AKT IS REQUIRED FOR FSH-STIMULATION OF β-CATENIN ACCUMULATION IN BOVINE GRANULOSA CELLS

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GRANULOSA CELLS

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Abstract: Folliculogenesis is a multifaceted process in which follicles mature and produce estradiol (E_2) and ovulate an oocyte. β-catenin (CTNNB1) is required as a transcriptional co-factor for FSH-simulated E_2 production. In bovine large antral follicles, greater concentrations of intra-follicular E_2 are associated with increased abundance of CTNNB1 protein. Likewise, bovine granulosa cells (GC) in culture, have increased CTNNB1 and protein kinase B (AKT) protein, and *WNT2* mRNA expression following FSH stimulation. These data indicate that FSH regulates CTNNB1 through the canonical WNT or AKT signaling pathways. AKT and WNT signaling pathways are both capable of phosphorylating GSK3-β, a component of the CTNNB1 degradation complex. Immunofluorescence FSH time course in bovine GCs from 0 to 48 h was conducted to evaluate localization of CTNNB1 from the cell junctions to the cytoplasm and nucleus. β-catenin fluorescence into the cytoplasm and nucleus was greatest at 6 h and remained the same through 24 h, and was slightly reduced at 48h. The objective of following studies was to elucidate AKT's role in CTNNB1 accumulation. Preliminary studies for AKT inhibitor (LY294002) and (insulin-like growth factor 1; IGF-I) dose response were conducted to determine the optimal response. Inhibition of the AKT pathway with LY294002 was confirmed by the reduction in phosphorylated AKT (pAKT). Inhibition of AKT reduced FSH-mediated CTNNB1 accumulation and subsequent E_2 production in bovine GCs. This suggests that FSH-stimulation of CTNNB1is mediated through the AKT signaling pathway. Another known AKT stimulator, IGF-I, was investigated for its ability to regulate CTNNB1. Insulin-like growth factor 1 increased pAKT compared to control and FSH, confirming AKT activation. Phosphorylated GSK3-β was below control and FSH levels for the IGF-I treated GCs, and IGF-I did not stimulate CTNNB1 accumulation above control. However, GC production of P_4 and E_2 were increased as expected. Suggesting IGF-I activation of AKT stimulates activation of other pathways, and CTNNB1 may not be required for IGF-I stimulation of steroid production. Stimulation with FSH increased accumulation of CTNNB1 protein and E_2, and requires AKT. AKT is required for steroid production, but is not sufficient to stimulate CTNNB1 accumulation.

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CHAPTER I

INTRODUCTION

The ovary is a dynamic reproductive organ that undergoes numerous changes during maturation. Follicular development or folliculogenesis, is the process in which follicles on the ovary grow and mature in response to endocrine regulation of the hypothalamic-pituitary-gonadal axis and local, intraovarian factors. Endocrine regulation of follicular development starts with production of gonadotropin-releasing hormone (GnRH) from the hypothalamus. In the female, the pulsatile release of GnRH and subsequent stimulation of the anterior pituitary regulates hormone production and release of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Nett et al., 2002). Stimulation of follicle growth, and regulation of gene transcription through FSH is important for follicular maturation and estradiol production. Luteinizing hormone initiates steroid hormone production in the follicle by regulating theca cell synthesis of testosterone, the precursor of estradiol (Carson and Smith, 1986). Additionally, a surge of LH results in ovulation of the dominant follicle and terminal differentiation of the follicle to form the corpus luteum (CL). Together the gonadotropins stimulate steroid hormone production (steroidogenesis) in the ovary of the female, by regulating specific cell types.

The outer most layer of the follicle is made of theca cells, which contain LH receptors, while granulosa cells inside the basement membrane contain FSH receptors. Together the theca and granulosa cells produce enzymes that cleave cholesterol into steroid hormones, and a final product of estradiol (E_2) (Carson and Smith, 1986; Gudermann et al., 1995).

Local intraovarian factors, acting in an autocrine or paracrine manner, also play a role in regulation of ovarian follicles. Recently, a requirement for β-catenin (CTNNB1) has been recognized as a key component for maximal FSH stimulated aromatase and estradiol production (Parakh et al., 2006; Hernandez Gifford et al., 2009). β-catenin is a transcriptional co-factor of the wingless-type mouse mammary tumor virus integration site (WNT) canonical signaling pathway. Numerous developmental processes including ovarian follicle development in the adult mammal are regulated by locally secreted WNT molecules. The canonical WNT signaling pathway requires regulation of CTNNB1 via a degradation complex comprised of glycogen synthase kinase-3 (GSK-β), adenomatous polyposis coli (APC), and axin. Other intraovarian factors, including protein kinase B (AKT) and insulin-like growth factor 1 (IGF-I), also contribute to follicular development and growth during the estrous cycle. AKT is important for cell survival and inducing protein synthesis and is known to interact with WNT signaling pathway. Additionally, IGF-I is recognized for its ability to synergize with gonadotropins to increase steroidogenic output (Armstrong and Webb, 1997). Insulin-like growth factor 1 stimulates granulosa cell proliferation, differentiation, and cell survival (Davidson et al., 2002). Synergy of FSH and IGF-I increases E_2, and signals through AKT (Gonzalez-Robayna et al., 2000). Furthermore, data from our lab highlight FSH regulation of both

CTNNB1 and AKT protein in steroid production in bovine granulosa cells. These results were demonstrated in large dominant bovine follicles with high intrafollicular fluid concentrations of E_2, which have greater accumulation of CTNNB1, indicating the importance of CTNNB1 in bovine folliculogenesis and possibly follicular selection. Similarly, cultured granulosa cells treated with FSH had an increased accumulation of CTNNB1 and AKT protein, and up-regulation of *WNT2* mRNA compared with vehicle treated controls (Castanon et al., 2012). It remains unclear whether FSH regulation of CTNNB1 accumulation is through the regulation of AKT or WNT signaling pathways. The current study was designed to elucidate AKT's role in the accumulation of CTNNB1.

CHAPTER II

REVIEW OF LITERATURE

Hypothalamus

Female ovarian function and male reproductive function is initiated through influence of the hypothalamus and pituitary (Fink, 1979). The hypothalamus is located at the base of the brain and is a dynamic link between the nervous system and endocrine system. Normal reproductive physiology requires communication and coordination between the hypothalamus, pituitary, and gonads. Signaling interaction of these tissues is called the hypothalamic-pituitary-gonadal axis (HPG axis). The hypothalamus makes up the floor and walls of the third ventricle, and is comprised of neuronal cell bodies that produce releasing and inhibiting-hormones (Ingram, 1939). Numerous physiological functions are regulated by the hypothalamus including; circadian rhythm, heart rate, blood pressure, appetite, body temperature, and reproduction among others. The hypothalamus communicates with the pituitary via its axons. These axons extend to the median eminence in the pituitary stock and are a part of neurons located higher in the hypothalamus, which release neurohormones into capillary beds of the primary portal plexus. The primary portal plexus is the beginning of the hypothalamo-hypophyseal portal system (Popa, 1930).

The releasing-hormones are immediately transferred via portal vein to the second portal plexus to the anterior pituitary. The hypothalamo-hypophyseal portal system is important because releasing-hormones from the hypothalamus are peptides that have a short half-life and are released in minute quantities. This portal system prevents dilution of the neurohormones by directly delivering them to the anterior pituitary. Production of specific releasing-hormones from the hypothalamus occurs as part of feedback regulation. Positive feedback of estradiol (E_2) in the female positively influences ovulation of an oocyte as it acts on the pituitary to up-regulate GnRH receptors (GnRHr) and enhance responsiveness of GnRH (Reeves et al., 1971; Crowder and Nett, 1984). Conversely, the increase E_2 for an extended time down regulates GnRHr through negative feedback.

Gonadotrophin-releasing hormone (GnRH) stimulates the production and release of hormones in the anterior pituitary. Gonadotropin-releasing hormone is a decapeptide produced in the ventromedial and arcuate nucleus (tonic center) and surge center (preoptic nucleus) of the hypothalamus (Gorski, 1970), which is responsible for initiating the cascade of events that regulate the gonads. GnRH is released in a pulsatile fashion to stimulate the production and release of hormones in the pituitary (Schneider et al., 2006). The sequence of GnRH determined by (Schally et al., 1971) pyro-Glu1-His2-Trp3 –Ser4 –Tyr5 –Gly6 – Leu7– Arg8 –Pro9 –Gly10 – NH_3. In response to pulsatile release of GnRH, the pituitary gonadotropes synthesize and release FSH and LH into circulation. Upon binding GnRHr G-protein coupled receptor activates long activated calcium channels, causing influx of calcium activation phospholipase C (PLC). Subsequently, phosphatidylinositol 4,5- bisphosphate (PIP_2) is cleaved by PLC to inositol triphosphate (IP3) and diacyl glycerol diffuses to endoplasmic reticulum and binds to IP3 receptor to cause release of calcium ions into the cytoplasm (Naor, 1990). Diacylglycerol (DAG) remains in the membrane and together DAG and calcium influx leads to the activation of protein kinase C (PKC). Activated PKC phosphorylates and activates proteins such as mitogen-activated

protein kinase (MAPK). There are at least 75 genes that are transcribed from GnRH signaling through MAPK (Salisbury et al., 2008). Four of the hallmark genes regulated by GnRH are chorionic gonadotropin alpha (*Cga),* LH β-unit (*Lhchb),* FSH β-unit (*Fshb*), and *Gnrhr* (Salisbury et al., 2008).

As previously mentioned, gonadotropin-releasing hormone's release is controlled through positive and negative feedback systems to result in stimulation of LH and FSH, which regulate steroid production in the ovary. Estradiol increases GnRH and progesterone (P_4) decreases pulsatile release of GnRH. In the midluteal phase of the bovine estrous cycle, negative feedback of P_4 suppresses GnRH release and lowers the expression of GnRH mRNA (Weesner et al., 1993). Conversely, ovine pituitary cells treated with E_2 demonstrate an increase in synthesis and insertion of receptors for higher sensitivity to GnRH (Gregg and Nett, 1990). In bovine cultured pituitary cells, an increase of LH is increased at 26 h versus 4 h with in the high dose of E_2, however the low dose at 26 h is below the 4 h concentrations of LH (Padmanabhan et al., 1978). Steroid hormones also influence and alter the release pattern of GnRH that determines which gonadotropin, FSH or LH, are produced from the anterior pituitary (Nett et al., 2002). The female brain has both a surge and tonic center, which are developed during sexual differentiation. The female brain develops the surge center in the preoptic area, which in the male remains a tonic center due of testosterone crossing the blood brain barrier and subsequently being aromatized to E_2, which prevents formation of the surge center. The surge center will supply GnRH to stimulate the ovulatory surge of luteinizing hormone (LH). Negative feedback actions of increased levels of E_2 over a period of time suppress the GnRH frequency while increasing the amplitude (Schneider et al., 2006). Schneider et al. (2006) also demonstrates P_4 causes a lower frequency of GnRH release. The dominant follicle producing increasing amounts of E_2 stimulates GnRH to pulse and increase sensitivity of GnRHr in the anterior pituitary to stimulate LH production. The LH pulse will be high in amplitude from the stimulation of GnRH produced in

the surge center in the hypothalamus. Low amplitude pulses with short intervals from the tonic center are regulated by P_4 production from the corpus luteum, these pulses of GnRH produce FSH in the anterior pituitary (Rahe et al., 1980).

Gonadotropins

The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are heterodimeric glycoprotein hormones produced by gonadotropes in the anterior pituitary. Gonadotropins are important in the female to regulate ovarian follicle development (Vale et al., 1977). Follicle-stimulating hormone recruits secondary follicles and stimulates maturation of the follicle, and the ovulatory surge of LH results in release of the oocyte. The gonadotropins are composed of the same α subunit, and a unique β subunit. Production and secretion of unique β-subunit is controlled by estradiol activity that influences the pulsatility of GnRH release (Dalkin et al., 1989). The pulse frequency and amplitude of GnRH determines the regulation of gene transcription for FSH and LH β-subunits. An increase of in GnRH pulse amplitude leads to the production to LHβ-subunits and basal pulses lead to the production of FSHβ-subunits. The gonadotropins signal through a 7-transmembrane receptor, which activates heterotrimeric G-protein (Gudermann et al., 1995; Peirce and Kendrick, 2002). G-protein coupled receptor activates adenylyl cyclase by converting ATP to cAMP (Gudermann et al., 1995; Park et al., 2005). Protein kinase A (PKA) directly activates cAMP response element binding protein (CREB) to carry out follicular maturation (Hunzicker-Dunn, 1981). Follicle-stimulating hormone also signals through the phosphoinositide 3-kinase (PI3K) pathway that is also stimulated by the G_s-protein coupled receptor to subsequently activate protein kinase b (AKT).

Follicle-stimulating hormone is necessary to induce maturation of ovarian follicles, in the intact animal, resulting in the generation of mature oocytes and production of estrogen (Hunzicker-Dunn and Maizels, 2006). In rodents, FSH pulsatile release occurs approximately

every 20 min (Shupnik, 1990). Receptors for FSH are located exclusively on granulosa cells in the ovary and up-regulate genes that drive steroidogenesis and follicular maturity (Carson and Smith, 1986). Granulosa cells acquire FSH receptors (FSHr) as secondary follicles, prior to follicular recruitment (Evans and Fortune, 1997). Granulosa cell proliferation and steroid production in growing follicles are stimulated by FSH. Two hallmark genes of FSH stimulation are aromatase and LH receptors. Aromatase is the enzyme responsible for enzymatically converting testosterone into estradiol. The ovarian follicle is the major site for estrogen production (Hillier, 1985). Estradiol 17-β is the most potent estrogen and is at its highest concentration during estrus. In response to FSH, granulosa cells acquire LH receptors, which act similar to FSHr to activate adenylyl cyclase (Kolena and Channing, 1972). Up-regulation of LH receptor on granulosa cells of dominant follicles aids in the maturation and ovulation of the follicle. Follicle-stimulating hormone secretion is also regulated by ovarian hormones inhibin and activin. Activin up-regulates the FSH_{β} production by the pituitary (Mason et al., 1989; Weiss et al., 1995). Activin is a glycoprotein produced in the granulosa cells that stimulates FSH synthesis and secretion, and aids in cell proliferation, differentiation, and apoptosis. Female mice deficient in activin type II receptor have low FSH levels and are infertile (Matzuk et al., 1995). Inhibin is produced by granulosa cells within the developing follicles and suppresses FSH synthesis (De Jong and Sharpe, 1976), and may play a paracrine role in the ovary (Hsueh et al., 1987; Knight et al., 2012). Inhibin concentrations promote follicular atresia of non-dominant follicles and inhibit development of additional follicles (Bicsak et al., 1986). The inhibitory effects of inhibin where demonstrated by vaccinating ewes against this hormone. Inhibin vaccinated ewes, which successfully produced antibody response to inhibin had a yearly increase in offspring and ovulation rate (Glencross et al., 1994). Another regulator of FSH stimulation, follistatin, produced in the pituitary, is an activin-binding protein that sequesters activin to lower levels of FSH. Estradiol inhibits the expression of activin lowering the production of FSH.

Luteinizing hormone stimulation of theca cells is required for ovulation of the oocyte from the dominant follicle and stimulatory effect of corpus luteum (CL). Theca cells produce androgen substrates, which will be converted to E_2 in the granulosa cell is a key regulator of the estrous cycle. Ovarian steroidogenesis starts in the theca cells when LH binds its receptor and subsequently produces testosterone. Steroid production from the ovary requires input from both theca and granulosa cells in a model known as two-cell theory of steroidogenesis (Falck, 1959). Theca cells contain LH receptors, LH binding causes a gene transcription of enzymes that enzymatically cleave cholesterol to testosterone, that is converted E_2 by enzyme aromatase in granulosa cells containing FSHr (Carson and Smith, 1986). Antral follicles in which granulosa cells have acquired LHCGR, the stimulation of LH matures the oocyte and prepares the cascade of genes prepare the dominant follicle for ovulation. Ovulation is the result of rupturing of a mature follicle, which releases antral fluid and eventually the oocyte. Luteinizing hormone signaling induces release of enzymes by granulosa cells leading to follicular rupture and theca cell luteinization (Espey, 1974; Brailly et al., 1981). After ovulation, the follicle differentiates into a corpus lutea (CL), which can respond to LH producing P_4. Rahe et al. (1980), characterized LH release in bovine estrous cycle by collecting serial blood samples on designated days of the estrous cycle to establish pulse frequency and amplitude changes of LH. Plasma concentrations of LH fluctuate in a pulsatile fashion during all phases of the estrous cycle and surge to cause ovulation on day 1. On day three, during the early luteal period; LH pulses exhibit low amplitude and high frequency, showing cycle dependent changes in the cow. During the midluteal phase (d 10), the pulses of LH have high amplitude and low frequency, whereas on Day 18 and 19, LH plasma patterns have both high frequency and high amplitude resulting in the preovulatory surge of LH (Rahe et al., 1980). Nett et al. (2002) showed 75% of stored LH is released in response to GnRH to cause the ovulatory surge. Down regulation of GnRH receptors leads to the termination of LH surge as well as depletion of LH (Crowder and Nett, 1984). After ovulation theca and granulosa cells differentiate in to luteal cells that produce P_4. Follicle-

stimulating hormone receptors decline after the LH surge, while LH receptors are first desensitized and mRNA degraded during the process of luteinization (Camp et al., 1991; Lu et al., 1993). After the LH surge many genes are up regulated including P_4 receptor to allow the development of a functional corpus lutea (CL) (Park and Mayo, 1991; Natraj and Richards, 1993).

Estrous Cycle

The estrous cycle is defined as the period from the onset of heat to the next demonstrated heat and relies on hormonal regulation of the HPG axis. The estrous cycle's distinct phases are illustrated and identified by the hormone profiles represented at different times of the cycle. The bovine estrous cycle ranges from 19-23 d and is divided into two phases, the luteal phase and the follicular phase. In cattle the luteal phase comprises 80% of the estrous cycle and during this time the CL is the dominant structure on the ovary. The remaining 20% of the estrous cycle is the follicular phase characterized by a large dominant follicle on the ovary, thus the largest amounts of LH and E_2 are being produced in this phase (Wettemann et al., 1972). The luteal and follicular phases can each be further divided into two stages, which are defined by hormone profile (steroidogenesis) and ovarian structures (folliculogenesis). The follicular phase last approximately three days and eighteen hours and includes proestrus and estrus stages starting d 17- d 1 of the cycle. Proestrus is the phase that the CL is regressing, so P_4 is declining while the dominant follicle is rapidly growing and producing increasing amounts of E_2. The increasing E_2 influences the cow to show heat, this is called estrus. During estrus, LH will begin to increase in pulse frequency and amplitude. Ovulation occurs in metesrus that last 5 d, and is part of the luteal phase. Diestrus (around 12 d) is also part of the luteal phase, and the main structure on the ovary is the CL that produces the characteristic high levels of P_4. Steroidogenesis is a process by which the structures on the ovary are influenced by gonadotropins to produce steroid hormones.

Interaction of the HPG axis and intraovarian signaling pathways are critical to maintain normal estrous cycle and reproductive health.

Steroidogenesis

Steroidogenesis in the ovary regulates numerous developmental and physiological processes within the body. Estradiol and P_4 are the major female sex hormones, which regulate the estrous cycle, and subsequently reproduction. Development of the ovarian follicle is vital in female reproduction as the follicle serves as the major source of steroid hormones. The precursor to all steroid hormones is cholesterol, which is composed of lipid 27-carbon (C) structure with four hydrocarbon rings. Steroid hormones are fat soluble and thus easily pass through the plasma membrane by simple or facilitated diffusion to then bind receptors which are located in the cytoplasm and nucleus (Mangelsdorf et al., 1995).

Activation of the enzyme cholesterol esterase, which hydrolyzes cholesterol ester to cholesterol, is through the activation of LH binding its receptor on the theca cell. Inside the theca cells, 27- C cholesterol is subjected to enzymes that cleave specified carbons to produce steroid hormones. Cholesterol is delivered to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR). Up-regulation of StAR is obligatory for steroidogenesis, and is an important part of delivering cholesterol to the mitochondria. Within the mitochondria the first enzymatic cleaving of carbons will occur, starting with cytochrome P450 side chain cleavage formally *Cyp11a1* (Hanukoglu et al., 1990; Amsterdam et al., 1992; Amsterdam and Selvaraj, 1997). In the mitochondria of the theca cell and granulosa cell, cholesterol is converted to pregnenolone, a 21- C structure, by enzyme P450 side chain cleavage (P450scc; formally *Cyp11a1)*. Theca cells, but not granulosa cells, produce enzyme, 17 beta-hydroxysteroid dehydrogenase forms 19- C testosterone from P_4. Testosterone is diffused from theca cells to granulosa cells, which make up a majority of the follicular cell composition in the mammalian

ovary (Carson and Smith, 1986). The granulosa cells then convert testosterone to 18-C E_2 via cytochrome P450 aromatase (*Cyp19a1*). Follicle stimulating hormone signals through cAMP to regulate transcription of aromatase and subsequent biosynthesis of E_2 (Thompson and Siiteri, 1974). Increasing amounts of E_2 lead to the ovulatory surge and follicle differentiation and formation of the CL, as described above. The reproductive system remains poised and ready for new cycle with continuous development of follicles.

Folliculogenesis

Before steriodogenesis can occur the follicles must develop and mature into secondary follicles. Development and maturation of ovarian follicles are also associated with changes in hormone production coordinated by the HPG axis. The ovary is a key component for reproduction because it contains all of the oocytes or females sex cells. Mammals are born with a finite number of oocytes. During embryonic development, primordial germ cells form and migrate from the genital ridge to the gonad. Once germ cells reach the gonad they are called oogonia and develop into clusters of cells called germ line cysts or an oocyte nest (Pepling, 2006). In mammals, the developing oocyte is contained within the follicle providing the optimal environment for maturation. Ovarian folliculogenesis is the process by which primordial follicles arise from somatic cells in the ovary and develop into preovulatory follicles for the possibility of fertilization (Webb et al., 1992). Cattle have two to three waves of follicular growth per estrous cycle (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989). Folliculogenesis can be divided into three distinct stages; recruitment, selection, and dominance. These stages of folliculogenesis are happening in waves of cohort follicles. Before recruitment, follicles from the primordial pool will grow together at a rapid pace and increase in size. Primary follicles will then degenerate or develop into a secondary/preantral follicle independently of hormones (Cortvrindt et al., 1997; Fair, 2003). The primordial follicle is the most immature stage and is compromised

of an oocyte and a single layer of flattened granulosa cells. The follicles contain an immature quiescent egg or oocyte and are suspended in prophase 1 of meiosis. The granulosa cells become cuboidal, reaching the classification of primary follicle (Braw-Tal and Yossefi, 1997; Aerts and Bols, 2010). During the development of primary to secondary follicles, FSH levels are high, although early stage follicles develop without hormonal input. The follicle becomes a secondary follicle when there are two or more layers of granulosa cells. Through classic endocrine mechanisms, development of the secondary follicle is influenced by gonadotropins to regulate growth (Armstrong and Webb, 1997). The rapid and continual growth of the oocyte increases volume expansion, and the zona pellucida develops between the oocyte and granulosa cells (van Wezel and Rodgers, 1996). Graafian/tertiary follicles have a well-characterized antrum containing fluid. Numerous follicles reach the antral stage, characterized by the formation of an 'antral' cavity, however as with previous stages, most will under go apoptosis. During recruitment, cohort (6 to 8) of antral follicles responds to FSH and develops LH receptors. Following recruitment, the second stage of folliculogenesis is selection a process in which one or two select antral follicles are allowed to continue to grow into a Graafian follicle while the rest will regress through apoptosis. During selection, continued growth requires the correct receptor type to respond to the hormone stimuli. Cohorts to the dominant follicle regress in the case of a mono-ovulatory species, and only one becomes a dominant follicle. Through the selection phase, FSH concentrations are low, LH concentrations are moderate, and inhibin is rising to further decrease FSH concentrations. The follicles are now producing increased amounts of E_2. The final stage is dominance, where the dominant follicle(s) undergo rapid development, while the growth of the remainder subordinate follicles are suppressed (Goodman and Hodgen, 1983). Follicle development patterns are associated with changes in expression of mRNA encoding gonadotropin receptors (Xu et al., 1995) and steroidogenic enzymes to allow selected follicles to mature and ovulate in response to the preovulatory LH surge (Armstrong and Webb, 1997). Estradiol levels are increasing during selection resulting in increased GnRH sensitivity in the

anterior pituitary and subsequently an increase in LH to provide the LH surge for ovulation. Follicular fluid of dominant follicles contains higher concentrations of E_2, insulin-like growth factors-binding protein (IGFBP) protease activity and free IGF, β-catenin (CTNNB1), AKT, and lower IGFBP4 concentrations (Mihm et al., 2000; Fortune et al., 2004).

Intraovarian growth factors

There are many intraovarian factors that mediate folliculogenesis and steroidogenesis. Two key components for follicular maturation are WNTs and IGF-I. Follicular fluid from large bovine follicles that produced the greatest estradiol had an increase of CTNNB1 and AKT and cultured bovine granulosa cells treated with FSH also demonstrated an increase in CTNNB1, AKT protein and *WNT2* mRNA expression (Castanon et al., 2012). Accumulation of CTNNB1 leads to the maximal production of E_2. Both *WNT2* and AKT inactivate GSK3-β to allow for this accumulation of CTNNB1.

β-Catenin

Beta-catenin was initially described as a cell-to-cell adhesion molecule, and later identified as a transcriptional co-factor (Kemler, 1993; Peifer, 1995). The β-catenin protein (CTNNB1) is 781 amino acids in length in the bovine and is regulated by a degradation complex consisting of glycogen synthase kinase-3 (GSK3), adenomatous polyposis coli (APC), and axin (Peifer et al., 1994; Zeng et al., 1997; Liu et al., 2002). The degradation complex marks CTNNB1 for ubiquitination and cellular proteosomal machinery then degrades the CTNNB1. Disassociation or inactivation of the degradation complex leads to CTNNB1 accumulation and translocation to the nucleus. The mechanism by which CTNNB1 crosses into the nucleus is not entirely understood. Current it is understanding is that CTNNB1 binds to the nuclear pore

machinery and requires GTP hydrolysis for import (Fagotto et al., 1998). Nuclear location allows for the interaction with nuclear transcription factors to regulate gene transcription. Accumulation of CTNNB1 can be through subsequent WNT or AKT signaling through FSH-stimulation as both signaling pathways can deactivate the CTNNB1 degradation complex.

Wingless-Type Mammary Tumor Virus Integration-Site Signaling

The term "WNT" is the fused names of two orthologous genes: Wg (wingless) in *Drosophlia* segmented polarity gene and of *Int-1* (integration), a mouse protooncogene (Nusse and Varmus, 1982). This is a large family of locally secreted glycoproteins, and there are currently 19 known mammalian WNTs (Giles et al., 2003). These signaling molecules are involved in various events during development, such as cell fate specification, cell proliferation, differentiation, survival and apoptosis, polarity and migration (Hoppler and Kavanagh, 2007). Granulosa cells express specific WNTs, some of which are hormonally regulated during follicle development, ovulation, and luteinization (Hsieh et al., 2002; Ricken et al., 2002; Wang et al., 2010). Expression of WNT2 mRNA occurs within the follicle at all stages and signal strength in granulosa cells of atretic follicles is reduced (Ricken et al., 2002). In bovine granulosa cells, *WNT2* mRNA and CTNNB1 was increase with FSH stimulation (Castanon et al., 2012)

WNT glycoproteins signal through one of 3 pathways; canonical (WNT/β-catenin), WNT/Ca^{2+}, and planar cell polarity pathways (Kohn and Moon, 2005; Gordon and Nusse, 2006). The canonical pathway uses β-catenin to interact with its transcription factor. Transduction of WNT activates co-receptors, Frizzled (FZD) which are 7 transmembrane G protein-coupled receptors and LDL-receptor-related protein (LRP) molecules, leading to phosphorylation of LRP6 to later recruit axin (Nusse, 2005). Low density lipoprotein-receptor-related protein phosphorylates the protein disheveled (DSH), and DSH recruits axin for disassociation of the β-catenin degradation complex. The multi-protein degradation complex marks β-catenin for

ubiquitination and is degraded by cellular proteosomal machinery (Giles et al., 2003; Logan and Nusse, 2004). Glycogen synthase kinase-3β (GSK3β), adenomatous polyposis coli (APC), and axin comprise the degradation complex (Cadigan and Nusse, 1997). Once axin is recruited, the degradation complex is disassociated allowing β-catenin to accumulate in the nucleus (Mao et al., 2001).

Normal rodent fetal development, sex determination, fertility, and formation of the internal and external genitals require WNT, as discovered in a pioneer study through the Wnt4 knockout mouse (Vainio et al., 1999). Vainio et al. (1999) knockout Wnt4 demonstrated the function of normal female tract development; the mice die within 48 h of birth due to renal failure, not allowing observations of sexually mature rodents. However, the study showed that development of the Müllarian ducts, feminization, and ductile side branching of mammary glands is mediated by WNT4. Mutant knockout Wnt4 mice are partially sex reversed and have masculinized proximal sex ducts, resembling epididymal region of the male, being highly convoluted, as well as a role in the depletion of oocytes prior to birth. A recent study using gene specific Wnt4 knockout mice (only knocking out Wnt4 in the reproductive tract) show Wnt4 is required for steroidogenesis to produce healthy ovarian follicles and cell differentiation to produce healthy CL that aids in female fertility (Boyer et al., 2010a). Wnt4 mutant mice demonstrated a decrease in ovary size, with a normal amount of follicles that developed to the secondary follicle stage and the ability to progress to normal follicular development, but with a reduced ability to produce E_2 (Boyer et al., 2010b). β-catenin is to allow steroidogenic factor 1(SF1) to respond to FSH and regulates the biosynthesis of E_2 through gene expression of *Cyp19a1* through functional interactions with SF1 (Parakh et al., 2006). β-catenin activates signaling pathways of FSH and WNT to enhance FSH action and promote preovulatory follicular growth and survival (Fan et al., 2010).

Protein Kinase B (AKT)

Protein kinase B, officially designated AKT, is a kinase important for follicle survival, as it blocks apoptosis and induces protein synthesis pathways. Signaling from phoshatidylinositide 3- kinase (PI3K) to activate AKT can be through both G-coupled protein receptor and tyrosine kinase receptors. Activation of AKT though the phosphorylation of Thr-306 and Ser-437 by phosphoinositide-dependent kinase-1 (PDK1) causes inhibition of pro-apoptotic factors such as foxhead transcription factors and caspase 9, both are known to mediate apoptosis (Cardone et al., 1998; Brunet et al., 1999). The AKT/PI3K signaling pathway activation leads to the interaction with GSK3-β, a member of the β-catenin degradation complex. To investigate the AKT/PI3K Signaling pathway, the inhibitor LY294002 is commonly used. Protein kinase B is an essential part of FSH-induced aromatase and E_2 production (Zeleznik et al., 2003). Follicle-stimulating hormone-mediated AKT phosphorylation/activation in rat granulosa cells (GC) is detected by 10 min, peaks at 1 h, and is undetectable 4 h after FSH treatment (Alam et al., 2004). FSH activation in granulosa cells leads to the phosphorylation of GSK3-β on Ser-9, which subsequently disassociates the β-catenin degradation complex (Fang et al., 2000). Insulin-like growth factor 1 and FSH are known stimulators of AKT present in the ovary. When inhibiting the AKT pathway in sheep on the first follicular wave, further follicular growth and E_2 production are inhibited (Ryan et al., 2008). Studies in bovine GC stimulated with IGF-I and PI3-K/AKT inhibitor produced a modest reduction in pERK1/2 in mammary epithelia cells (Mani et al., 2010), supporting previous suggestion that cross talk between AKT and MAPK signaling pathways exist (Kumar et al., 2008). The PI3-K pathway was required for protection against apoptosis with IGF-I stimulation (Carson and Smith, 1986; Parakh et al., 2006). Protein kinase B has already been demonstrated to be essential component of FSH-induce aromatase and E_2 production in rodents (Zeleznik et al., 2003). The ability of AKT to inactivate GSK3-β to allow

CTNNB1 accumulation indicates AKT's importance in the regulation of follicle development (Wu and Pan, 2010) and has recently been shown in in bovine GC (Castanon et al., 2012).

Insulin-like growth factor 1 (IGF-I)

Insulin like growth factor 1 plays leading role through interactions with gonadotropins in relation to steroidogenesis and survival response of the follicle (Armstrong and Webb, 1997). Production of the peptide IGF-I occurs in important reproductive organs the hypothalamus, ovaries, oviducts and uterus (Zhou et al., 1997; Velazquez et al., 2008). Insulin like growth factor 1 is one of the numerous intraovarian regulators of follicle development (Kemler, 1993). The most important role of IGF's appears to be the ability to synergize with gonadotropins and amplify the effect of gonadotropins (Adashi et al., 1985; Veldhuis et al., 1986). Follicular growth in bovine can be linked with and steroid production with metabolic status of the animal, making IGF-I and insulin key growth factors (Spicer and Echternkamp, 1995). Cows in a negative energy balance will have low concentrations of circulating of IGF-I and/or insulin, and therefore the follicular development is compromised (Perks et al., 1999; Webb et al., 2004). Insulin-like growth factor-1's effects are mediated through type-1 IGF receptor (IGF-IR), which is a transmembrane tyrosine kinase receptor, leading to the activation of the PI3K pathways. Insulin-like growth factor 1 actives two different pathways, depending on the cell type PI3K and/ or MAPK pathway (Butt et al., 1999; Poretsky et al., 1999). Protein kinase B (AKT), is activated by the PI3K pathway, and is an important mediator of proliferation and cell survival. The MAPK family, extra cellular-regulated kinase (ERK) also regulates proliferation, differentiation, and cell survival. During the selection of the dominant follicle both AKT and ERK signal pathways are up regulated in follicles of cattle and sheep (Ryan et al., 2007). Insulin-like growth factor 1 is also known to stimulate proliferation of granulosa cells to enhance steroid production (Spicer and Echternkamp, 1995; Davidson et al., 2002). Follicle-stimulating hormone and IGF-I have shown

to act synergistically to enhancing follicular development, in rat GC through AKT (Gonzalez-Robayna et al., 2000) and bovine GC (Ryan et al., 2008), however, the mechanisms behind the interaction remains unclear (Richards et al., 2002; Velazquez et al., 2008). Estradiol treatment can increase IGF-IR in granulosa cells from small bovine follicles (Spicer et al., 1994). Spicer and Aad (2007) and Mani et al. (2010) demonstrate IGF-I synergizes with FSH to increase *CYP19A1* mRNA. Genes important for the regulation of steroidogenesis: *CYP19A1, CYP11A1* and *HSD3B1* mRNA are up-regulated by IGF-I in bovine granulosa cells, and inhibition of the AKT pathway reduces IGF-I stimulated mRNA expression (Mani et al., 2010). Also, FSH and IGF-I work synergistically through PI3K-AKT pathways to stimulate steroid production (Mani et al., 2010).

Conclusion

Regulation of folliculogenesis and steroidogenesis require a coordinated effort of gonadotropins and intraovarian signaling pathways. The hypothalamic-pituitary-gonadal axis regulation of hormones has been identified as controllers of ovarian cyclicity. However, mechanisms of intraovarian signaling pathways stimulated by FSH are not fully understood. The recent findings in our lab using bovine granulosa cells indicate that both AKT protein and *WNT2* mRNA are induced in FSH-stimulated cells. As CTNNB1 was also increased during FSH-stimulation, and both AKT and canonical WNT signaling pathways have the ability to regulate CTNNB1 accumulation. The focus of this thesis will be to reveal AKT's role in the accumulation of CTNNB1.

CHAPTER III

AKT IS REQUIRED FOR FSH-STIMULATION OF β-CATENIN ACCUMULATION IN BOVINE GRANULOSA CELLS

ABSTRACT: Regulation of estradiol (E_2) biosynthesis by FSH requires the transcriptional co-factor beta-catenin (CTNNB1). Increased abundance of CTNNB1 is demonstrated in large antral follicles with greatest concentrations of intra-follicular E_2. In bovine granulosa cells (GC), FSH increases CTNNB1 and protein kinase B (AKT) protein, and *WNT2* mRNA expression. These data indicate FSH regulates CTNNB1 through the canonical WNT or AKT signaling pathways. The objective of this study was to elucidate AKT's role in CTNNB1 accumulation. Bovine GC were pre-incubated with AKT inhibitor (LY294002; LY) for 30 min, then cultured with or without FSH for 24 h (n = 4). Total protein was collected for analysis by Western blot. Relative abundance of protein was analyzed using one-way ANOVA procedure of SAS. The AKT inhibitor LY reduced total AKT abundance ($P = 0.02$) and ablated phosphorylated AKT protein ($P < 0.001$). Inhibition of AKT signaling with LY alone ($P = 0.05$) or in combination with FSH ($P = 0.02$) reduced CTNNB1 protein compared to FSH-treated GC. Progesterone (P_4) media concentrations did not differ among treatment groups ($P = 0.18$). Inhibition of AKT reduced ($P = 0.02$) FSH-mediated E_2 production to levels similar to controls. A subsequent study

was conducted to investigate whether a known AKT stimulator, IGF-I, could also mediate CTNNB1 accumulation (n = 3). Bovine GC treated with FSH had greater CTNNB1 compared to control and IGF-I groups ($P = 0.01$). FSH did not increase P_4 ($P = 0.46$) above controls, however, IGF-I alone or in combination with FSH increased ($P < 0.001$) P_4 (161.8 and 185.9 ± 13 ng/mL, respectively). Estradiol concentrations were increased in FSH treated GC (140 ± 65 pg/mL) compared to control treated GC (93 ± 65 pg/mL), and addition of IGF-I alone or in combination with FSH increased E_2 (280 and 343 ± 65 pg/mL, respectively; $P = 0.06$). Data demonstrate that AKT is required for FSH-induced accumulation of CTNNB1 and steroid synthesis. IGF-I stimulation of AKT increased steroid synthesis but not CTNNB1 accumulation indicating AKT is necessary for FSH-induced accumulation of CTNNB1, and CTNNB1 is not required for IGF-I induction of steroidogenesis.

INTRODUCTION

The ovary is a dynamic reproductive organ that undergoes numerous changes during follicular maturation. During folliculogenesis ovarian follicles grow and mature in response to endocrine regulation of the hypothalamic-pituitary-gonadal axis and local, intraovarian factors such as insulin-like growth factor 1 (IGF-I) and AKT. The pituitary hormone, FSH stimulates follicle growth and regulates granulosa cell (GC) gene transcription important for follicular maturation and estradiol production. More recently, the transcriptional cofactor β-catenin (CTNNB1) has been recognized as a key component for maximal FSH–stimulated aromatase and estradiol production in rodents (Parakh et al., 2006; Hernandez Gifford et al., 2009). Regulation of CTNNB1 occurs via a degradation complex comprised of glycogen synthase kinase-3 (GSK3-β), adenomatous polyposis coli (APC), and axin. Recent data from our lab demonstrates that

FSH regulates both CTNNB1 and protein kinase B (AKT) in bovine GC steroid production. Large bovine follicles containing high intrafollicular concentrations of estradiol have greater accumulation of CTNNB1, indicating the importance of CTNNB1 in folliculogenesis and possibly follicular selection (Castanon et al., 2012). Similarly, GCs treated with FSH have elevated CTNNB1 and AKT protein, and increased expression of *WNT2* mRNA (Castanon et al., 2012). Follicle-stimulating hormone induced aromatase and estradiol production also relies on AKT (Zeleznik et al., 2003). Furthermore, AKT is important for cell survival and protein synthesis, and regulates the deactivation of GSK3-β, a component of the CTNNB1 degradation complex. Insulin-like growth factor 1(IGF-I) is recognized for its ability to synergize with gonadotropins to increase steroidogenic output and also signals though the PI3-K/AKT signaling pathway (Kemler, 1993; Richards et al., 2002; Mani et al., 2010). Bovine GC treated with FSH and IGF-I synergistically increase *CYP19A1* mRNA and signal through the AKT/PI3-K pathway (Mani et al., 2010). Suggesting AKT is important for steroidogenesis and may play a role in FSH-stimulated CTNNB1 that is required for E_2 production (Hernandez Gifford et al., 2009).

It remains unclear whether FSH regulation of CTNNB1 accumulation is through the regulation of AKT or WNT*2* signaling pathways in bovine granulosa cells (GC). The current study will examine AKT's role in the accumulation CTNNB1.

MATERIALS AND METHODS

Tissue Collection

Bovine ovaries were collected at a local abattoir (Creekstone Farms, Arkansas City, KS) from natural, non-pregnant cows and heifers not exposed to implants, ionophores or antibiotics. For the first study, 40 pair of bovine ovaries at random stages of the estrous cycle were collected

to evaluate FSH regulation of AKT meditated CTNNB1, pAKT, and AKT protein accumulation utilizing an AKT inhibitor. A second study was conducted to evaluate the ability other known AKT stimulators on CTNNB1 accumulation. For the second study, 60 pair of bovine ovaries at random stages were collected for the evaluation of CTNNB1, pAKT, pGSK3-β, and AKT. Ovaries were rinsed with 0.9% saline, followed by a second rinse with 70% ethanol and placed in ice cold antibiotic saline solution containing 0.15 M NaCl with 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Grand Island, NY) for transport to the laboratory. Upon arrival, antibiotic solution was removed and a replaced with fresh antibiotic saline solution at room temperature. Granulosa cells from small follicles (1 to 5 mm) were isolated by follicular fluid aspiration, using a 3-mL syringe fitted with a 20-gauge needle. The suspension was centrifuged at 220 x g for 7 min at 4° C to recover GC.

Granulosa Cell Culture

To determine the role of AKT on CTNNB1 accumulation and subsequent steroidogenesis, isolated GCs were cultured using methods previously described (Castanon et al., 2012). Follicle size was selected based on indications that follicular recruitment occurs when follicles are 4 to 6 mm in diameter in cattle (Savio et al., 1988; Sirois and Fortune, 1988) and granulosa cells acquire FSH receptors prior to follicular recruitment (Xu et al., 1995). Furthermore, there is no detection of *LHCGR* mRNA before expression of steroidogenic enzyme mRNAs in GC of recruited follicles (Bao et al., 1997). Isolated GCs were re-suspended and washed twice in short-term medium (1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 containing 0.12 mM gentamycin, 2.0 mM glutamine, and 38.5 mM sodium bicarbonate obtained from Sigma-Aldrich, St. Louis, MO). Following the final wash, cells were re-suspended in 8 mL of re-suspension medium (serum free medium with 2.5 mg/mL collagenase and 1 mg/mL DNase, to prevent cell clumping) (Sigma-Aldrich), and cell number

and viability were determined by trypan blue exclusion using a hemocytometer. Dose response for LY294002 (Sigma-Aldrich) and IGF-I were conducted in 6-well plates, cells were seeded at 9.0 x 10^5 in complete medium (1:1 DMEM and Ham's F-12 containing 10% fetal bovine serum, 0.12 mM gentamycin, 2.0 mM glutamine, and 38.5 mM sodium bicarbonate). For experiment 1 and 2, cells were seeded at a density of 9.3 x 10^5 in complete medium in 60-mm culture dishes for protein. For hormone analyses 35-mm culture dishes were seeded at 4.0 x 10^5 in DMEM-F12 complete medium. Granulosa cells were incubated for 22 to 26 h at 38.5° in 5% CO_2. Medium and unattached cells were removed, wells were washed with warm 1x PBS, and fresh complete medium was replaced. The AKT inhibitor LY294002 (Sigma-Aldrich) dose response study the GC at a confluency of 70-85% were washed to remove medium and unattached cells, GC were then treated with LY294002 at 10, 20, 30, or 40 μM or dimethyl sulfoxide (DMSO) for 24 h in serum-free medium. The 30 μM dose of LY294002 was chosen based on greatest effect on CTNNB1 accumulation with out resulting in cell death, which was seen at the highest dose of LY294002. For experiment (1) GC at 70-85% confluency, were washed to remove medium and unattached cells, and GC were then treated with LY294002 (Sigma-Aldrich) at 30 μM or DMSO for 30 min in serum-free medium supplemented with 10^{-7} M testosterone propionate (Sigma-Aldrich). Following pretreatment 100 ng/mL of purified human FSH (S1AFP-B-3; National Hormone and Peptide Program, National Institutes of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD) or vehicle was added directly to the well for 24 h. Medium was collected from the 60-mm dishes for steroid analyses and bovine GCs were collected in 250 μL of mammalian-protein extraction reagent (M-PER) lysate buffer (Thermo-Scientific, Rockford, IL), and samples were stored at -20° C until analysis. For experiment (2) a dose response for IGF-I was conducted, at 70% confluency, medium and unattached cells were removed, and GCs were then treated with 0, 10, 25, 50, or 100 ng/mL of human insulin-like growth factor 1 (IGF-I; Sigma-Aldrich). An initial dose response study provided that at 50 and 100 ng/mL of IGF-I would maximize the CTNNB1 accumulation above controls, thus a dose of

50 ng/mL IGF-I was chosen for treatments. For experiment 2, GC at a confluency of 70% were treated with or without 100 ng/mL of hFSH as previously described by Castanon et al. (2012). Directly following hFSH treatment, IGF-I was added at 0 or 50 ng/mL in serum-free medium supplemented with 10^{-7} M testosterone propionate (Sigma-Aldrich) for 24 h. Bovine GC in 60-mm were collected in 250 μL of M-PER (Thermo-Scientific), medium was collected from 35-mm plates and medium stored at -20˚C until samples were analyzed. Following a wash with 1x PBS, cells were removed from plate 35-mm plate using 1 mL of TrypLE Select (Invitrogen, Grand Island, NY). TrypLE Select was incubated for 5 min at 38.5° in 5% CO_2. Cells were centrifuged at 220 x g for 7 min at 4° C. Cells were reconstituted with 500 μL of 1x PBS. Granulosa cell numbers were determined by trypan blue exclusion using a hemocytometer.

Indirect Immunoflorescence

Indirect immunofluorescence was performed to observe the translocation of CTNNB1 in a FSH time course study. Prior to seeding GCs, cover slips (Fisher Scientific) were flamed and treated with Poly-Lysine (Fisher Scientific) to allow attachment of cells to the cover slips. Granulosa cells were counted, and seeded at 1.0 x 10^6 in complete medium. When cells reached 70-85% confluency, GC were washed to remove medium and unattached cells, GC were then treated with or without 100 ng/mL of purified human FSH in serum-free medium supplemented with 10^{-7} M testosterone propionate (Sigma-Aldrich) prior to immunofluorescence. Medium was collected from the 3-mm dishes for steroid analysis, and washed with PBS. Bovine granulosa cells were fixed at 0 min, 30 min, 3 h, 6 h, 12 h, 18 h 24 h, and 48 h. Bovine GCs were fixed with 4 % paraformaldehyde for 20 min, fixative was aspirated and wash 3 times with PBS for 5 min each on rocking platform. Next, cells were permeabilized for 5 min with 0.1 % triton x 10 diluted in 1x PBS at room temperature. Cells were subjected to 3 washes with 1x PBS for 5 min each on a rocking platform. Granulosa cells were then incubated in 50 mM ammonium chloride for 15

min to quench free aldehyde groups from PFA fixation. The GC on the cover slips were then placed in a humid light-tight box for the remainder of processes. The GC were blocked in 10% goat serum in 1x PBS for 30 min. Primary antibody *a-mouse β-catenin* at 1:1000 was pipetted directly onto each slip and a piece of parafilm was placed on the slip with the antibody to prevent evaporation. The fixed bovine GCs incubated overnight with the primary antibody. Bovine GC were washed 3 washes with 1x PBS for 5 min each. Subsequently, keeping the cells in the dark, secondary antibody of AlexaFlour 488-goat-α-mouse and fluorecein isothiocyanate (FITC) at a concentration of 1:1000 was added directly to the slip and covered with parafilm. Secondary antibody incubated for 2 h at room temp, followed by 3 washes with 1x PBS for 5 min each. Bovine GC nuclei were stained with 1 μg/mL DAPI (Invitrogen). The excess moisture was removed. Cover slips were then mounted to a glass slide by placing cell side down on a drop of Vectasheild mounting medium (Vector Labs). Excess mounting medium was removed and after 10 -15 min of drying in a dark drawer, clear nail polish was used to seal the edges. Slips remained in the dark until fluorescence was detected.

Western Blot

Total protein lysate was isolated from GC collected in M-PER lysate buffer, according to manufacturer's protocol. Protein concentrations from primary cultures of bovine GC incubated with treatments as described above, were quantified using bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL). Protein lysates from granulosa cells (10 μg) were separated by 10% SDS-PAGE Tris-HCL gels and transferred to Hybond-C Extra nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ). Protein concentrations were chosen on the ability to detect the greatest measurable differences (pAKT and pGSK3-β). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h. The membrane was incubated in primary rabbit anti-β-actin at

1:10,000. β-actin was used as loading control for total protein. The membrane was rocked at room temperature for 1 h with secondary antibody, horseradish peroxides-conjugated (HPR) goat anti-rabbit (Thermo-Scientific) at a concentration of 1:10,000. Antibody was detected using chemiluminescence with immoblotin detection reagent (Millipore, Billerica, MA) and visualized via autoradiograph. The same process was used for all blots and membrane was probed for protein of interest. β-catenin was detected using primary mouse anti-β-catenin 1: 10,000 (BD Transduction Laboratories, San Diego, CA) and secondary goat antimouse 1:10,000 (Thermo-Scientific). Rabbit anti-phosphoAKT and rabbit anti-phosophoGSK3-β (Cell Signaling Technology, Danvers, MA) final concentration was 1:1000, and HPR-conjugated secondary antibody 1:3000 (Thermo-Scientific). Rabbit anti-AKT concentration was 1:2000 with HPR-conjugated secondary antibody 1:5000 (Thermo-Scientific). Relative abundance of proteins was quantified using AlphaEaseFC image acquisition system (Alpha Innotech, San Leandro, CA). Immunoreactive band intensity was determined in relation to the β-actin loading control. If no band was present in target protein, a value of zero was assigned to allow for statistical analysis.

Radioimmunoassays

Granulosa cell culture medium were analyzed for E_2 and P_4 by solid phase RIA using components of commercial kits manufactured by Siemens Medical Diagnostics Corp. (Los Angeles, CA) as previously described (Castanon et al. 2012). The E_2 concentration in samples for cell culture medium was determined in 200 μL medium. The specific binding was 62.5%, and detection limit (95% of maximum binding) of the assay was 2 pg/mL. Intra-assay CV for E_2 was 6.5%. For P_4 analysis, samples of GC culture medium were diluted 1:10 in assay buffer and assayed at 100 uL. The specific binding for P_4 was 58.8%, and detection limit (95% of maximum binding) of the assay was 0.1 ng/mL. Intra-assay CV for P_4 was 4.1%.

Statistical Analysis

For experiment 1, AKT was inhibited in bovine GCs. Medium E_2 and P_4 concentrations are representative of entire cell population of 60-mm plates. Steroid production and protein abundance were analyzed using the 1-way ANOVA procedure of SAS (Version 9.3; SAS Institute, Inc., Cary, NC). Experiment 2 bovine GC were treated with AKT stimulators FSH and IGF-I. IGF-I is a mitogen, to account for cell proliferation; steroid production was expressed as pg/mL (E_2) or ng/mL (P_4) per 10^5 GC (after cell culture) or as total (entire cell population in 30-mm dish) pg/mL (E_2) or ng/mL (P_4). Medium steroid concentration and protein abundance for experiment 2 were analyzed using the 1-way ANOVA procedure of SAS (Version 9.3; SAS Institute, Inc., Cary, NC). For all experiments significance set was at $P = 0.10$ and tendency at $P = 0.15$.

Results

LY294002 dose response in bovine GCs

The AKT inhibitor LY reduced healthy GC numbers and confluency in all doses tested (10, 20, 30, and 40 μM). The highest doses of 40 μM had low concentrations of protein due to the high rate of cell death. The remaining dose of LY294002 (10, 20, and 30 μM) appeared to decrease CTNNB1 accumulation compared to control GCs. The dose of 30 μM was chosen for future experiments, because it had the greatest reduction in CTNNB1 compared to control, 10 and 20 μM of LY294002, and had reduced cell death (reduced floating cells and an increased confluency) compared to the 40 μM LY294002 treatment group (Figure 1A).

IGF-I dose response in bovine GCs

A single dose response study was conducted on GC were treated with IGF-I at 0 (control), 10, 25, 50, 100 ng/mL of IGF-I based on previous literature. β-catenin accumulation in the low doses (10 and 25 ng/mL) slightly increased, but comparable to control treated GC. Insulin-like growth factor 1 at 50 and 100 ng/mL appeared to increase CTNNB1 above control, suggesting IGF-I regulation of CTNNB1 (Figure 1B).

FSH Immunofluorescence time course in bovine GCs

The accumulation and localization of CTNNB1 was evaluated through immunofluorescence. Immunofluorescence allows qualitative analysis of CTNNB1 location. β-catenin is highly expressed in cell to cell junctions in the controls. β-catenin fluorescence in GC appears to be increased as early as 3 h after FSH treatment compared to the 0 h control bovine GCs. There appears to be an increase in β-catenin in the cytoplasm and in proximity to the nucleus at 3 h and to an even greater extent at 6 h after FSH-stimulation and this remained consistent through 24 h. β-catenin was located in the cell to cell junctions as well as the cytoplasm, and in some cases the nucleus of the bovine GCs. After 48 h of FSH stimulation β-catenin was reduced with no defined location (Figure 2).

AKT is required for CTNNB1 accumulation

Inhibition of AKT via the AKT inhibitor LY reduced the accumulation of AKT (Figure 3A) in GC treated with LY alone ($P = 0.02$) or in combination with FSH ($P < 0.01$) compared to control treated cells. Granulosa cell accumulation of AKT was not different between control and FSH treated groups ($P = 0.24$; Figure 3A). Treatment with the AKT inhibitor ablated pAKT, the active form of AKT (Figure 3B), compared to control and FSH treated GC ($P < 0.01$). Control

GC have an increased accumulation of pAKT compared to FSH alone ($P < 0.01$) when averaged across three experiments. Inhibition of AKT signaling alone reduced β-catenin accumulation compared to the non-inhibited groups ($P = 0.02$; Figure 3C). No change was detected between control as compared to FSH. Treatment of LY reduced the accumulation of CTNNB1 compared to control GC ($P = 0.04$). β-catenin accumulation was reduced in AKT inhibited GC treated with FSH compared to FSH alone ($P = 0.02$; Figure 3C).

AKT inhibition reduces steroid production

Steroid production in response to AKT inhibition was determined in medium samples of cultured GCs via RIA. Progesterone production did not differ among the treatment groups ($P = 0.18$; Figure 4A). Medium E_2 production increased only in FSH-treated cells ($P = 0.02$). FSH-stimulated estradiol (67.7 ± 9.6 ng/mL) production was reduced in AKT inhibited cells and in combination with FSH (20.2 ± 9.6 ng/mL) as depicted in Figure 4B.

FSH and IGF-I stimulation increases E_2 and P_4 production

In experiment 2, known AKT stimulators FSH and IGF-I, were employed to determine their ability to facilitate CTNNB1 accumulation and subsequent steroidogenesis in bovine GC (Table 1). Progesterone concentrations increased in medium of bovine GC treated with IGF-I and co-incubation with FSH + IGF-I compared to control and FSH ($P < 0.01$). A similar pattern was demonstrated in medium E_2 concentrations as bovine GC treated with FSH in combination with IGF-I produced an increase of E_2 compared to control and FSH ($P = 0.04$). Control (93.2 ± 65.1 pg/mL) and FSH (140.5 ± 65.1 pg/mL) treated cells did not significantly differ, as depicted in Table 1.

FSH increases β-catenin but not pAKT, while IGF-I increase pAKT does not regulate β-catenin

In experiment 2, AKT accumulation did not differ among treatment groups (P = 0.72; Figure 5A). Granulosa cells treated with FSH, IGF-I, or the combination did not stimulate pAKT accumulation (*P* = 0.32; Figure 5B). Accumulation of pGSK3-β, was greater in control and FSH-treated GC than IGF-I treated (*P* = 01; Figure 5C). The inactive GSK3-β accumulation is decreased by 9 fold in GC treated with FSH in combination with IGF-I compared to control and FSH (*P* = 0.01). Total β-catenin protein in bovine GC was increased in FSH compared to control and IGF-I (*P* < 0.01; Figure 5D). The accumulation of β-catenin was comparable between control and IGF-I, but IGF-I and FSH-induced accumulation of CTNNB1 is reduced (*P* = 0.02) compared to FSH alone.

Discussion

The hypothalamic-pituitary-gonadal axis is vital for the regulation of folliculogenesis and steriodogenesis. Follicle-stimulating hormone from the pituitary regulates follicle growth and activates intraovarian signaling pathways in GC resulting in CTNNB1 accumulation. The canonical WNT signaling pathway is known for regulating CTNNB1 accumulation, the disassociation of the CTNNB1 degradation complex, allows CTNNB1 to interact with transcription factors in the nucleus. β-catenin interacts with transcription factors SF1 to transcribe genes, namely *Cyp19a1* (Parakh et al., 2006). Aromatase is required for enzymatic conversion of testosterone to E_2. The transcriptional co-activator CTNNB1 is required for maximal FSH treatment of *Cyp19a1* mRNA expression, and subsequent E_2 production in rodents (Hernandez Gifford et al., 2009). The WNT signaling pathway is important in embryonic development and for normal follicular development has been demonstrated in rodents (Brunet et

al., 1999; Boyer et al., 2010b). In bovine embryos AKT is important for the inactivation of GSK3-β, a reduction in pGSK3-β decreased embryo development and quality, also implicated involvement in WNT signaling (Aparicio et al., 2010). Recent studies in large bovine ovarian follicles having the greatest E_2, also had an increase in CTNNB1 (Castanon et al., 2012). Bovine GC cultured in the presence of FSH have an increased accumulation of CTNNB1, as well as AKT protein and *WNT*2 mRNA expression indicating their importance during late follicle development (Castanon et al., 2012). However, the mechanisms of CTNNB1 accumulation are not fully understood. The present study extends these finding to demonstrate that AKT is important for downstream activation of FSH activated CTNNB1 accumulation and subsequent E_2 production in bovine GC, as demonstrated through AKT inhibitor studies. AKT is needed in the bovine follicle to mediate FSH and IGF stimulation of inhibin-A, E_2, and P_4 secretion (Kemler, 1993). Follicle-stimulating hormone activates AKT, which aids in disassociation of the CTNNB1 degradation complex. Data from experiment 1 (Figure 3C) demonstrated that AKT is required for FSH-mediated CTNNB1 accumulation. AKT inhibitor LY294002 reduces FSH activation of AKT in porcine GC (Johnson et al., 2001). In the first study, data demonstrated AKT protein was greater in control compared to FSH alone; this is likely attributed to DMSO, which serves as the vehicle control. Naito et al. (2005) demonstrated that DMSO was able to increased pAKT and CTNNB1 in caridomyiod cells. Stimulation of FSH leads to the phosphorylation of AKT, which deactivates GSK3-β through phosphorylation on Ser-9 (Uzbekova et al., 2009). Deactivation of GSK3-β leads to the accumulation of CTNNB1, as GSK3-β is part of the CTNNB1 degradation complex. The CTNNB1 degradation complex is comprised of GSK3-β, APC, and axin (Cadigan and Nusse, 1997). Follicle-stimulating hormone would increase pGSK3-β and subsequently CTNNB1 protein accumulation, and E_2 concentrations. In the current study, inhibition of AKT via LY294002 ablates pAKT and reduces CTNNB1 accumulation. AKT inhibited cells had reduced FSH-mediated steroid production concentration in the medium. However, data from experiment 2, deactivation of GSK3-β was not increased above controls. The phosphorylation of

GSK3-β is expected to allow CTNNB1 to escape phosphorylation and ubiquitination, which can then accumulate and move to the nucleus. Thus expecting an increase of pGSK3-β in treatment groups with increased CTNNB1.

Since FSH increases CTNNB1 accumulation in bovine GCs at 24 h (Castanon et al., 2012), and our data shows AKT is mediating CTNNB1 accumulation down stream of FSH-stimulation. The next studies explored another known AKT stimulator IGF-I, to investigate its ability to regulate CTNNB1. Insulin-like growth factor 1 stimulation of CTNNB1 levels were not greater than control at 24 h of stimulation, however, in a preliminary IGF-I dose response study GC treated with 50 ng/mL of IGF-I appeared to regulate CTNNB1. Among individual studies, IGF-I mediated CTNNB1 protein abundance varied from control (lowly abundant) to FSH (highly abundant). The activated form of AKT (pAKT) was not different among any group. Also, IGF-I is not deactivating GSK3-β through the phosphorylation of Ser-9, which can lead to CTNNB1 accumulation. Mani et al. (2010) showed an increase pAKT to AKT ratio accumulation in IGF-I-treated GC compared to FSH-treated bovine GC, suggesting IGF-I does signal through AKT. Insulin-like growth factor 1 activation of AKT increases expression of steroidogenic genes *CYP11A1*, *HDS3B1*, and *CYP19A1*, which produces enzymes active in the conversion of cholesterol to estradiol (Mani et al., 2010). Follicle-stimulating hormone and IGF-I treatments together synergize to increase *Cyp19a1* mRNA and E_2 production (Spicer and Aad, 2007; Mani et al., 2010). In the current study, P_4 and E_2 for total and per10^5 cell production respectively follow the same trend. Progesterone concentrations in medium were increased in IGF-I alone and combined with FSH compared to control and FSH treated GC. This is expected as IGF-I influenced gene transcription of earlier steroidogenic genes that produce P_4, compared to FSH-stimulation of aromatase that converts androgens to E_2 in the GC. There was also an increase in E_2 production in the IGF-I treated GC compared to control and FSH treated cells. Follicle-stimulating hormone stimulation of CTNNB1 protein (expressed as fold of control) was lower in current study than those demonstrated by Castanon et al. (2012). This suggests that in

order for FSH alone to increase aromatase, a minimum CTNNB1 response is required. Further research will be required to verify this suggestion.

Data for the second study show that pAKT accumulation at 24 h is reduced in FSH treated GC compared to control GC, however IGF-I treated GC appear to increase pAKT. Activation of AKT through FSH and IGF-I stimulation maybe observed through collecting cells at 3 h or after 48h. The phosphorylation of AKT through IGF-I in bovine GC occurs as early as 5 min, and pAKT/AKT ratio is greatest at 15 min and the ratio decreases, but remains above the initial 5 min activation (Mani et al., 2010). Ryan and colleagues (2008) show that sheep GC stimulated with FSH accumulated more pAKT than IGF-I alone, however the cells were in culture for 144 h, 68 h longer than the present study. Follicle-stimulating hormone-mediated AKT phosphorylation/activation in rat GCs is detected by 10 min, peaks at 1 h, and is undetectable 4 h after the FSH treatment and has a biphasic increase (below previous high accumulation) around 24-48 (Gonzalez-Robayna et al., 2000; Alam et al., 2004). In the current studies, FSH-stimulation of GCs did not increase total AKT accumulation as previously seen in bovine GC at the 24 h time point (Castanon et al., 2012) and sheep GCs (Kemler, 1993). Additionally, phosphorylation of GSK3-β did not increase in FSH-stimulated GC as we hypothesized; we expected an increase in pGSK3-β accumulation to follow the increase of CTNNB1. There is an increase of pGSK3-β in IGF-I-treated samples compared to FSH and IGF-I, while the bovine GCs treated in combination of FSH and IGF-I had an increase of CTNNB1 protein. A decrease in CTNNB1 accumulation in GC co-incubated in FSH and IGF-I suggests the synergy/additive effect of FSH and IGF-I is not entirely through the AKT's accumulation of CTNNB1.

In conclusion, results from the current study demonstrate that AKT is required for FSH-induced accumulation of CTNNB1 and steroid synthesis. Further studies on the ability of IGF-I regulate CTNNB1 accumulation in bovine GC are required. AKT is necessary for FSH-induced

accumulation of CTNNB1, and AKT alone is not responsible for FSH and IGF-I induced synergy of steroidogenesis.

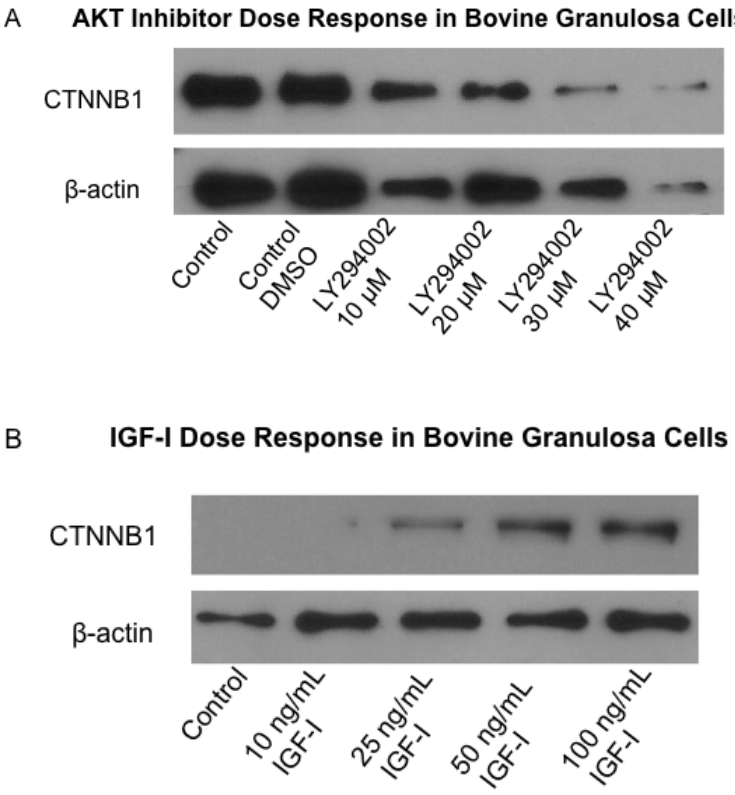
A
AKT Inhibitor Dose Response in Bovine Granulosa Cells
CTNNB1
β-actin
Control
Control DMSO
LY294002 10 μM
LY294002 20 μM
LY294002 30 μM
LY294002 40 μM
B
IGF-I Dose Response in Bovine Granulosa Cells
CTNNB1
β-actin
Control
10 ng/mL IGF-I
25 ng/mL IGF-I
50 ng/mL IGF-I
100 ng/mL IGF-I

Figure 1.

Figure 1. β-catenin accumulation Western blot (WB) analysis was performed to measure CTNNB1 accumulation. Effect of AKT inhibitor or stimulator IGF-I on protein in cultured bovine granulosa cells (GC) from small follicles (1 to 5 mm). **(A)** Bovine GC were pre-treated with AKT inhibitor LY294002 at doses of 10, 20, 30, or 40 μM or DMSO for 30 min, and incubated for 24 h prior to collection. Using M-PER, total protein was collected. β-actin was used as the loading control for proteins of interest. **(B)** Granulosa cells were treated with 0, 10, 25, 50, and 100 ng/mL IGF-I for 24 h, total protein was also collected using M-PER.

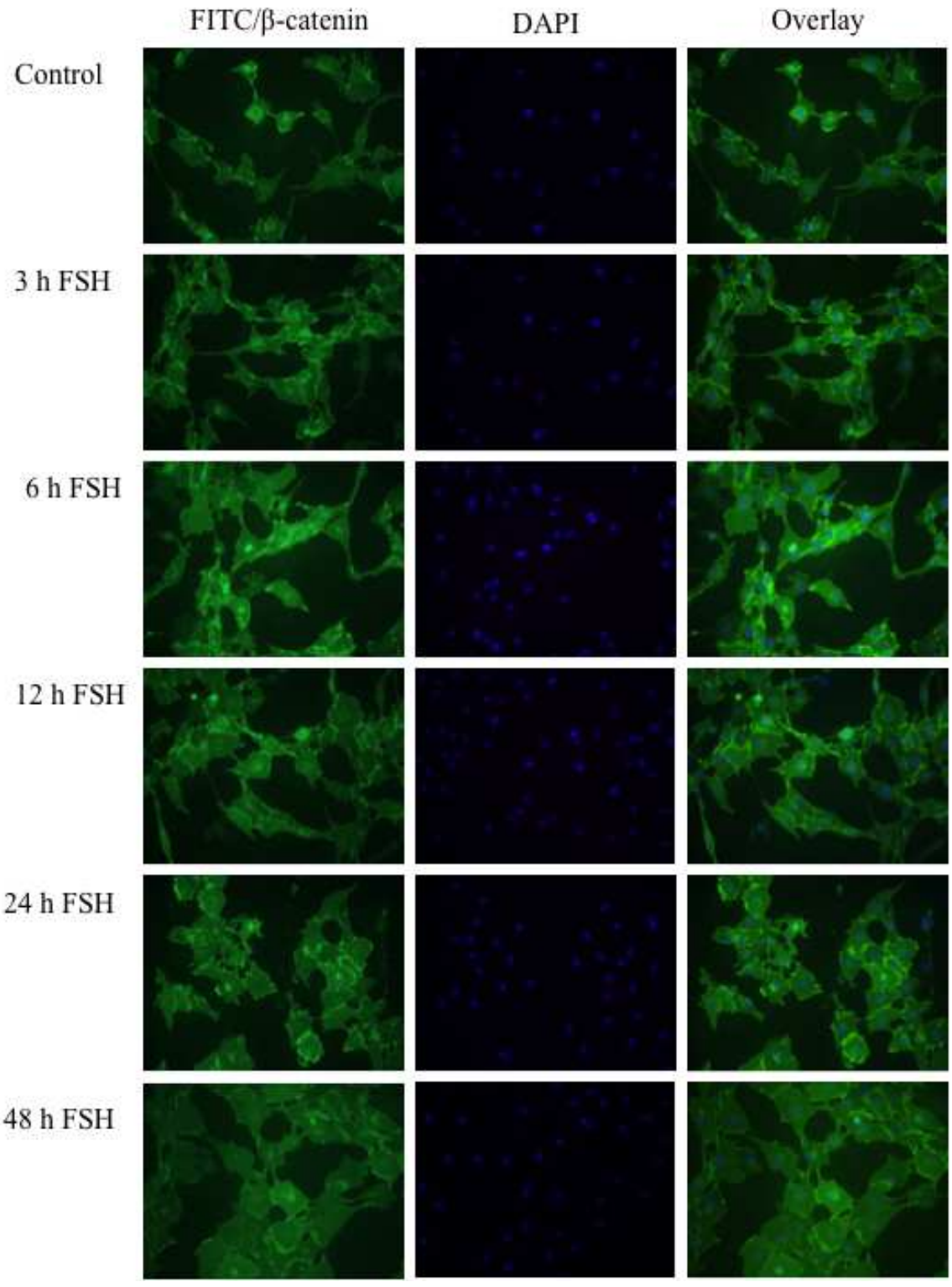
FITC/β-catenin
DAPI
Overlay
Control
3 h FSH
6 h FSH
12 h FSH
24 h FSH
48 h FSH

Figure 2.

Figure 2. Effect of FSH treatment intervals on cellular localization of CTNNB1. Bovine granulosa cells were fixed and stained at 0, 3, 6, 12, 24, and 48 h after FSH treatments. β-catenin protein is represented in the green florescent (FITC)**,** the middle panel references the cell's nucleus (DAPI), and the third column is a merge of FITC and DAPI to show location and movement of CTNNB1 after FSH stimulation. An increase in CTNNB1 can be observed at 3 h of FSH with stronger fluorescence at 6 h of stimulation; at 24 and 48 h CTNNB1 appears reduced compared to 6 h.

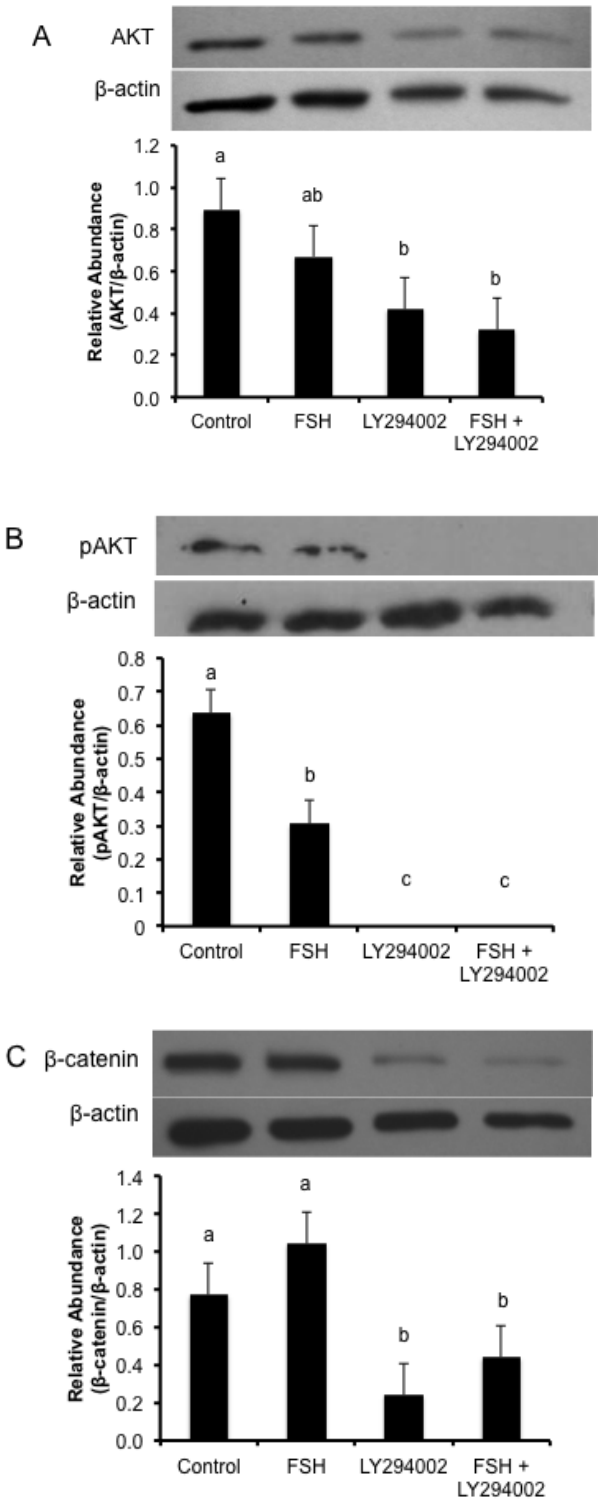
A
AKT
β-actin
Relative Abundance (AKT/β-actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
ab
b
b
Control
FSH
LY294002
FSH + LY294002
B
pAKT
β-actin
Relative Abundance (pAKT/β-actin)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
c
c
Control
FSH
LY294002
FSH + LY294002
C
β-catenin
β-actin
Relative Abundance (β-catenin/β-actin)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
b
Control
FSH
LY294002
FSH + LY294002

Figure 3.

Figure 3. Effects of AKT inhibitor LY294002 on bovine granulosa cells (GC) with and without FSH stimulation were analyzed using Western blot (WB). Total protein was collected from freshly isolated GC from isolated small follicles (1 to 5 mm). The bovine GC were pre-treated with AKT inhibitor LY294002 (30 μM) or vehicle for 30 min, and subsequently treated 100 ng/mL of FSH for 24 h. Using M-PER a lysate buffer, total protein was collected and β-actin was used as the loading control for proteins of interest. **(A)** Representative WB showing reduced accumulation of AKT in LY294002 treated cells. Quantitative analysis of AKT protein abundance, indicates successful inhibition of the AKT pathway ($P = 0.02$). In GC treated with LY alone ($P = 0.02$) or in combination with FSH ($P < 0.01$), AKT tended to be reduced compared to control. **(B)** The activated form of AKT (pAKT) protein abundance showed a dramatic decrease in LY-treated cells indicating AKT signaling inhibition ($P < 0.01$). Lanes with no band were assigned zeros, with no difference among treatment group; there is no error to report. **(C)** FSH-mediated CTNNB1 accumulation was reduced in the AKT inhibited GC compared to FSH treated GC ($P = 0.02$). Treatment effect of LY compared to control and FSH ($P \leq 0.05$ and 0.01) respectively. β-actin served as a loading control. Columns represent the mean ± SEM, n = 4. Columns with different letters differ ($P \leq 0.10$).

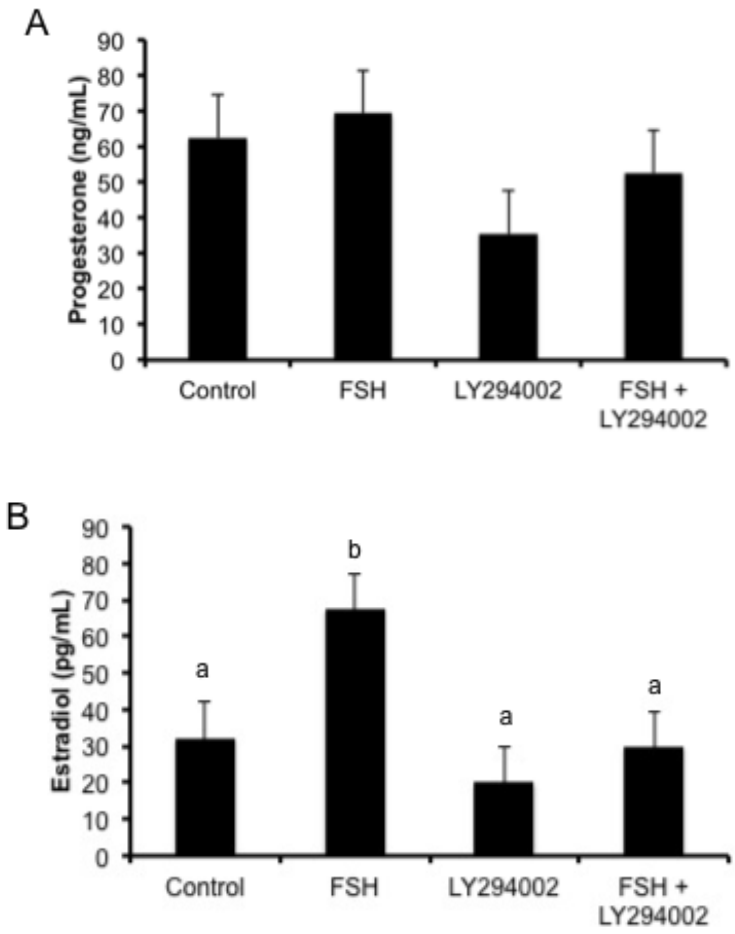
A
Progesterone (ng/mL)
90
80
70
60
50
40
30
20
10
0
Control
FSH
LY294002
FSH +
LY294002
B
Estradiol (pg/mL)
90
80
70
60
50
40
30
20
10
0
a
b
a
a
Control
FSH
LY294002
FSH +
LY294002

Figure 4.

Figure 4. Inhibition of AKT on P_4 and E_2 production by cultured granulosa cells (GC). Bovine GC pretreated 30 min with LY294002 or vehicle were subsequently treated with 100 ng/mL of FSH or vehicle for 24 h prior to medium collection. **(A)** Progesterone production by bovine GC pre-treated 30 min with vehicle or AKT inhibitor LY294002 (30μM) and subsequent 24 h treatment with vehicle or FSH (100 ng/mL) was unaffected by treatments ($P = 0.18$). **(B)** Estradiol production by GC was increased in FSH treated GCs versus AKT inhibited GC subsequently treated with FSH, suggesting AKT is important FSH mediated E_2 production. Columns represent the mean ± SEM, n = 4. Columns with different letters differ ($P \leq 0.10$).

Media concentrations of progesterone and estradiol in bovine granulosa cells

	Progesterone		Estradiol	
	P_4, ng/mL	$P_4/10^5$ cells**	E_2, pg/mL	$E_2/10^5$ cells**
Control	$72.7^{a} \pm 13.3$	26.4 ± 16.1	$93.0^{a} \pm 65.1$	$26.7^{a} \pm 21.4$
FSH	$87.7^{a} \pm 13.3$	24.9 ± 16.1	$140.7^{a} \pm 65.1$	$37.8^{a} \pm 21.4$
IGF-I	$161.8^{b} \pm 13.3$	54.3 ± 16.1	$280.2^{b} \pm 65.1$	$76.3^{ab} \pm 21.4$
FSH + IGF-I	$185.9^{b} \pm 13.3$	72.0 ± 16.1	$343.5^{b} \pm 65.1$	$115.7^{b} \pm 21.4$

[a,b] Within a column, means with different subscripts differ $P = 0.10$, n = 4

**Steroid concentration was divided by cell numbers

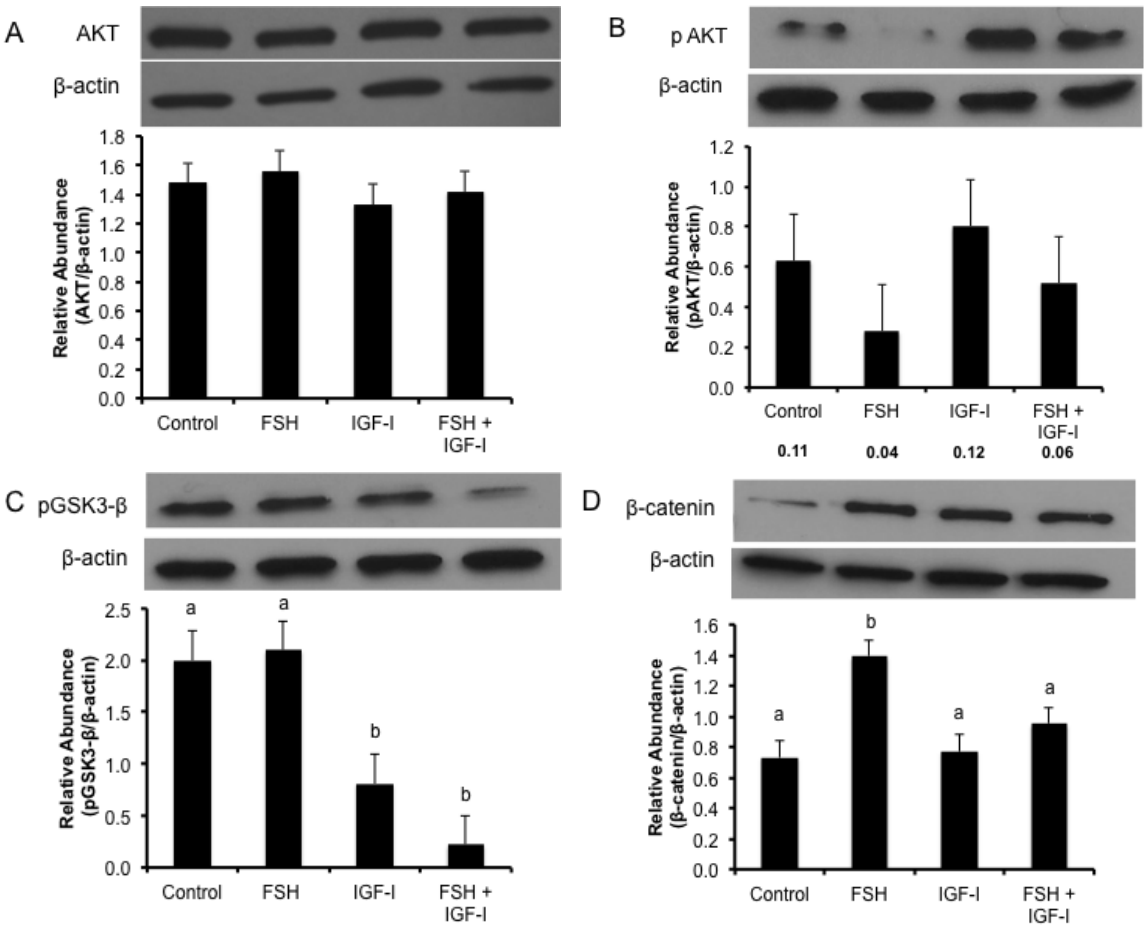
A
AKT
β-actin
Relative Abundance (AKT/β-actin)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
FSH
IGF-I
FSH + IGF-I
B
p AKT
β-actin
Relative Abundance (pAKT/β-actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
FSH
IGF-I
FSH + IGF-I
0.11
0.04
0.12
0.06
C
pGSK3-β
β-actin
Relative Abundance (pGSK3-β/β-actin)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
b
Control
FSH
IGF-I
FSH + IGF-I
D
β-catenin
β-actin
Relative Abundance (β-catenin/β-actin)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
a
Control
FSH
IGF-I
FSH + IGF-I

Figure 5.

Figure 5. Effect of FSH and/or IGF-I stimulation on accumulation of AKT, pAKT, pGSK3-β, and CTNNB1 protein levels in bovine granulosa cells (GC). Total protein was collected with reagent M-PER from cultured GC from small follicles (1 to 5 mm) after 24 h treatment with FSH (100 ng/mL), IGF-I (50 ng/mL) or co-incubation. Western blots (WB) were performed for analysis, using β-actin as loading control. **(A)** Representative WB of AKT accumulation in bovine GC of AKT accumulation after FSH and/or IGF-I treatment of GC ($P = 0.72$). **(B)** Representative WB of the activated form of AKT, pAKT accumulation and quantitative analysis of pAKT, after FSH and/or IGF-I treatment of GC ($P = 0.49$). Number at the bottom of the column is pAKT to AKT ratio. **(C)** Phosphorylation of GSK3-β accumulation was decreased in IGF-I treated GC. **(D)** Accumulation of CTNNB1was increased in FSH treated GC ($P = 0.01$), whereas IGF-I alone did not affect CTNNB1, but reduced the FSH-induced CTNNB1 levels. Columns represent the mean ± SEM, n = 3. Columns with different letters differ ($P \leq 0.10$).

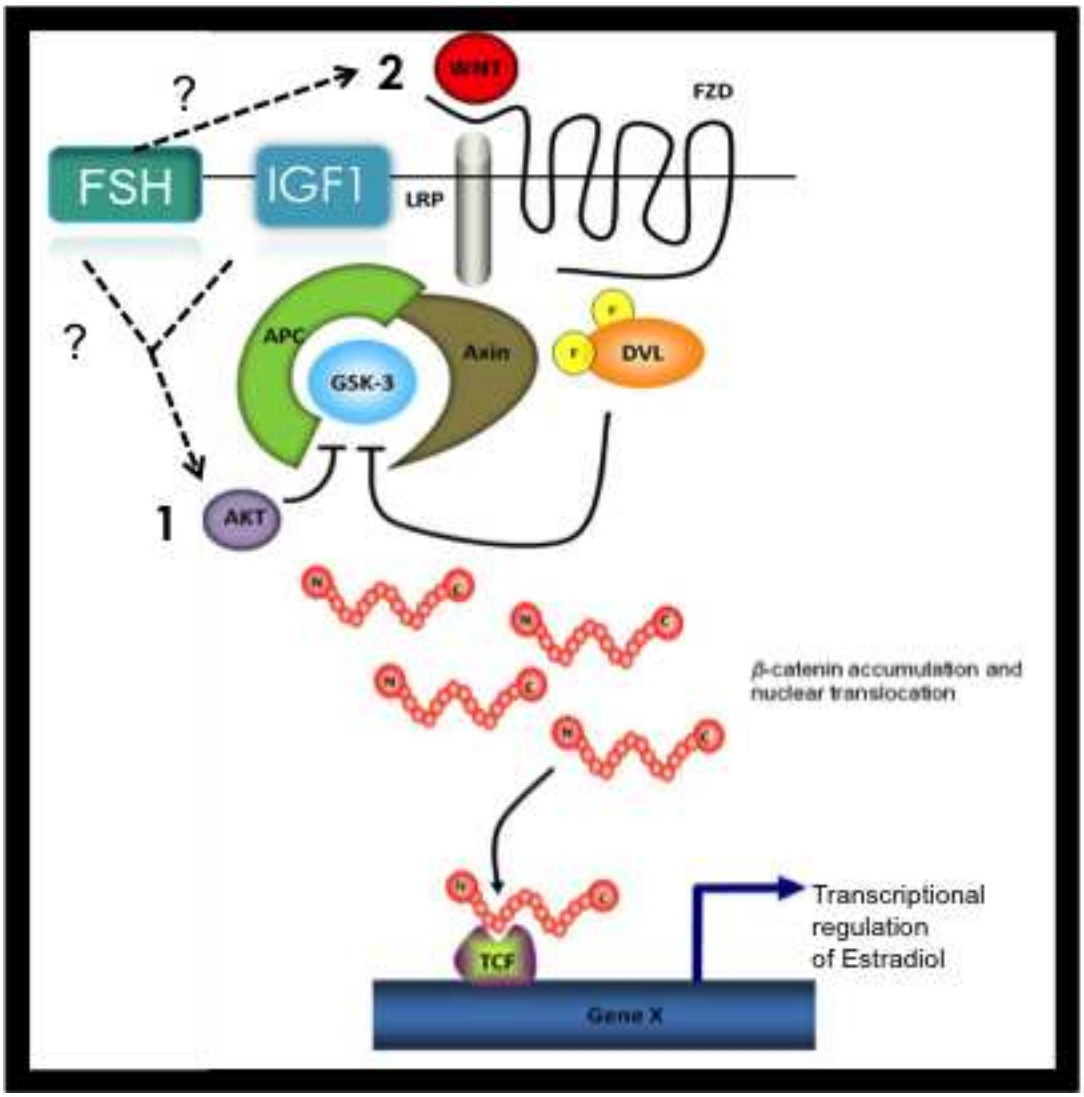
?
2
WNT
FZD
FSH
IGF1
LRP
?
APC
Axin
GSK-3
DVL
1
AKT
β-catenin accumulation and
nuclear translocation
TCF
Transcriptional
regulation
of Estradiol
Gene X

Figure 6.

Figure 6. β-catenin accumulation is regulated by the AKT signaling and canonical WNT signaling pathway. Follicle-stimulating hormone activates AKT via phosphorylation in the disassociation of the CTNNB1 degradation complex allowing for the accumulation of CTNNB1 and transcriptional regulation, which is required for E_2 production. WNT binding co-receptors Frizzled and LRP leads to the phosphorylation of GSK3-β to disassociate the multi-protein complex to allow for CTNNB1 accumulation and translocation into the nucleus. β-catenin within the nucleus interacts with TCF/LEF for transcription of aromatase. Aromatase converts testosterone into E_2.

CHAPTER IV

SUMMARY AND CONCLUSION

Establishing potential mechanisms involved in bovine fertility is important for cattle producers to produce progeny as well as for scientists in pursuit of creating methods to regulate the cow's estrous cycle and establish normal intraovarian activity. Signaling pathways, pathway interactions, and physiological mechanisms in female reproduction are continually being discovered and differences between species is recognized. Similar among species, FSH stimulation of the GC converts testosterone into E_2. β-catenin accumulation through FSHmediation is needed for maximal E_2 and *Cyp19a1* mRNA production in rodents (Hernandez Gifford et al., 2009). Large bovine ovarian follicles with increased E_2 also have increased production of CTNNB1 and FSH-stimulation of bovine GC increased CTNNB1 and AKT proteins and *Wnt2* mRNA (Castanon et al., 2012).

The WNT and AKT signaling pathway are both known for the ability to phosphorylate GSK3-β, rendering the CTNNB1 complex inactive. β-catenin degradation complex is comprised of GSK3-β, APC, and axin. The phosphorylation of GSK3-β on Ser 9 makes the protein inactive, and disassociates the multi-protein complex. Inactivating the CTNNB1 degradation complex through FSH activating AKT signaling, in turn phosphorylating GSK3-β, as does WNT signaling through co-receptors Frizzled and LRP. Both AKT and WNT signaling pathways increase cytoplasmic CTNNB1 and translocation to the nucleus. Accumulation of CTNNB1 in the

nucleus is regulated by CTNNB1 escaping phosphorylation by the multi-protein degradation complex. The accumulation of CTNNB1 in the nucleus allows interaction with transcription factors (TCF/LEF and SF1) and subsequent gene transcription. The focus of these studies was to investigate AKT's role in CTNNB1 accumulation.

Our data shows normal production of E_2 and accumulation of CTNNB1 requires the AKT signaling pathway. Inhibition of AKT using LY294002 reduces FSH-mediated CTNNB1 accumulation and production of E_2. These data show for the first time that AKT is necessary for FSH-mediated accumulation of CTNNB1. IGF-I, another AKT stimulator may increase CTNNB1, however, IGF-I stimulation of AKT to stimulate steroid production does not require CTNNB1.

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