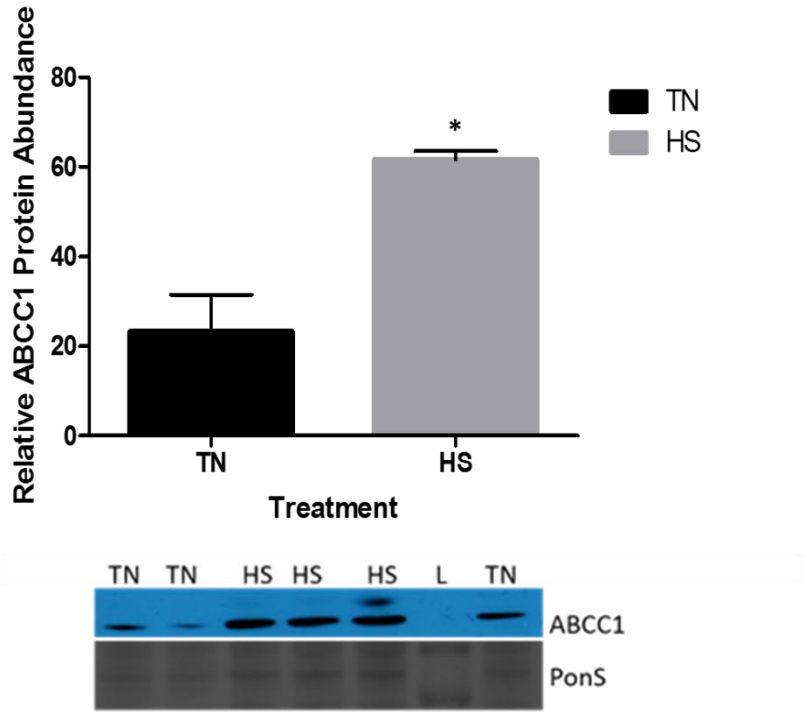

Figure 3.4. Chronic HS increased ovarian ABCC1 protein abundance in pre-pubertal gilts.

Ovarian ABCC1 protein was quantified by western blotting from protein homogenates isolated from pre-pubertal gilt ovaries exposed to thermal neutral (TN; 20°C; 35-50% humidity; n = 5-6) or heat stress (HS; 35°C; 20-35% humidity; n = 5-6) conditions for 35 (n = 3 per treatment) days. Data represents relative protein abundance between treatments. The western blotting image as well as the ponceau S (PonS) total protein staining is provided. * indicates different ABCC1 protein abundance between treatments; $P = 0.01$.

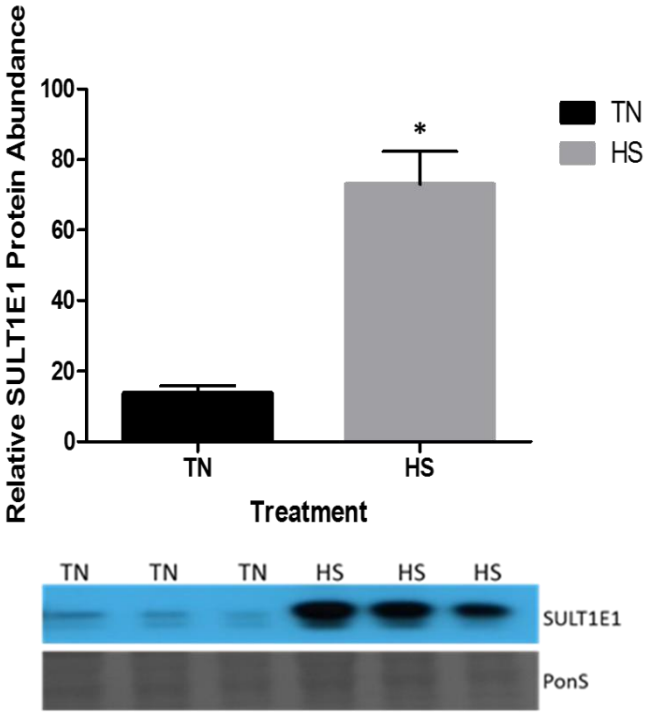

Figure 3.5. Porcine ovarian SULT1E1 induced by HS in pre-pubertal gilts.
Ovarian SULT1E1 protein was quantified by western blotting from protein homogenates isolated from pre-pubertal gilt ovaries exposed to thermal neutral (TN; 20°C; 35-50% humidity; n = 5-6) or heat stress (HS; 35°C; 20-35% humidity; n = 5-6) conditions for 35 (n = 3 per treatment) days. Data represents relative protein abundance between treatments. The western blotting image as well as the ponceau S (PonS) total protein staining is provided. * indicates different SULT1E1 protein abundance between treatments; P = 0.003.

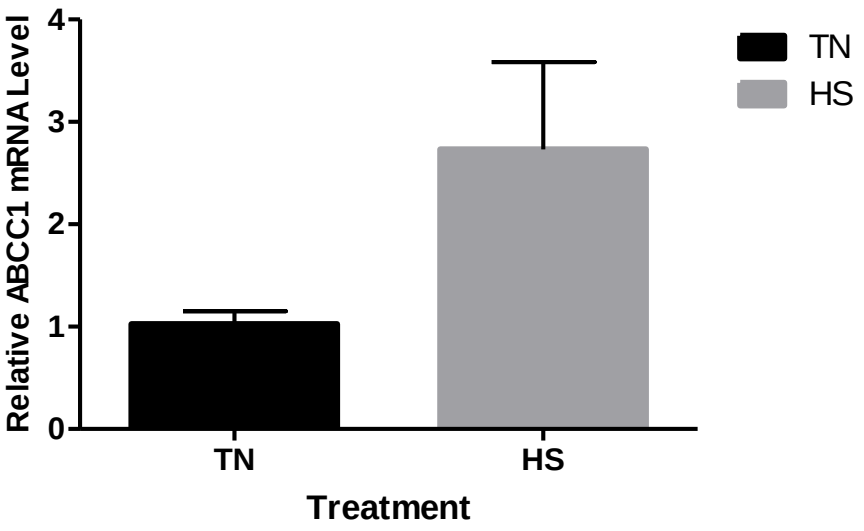

Figure 3.6. *ABCC1* mRNA level not altered in the post-pubertal gilt due to HS. Post-pubertal gilts were synchronized in estrous by Matrix® feeding for 14 days. On the day after Matrix® withdrawal, gilts were exposed to TN (n = 6, 20.3°C ± 0.1°C) or HS (n = 6, 26-32°C) conditions for 5 d during the follicular phase. *ABCC1* mRNA was quantified from total ovarian RNA by RT-PCR and data is expressed as mean fold change between treatments; $P = 0.09$.

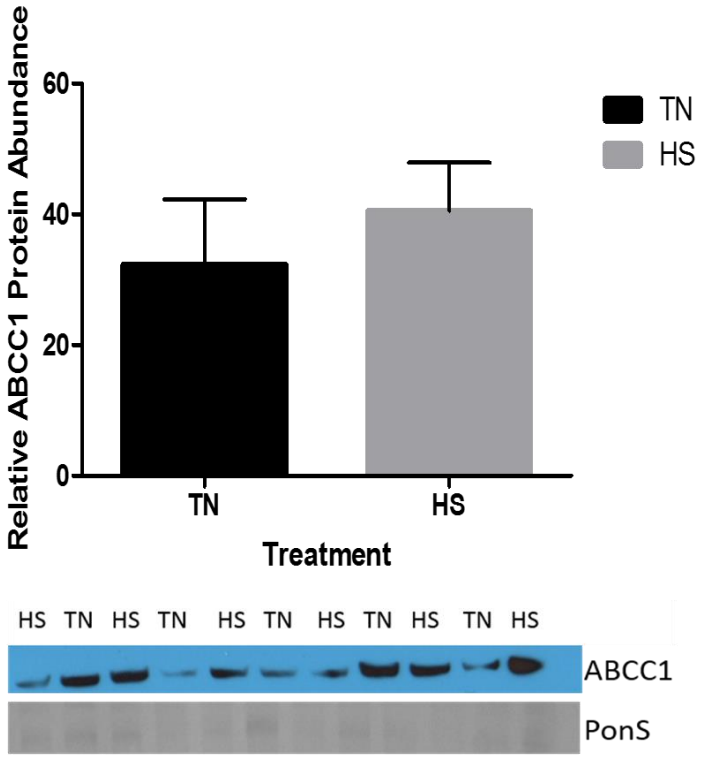

Figure 3.7. HS does not impact ABCC1 protein abundance in the post-pubertal gilt.

Post-pubertal gilts were synchronized in estrous by Matrix® feeding for 14 days. On the day after Matrix® withdrawal, gilts were exposed to TN (n = 6, 20.3°C ± 0.1°C) or HS (n = 6, 26-32°C) conditions for 5 d during the follicular phase. ABCC1 protein was quantified from total ovarian protein homogenates by western blotting and data is expressed as relative protein abundance between treatments; $P = 0.51$.

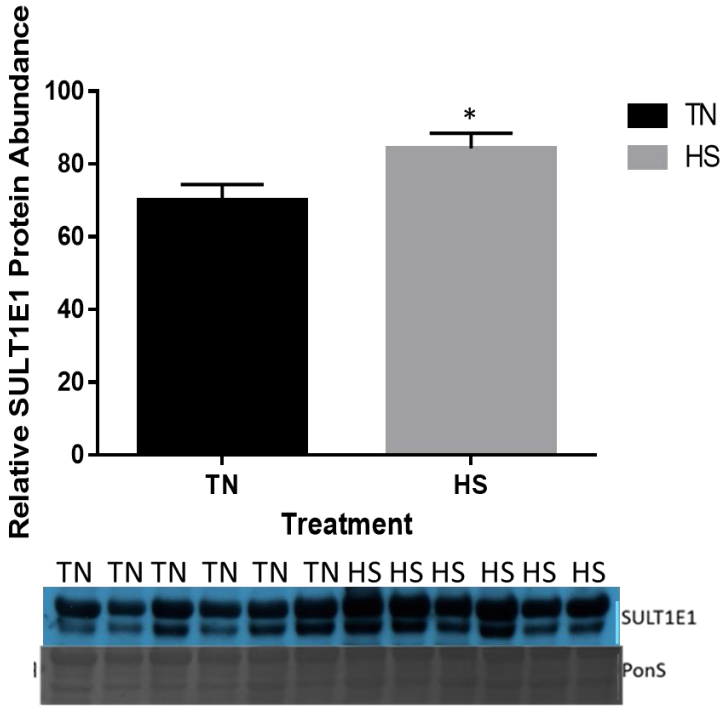

Figure 3.8. HS increased SULT1E1 protein abundance in post-pubertal gilts. Post-pubertal gilts were synchronized in estrous by Matrix® feeding for 14 days. On the day after Matrix® withdrawal, gilts were exposed to TN (n = 6, 20.3°C ± 0.1°C) or HS (n = 6, 26-32°C) conditions for 5 d during the follicular phase. SULT1E1 protein was quantified from total ovarian protein homogenates by western blotting and data is expressed as relative protein abundance between treatments. * indicates different SULT1E1 protein abundance between treatments; $P = 0.04$.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

The reviewed literature and data offered in this thesis highlight the importance of intact metabolic processes for conservation of life. As the mammalian ovary is comprised with a finite primordial follicular pool that could result in sterility if depleted, it is imperative that appropriate defense mechanisms are opposed to the effects of ovotoxicants. Investigations of potential detoxification regulatory pathways and biotransformation enzymes were conducted in this study as well as regulation of homeostatic physiological processes that can be negatively influenced by toxicants or natural environmental occurrences. Chapter 4 will contain the author's inclusive interpretation of the literature reviewed, experimental studies, and future research considerations.

Impact of PM on ABC drug transporters

Phosphoramide Mustard (PM) is the ovotoxic metabolite of the chemotherapeutic reagent cyclophosphamide (CPA) [1]. CPA-induced ovotoxicity resulted in depletion of follicles by targeting granulosa cells in a mouse model [2]. We previously demonstrated that PM (60 µM) exposure in a postnatal day 4 (PND4) cultured rat ovarian model reduced all follicle types by day 4 of treatment [3]. The ovotoxicant also has the capability to disrupt DNA integrity to alter the follicular reserve by depleting primordial and primary follicles [4]. Loss of the ovarian follicular reserve leads to premature ovarian failure (POF) in

females [5], being that this destruction is irreversible. In this thesis, ovarian detoxification and defense mechanism for cellular protection were examined. We investigated the effect of PM exposure on adenosine triphosphate (ATP) binding cassette drug transporters, ABCB1 and ABCC1. As previously discussed, these phase III biotransformation enzymes were discovered on the basis of their conferring multidrug resistance to cancer cells reducing the efficacy of chemotherapy treatment [6, 7]. ABCB1 transports uncharged or positively charged hydrophobic substrates converting them to hydrophilic forms by glutathione (GSH) conjugation, oxidative process with cytochrome P450 (CYP450) enzymes, or initial blockage from entering into cytoplasm of cell [6]. In contrast of substrate specificity, ABCC1 transports anionic compounds, conjugated GSH substrates, sulfates, and hydrophobic constituents [8-14]. In this study, two different models were used that included a cultured rat spontaneously immortalized granulosa cell (SIGC) line and postnatal day 4 whole ovarian culture. SIGCs were used because somatic cell signaling is vital for follicle development as they contain follicle stimulating hormone (FSH) receptors [15]. The *in vitro* whole ovarian model allowed greater insight of intracellular interaction with the oocyte and granulosa cell in response to toxicants and the chemical response within different follicular stages.

PM exposure to SIGCs or PND4 rat ovaries did not affect ABCC1 protein abundance compared to the control. However, a biological reduction in ABCB1 protein expression was observed in PND4 ovaries after PM exposure. Though both drug transporters have GSH substrate specificity, ABCB1 undergoes an oxidative process via CYP450 activity and studies have indicated that overexpression of CYP450 enzymatic activity potentially

inactivates anticancer drugs, as the presence of these enzymes are seen in tumors [16-19]. CPA undergoes hepatic biotransformation via CYP450 enzymes suggesting that ABCB1 could potentially bind to PM and export it out the cell decreasing its expression or that the drug transporter viability is susceptible to PM-induced reduction in non-cancerous cells. Danker *et al.* (2013) demonstrated decreased ABCB1 and ABCC1 transport activity with the endocrine disruptors' tetrabromobisphenol A (TBBPA) and perfluorooctanoic acid [20]. Protein abundance of ABCC1 may not have been relevant to PM exposure as it belongs to a separate subfamily of ABC superfamily demonstrating that ABCB1 may have a higher binding affinity for PM [21].

Localization of ovarian ABCB1 and ABCC1 in PND4 ovaries was also investigated to understand regional expression in various follicular stages. ABCC1 was localized in the oocyte nucleus of small follicles in PND4 ovaries, while increased expression was observed in maturing follicles. ABCB1 expression was confined to the oocyte cytoplasmic region and increased abundance was observed in growing pre-antral follicles. Similarly, starfish and porcine somatic cells and oocyte demonstrated increased expression of ABCB1 in developing follicles [22-25]. ABC proteins transport function relies on hydrolysis of ATP with cytoplasmic domains [6]. *In vivo* PM exposed adult mice expressed ABCC1 protein in interstitial tissue, oocyte cytoplasm, nucleus of oocyte and oocyte peri-cytoplasmic and peri-nuclear membrane of control treated mice in pre-ovulatory follicles. However, peri-cytoplasmic and peri-nuclear ABCC1 fluorescence was not observed in PM-exposed ovaries, likely due to the severity of PM-induced follicle damage that was evident. ABCB1 expression in this model was confined to granulosa cells, interstitial

tissue, oocyte, peri-nuclear and peri-cytoplasmic region of pre-ovulatory follicles post-PM exposure. Localization of ABCB1 was found in the peri-nuclear area of the cytoplasm in non-metaplastic chronic gastric human fetuses [26].

It was quite interesting that ABCB1 protein abundance of PND4 was altered by PM exposure as it was also restrained to the oocyte's cytoplasmic region, while, ABCC1 protein levels were unaltered and localized to the nucleus of the oocyte. This information may suggest that ABCB1 plays a role in regulation of PM transport from the cytoplasm as a defensive mechanism.

Regulation of phase III biotransformation enzymes via PI3K signaling

Phosphatidylinositol-3 kinase signaling (PI3K) is an intracellular pathway that promotes cell proliferation. Primordial viability and follicular recruitment depend on this pathway [27-30]. PI3K activation is by binding the oocyte c-kit receptor by kit ligand (KL), that is formed in the granulosa cells. KL plays a vital role in oogenesis and folliculogenesis in the preantral stage [31]. It is expressed in granulosa cells of several species, including the rat, ovine, and human [31-33]. KL can be expressed as a membrane-associated protein (KL-2) or soluble protein (KL-1) [31, 34] but translation results in membrane-associated products [31]. KL-2 is the main isoform for growth and oocyte survival [31] and both forms are present in the rodent model [31, 32]. The oocyte receptor, c-Kit, is present during postnatal ovarian development of all follicle stages [31]. PI3K is involved in regulation of ovarian chemical biotransformation enzymes. Our previous studies displayed that PI3K inhibition altered the mRNA and protein abundance of Nrf2, Gstp, AhR, and Gstm,

implying that PI3K has a regulatory role in phase I and phase II chemical biotransformation [35, 36].

We investigated regulation of ABCC1 and ABCB1 via the PI3K signaling pathway. PND4 rat ovarian culture model was used, so ovaries were comprised of primordial and small primary follicles. Ovaries were treated with the PI3K inhibitor, LY294002, and the PI3K activator, KL. PI3K signaling was observed to regulate ABCC1 and ABCB1 protein in PND4 rat ovaries. Inhibition of PI3K nearly depleted the total abundance of ABCC1 while PI3K activation did not alter the drug transporter when compared to the control. Deficiency of PI3K also reduced ABCB1 protein level, though not to the extent of ABCC1, and again KL treatment did not impact ABCB1. Imai *et al*., demonstrated inhibition of ABCB1 and ABCC1 in prostate cancer cells following LY294002 treatment [37, 38]. Surprisingly, no impact on ABCC1 or ABCB1 protein was noted due to overactivation of PI3K with KL. An *in vitro* study showed that KL (100 ng/ml) increased follicular growth of rat primordial to primary follicles after 5-14 d of culture [31, 39]. They suggested that low levels of KL may not be sufficient to promote follicular development. In this particular study 400ng/ml of KL was used in the cultured system ensuing PI3K activation and follicular growth as this had previously been demonstrated to prevent VCD-induced follicle depletion in the cultured rat ovary system [40].

Though KL treatment had no impact on phase III biotransformation enzymes, inhibition of PI3K signaling demonstrated that ABCB1 and ABCC1 are downstream of the pathway. Additional information on phase III biotransformation enzymes will aid in gaining more

insight about detoxification and defensive mechanisms of the ovary that would further our understanding of reproductive science.

Impact of HS on ovarian ABCC1 and SULT1E1 in the pig

The porcine reproductive cycle, which is very similar to the human, can be negatively affected disrupted by heat stress (HS). HS is a biological response to increased body temperature [41-43] which impacts agriculture livestock production [44]. HS disrupts the development and function of mammalian reproduction by impairing hormone production secretions, embryo development [15, 16], ovarian folliculogenesis [16, 17], and uterine environment [18, 19]. Studies have reported seasonal infertility, reduced implantation rates, impairment of embryo development, and prolonged weaning related to HS in gilts and sows [14, 20-22]. Our research group has previously demonstrated that HS altered PI3K signaling, which is involved in primordial follicle activation, maturation, and ovarian steroidogenesis [45]. Also, 7 or 35 d of HS in pre-pubertal gilts increased aromatase (CYP19a1) and steroidogenic acute regulatory protein (STAR) both transcriptionally and at the protein level [45].

We hypothesized that HS alters balance of the steroidogenic process and the hypothalamic pituitary gonadal axis (HPG) potentially disrupting 17β-estradiol. SULT1E1 is an enzyme that catalyzes sulfonation of estrogens, converting them into an inactive form which is transported extracellularly by ABCC1. Although, SULT1E1 had not previously been described in the ovary of swine, it had been characterized in the porcine uterine endometrium [46]. We made the novel finding that SULT1E1 is also expressed in the

ovary. The two pig models that were included in this investigation were pre-pubertal gilts and post-pubertal gilts that were estrus synchronized. Post-pubertal gilts were synchronized to ensure they were at the same follicular stage of development, thereby eliminating variables that could alter observed data. To ensure elimination of bias of dissimilar feed intake, in the pre-pubertal 7 d gilts, an additional group of thermoneutral gilts were pair-fed to their HS cohorts. Additionally, in the post-pubertal gilts, all received the same feed amount, regardless of treatment. We demonstrated no changes in ovarian ABCC1 protein or mRNA level due to 7 d of HS. Chronic HS for 35 d increased ABCC1 and SULT1E1 in pre-pubertal gilts compared to relative controls. Up-regulation of both SULT1E1 and ABCC1 could be indicative of increased 17β-estradiol potentially impairing the steroidogenic response leading to poor reproductive function by decreasing fertilization, oocyte viability, impairment of fetal growth, and spontaneous abortions that are indeed phenotypes observed in HS animals. It could also be proposed that ABCC1 detoxifies the sulfate conjugated 17β-estradiol out of the ovary thereby sending a feedback signal to the HPG axis. Post-pubertal gilts *ABCC1* mRNA levels or protein levels were not altered following 5 d of HS exposure, however SULT1E1 protein expression was increased. The data generated from 5 d HS post-pubertal gilts were variable which is surprising considering that ovaries were collected at the same stage of the estrous cycle. The changes could be from different follicle stages therefore immunostaining to localize SULT1E1 and ABCC1 is a future experiment that is warranted.

As previously discovered by our research group [45], increased levels of CYP19A1 and STAR in pre-pubertal gilts following 7 and 35 d of HS and the findings in this thesis of

significant increases in SULT1E1, the sulfonation catalytic enzyme, and up-regulation of ABCC1, transporter of sulfonated substrates, provide additional evidence that HS has the potential to be an endocrine disruptor. Though little information is known about the regulation and elimination of ABCC1 and its substrates, further investigations are required.

Future Research

The course of this experimental data expressed novel findings, however there are unknown variables that need to be addressed. We demonstrated no impact of PM on ABCC1 protein abundance in PND4 rat ovaries, however there was a slight reduction in ABCB1 abundance. An impact could have been observed in ABCC1 protein abundance with extended PM exposure to PND6 and possibly a more significant alteration in ABCB1 trend. Being that both drug transporters are GSH substrates it would have been interesting to follow up the previous study from our group [47] that demonstrated GSH supplementation during PM exposure reduced PM-induced follicle loss in all follicle types and GSH depletion during PM exposure resulted in depletion of all follicle stages. Investigation of ABCC1 and ABCB1 metabolic roles with GSH supplementation or depletion during PM exposure on follicle loss and protein abundance could instigate better detoxification techniques for preservation of the ovarian follicular reserve and improve understanding of biotransformation enzymatic activity. A transport interaction assay of ABCC1 and ABCB1 could also expand knowledge regarding interaction with PM and functional susceptibilities to the compound, as demonstrated by [20].

Phase III biotransformation enzymes are regulated via the PI3K signaling pathway. Inhibition of PI3K resulted in reduced protein abundance of ABCC1 and ABCB1, however, PI3K activation via KL had no impact. There are two isoforms of KL, KL-1 being soluble and KL-2 that remains membrane bound. KL-2 is the main isoform that is for growth and survival of the oocyte [31, 48]. Alteration to the KL-2 isoform used as the activator of PI3K could help resolve this confounding discovery. Studies have shown that even though *Kl* expression is high in at the transcriptional level in primordial follicles obtained at birth, it is not present at the translational level suggesting that KL-treated PND4 rat ovaries did not have the capability of proper activation resulting in unaltered levels of ABCB1 and ABCC1. We could also examine c-Kit expression in the KL-treated ovarian culture to ensure that system did not defect the functionality of the oocyte receptor. A more economical approach to determining PI3K activation in the PND4 ovarian culture is also to count the number of developing follicles compared to the control as the KL treatment should have initiated such process.

The investigation of HS in porcine ovarian steroidogenic response revealed no changes in SULT1E1 or ABCC1 protein or in their mRNA levels post 7 d of HS in pre-pubertal gilts. Chronic HS (35 d) did increase both substrates potentially indicating that HS could lead to impairment of steroidogenic production by altering 17β-estradiol or it could demonstrate that sulfonation and transportation via ABCC1 is a regulatory process for excessive active estrogens that could inflict poor reproduction in pre-pubertal gilts. Cyclic gilts resulted in variable data: increased SULT1E1 protein abundance post 5 d HS exposure, but no impact on *ABCC1* mRNA expression. For further investigation of this study, luteinizing hormone

(LH) levels should be determined as it increases 17β-estradiol leading to problematic reproductive consequences. Immunohistochemistry (IHC) of ABCC1 and SULT1E1 of porcine ovarian tissue slices would have provided visual evidence of localization and abundance of these proteins.

Information regarding phase III ovarian biotransformation enzymes is scant and expanded knowledge on this topic could shed light on possible mechanisms and techniques that can preserve the quality of the ovary and life of women who are exposed to detrimental senescence-induced ovotoxicants. Further characterization and investigation of SULT1E1 and ABCC1 function and substrate transportation could relieve HS abnormalities that are seen in livestock production that leads to huge economic losses. In conclusion, the collective purpose of the research described in this thesis was to increase our understanding regarding harmful toxicants and unbalanced environmental conditions to enhance reproductive health in humans and animals.

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APPENDIX: SUPPLEMENTAL DATA

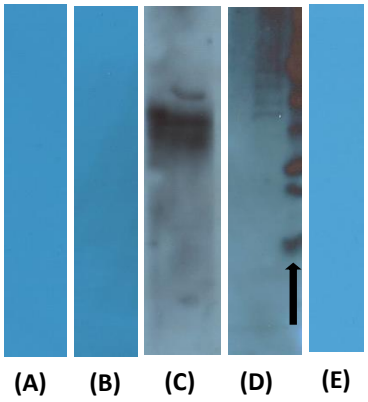

Figure 1.1. Antibody negative controls for western blots.
PND4 rat ovaries were cultured for 4 d in media containing vehicle control (CT; DMSO) or gilts exposed to control conditions (TN; 20.3°C) followed by total protein isolation and western blotting. Letters indicate specific antibody tested: (A): ABCC1, 1° only; (B): ABCB1, 1° only; (C): Goat Anti-Rabbit HRP, 2° only; (D): Normal Goat IgG/Goat Anti-Rabbit HRP, 1° and 2°; (E): SULT1E1, 1° only. Antibodies in lanes A-D were tested against rat ovarian tissue and lane E utilized porcine ovarian tissue. Black block arrow indicates the protein ladder residue present on blot.