DEVELOPMENT AND APPLICATION OF A BIOASSAY FOR FOLLICLE-STIMULATING HORMONE

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Wayne Young
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Abstract

Follicle-stimulating hormone (FSH) is involved in the regulation and maintenance of vital reproductive processes, such as gametogenesis, follicular development and ovulation. Produced in the anterior pituitary, FSH is a glycoprotein hormone that exists as a family of isohormones. Follicle-stimulating hormone concentrations have traditionally been measured by radioimmunoassay (RIA). However, results generated using RIA are a determination of the immunological activity of FSH. The potential of FSH to generate a biological response cannot be measured by RIA. Therefore, the identification of physiologically significant differences in the activity of these isoforms requires the use of assay systems that can differentiate between the biological activity of the FSH isoforms.

Commonly used assays for measuring the biological activity of FSH are based on the measurement of aromatase activity in cultured rat Sertoli cells following stimulation with FSH. However, these assays have an inherently high ethical cost involved due to the use of primary tissue culture. In addition, the variation in these assays associated with differences between animals is difficult to eliminate. Recently a bioassay for human FSH has been described based on FSH stimulation of cyclic AMP production by a Chinese hamster ovary (CHO) cell line stably expressing the human FSH receptor (FSH-R). The purpose of this study was to evaluate the potential usefulness of this CHO FSH-R cell line expressing the human receptor for FSH to be used as a bioassay to measure the biological activity of ovine FSH. The receptor cell line bioassay described in this study is based on the ability of FSH to stimulate cAMP production by cultured CHO FSH-R cells. Optimisation of the culture system to enable the bioactivity of ovine FSH to be measured by bioassay was undertaken. This involved optimising the density of cultured cells, the time in culture and time exposed to FSH and the most suitable dose range for FSH. The influence of matrix effects, such as those exerted by serum was also investigated. The specificity of the assay towards FSH was also determined as was the sensitivity, accuracy and precision of the assay. No stimulation of cAMP production was seen in CHO FSH-R cells following treatment with α-FSH, β–FSH, LH, TSH, GH, prolactin or vasopressin at concentrations up to 10 μg/ml.

Although the methodology used differed slightly depending on the presence or absence of serum, all assayed were performed using the following methods and materials. Freshly thawed FSH-R cells were bulked up in culture, and aliquots of 1×10^5 to 5×10^5 cells/well dispensed into 48 well

culture dishes and incubated overnight at 37°C. The assay culture media was then replaced with 0.25 ml fresh media (α-MEM + 0.1% BSA + 0.25 mM 3-isobutyl-1-methyl-xanthine) containing varying doses of NIH-FSH-RP2 (RP2) FSH preparations or FSH containing samples, and the cells incubated for 4 hours at 37°C. The assay culture media was then removed and stored frozen at -20°C until assayed for cyclic adenosine monophosphate (cAMP) by RIA.

Once optimal assay conditions were determined, the CHO FSH-R cell bioassay was used to measure FSH concentrations in ovine serum, pituitary extracts and medium from cultures of ovine pituitary cells. It was found that the concentrations of FSH in serum from intact sheep was close to the detection limit of the assay. Thus, while FSH concentrations could be measured in serum from some sheep, other animals had concentrations that were too low to be accurately measured by the bioassay in its present form. The assay was, however, well suited to measuring FSH concentrations in serum from sheep that had elevated concentrations of FSH. In one study, FSH concentrations measured by the bioassay were compared to those measured by RIA in sheep that had been ovariectomised and then hypophysectomised. It was found that the profile of FSH concentrations following hypophysectomy was similar whether measured by RIA or by bioassay (R^2=0.7513), though absolute concentrations sometimes differed. This suggested that the immunoassay and bioassay were not always measuring the same characteristics of FSH. The assay was also used to measure FSH concentrations in samples of ovine hypophyseal venous blood. However, the results obtained for these samples indicated a poor correlation between FSH concentrations obtained by bioassay and RIA. Levels of bioactive FSH in hypophyseal venous blood fluctuated markedly and were up to 10-fold higher than the associated RIA concentrations.

The CHO-cell bioassay was also found to be very suitable for measuring pituitary concentrations of FSH. In one study, pituitary extracts underwent chromatography and the separated isoforms of FSH were analysed by bioassay and RIA. Again, there was excellent correlation (R^2=0.9328) between the concentrations of FSH measured both assay types. However, some differences were apparent suggesting a discrepancy in the biological and immunological characteristics of different FSH isoforms. The bioassay was also used to measure FSH concentrations in media from pituitary cells in tissue culture where serially diluted samples displayed good parallelism with the RP2 FSH standard curve.

Results of this study demonstrate that the CHO FSH-R cell bioassay is suitable for measuring the biological activity of ovine FSH in a variety of biological fluids. The use of a permanent cell line

eliminates the high ethical cost associated with primary tissue culture that other bioassay systems have. The inherent variation associated with culture systems utilising tissue from different sources is also avoided. The sensitivity of the bioassay is suitable for measuring FSH in surgically altered sheep or hypophyseal blood concentrations where FSH levels are generally higher than those in the peripheral circulation. In addition to blood samples, the bioassay is also excellent for monitoring FSH activity in pituitary extracts and in media from tissue culture. However, the sensitivity of the bioassay currently does not always allow measurement of bioactive FSH concentrations in serum samples with low FSH levels.

In summary, the CHO FSH-R cell bioassay described in this study offers a useful alternative to RIA and other bioassays for monitoring the biological activity of ovine FSH and its isoforms in various biological fluids. It is concluded that this convenient and robust bioassay may have considerable application in future investigations of ovine FSH bioactivity.

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Chapter One

INTRODUCTION

1 Introduction

Follicle-stimulating hormone (FSH) is a key glycoprotein hormone involved in the regulation and maintenance of essential reproductive processes, including gametogenesis, follicular development and ovulation (Scaramuzzi *et al*, 1993; Linsay, 1991) and acts by binding to specific receptors localized in the gonads. In the male, FSH acts on the Sertoli cells in the seminiferous tubules of the testis, to stimulate spermatogenesis. However, the mechanisms of FSH stimulation of spermatogenesis remains unclear. The target for FSH action in the female are the granulosa cells of follicles, where it stimulates production of estrogens and progestins, as well as various nonsteroidal substances (Dahl and Hsueh, 1988).

1.1 Structure of FSH

The two pituitary gonadotrophins, FSH and luteinizing hormone (LH) are members of the glycoprotein protein hormone family, which also include pituitary thyroid stimulating hormone (TSH), and the placental gonadotrophin chorionic gonadotrophin (CG), found only in equids and primates. All glycoprotein hormones are composed of a common α-subunit that is noncovalently associated with a hormone-specific β-subunit. In addition, all the glycoprotein hormones consist of a mixture of molecular isoforms. The nature of the microheterogeneity of glycoprotein hormones arise from differences in glycosylation of the hormones, including the degree of terminal sialylation and/or sulfation (Ulloa-Aguirre *et al*, 1995). The resulting acidic-base charge provides the major basis for separation by chromatographic techniques, such as isoelectric focusing or chromatofocusing (Simoni *et al*, 1994). Therefore, oligosaccharide chains of varying stages of completion, and different oligosaccharide structures are found on a single gonadotrophin. Although the heterogeneity of FSH is well established, regulation of FSH heterogeneity in the circulation is poorly understood. In addition, the functional significance of such a variety of isoforms for FSH remains unclear. Structural differences in these isoforms alter a number of physical and physiological properties of the hormone, including changes in receptor-binding activity, plasma half-life, and *in vivo* and *in vitro* bioactivity. Therefore a mixture of circulating FSH isoforms reaches target tissues within the gonads to influence a variety of physiological endpoints.

1.1.1 Glycosylation

Unlike other hormones now known to be glycoproteins, in which a single protein chain is glycosylated, both the α– and β-subunits of the glycoprotein hormones, including FSH, are glycosylated. For the α subunit of FSH (92-96 amino acids in length), the pattern of glycosylation is very consistent, with two N-linked glycosylation sites at asparagine (Asn) 52 or 56 and Asn78 or 82. Patterns of glycosylation on the β-subunit, however, are more variable than those found on the α-subunit. The β-subunit, comprised of 111-112 amino acids, exhibits two glycosylation sites at Asn6, 7, or 13 and Asn23, 24, or 30 (Ulloa-Aguire *et al*, 1995). Glycosylation and the processing of incorporated oligosaccharides involves a complex biosynthetic pathway that begins in the rough endoplasmic reticulum, and continues through the Golgi apparatus until the mature hormone is transported to secretory granules. The numerous carbohydrate intermediates resulting from processing in the Golgi apparatus are responsible for many of the FSH glycoforms secreted by the pituitary.

The oligosaccharide structures on FSH are highly variable and play a key role in determining the biological properties of the hormone. Most oligosaccharide chains in FSH are double branching structures with one end terminating in a negatively charged group (GalNAc-SO_4 or Gal-sialic acid) and the other branch terminating in either a negatively charged group or in non-charged mannose (Ulloa-Aguire *et al*, 1995). Tribranched oligosaccharides of different types containing sialic acid residues are also found. The main chemical basis for the isoform differences and extensive charge heterogeneity of FSH is attributed to variations in the structure and distribution of sialylated oligosaccharides. Follicle-stimulating hormone, in general, is rather acidic (Simoni *et al*, 1994), with a median isoelectric point (pI) approximately 4-5. Enzymatic desialylation of ovine pituitary FSH results in a redistribution of the isohormones towards less acidic forms (Keel and Schanbacher, 1988). Isoforms of FSH identified by charge-based fractionation differ from each other not only with respect to the degree of terminal sialylation and sulphation, but also in the inner carbohydrate moieties of the oligosaccharide side chains. Therefore, many FSH isoforms may have the same charge yet differ in oligosaccharide composition.

1.1.2 FSH Isoform Distribution

Although the factors affecting final gonadotrophin isoform distribution are complex and multifactorial, endocrine changes clearly regulate the proportions of FSH isoforms both within the pituitary and in the peripheral circulation. Differences in circulating FSH iosforms may be mediated by a number of different factors such as: 1) regulated preferential secretion of one or more FSH isoforms; 2) differences in the removal of carbohydrate moieties from the FSH molecule by circulating enzymes; and 3) changes in metabolic clearance (Ulloa-Aguire *et al*, 1995).

1.2 Synthesis and Release of FSH

Follicle stimulating hormone is the primary hormone involved in the emergence of waves of follicles, and its decline is associated with selection of a dominant follicle. The dominant follicle then becomes dependent on LH for further development when concentrations of FSH are minimal. The patterns of secretion of the two gonadotrophins are highly divergent, yet they can be produced in the same cell under the control of a single releasing hormone. The synthesized gonadotrophins are stored in secretory granules within the cytoplasm, and are secreted by exocytosis (Roche, 1996).

In the pituitary, the major hormone responsible for stimulating gonadotrophin release is gonadotrophin-releasing hormone (GnRH). Specific receptors on gonadotrophs respond to GnRH, triggering the release of intracellular Ca^{2+}, causing a transient release of both FSH and LH. The gonadotrophs become insensitive to GnRH, therefore the pulsatile nature of its release is necessary to prevent down regulation of its receptors. Without continual pulsatile stimulation by GnRH, secretion of LH and FSH declines rapidly, as do concentrations of messenger RNAs for the subunits of gonadotrophins in the anterior pituitary gland.

Other hormones that influence the secretion of FSH include estradiol, progesterone, inhibin; and activin. Inhibin and estradiol act directly at the anterior pituitary to decrease expression of the gene encoding the FSH subunit by reducing both the transcription and stability of mRNA, thereby negating the GnRH effects on FSH release. Estradiol causes a major decrease in FSH, while LH is initially decreased and then increased. Inhibin also suppresses FSH release, but without affecting LH. A summary of factors that influence FSH secretion is shown in Figure 1.1

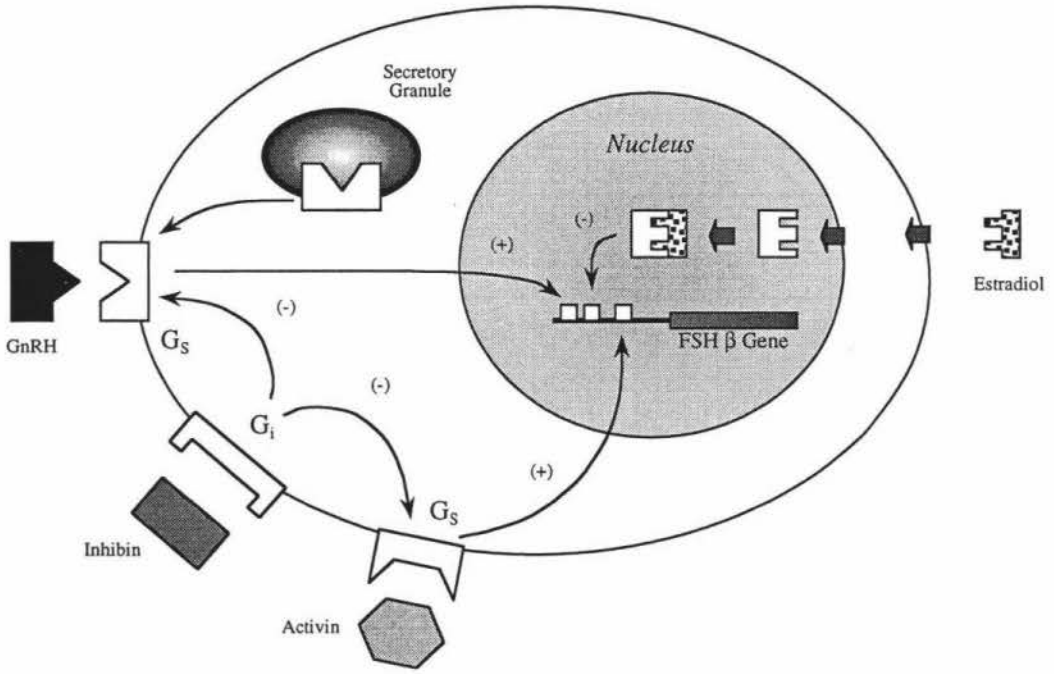

Fig 1.1 Schematic representation of factors known to regulate synthesis and secretion of FSH. Binding of GnRH to its receptors stimulates both protein kinase C (PK_C) and protein kinase A (PK_A) second messenger systems, thereby activating the stimulatory G protein (G_s). This, in turn, leads to the activation of the genes encoding α- (not pictured) and β- FSH subunits. Inhibin likely binds with an inhibitory G protein (G_i) coupled receptor. This may lead to inhibition of the stimulatory activity of GnRH and activin on FSH synthesis and secretion. Interaction of estradiol with its receptor inhibits transcription of the gene for β-FSH subunit. However, activation of the estradiol receptor leads to an increase in transcription for the gene encoding the receptor for GnRH (not shown) (Nett, 1990).

1.3 Physiology of FSH

In the female, the primary role of gonadotrophins is to initiate and sustain follicular functions. The initial stages of folliculogenesis occur independently of gonadotrophic hormones. In the adult ovary, folliculogenesis starts when follicles leave the pool of resting follicles to enter the growth phase. From there, the development of the follicle is characterized by a dramatic course of cellular proliferation and differentiation.

1.3.1 Follicular Development

Follicles in the ovary begin to form during fetal development and most remain in a resting stage until they either degenerate or are activated by various signals to enter the growth phase. These follicles constitute the stock of the resting follicles. Three types of resting follicles (primordial, intermediary, small primary) are evident in most mammals and can be distinguished by the size of the follicle and the structure and number of the granulosa cells surrounding the oocyte. With increasing age, the population of resting follicles progressively decreases. The age-related depletion of the resting follicle stock occurs as a result of two processes: atresia and entry into the growth phase. In sheep, the first follicles begin development at day 70 of gestation and by birth, at day 148, antral follicles are well developed. In adult ewes the estimated growth time for follicles is six months. Formation of the antrum occurs in ovine follicles when they reach 0.2mm in diameter and further increase in follicle size is attributed to an increase in granulosa cell numbers and a build up of follicular fluid. The granulosa cells reach a maximum growth rate when the follicle is approximately 1.0mm in diameter, after which the number of cell divisions declines until there is almost no mitotic activity in the mature preovulatory follicle. The follicular fluid is a derived from serum and is enriched with secretory products from follicle cells such as glycosaminoglycans, insulin-like growth factor I (IGF-1), inhibin, transforming growth factor β (TGF_{β}), prostaglandins and steroids. These substances may amplify or inhibit the actions of gonadotrophins such as LH and FSH.

Models of follicular growth and development based primarily on follicle size, number and morphological characteristics have been superseded by those based more on the functional capacities of the functional cells of the follicle. In one such model (Scaramuzzi *et al*, 1993),

follicles can be quiescent (i.e. primordial), committed to growth, ovulatory or atretic. Committed follicles can be non-responsive to gonadotrophins, and those that are responsive can be independent of or dependent on FSH and LH.

The role of gonadotrophins in initiating follicular growth is unclear. However, at advanced stages of follicular growth, antral follicles become dependent on FSH support for terminal follicular growth, leading to the emergence of waves of follicles. At this stage, an inappropriate stimulation, particularly by LH, can cause atresia. (Findlay, 1994). The decline of FSH is associated with selection of a dominant follicle that becomes dependent on LH for ovulation when concentrations of FSH are minimal. In addition, the reinitiation of growth in primordial follicles and acquisition of responsiveness of granulosa cells to FSH can occur independently of gonadotrophin support, indicating a role of autocrine and paracrine factors in folliculogenesis

In this model, the follicles committed to growth can be differentiated along a hierarchy according to their developmental status. Granulosa cells clearly undergo differentiation as the committed follicle move through the functional stages towards ovulation or atresia (Findlay, 1994). The stages of differentiation can be determined using parameters such as expression of gonadotrophin receptors, production of cyclic adenosine monophosphate (cAMP) in response to secretagogues, and production of steroids (estradiol, progesterone and 20α-hydroxyprogesterone) and peptide hormones (inhibin, oxytocin). Relatively undifferentiated granulosa cells, found in early committed follicles, lack any significant differentiation in terms of ability to respond to FSH and LH and to produce steroids and peptides (Scaramuzzi *et al*, 1993). Granulosa cells of small-medium bovine follicles demonstrate responsiveness to FSH and LH, but will not spontaneously luteinize *in vitro* unless treated with gonadotrophin. However, fully differentiated granulosa cells found in ovulatory follicles are generally capable of spontaneous luteinization *in vitro* (Findlay, 1994).

1.3.2 Control and Regulation of Folliculogenesis

The initiation of follicular growth is still not fully understood and there are two schools of thought on whether the initial stimulus for growth comes from the oocyte or the granulosa cells. While FSH is thought to influence the number of follicles that commence growth, gonadotrophins may not be essential in initiating follicle growth as follicles with four or five layers of granulosa cells have been observed in sheep following hypophysectomy. Although it is uncertain whether preantral follicles require gonadotrophins for follicular growth, it is evident that growth related processes are slowed down in their absence (Peters and McNatty, 1980).

1.3.2.1 Role of Estrogen in Follicles

Follicle-stimulating hormone is thought to stimulate the differentiation of the granulosa cells and promote antrum formation. To prevent the follicle from undergoing atresia, continuous presence of FSH is required. In human and sheep, FSH is present in some, but not all antral follicles. In follicles which have a higher concentration of androgen than estrogen (androgenic follicles), FSH is not present in detectable amounts. These follicles always undergo atresia.

Estrogen, primarily in the form of 17β-estradiol, is found in antral follicles arising mainly from the theca and granulosa cells. In the sheep ovary, over 95% of estradiol produced originates from granulosa cells, while the output of estradiol from theca interna cells is negligibl (McNatty and Henderson, 1987). Estradiol in conjunction with FSH enhances mitotic activity and influences the number of LH and prolactin receptors on granulosa cells. The concentration of estradiol in some follicles can be as high as 40,000 times that of estradiol in plasma in sheep. The estradiol/androgen balance in the follicle is kept in favour of estradiol by synthesis of estradiol from androgen. This ability to metabolize androgen to estrogen is maintained by FSH, thus follicles deficient in FSH lose this ability and eventually become androgenic and degenerate. Follicles which have been FSH-estradiol primed can then respond to a preovulatory rise in LH which subsequently leads to ovulation and corpus luteum formation.

As a result of the elevated levels of circulating estrogen, FSH production from the pituitary falls resulting in lowered peripheral concentrations of FSH. Without exposure to sufficient levels of FSH, the smaller antral follicles are no longer able to maintain their levels of estrogens and consequently undergo atresia. Therefore the dominant follicle stimulates its own growth and simultaneously deprives the others of FSH, accelerating their demise. The remaining healthy follicles already have high level of estrogens stored and continue to accumulate FSH.

Eventually the heightened level of circulating estrogen, secreted by the developing follicle, stimulates an increase in LH production, which accumulates in the follicle. Exposure of the follicle to high levels of LH initiates the final stages of oocyte maturation, inhibition of estrogen secretion and further granulosa cell divisions. Increased prostaglandin synthesis and initiation of the corpus luteum formation from the granulosa cells also occur due to the presence of LH.

1.3.2.2 Activin and Inhibin in Follicles

Inhibin, activin, and their binding protein, follistatin, have direct and indirect effects on granulosa and theca cells that can influence follicular development and steroidogenesis (Roche, 1996). Inhibin is a glycoprotein that has been isolated from follicular fluid as a heterodimer consisting of a common α-subunit combined with one of two β-subunits, βA (inhibin-A) or βB (inhibin-B) (Gougeon, 1996). The βA- and βB-subunits combine to give three forms of activins: activin (βAβB), activin A (βAβA), and activin B (βBβB). Follistatin is a monomeric glycosylated protein that is the major high-affinity binding protein for both inhibin and activin in follicular fluid.

A characteristic feature of activin and inhibin are the opposite effects that these peptides exert on certain follicular functions. On one hand, inhibin stimulates androgen production in theca interna cells, while activin exerts a negative influence on androgen production in the same cells. On the other hand, inhibin appears to be a potent inhibitor of ovarian cell proliferation in mice, whereas activin has been shown to stimulate *in vitro* proliferation of immature rat granulosa cells (Li *et al*, 1995). The effects of follistatin on steroid synthesis by preovulatory granulosa cells are indirect. Follistatin inhibits the suppressive effects of activin, as it binds with high affinity to activin and with lower affinity to inhibin (Gougeon, 1996).

Activin has been shown to play an important role in regulating granulosa cell development and functional integrity in ovarian follicles. Receptors and mRNA for activin have been found in oocytes, granulosa cells and theca interna cells of the rat (Cameron *et al*, 1994). Activin increases the number of FSH receptors in rat granulosa cells and stimulates the reorganization of follicular structures from monolayer cultures of immature granulosa cells in both rats and humans (Li *et al*, 1995). Inhibin has both autocrine and paracrine effects; it increases LH-induced androgen synthesis in thecal cells, and production of inhibin is stimulated by steroids and FSH (Roche, 1996). Thus, inhibin and activin provide a mechanism by which follicles can communicate directly with the pituitary in a feedback relationship with FSH. Therefore, within individual follicles a sequential change of inhibin, activin and their binding proteins may ultimately determine the different fates of the follicles committed to growth.

1.4 Biological Features of FSH Isoforms

Due to structural differences, FSH isoforms differ in their ability to bind to target cell receptors, survive in the circulation, and stimulate a biological response *in vitro* and *in vivo*.

Removal of the carbohydrate moieties in FSH has been found to increase the receptor binding ability and immunological activities of the hormone in radioreceptor and radioimmunoassays (Manjunath *et al*, 1982). In contrast, the deglycosylated FSH molecules have a greatly diminished capacity for stimulation of cAMP by gonadal cells (Manjunath *et al*, 1982). In addition, when combined with intact FSH, the deglycosylated FSH has been shown to interfere with the ability of the intact hormone to elicit steroidogenesis and these hormones are potent and specific antagonists in *in-vitro* (Manjunath *et al*, 1982).

The protein domain of the FSH molecule binds to specific plasma membrane FSH receptors, while the carbohydrate moieties of FSH are believed to interact with membrane components either within or adjacent to the receptor molecule. In general, certain peptide regions are thought to be critical for receptor binding, whereas the oligosaccharides are thought to be important for signal transduction. Indeed, receptor binding and signal transduction has been shown to be dissociable functions involving different sites on the FSH glycoprotein (Valove *et al*, 1994).

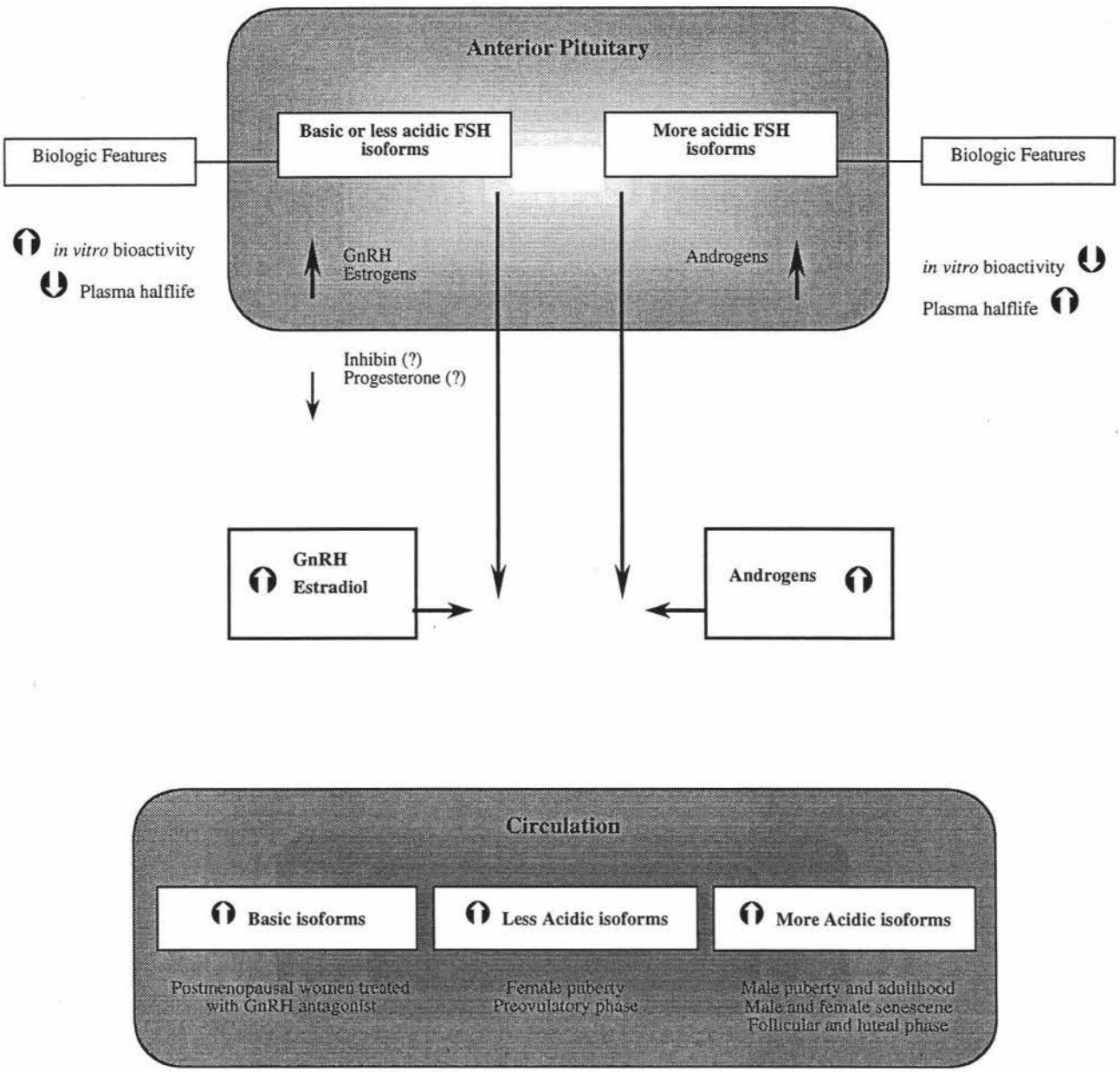

Fig 1.2 Hormonal factors influencing the synthesis and release of different FSH isoforms from the pituitary gland and the distribution of these isoforms associated with various physiological conditions (Roche, 1996).

Studies comparing in vitro bioactivity with immunoreactivity appear to indicate that less acidic, less glycosylated FSH isoforms have a higher biopotency *in vitro*, but have a shorter half-life (Stanton *et al*, 1992). Although heterogeneity has been studied extensively in the pituitary, regulation of FSH heterogeneity in the circulation is poorly understood due in part to the relatively low concentrations of circulating FSH. Despite this limitation, studies examining the correlation between qualitative differences in pituitary FSH content with age, sex and stage of the estrous cycle indicate that endocrine changes clearly regulate the proportions of FSH isoforms both within the pituitary and in the peripheral circulation (Roche, 1996). A majority of studies show that estradiol is associated with increased release of less acidic isoforms of FSH, and androgens are associated with increased release of more acidic forms of FSH. The onset of GnRH induced puberty in sheep and the preovulatory phase in normal cycling women is accompanied by major shifts in the distribution pattern of circulating FSH isoforms, and an associated increase in FSH bioactivity (Padmanabhan *et al*, 1992). A summary of factors thought to influence the synthesis and distribution of different FSH isoforms is shown in Figure 1.2.

1.5 FSH Mode of Action

Follicle-stimulating hormone, like all protein hormones, mediates its effects on target cells via specific protein receptors located on the cell surface. Receptors for FSH have been studied in both males and females. The primary location for FSH receptors in males is the Sertoli cells within the seminiferous tubule. In females, the major site of FSH receptor expression is the granulosa cells.

In both sexes, the secondary messenger cAMP mediates the action of FSH through its receptors. Treatment with membrane soluble cAMP analogs or cAMP-inducing agents stimulates multiple physiological responses in granulosa cells. Furthermore, the action of FSH in granulosa cells is enhanced with the addition of phosphodiesterase inhibitors that reduce cAMP breakdown (Welsh *et al,* 1985; Simoni *et al*, 1997). Follicle-stimulating hormone induced increase of intracellular cAMP in granulosa or Sertoli cells activate PKA, which results in subsequent phosphorylation of structural proteins, enzymes, and transcriptional activators.

Binding of FSH to its receptors *in vitro* is a process highly dependent on temperature and salt concentration and slowly becomes non-reversible at 37°C (van Loenen *et al*, 1994a). However, only a few minutes are required to attain steady state receptor activation, as measured by cAMP production in Sertoli cells, whereas the development of high-affinity receptor binding requires several hours (van Loenen *et al*, 1994b). The binding of FSH to granulosa cell membrane preparations is fully reversible only when hormone-receptor association occurs at 4°C and requires strong acidic treatment. The molecular basis for the slow formation of an irreversible interaction between FSH and its receptors is unknown. Whether the stabilization of FSH receptors occurs *in vivo* is also not known, although, in such a case, it could be an important step for hormone-receptor internalization.

1.5.1 FSH Receptor Structure

FSH receptors belong to the family of G protein-coupled receptors, characterized by complex transmembrane proteins consisting of seven hydrophobic helices inserted into the plasmalemma and by intracellular and extracellular domains of variable dimensions depending on the ligand (Simoni *et al*, 1997). Early experiments indicate that FSH receptors are oligomeric glycoproteins, consisting of four disulfide-linked monomers (Reichert and Dattatreyamurty, 1989). It is thought that FSH receptor binding is dependent on the integrity of the disulfide bonds, which stabilize the receptor conformation. The intracellular portion of FSH receptors is coupled to a G Protein and, upon receptor activation of the extracellular domain, initiates a cascade of events that ultimately leads to the specific biological effects of FSH in target tissues.

Depending on the species, the calculated molecular mass of the mature receptor protein ranges between 75 and 76.5 kDa, based on the cDNA sequence (Simoni *et al*, 1997). Further characterization of the amino acid sequence and hydropathy plot analysis suggests that FSH receptors consist of a large hydrophilic domain followed by hydrophobic regions 21-24 amino acids long, which span the plasma membrane seven times. At the C terminus, the sequence predicts a highly basic cytosolic segment.

Beside the transmembrane domain with the typical seven membrane-spanning helices, the glycoprotein hormone receptors are characterized by a very large extracellular domain in the N

terminus region, required for interaction with complex ligands. The extracellular domain displays several significant primary and secondary structural features. It is composed of several imperfectly replicated leucine-rich units of approximately 24 residues each, which, because of there amphiphatic character, are thought to mediate interaction between the hydrophilic hormone and the hydrophobic membrane (Gromoll et al, 1996). The secondary structure is determined by eight highly conserved cystein residues, a structural feature that can also be found in LH receptors and TSH receptors. This suggests a crucial role for the cystein residues in maintaining conformational (Gromoll et al, 1996). The extracellular domain of FSH receptors also has three potential sites for N-linked glycosylation conserved in all species at amino acid positions 191, 199, and 293. Additional species specific glycosylation sites may also be present. Although it was suggested that the glycosylation pattern of FSH receptors may affect hormone binding, recent studies indicate that glycosylation is rather required for correct folding of the receptor protein and transportation to the membrane (Davis *et al*, 1995).

1.5.2 Signal Transduction

FSH stimulation of cAMP production involves three distinct plasma membrane components: the hormone receptor, the nucleotide regulatory or G protein (guanine nucleotide binding protein), and the catalytic moiety of adenylyl cyclase. The receptor and cyclase are responsible for hormone recognition and enzymatic activity, respectively, and the G protein, in the presence of GTP, modulates their activity. After interaction with the hormone, the receptor becomes coupled to the G protein. The FSH activated G protein, termed G_s, stimulates adenylyl cyclase activity and, in turn, production of cAMP (Zhang *et al*, 1991). Association of the receptor to G_s can only occur when the receptor is in an active state. This implies that the receptor exists in an equilibrium between two forms, inactive and active, the second resulting in a conformational change of the inactive form. A summary of events that take place following receptor binding and activation is detailed in Figure 1.3. The interaction between FSH and its receptor determines receptor isomerisation to the active form and initiates a cascade of events collectively indicated as signal transduction.

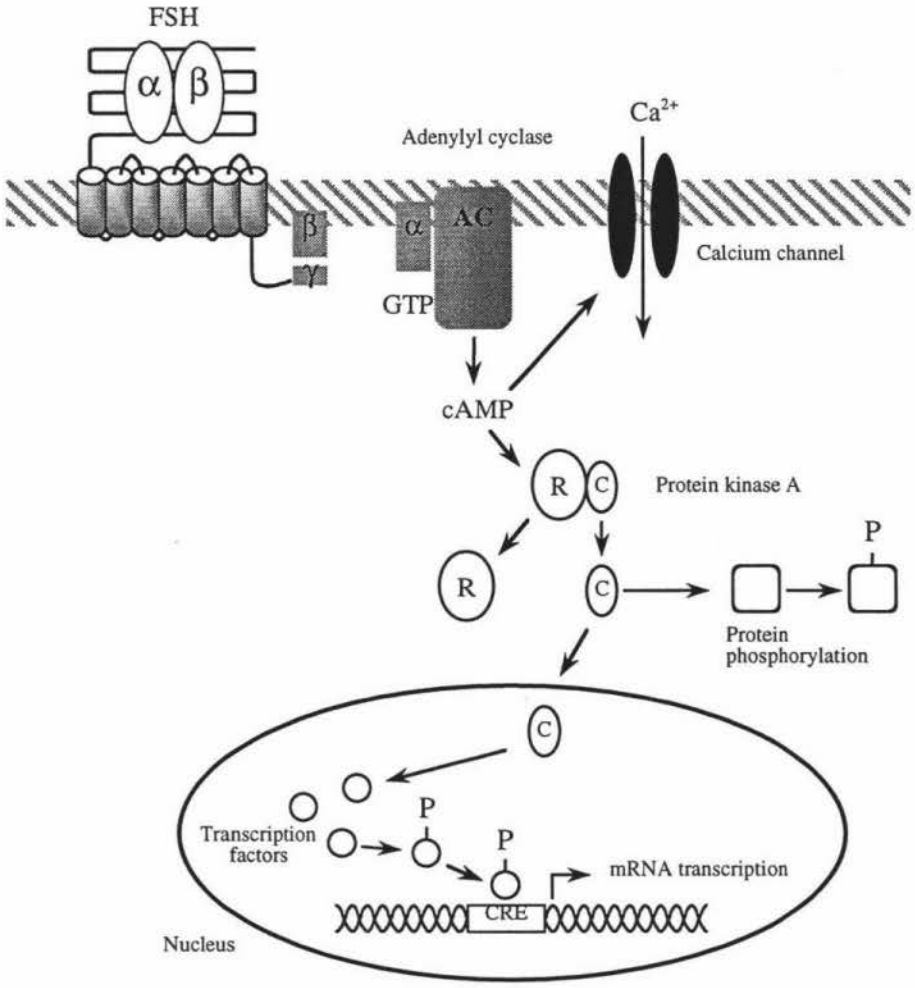

Figure 1.3 Model of signal transduction pathways of the FSH receptor. Upon binding of FSH to the FSH receptor, the G_{sa} subunit dissociates. In conjunction with GTP, this complex directly activates adenylyl cyclase, which produces cAMP from ATP. PKA is activated by cAMP, which leads to the dissociation of the catalytic subunit (C) from the regulatory subunit (R). The active catalytic site can activate proteins and transcription factors by phosphorylation. In addition, the production of cAMP leads to a rise in intracellular Ca^{2+} via the gating of calcium channels

On stimulation by the occupied and activated receptor molecule, the G protein releases GDP and binds GTP. In its GTP-bound conformation, the activated G protein is capable of stimulating adenylyl cyclase. This active complex exhibits GTPase activity, and hydrolysis of the bound GTP to GDP terminates the action of the G protein.

The activation of adenylyl cyclase by FSH and the subsequent synthesis of cAMP therefore triggers a cascade of events that ultimately lead to numerous physiological events in the target tissues including stimulation of granulosa cell proliferation, antrum formation, steroidogenesis, inhibin production, and accumulation of plasminogen activator (Dahl and Hsueh, 1988).

1.5.3 Receptor Desensitization

In the absence of FSH, cultured granulosa cells rapidly lose FSH binding sites, but incubation in the presence of FSH also results in a dose- and time-dependent loss of receptors in a process referred to as down-regulation. Sertoli cells incubated in the presence of FSH exhibit a decrease in adenylyl cyclase sensitivity with a decrease of cAMP production preceding the FSH receptor loss. By modulating the number and functionality of FSH receptors, depending on the presence or absence of FSH, target cells are able to protect themselves from overstimulation.

In addition to an increase in phosphodiesterase activity and consequent cAMP degradation, an early event after receptor stimulation is agonist-induced receptor desensitization, due to uncoupling of the FSH receptor from G_s (Ford and LaBarbera, 1988). Uncoupling occurs through enzymatic phosphorylation of the C-terminal, intracellular domain of G protein-coupled receptors.

As part of the desensitization process, FSH receptor numbers decrease through internalization of hormone-receptor complexes or reduced receptor protein synthesis. Receptor internalization is evident after 1-4 hours and precedes the decrease of receptor protein synthesis. Over longer periods of time, the presence of FSH results in a dose- and time-dependent decrease of functional FSH binding sites in porcine granulosa cells maintained in culture for up to 6 days (Simoni *et al,* 1997). However, at the end of this period, levels of FSH receptor mRNA increase.

1.6 Expression of FSH Receptor in Cell Lines

The use of recombinant DNA technology led to major advances in the understanding of the structure of FSH receptors. Due to the similarity of cloned LH receptors and TSH receptors and the observation that all three glycoproteins act on their respective receptors via the cAMP pathway, it was assumed that the structural design of FSH receptors should also be similar to that of the other glycoprotein hormone receptors. Since Sertoli cells are a specific target of FSH action and do not bind LH, cDNA probes corresponding to selected regions of the LH receptor were used to screen a rat Sertoli cell cDNA library. The isolated cDNA were then transfected into human embryonic kidney cells, which then displayed an FSH-dependent and saturable increase in intracellular cAMP. In contrast, no cAMP stimulation was observed when using human (h) CG or hTSH, indicating the successful cloning of rat FSH receptor (Sprengel *et al,* 1990).

After cloning of the cDNA, the FSH receptors were expressed in a number of cell lines. Both rat and human receptor cDNA were successfully introduced into eukaryotic cell lines and shown to be functional, thus producing a number of recombinant lines useful for studying FSH receptor properties such as hormone binding, signal transduction, and desensitization. Thus, with the advent of FSH receptor cell lines the establishment of novel bioassays for FSH has been made possible.

1.6.1 Assays for FSH

The development of a detection and measurement system is a critical step in identifying hormones and understanding their functions. Generally, the first assays developed were performed *in vivo*, as functions that were possibly regulated by the hormone could be used as endpoints in assays to monitor the purification of that hormone. The major disadvantage of *in vivo* assays the lack of precision, as animal to animal variance is usually high. However, testing hormone preparations in live animals is extremely important in that their functions may be modified by unknown factors *in vivo*, which cannot be predicted by other methods. The standard *in vivo* assay for FSH is the ovarian augmentation test whereby rat ovaries are collected after FSH

treatment and their weights measured. Any increase in ovarian weight is presumed to be due to follicular development induced by FSH (Mannaerts *et al*, 1991).

1.6.1.1 Radioimmunoassay vs. Bioassay

The development of radioimmunoassays (RIA) for gonadotrophins has proved to be a valuable tool for understanding the role of these hormones in reproduction. The major components of an RIA are (1) an antibody that is specific for the hormone to be examined, (2) a radioactively labeled hormone, and (3) the unknown sample or standard. Through competitive binding of the labelled hormone and the hormone in the sample to the specific antibody, the amount of hormone in the unknown sample can be quantified. However, immunological activity may not always correlate with biological activity as antibodies generated against FSH may not recognize all isoforms equally and different antibodies may also recognize different isoforms preferentially. Despite these disadvantages, RIA's are usually far more sensitive than bioassays and do not require the use of animals for primary tissue culture. Therefore, both RIA and specific *in vitro* bioassays are required to further understand gonadotrophin potency and physiology (Ulloa-Aguirre *et al*, 1995).

Considering that neither receptor binding assays (RBA) nor RIAs measure postreceptor events, *in vitro* bioassays have been used to study the biopotency of FSH isoforms at the target cell level. These assays make use of culture systems involving either primary cell cultures or immortalized cell lines. One advantage of using *in vitro* bioassays compared to *in vivo* assays is that many more experimental tests can be performed per experimental animal, as only a small amount of tissue is normally required for each test. In addition, the precision of *in vitro* assays is usually much higher than in *vivo* assays (Mannaerts *et al*, 1991; Braileanu *et al*, 1998).

To evaluate the potency of individual FSH isoforms, bioactivity and activities in either RIAs or receptor binding activity have been compared as the receptor-binding activity versus immunoactivity (RBA/RIA) or the bioactivity versus immunoactivity (B/I) ratio. In early studies, incubation of rat granulosa cells with equivalent concentrations of six different hamster FSH isoforms showed that the less acidic isoforms stimulated plasminogen activator release more than the highly acidic isoforms (Ulloa-Aguirre *et al*, 1988).

1.6.1.2 FSH *in vitro* Bioassays

Numerous *in vitro* assays for FSH have been developed utilizing testicular tissue or granulosa cell preparations for binding assays. These include the stimulation of plasminogen activator in granulosa *cells* (Beers and Strickland, 1978) and estrogen production by Sertoli cells controlled by FSH sensitive aromatase (Padmanabhan *et al*, 1987). However, these assays are ethically costly, as large numbers of animals are required to provide tissue for primary cell culture. In addition, due to the requirements of primary cell cultures, disparities between cultures are difficult to avoid because of variation between individual animals. As a result, an acceptable level of sensitivity may be difficult to obtain in such bioassays.

Recently, the development of a Chinese hamster ovary cell line that stably expresses the human FSH receptor has enabled the development of an FSH bioassay for human FSH (Albanese *et al*, 1994). This bioassay effectively overcomes the main problems associated with more traditional *in vitro* bioassays, namely the ethical cost involved and sensitivity. Therefore, the feasibility of using this cell line for the development of an *in vitro* bioassay for ovine FSH will be examined in this study.

Given the disadvantages of FSH bioassay systems requiring primary tissue culture, this study examines the use of an alternative bioassay system that does not suffer from the same drawbacks. Therefore, described in this study are preliminary experiments used to investigate the FSH stimulated cAMP response of a receptor cell line described by Albanese *et al* (Albanese *et al*, 1994) followed by the validation of an *in vitro* FSH bioassay using ovine sera, purified ovine pituitary extracts, and pituitary cell culture media. The potential use of the bioassay as a tool for investigating heterogeneity in FSH is examined.