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P450 AROMATASE EXPRESSION AND ESTRADIOL SECRETION IN BOVINE GRANULOSA CELLS IN VITRO.

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ABSTRACT

Folliculogenesis is a highly complex process which involves hormone-induced proliferation and differentiation of both theca and granulosa cells, leading ultimatelly to the increased ability of follicles to produce estradiol and to respond to gonadotropins. The ability to synthesize estradiol is an essential characteristic of a healthy ovarian follicle. Estradiol is synthesized in granulosa cells from thecal androgens; a process catalized by the enzyme P450 aromatase ($P450_{arom}$). In cattle, the study of the hormonal regulation of $P450_{arom}$ gene expression and estradiol secretion has been hampered because of the lack of a cell model in which estradiol secretion can be maintained. Recently, a cell culture system has been developed in which bovine granulosa cells maintain aromatase activity in vitro. Thus, the aim of the present study was to examine the mechanisms involved in the regulation of $P450_{arom}$ gene expression and estradiol secretion by FSH, insulin and IGF-I in bovine granulosa cells in vitro.

The objective of the first study was to study the time course and the effects of FSH on $P450_{arom}$, $P450_{scc}$ mRNAs, and estradiol and progesterone secretion. Estradiol secretion increased with time of culture, and this was correlated with an increase in $P450_{arom}$ mRNA abundance. Progesterone secretion also increased with time of culture, but $P450_{scc}$ mRNA did not. Low FSH concentrations (1 ng/ml) increased $P450_{arom}$ mRNA and estradiol secretion, whereas higher FSH concentrations (100 ng/ml) were inhibitory to $P450_{arom}$ mRNA but not estradiol secretion. Contrary to $P450_{arom}$ mRNA, $P450_{scc}$ mRNA was increased with high FSH concentrations (100 ng/ml). Insulin at 100 ng/ml,

increased $P450_{arom}$ mRNA and estradiol secretion, but was without effect on $P450_{scc}$ mRNA and progesterone secretion. We conclude that in the present culture cell model, $P450_{arom}$ mRNA and estradiol secretion can be maintained with low FSH concentrations, whereas higher doses of FSH downregulated $P450_{arom}$ mRNA levels. Contrary to $P450_{arom}$ mRNA, $P450_{scc}$ mRNA and progesterone secretion increased with highest FSH concentrations.

The objective of the second study was to determine the relative importance of FSH, IGF-I and insulin to maintain $P450_{arom}$ mRNA and estradiol secretion in cultured bovine granulosa cells. Granulosa cells were stimulated with insulin (insulin-stimulated cells; 100 ng/ml), when insulin was withdrawn from the medium, $P450_{arom}$ mRNA could not be detected by Northern blotting, and estradiol secretion declined dramatically. The replacement of medium with FSH, could not maintain $P450_{arom}$ mRNA, nevertheless estradiol secretion was maintained at control (insulin-stimulated) levels. Contrary to $P450_{arom}$ mRNA, $P450_{scc}$ mRNA and progesterone secretion increased by increasing doses of FSH, irrespective of the withdrawal of insulin. In FSH stimulated granulosa cells, we showed that both FSH and insulin at physiological concentrations were nessesary to maintain $P450_{arom}$ mRNA. In the absence of FSH or insulin, $P450_{arom}$ mRNA abundance, was not detectable by Northern blot. The absence of IGF-I in the medium was without effect on $P450_{arom}$ mRNA when insulin and FSH were present. Estradiol secretion was maintained by the presence of FSH alone but not in its absence. In FSH-stimulated cells, progesterone secretion

was not affected by the withdrawal of IGF-I, insulin and/or FSH, whereas $P450_{scc}$ mRNA was reduced by the withdrawal of FSH or both insulin and IGF-I from the medium. We conclude that the presence of FSH and insulin at physiological concentrations were necessary to maintain $P450_{arom}$ mRNA levels, whereas the sole presence of FSH was sufficient to maintain estradiol secretion, suggesting that $P450_{arom}$ mRNA and estradiol secretion are regulated differentially.

The objective of the third study was to understand the intracellular mechanisms utilized by insulin and FSH to induce $P450_{arom}$ mRNA and estradiol secretion in granulosa cells. At day four of culture, specific inhibitors of PI3K (phosphatidylinositol 3-kinase), PKC (protein kinase C), PKA (protein kinase A) and MEK (MAP kinase kinase activation) were added to the cultures, and granulosa cells were recovered at day six of culture. In insulin-stimulated cells, PI3K and PKC inhibitors decreased $P450_{arom}$ mRNA abundance and estradiol secretion, whereas the inhibition of PKA was without effect. In FSH-stimulated cells the PKA inhibitor reduced $P450_{arom}$ mRNA abundance. The inhibition of PI3K and PKC did not affect $P450_{arom}$ mRNA in FSH-stimulated cells, although estradiol secretion was also inhibited by PI3K and PKC inhibitors. Inhibition of MEK pathway significantly increased $P450_{arom}$ mRNA abundance in both insulin and FSH-stimulated cells, although estradiol secretion was not affected. In both insulin and FSH-stimulated cells $P450_{scc}$ mRNA abundance and progesterone secretion were not affected by any inhibitor. We conclude that $P450_{arom}$ mRNA

and estradiol secretion are regulated by different intracellular mechanisms in bovine granulosa cells.

The results of these studies showed that $P450_{arom}$ mRNA abundance and estradiol secretion can be induced and maintained by physiological FSH and insulin concentrations, and they are differentially regulated in bovine granulosa cells in vitro.

Key Words: Bovine, granulosa cells, $P450_{arom}$ mRNA, $P450_{scc}$ mRNA, estradiol, progesterone, insulin, IGF-I, FSH, second messengers.

RÉSUMÉ

La folliculogenèse est un processus très complexe déclenchée par la prolifération et la différentiation des cellules de la thèque et de granulosa induites par les hormones, et qui provoque la production d'une grande quantité d'oestradiol par les follicules en réponse aux gonadotrophines. La capacité de synthétiser l'oestradiol est une caractéristique essentielle des follicules ovariens. L'oestradiol est synthétisé par les cellules de granulosa à partir d'androgènes d'origine thécale par l'action de l'enzyme aromatase P450 ($P450_{arom}$)

Chez l'espèce bovine, l'étude de la régulation hormonale de la $P450_{arom}$ dans les cellules de granulosa était très difficile en raison d'un système de culture capable de maintenir l'activité $P450_{arom}$ in vitro. On a récemment développé un système de culture qui maintient l'activité $P450_{arom}$ dans les cellules de granulosa bovine in vitro. L'objectif de cette étude a été d'étudier la régulation du gène codant pour la $P450_{arom}$ dans les cellules de granulosa bovine in vitro.

Dans la première étude, nous avons demontré que l'ARNm de la $P450_{arom}$ était induit dans un système de culture à long terme. La sécrétion d'oestradiol augmentait avec le temps de culture et corrélait à une augmentation de l'ARNm de la $P450_{arom}$. La sécrétion de progestérone augmentait également avec le temps de culture, mais pas la concentration de l'ARNm de $P450_{scc}$. À faible concentration (1 ng/ml), la FSH a augmenté le taux d'ARNm de la $P450_{arom}$ et la sécrétion d'oestradiol. Par contre, une forte concentration de FSH (100ng/ml) a eu un effet inhibiteur sur la concentration d'ARNm de la $P450_{arom}$,

mais pas pour la sécrétion d'oestradiol. Au contraire, l'ARNm de la $P450_{scc}$ a augmenté avec les plus hautes concentrations de FSH (100 ng/ml). L'insuline à 100 ng/ml comparativement à 10 ng/ml a augmenté la concentration d'ARNm de la $P450_{arom}$ et la sécrétion d'oestradiol, mais n'a pas eu d'effet sur l'ARNm de la $P450_{scc}$ et la sécrétion de progestérone.

Dans la deuxième étude, nous avons déterminé l'importance relative de la FSH, de l'IGF-I et de l'insuline utilisés dans le milieu de culture pour maintenir l'ARNm de la $P450_{arom}$ et la sécrétion d'oestradiol par les cellules de granulosa. Quand les cellules ont été stimulées à l'insuline (100 ng/ml) suivi par son retrait du milieu de culture, l'ARNm codant pour la $P450_{arom}$ n'a pas pu être détecté par "Northern blot", et la sécrétion d'oestradiol a chuté jusqu'à un taux presque indétectable. Cependant, quand l'insuline a été enlevée et remplacée par la FSH, même si l'ARNm codant pour la $P450_{arom}$ n'était toujours pas détectable par "Northern blot", la sécrétion d'oestradiol s'est maintenue à un niveau semblable à celui des cellules stimulées à l'insuline. Dans les mêmes conditions, l'ARNm codant pour la $P450_{scc}$ et la sécrétion de progestérone ont été augmentés par des doses croissantes de FSH, en dépit du retrait de l'insuline. Dans les cellules stimulées à la FSH, nous avons démontré que la FSH (1 ng/ml) et l'insuline (10 ng/ml) sont toutes deux suffisantes et nécessaires aux concentrations physiologiques pour maintenir l'ARNm codant pour la $P450_{arom}$. En l'absence de FSH ou d'insuline, l'ARNm codant pour la $P450_{arom}$ n'était pas détectable par "Northern blot". L'absence d'IGF-I dans le milieu était sans effet sur le niveau d'ARNm codant pour la $P450_{arom}$ quand l'insuline et la

FSH étaient présentes. La présence de FSH seule était suffisante et nécessaire au maintien de la sécrétion d'oestradiol. Dans les cellules stimulées à la FSH, la sécrétion de progestérone n'était pas affectée par le retrait de cette hormone, tandis que le niveau d'ARNm codant pour la $P450_{scc}$ a subi une diminution à la suite de l'enlèvement de la FSH seule, mais également à la suite du retrait de l'insuline et de l'IGF-I.

La troisième étude ciblait une compréhension de la voie intracellulaire utilisée par l'insuline et la FSH pour l'induction l'ARNm codant pour la $P450_{arom}$ et la sécrétion d'oestradiol. Pour ce faire, nous avons utilisé des inhibiteurs spécifiques des enzymes PI3K (phosphatidylinositol 3 kinase), PKC (protéine kinase C), PKA (protéine kinase A) et MEK (*MAP kinase kinase activation*) dans le milieu de culture. Dans les cellules stimulées à l'insuline, les inhibiteurs de PI3K et PKC ont diminué le taux d'ARNm codant pour la $P450_{arom}$ et la sécrétion d'oestradiol, alors que l'inhibition de PKA était sans effet. Dans les cellules stimulées à la FSH, l'inhibiteur de la PKA a diminué le taux d'ARNm codant pour la $P450_{arom}$ et la sécrétion d'oestradiol. L'inhibition de la PI3K et de la PKC n'a pas affecté le taux d'ARNm codant pour la $P450_{arom}$ dans les cellules stimulées à la FSH, bien que la sécrétion d'oestradiol a aussi été inhibée par les inhibiteurs de la PI3K et de la PKC. L'inhibition de la MEK a augmenté le niveau d'ARNm codant pour la $P450_{arom}$ dans les cellules stimulées à l'insuline ou à la FSH, mais sans avoir d'effet sur la sécrétion d'oestradiol. Dans les deux expériences, les concentrations d'ARNm codant pour la $P450_{scc}$ et la sécrétion de progestérone n'ont pas été affectées. Nous concluons que l'accumulation

d'ARNm codant pour la $P450_{arom}$ est contrôlée par l'insuline et la FSH par deux mécanismes intracellulaires différents, tandis que la sécrétion d'oestradiol est exclusivement contrôlée par la PIK3 et la PKC dans les cellules de granulosa bovine in vitro.

L'ensemble des résultats de cette étude démontrent que l'accumulation d'ARNm codant pour la $P450_{arom}$ et la sécrétion d'oestradiol dans les cellules de granulosa bovine in vitro peuvent être induites et maintenues par des concentrations physiologiques d'insuline et de FSH. Ils démontrent également que ces hormones agissent par des voies de transduction différentes et que l'induction de l'ARNm codant pour la $P450_{arom}$ répond à une voie de signalisation intracellulaire différente de celle qui stimule la sécrétion d'oestradiol.

Mots clés: bovine, cellules de granulosa, $P450_{arom}$, $P450_{scc}$, oestradiol, progestérone, insuline, IGF-I, FSH, seconds messagers.

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LIST OF ABBREVIATIONS

cAMP	Cyclic adenosine 3',5'monophosphate
cDNA	Complementary deoxyribonucleic acic
CRE	cAMP-response element
CREBP	cAMP response element-binding protein
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehidrogenase
CL	Corpus luteum
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
eCG	Equine chorionic gonadotropin
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
FSHr	Follicle-stimulating hormone receptor
HDL	High density lipoproteins
IGF-1	Insulin-like growth factor 1
IGFBPs	Insulin like growth factor-binding proteins
IL	Interleukin
Insp3	Inositol trisphosphate
IP3K	Inositol trisphosphate 3 kinase

LDL	Low density lipoproteins
LH	Luteinizing hormone
LHr	Luteinizing hormone receptor
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$P450_{arom}$	Cytochrome P450 aromatase
$P450_{17OH}$	Cytochrome P450 17_α hydroxylase
$P450_{scc}$	Cytochrome P450 side chain cleavage
PDGF	Plateled derived growth factor
PGF2α	Prostaglandin F2 alpha
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol myristate acetate
RNA	Ribonucleic acid
StAR	Steroidogenic Acute Regulatory Protein
SF1	Steroidogenic Factor 1
TPA	12-O-tetradecanoylphorbol-13-acetate
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha

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AVANT-PROPOS (PREFACE)

Cette thèse est présentée à la Faculté des études supérieures de l'Université de Montréal pour l'obtention du grade de *Philosophiae Doctor* en sciences vétérinaires, option reproduction. Elle est composée d'une introduction générale, d'une revue de littérature générale; trois articles comprenant chacun: une introduction, une section matériel et méthodes, des résultats, une discussion et des références; une discussion générale, une conclusion générale ainsi que des references générales.

This thesis is presented to the *Faculté des études superieures de l'Université de Montréal* for the obtention of the *Philosophiae Doctor* degree in veterinary sciences, option reproduction. It composes a general introduction, a general literature review; three publishable articles, each of which contains a specific introduction, materials and methods, results, discussion and references; a general discussion, a general conclusion and general references.

INTRODUCTION

Reproduction in mammals is dependent on regular and cyclic ova release by ovarian follicles (Picton *et al.*, 1998). Folliculogenesis is the process by which primordial follicles, which are formed early during fetal development, are activated by an unknown mechanism to begin growing. These follicles increase in diameter owing primarily to increased proliferation and differentiation of granulosa cells. These follicles are collectively named preantral follicles (Hirshfield, 1991). In cattle, as follicular growth continues beyond 250 µm in diameter, liquid becomes present in the follicle to form an antrum; these follicles are called tertiary or antral follicles (Gosden *et al.*, 1988). Tertiary follicles develop throughout the life span of the female, and depending of the species, one or more follicles will ovulate during each estrous cycle. From the pool of primordial follicles, less than 1% are selected to develop fully and ovulate, the rest die by atresia (Monniaux *et al.*, 1997). In cattle, it is now well established that antral follicular development occurs in a pattern of waves (Pearson & Ginther, 1984; Sirois & Fortune, 1988; Savio *et al.*, 1988). Early during each estrous cycle, 5 or 6 small antral follicles are recruited from the pool of ovarian preantral follicles, and from these recruited follicles, one is selected to continue growing to become the dominant follicle. The remaining subordinate follicles will die by atresia (Fortune, 1994). The dominant follicle of the first follicular wave will also normally became atretic, due to the presence of an active corpus luteum, and a second follicular wave will emerge. If the dominance phase of a follicle coincides with the demise of the corpus luteum, the dominant follicle of

the second follicular wave will ovulate. If this is not the case, the dominant follicle will regress and a third follicular wave will emerge. The phases of follicular recruitment, selection and dominance, are collectively named follicular dynamics (Fortune, 1994).

The most striking characteristic of dominant follicles is the high capacity to synthesize and secrete estradiol (Lucy *et al.*, 1992). Estradiol is synthesized in granulosa cells from thecal androgens, by two successive enzyme-catalysed reactions (Fortune, 1986). In cattle, androstenedione is the preferred substrate, which is converted to estrone by the enzyme cytochrome $P450_{arom}$. Estrone is then converted to estradiol by the enzyme 17β-hydroxysteroid dehydrogenase.

Studies on $P450_{arom}$ gene expression in cattle in vivo, have shown that $P450_{arom}$ mRNA first appears during follicular recruitment of the first follicular wave (Bao *et al.*, 1997a). The induction of $P450_{arom}$ mRNA expression has been postulated to occur as a response to a transient increase in plasma FSH concentrations that precedes the emergence of each follicular wave (Adams *et al.*, 1992). This is supported by data from rat and human granulosa cell cultures, where FSH stimulates $P450_{arom}$ mRNA abundance (Fitzpatrick & Richards, 1991; Steinkampf *et al.*, 1987).

It has been suggested that the control of $P450_{arom}$ mRNA differs among rats, humans and cattle (Tian *et al.*, 1995; Hinshelwood *et al.*, 1997). The study of estradiol secretion in bovine granulosa cells has been hampered because bovine granulosa cells luteinize spontaneously in vitro and lose the capacity to

secrete estradiol (Henderson & Moon, 1979; Skinner & Osteen, 1988; Roberts & Echternkamp, 1994; Berndtson *et al.*, 1995b), and $P450_{arom}$ mRNA abundance is either not detectable (Voss & Fortune, 1993b) or declines rapidly (Berndtson *et al.*, 1996). Recently, Webb and colleagues have developed a long term culture system that permits the induction and maintenance of estradiol secretion in ovine (Campbell *et al.*, 1996) and bovine (Gutiérrez *et al.*, 1997b) granulosa cells.

The overall objective of the present study was to study the regulation of $P450_{arom}$ mRNA and estradiol secretion in bovine granulosa cells in vitro.

LITERATURE REVIEW

FOLLICULOGENESIS

Follicular Development

Successful reproduction in mammals requires the formation of an ovum ready to be fertilized by a spermatozoan. To accomplish this objective the female has to activate her ovarian folliculogenic machinery.

Ovarian follicular growth and maturation is a highly complex process, involving a series of sequential subcellular and molecular transformations of various components of the follicle such as the oocyte, and granulosa and theca cells (Picton *et al.*, 1998). In mammals folliculogenesis begins early in fetal life, when a pool of primordial follicles is formed, each consisting of an oocyte arrested in prophase I of meiosis and a single layer of flattened pre-granulosa cells (Hirshfield, 1991). Primordial follicles begin to grow gradually and continually leave the resting pool. Once the follicle begins to grow it meets one of two fates, ovulation or atresia; the first one being the exception, because more than 99 % of follicles undergo atresia (Baker & Spears, 1999).

Follicle growth involves hormone-induced proliferation and differentiation of both theca and granulosa cells, leading ultimately to the increased ability of follicles to produce estradiol and to respond to gonadotropins. Production of estradiol determines which follicle will gain the LH receptors necessary for ovulation and luteinization (Richards *et al.*, 1998). Mechanisms associated with

initiation of growth of follicles and regulation of development to ovulation or atresia are not fully understood.

Formation of primordial follicles

In cattle, as in the majority of mammals, the number of ovarian oocytes is established during prenatal development (Mauleon, 1967). Thus the number of oocytes present at birth represents the total number available during the lifespan of the animal. Oogonia originate from primordial germ cells that have an extragonadal origin (Motta *et al.*, 1997). These cells are found in the yolk sac endoderm and in the region of the allantoids arising from the primitive streak. Subsequently, primordial germ cells migrate, first by passive transfer into the endodermal epithelium of the hindgut and then by ameboid movement along the dorsal mesentery of the genital ridges found in the roof of the coelom, the site of gonad development (Erickson, 1974).

Mitotic division of oogonia takes place from about day 50 through day 130 of gestation in cattle as described by Mauleon (1967). Oogonia begin to undergo meiosis at approximately 80 days of fetal life in the calf, the ovigerous cords are formed, and the ovary becomes divided into cortical and medullary parts. By day 150, the ovigerous cords are disrupted, and by day 170 the ovary is characterized by a narrow cortical band of germinal tissue and a prominent medulla (Mauleon, 1967). With the disruption of the ovigerous cords, the vast majority of surviving oocytes, arrested at the diplotene phase of meiotic prophase I, acquire a single layer of flattened follicle cells and thus form

primordial follicles, which number, at birth, approximately 150,000 in cattle (Erickson, 1966).

Preantral stage

Once primordial follicles are established, they remain quiescent until a signal of unknown nature stimulates them to begin to grow and differentiate in an irreversible but apparently continuous process. This is believed to be gonadotropin independent, because follicular growth at this stage is not interrupted by the absence of gonadotropins (Campbell *et al.*, 1995). The cell type which provides the stimulus is unknown; it may be the oocyte, the granulosa cells or the ovarian stroma. The protein kit ligand (steel factor) from granulosa cells, and its receptor (c-kit) in the oocyte have been implicated in the initiation of follicular development (Manova *et al.*, 1993; Packer *et al.*, 1994). Conversely, a role of the oocyte in the initiation of granulosa cell proliferation remains hypothetical. A possible candidate is the transcription factor WT-1 (Wilms' tumor suppressor) reported in high abundance in primordial and primary follicles, whereas expression is lower in secondary follicles (Hsu *et al.*, 1995). Recently, it has been postulated that ovarian stroma exerts a negative effect on the initiation of primordial follicle growth in vitro (Wandji *et al.*, 1997). Follicular growth begins with transformation of primordial follicles to primary follicles, which are oocytes surrounded by a single layer of cuboidal granulosa cells (Cahill & Mauleon, 1981). By proliferation of the granulosa cells, secondary follicles with two or more layers of granulosa cells are formed. The size of the

oocytes increases, and the zona pellucida is formed between the oocyte and the granulosa cells. The stroma surrounding the secondary follicles begins to develop into a theca layer. Follicles in these two stages are collectively described as preantral (Hirshfield, 1991).

Although it has been shown that FSH receptors are present in primary and secondary bovine follicles, at least in fetal and neonatal calves (Wandji *et al.*, 1992b), growth can continue until follicles reach 4 mm in diameter in cattle in the absence of gonadotropins (Campbell *et al.*, 1995). In preantral follicles, follicular vascularization is poorly developed (Suzuki *et al.*, 1998), which suggests that paracrine rather than endocrine factors may control these stages of folliculogenesis. In particular, growth factors and cytokines may play an important role in regulating granulosa cell proliferation. In ovaries from bovine fetuses and neonatal calves, granulosa cells of preantral and small antral follicles express receptors for bFGF (basic fibroblast growth factor) and EGF (epidermal growth factor, Wandji *et al.*, 1992a).

Antral stage

Cavitation of the follicle to form the fluid filled antrum is the next stage in follicle development, and the consequent structures are known as tertiary or antral follicles. Antral follicles increase in size, and only one or some of them, depending of the species, will ovulate at the end of the estrous cycle (le Nestour *et al.*, 1993). Antral follicles exist in the bovine ovary with a range of diameters from 0.14 to 20 mm (Lussier *et al.*, 1987). Follicle growth rate, measured as

mitotic index is the slowest in small antral follicles over 27 days are required to attain a diameter of 0.67 mm from an early stage (approx 0.2 mm; Lussier *et al.*, 1987). Growth of antral follicles between 1 and 2 mm diameter is attributed to an increase in the number of granulosa cells and follicles larger than 2.5 mm results from antrum development and the most rapid growth appears to occur (Lussier *et al.*, 1987). Growth of an early antral follicle to preovulatory size requires approximately 42 days in cattle (Lussier *et al.*, 1987). In cattle, the diameter of the oocyte increases from 20-30 μm in primordial follicles to 120-150 μm in antral follicles (Erickson, 1966). Antrum formation is a gonadotropin-influenced event, and FSH is the principal hormone responsible (Gosden *et al.*, 1988).

Growth of the follicle after antrum formation results mostly from enlargement of the antrum, with some contribution from continued mitosis of the granulosa cells (Gosden *et al.*, 1988). Follicular fluid consists of substances derived from blood (electrolytes, growth factors, blood cells, etc) as well as from local secretion and metabolism (steroid hormones, inhibin, activin, follistatin, growth factors, etc) (Gosden *et al.*, 1988).

Follicular dynamics

In early studies, Rajakoski (1960), suggested that two waves of follicular growth occur during the cycle, the first wave emerging a few days after estrus, and the second follicular wave beginning around day 12-14 of the estrous cycle.

This concept was supported by Swanson *et al.*, (1972) and by Mariana (1972). On the other hand, other investigators concluded that follicular development is continuous and independent of the stage of the cycle (Choudary *et al.*, 1968; Donaldson *et al.*, 1968; Marion *et al.*, 1968; Dufour *et al.*, 1972). The "two wave" hypothesis was not tested for more than 20 years, and results of a histologic study (Matton *et al.*, 1981) were consistent with the two-wave hypothesis. Later in 1983 studies involving measurements of follicles and steroid assays of blood and follicular fluid, led to the conclusion that there were three follicular waves (Ireland & Roche, 1983). It was not until transrectal ultrasonic imaging technology (Pierson & Ginther, 1984) provided a means for repeated, direct, noninvasive monitoring of antral follicles in cattle, and it was found that most (81%) estrous cycles consisted of two follicular waves (Ginther *et al.*, 1989b). This technology was exploited by other groups, who found mostly (80%) three-wave cycles (Savio *et al.*, 1988; Sirois & Fortune, 1988). The power of ultrasound technology has been extended to other species including horses (Ginther & Pierson, 1984), sheep (Ginther *et al.*, 1995) and goats (Ginther & Kot, 1994). In cattle it is currently used to monitor the functional and endocrine status of the follicle, through the utilisation of computer-assisted quantitative echotexture analysis of ultrasound images (Singh *et al.*, 1998).

The appearance and regression of follicle waves is termed follicular dynamics (Figure 1). It is characterized by the initiation of growth of a cohort of 3-6 small antral follicles (2-4 mm) which are recruited out of the pool of smaller

antral follicles (<2 mm in diameter), this process is called recruitment (Lucy *et al.*, 1992). Selection is the process by which the appropriate number of follicles is selected from a cohort of growing follicles to develop to ovulatory competence. In monovular species such as cattle, a single follicle is chosen to continue growth after recruitment and has the potential to achieve ovulation (Fortune, 1994). Follicular dominance is the process by which a single selected follicle from the pool of growing follicles, continues its growth exerting an inhibitory effect on the the other follicles of the wave, which cease growing and undergo atresia (Lucy *et al.*, 1992; Fortune, 1994). The dominant follicle also inhibits the recruitment of a new cohort of follicles. Two to four waves of follicular development have been reported in cattle during the estrous cycle (Fortune, 1994). Follicle waves also occur during pregnancy (Ginther *et al.*, 1989a) and during the prepuberal period (Adams *et al.*, 1994). When the dominant follicle coincides with the presence of an active corpus luteum, the fate of this follicle is usually atresia, and a new follicular wave emerges. If luteal regression occurs when there is a dominant follicle present, this follicle will usually ovulate (Kastelic *et al.*, 1990a).

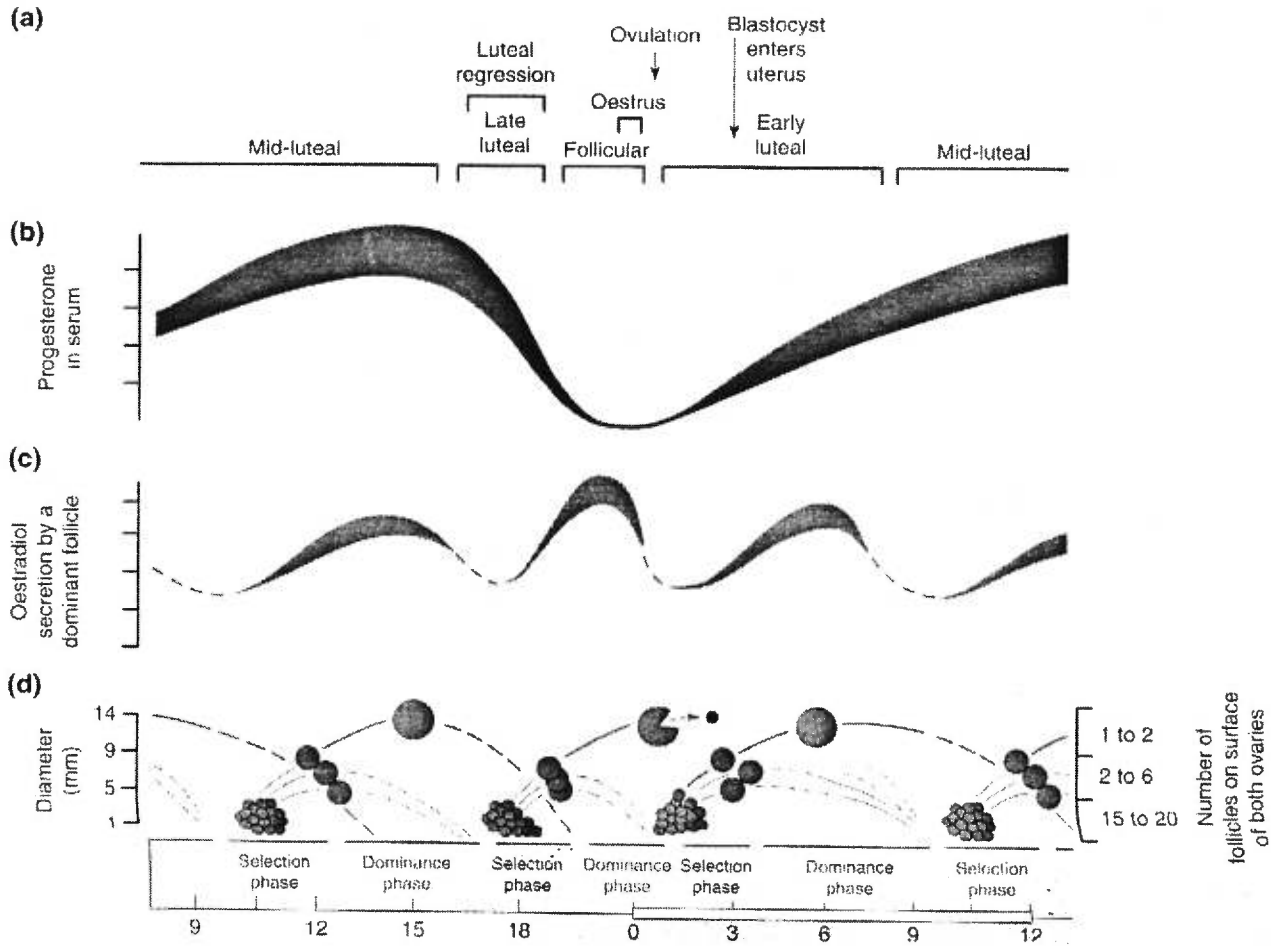


Fig 1. Follicular waves during the estrus cycle in the cow.

Atresia

Atresia is a physiological process by which 99% of growing follicles degenerate, losing their morphological integrity and their physiological and biochemical capabilities (Monniaux *et al.*, 1998). It is present throughout all stages of follicular development (Monniaux *et al.*, 1998), during gestation (Guilbault *et al.*, 1986) and in prepuberal animals (Dufour *et al.*, 1988) with the first histological signs of atresia appearing in oocytes of preantral follicles, and in granulosa cells of antral follicles (Blondin & Sirard, 1995). In cattle, the rate of atresia (percentage of atretic follicles) is low in preantral and small antral follicles, increases in medium antral follicles, and then remains high during the terminal phases of follicular growth. (Lussier *et al.*, 1987; Lussier et al.,1994).

Atresia is the fate of non-ovulatory dominant follicles as well as subordinate follicles of the bovine estrous cycle (Fortune, 1994). Early studies have attempted to characterize and classify the degree of atresia in antral follicles, using morphological, histological and endocrinological criteria. Morphologically healthy antral follicles have a highly vascularized theca, a dense granulosa layer and clear follicular fluid (McNatty *et al.*, 1984; Tsonis *et al.*, 1984). Based on histological examinations ovarian follicles can be classed as non-atretic, early atretic, and late atretic, according to the number of pycnotic nuclei, thickness of granulosa cell layer and basement membrane integrity (Grimes *et al.*, 1987; Spicer *et al.*, 1987). Nonatretic follicles have at least seven layers of granulosa cells, an intact basement membrane and few pycnotic nuclei; early atretic follicles had two to six layers of granulosa cells, numerous

pycnotic nuclei and a loosening of the basement membrane; and atretic follicles had fewer than two layers of granulosa cells and complete disruption of basement membrane integrity. (Grimes *et al.*, 1987; Spicer *et al.*, 1987). Hormone measurements have also been used to indicate the health of the follicle. Non-atretic follicles contain higher estradiol and lower progesterone concentrations than do atretic follicles (Webb & England, 1982; McNatty *et al.*, 1984). Total immunoreactive inhibin concentrations are higher in non-atretic than in atretic follicles (Martin *et al.*, 1991; Guilbault *et al.*, 1993) whereas dimeric inhibin concentrations are higher in atretic than in healthy follicles (Guilbault *et al.*, 1993).

The characterization of the early stages of atresia based on morphological and histological criteria has been difficult, because estradiol secretion in histologically early-atretic follicles does not differ from that of non-atretic follicles, (Tsonis *et al.*, 1984; McNatty *et al.*, 1984; Grimes *et al.*, 1987), although progesterone concentrations are higher in early atretic than in healthy follicles (Spicer *et al.*, 1987). Recently, it has been shown that histological indices of atresia did not closely correlate with either morphological or endocrinological measures of follicular atresia (Price *et al.*, 1995).

It is now established that granulosa cell death during follicular atresia occurs by a process called apoptosis (Jolly *et al.*, 1994). Apoptosis or programmed cell death is a widespread phenomenon that plays a crucial role in several physiological and pathological processes (Schwartzman & Cidlowski, 1993), and is characterized morphologically by loss of cellular volume

(cytoplasmic condensation) accompanied by nuclear pycnosis resulting from fragmentation of the chromatin. In the membrana granulosa in cattle, these nuclei are frequently crescent shaped and uniformly electron dense and are approximately the same size as healthy nuclei, all of which are typical of early apoptosis, these nuclei were within the membranes of a healthy granulosa cell, suggesting that phagocytosis by a neighboring granulosa cell is an unusually early event in the apoptotic pathway of granulosa cells (Van Wezel *et al.*, 1999). At the biochemical level, the most marked feature in apoptotic cells is the loss of DNA integrity following endonuclease-mediated fragmentation of the nuclear genomic pool (Kim *et al.*, 1999).

As follicle development and atresia are intimately related to steroid hormone production by the follicle, the major steps involved in steroidogenesis, are reviewed below.

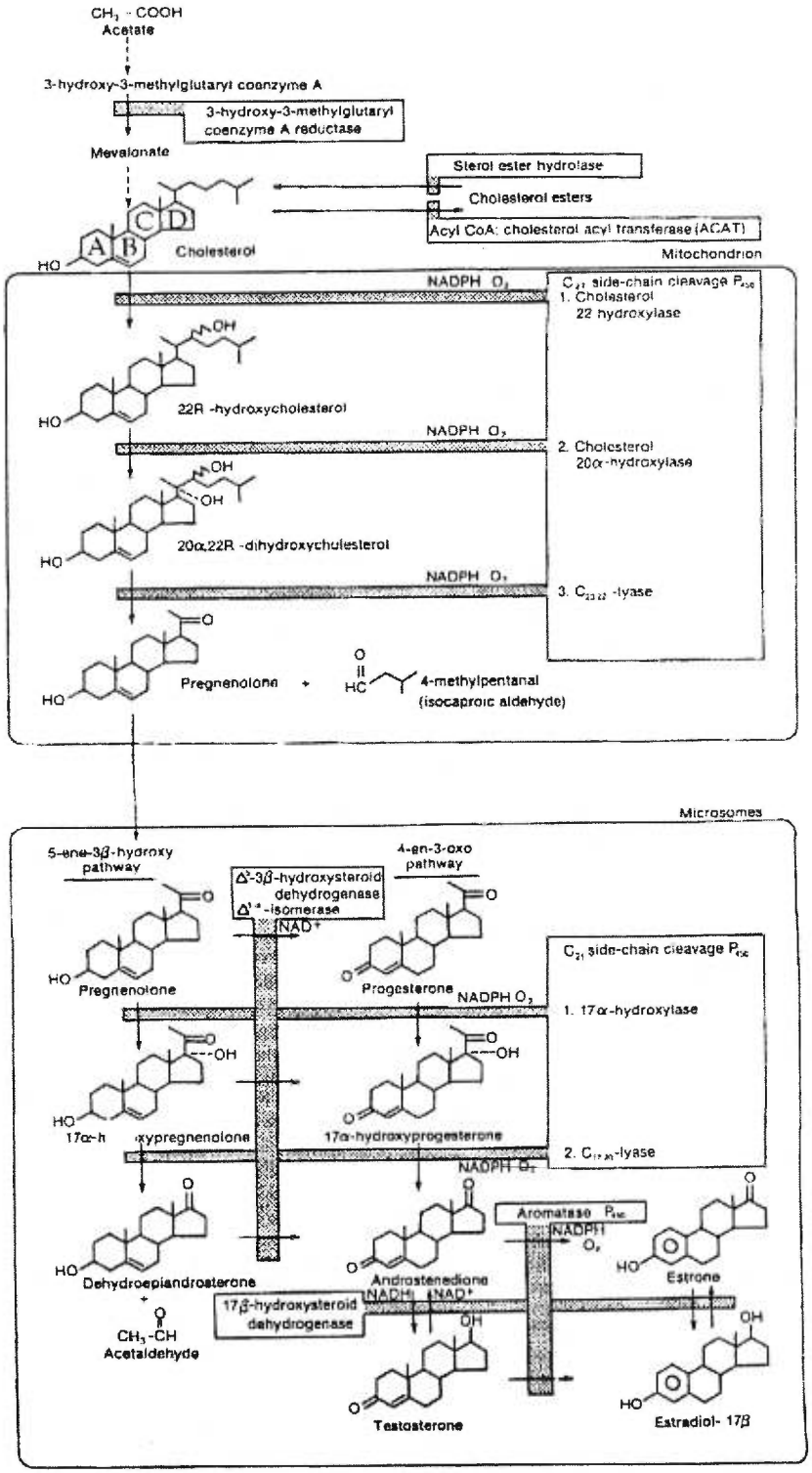
STEROIDOGENESIS IN OVARIAN FOLLICLES

The ovarian steroid hormones can be classified according to their biological activity and their number of carbon atoms. These are progestins, androgens and estrogens comprising 21, 19 and 17 carbons respectively, also designated as C_{21}, C_{19} and C_{17} steroids. They comprise a ring complex, formed of three cyclohexane rings (A, B, C) and a cyclopentane ring (D), referred to the perhydrocyclopentanophenanthrene nucleus (Gore-Langton & Armstrong, 1994).

The biosynthesis of ovarian steroids is catalyzed by up to three cytochrome P450 and two hydroxysteroid dehydrogenase enzymes (Gore-(Langton & *Armstrong.*, 1994; figure 2). The precursor of all steroids is cholesterol, which is firstly introduced into the inner mitochondrial membrane by the now well characterized steroidogenic acute regulatory protein (StAR) (Stocco & Clark, 1996). Once in the mitochondria, cholesterol is converted to pregnenolone. Pregnenolone in the microsomes can be metabolized using two different pathways, Δ^5 and Δ^4 (Gore-(Langton & *Armstrong.*, 1994). The Δ^4 pathway, involves the successive conversion of pregnenolone to progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone and estradiol.

In the Δ^5 pathway, pregnenolone can be metabolized without being converted to progesterone. Pregnenolone is successively converted to 17α-hydroxypregnenolone, dehydroepiandrosterone, androstenedione, estrone and estradiol. It has been suggested there exist differences between species in the utilization of steroidogenic pathways, and that the Δ^5 pathway is the preferred pathway in ruminants (Zuber *et al.*, 1986b).

FIG. 2 Steroidogenesis in ovarian follicles



Steroid precursor

Cholesterol is the precursor of all steroids, and the principal sources are a) lipoproteins from blood, b) ovarian cell stores such as free cholesterol in cell membranes, or cholesterol esters stored within cytoplasm lipid droplets, and c) cholesterol synthesized de novo in the ovarian cell from 2 carbon components (acetate) derived from metabolism of carbohydrate, fat or protein (Strauss *et al.*, 1981)

Data obtained from studies using extrafollicular tissues suggest that circulating lipoproteins are the most important source of steroidogenic cholesterol. Cholesterol is obtained from both low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Gwynne & Strauss, 1982). There are specific receptors for LDL lipoproteins located on the cell membrane. Once the LDL is bound to its ligand, the lipoprotein-receptor complex is internalized and free cholesterol is liberated from lipoproteins by lysosomal esterases. (Murphy & Silavin, 1989). HDL uptake is fundamentally different from that of LDL, and involves effecient transfer of the lipids, but not the outer shell proteins from HDL to cells (Gu *et al.*, 1998). The receptor for HDL is a class B type I scavenger receptor (SR-BI), which is expressed in non-luteinized theca cells, as well as in theca and granulosa cells during luteinization, and is regulated by gonadotropins in rats (Li *et al.*, 1998). There are species differences relative to the utilization of the two classes of lipoproteins; HDL seems to be most important in rodents, whereas LDL is the major circulating form of cholesterol in other species including cattle (Gore-Langton & Armstrong, 1994; Gwynne *et al.*, 1982) Once

cholesterol is in the interior of the cell, it can be stored within lipid droplets converted to esters of long-chain fatty acids, by the enzyme acyl coenzyme A: cholesterol transferase (ACAT), or it can be used immediately for steroid synthesis (Murphy & Silavin, 1989).

To be used in steroidogenesis, cholesterol must be transported from the cytoplasm to the inner mitochondrial membrane, where the enzyme P450 side chain cleavage ($P450_{scc}$), converts it to pregnenolone (Simpson, 1979). The first part of the process consists of the mobilization of cholesterol from cellular stores to the outer mitochondrial membrane (Liscum & Munn, 1999). Several factors may be implicated in this process, including sterol carrier protein 2 (SCP2, Pfeifer *et al.*, 1993). Recently, a role for Niemann-Pick C1 protein, the mutation of which causes a disease characterized by the lysosomal accumulation of low density lipoprotein-derived cholesterol, has also been implicated (Watari *et al.*, 1999). The second part of cholesterol transport consists of the transport of cholesterol from the outer to the inner membranes of the mitochondria. This step is the major barrier for the movement of cholesterol to $P450_{scc}$, because of the aqueous space between membranes (Schroeder *et al.*, 1991). Cholesterol is hydrophobic, and cannot across this barrier without some form of active transport (Phillips *et al.*, 1987). It is now well established that the acute transport of cholesterol from the outer to the inner mitochondrial membrane is mediated by the protein StAR (Stocco *et al.*, 1996). StAR is a 37 kDa protein which acts to form contacts sites between both mitochondrial membranes, allowing

cholesterol to pass into the mitochondria. (Stocco, 1998). This is the rate limiting step in steroidogenesis.

Steroidogenic enzymes

The cytochrome P450s constitute a diverse superfamily of constitutive and inducible hemoproteins (Nebert *et al.*,1989). They catalyze a variety of metabolically important oxidative reactions involving endogenous substrates (e.g., steroids and fatty acids) and xenobiotics (e.g., drugs, mutagens and carcinogens) (Lacroix *et al.*, 1990). The descriptions of P450 were based on the isolation of a red pigment inside the liver microsomes able to bind carbon monoxide (Porter & Coon, 1991)

The term cytochrome P450 refers to the spectral profile of the reduced form of carbon monoxide which strongly absorbs at a wavelength of 450 nm (Rodgers,1990). Cytochrome P450s are hemoproteins acting like terminal oxidases in the microsomes. In the steroidogenic cytochrome P450s, the oxidoreductase activity is achieved by two different enzymes, adrenodoxin and flavoprotein NADPH reductase (Rodgers, 1990).

All steroidogenic cytochrome P450s have regions of high homology, the heme-binding region, which is attributable to a conserved cysteine residue, the I-helix region that forms the active sites (Rodgers, 1990). The I-helix region is responsible for binding to the steroidogenic substrate, and the convertion to a product (Poulos & Howard, 1987). There is also a high degree of homology

between species for a given cytochrome P450 enzyme (Nishihara *et al.*, 1988; Mulheron *et al.*, 1989; Fevold *et al.*, 1989)

The hydroxysteroid dehydrogenases involved in ovarian steroid production are 3β-hydroxysteroid dehydrogenase-isomerase (Type I and II) and 17β- hydroxysteroid dehydrogenase (Type 1,2,3,4,5), of which the isoforms are products of different genes (Penning *et al.*, 1997; Andersson & Moghrabi, 1997).

P450 side chain cleavage ($P450_{scc}$)

Once cholesterol is inside the mitochondria, it is converted to pregnenolone by the $P450_{scc}$ complex, found in the interior of the mitochondria (Niswender *et al.*, 1994). Three different chemical reactions are involved in this process: 20α-hydroxylation, 22-hydroxylation, and the side chain cleavage of cholesterol between carbons 20 and 22 to form pregnenolone and isocaproic acid (Gore-Langton & Armstrong, 1994). The reaction utilizes nicotinamide adenine dinucleotide phosphate (NADPH) generated within the mitochondria by oxidation of Krebs cycle intermediates or fatty acids (Lieberman *et al.*, 1984). Three moles each of NADPH and of oxygen are utilized per mole of cholesterol undergoing side-chain cleavage. It has been suggested that free hydroxylated intermediates remain bound to the $P450_{scc}$ until the ultimate product, pregnenolone, is formed and released (Lieberman *et al.*, 1984).

The complementary DNA for $P450_{scc}$ has been cloned in several species, including cattle (Morohashi *et al.*, 1984). Northern analysis has revealed one transcript of approximately 2.0 kb.

3β-hydroxysteroid dehydrogenase

Pregnenolone metabolism is the cornerstone of androgen and estrogen synthesis in the gonads in mammals and it takes place inside the cellular microsomes (Conley & Bird, 1997b). Steroids can be produced using Δ^5 and Δ^4 pathways. The metabolites of the Δ^5 pathway, which include pregnenolone,

17α-hydroxypregnenolone, and dehydroepiandrosterone, can be converted to progesterone, 17α-hydroxyprogesterone, and androstenedione, respectively. The first conversion is made by the Δ^5-3β-hydroxysteroid dehydrogenase Δ^{5-4} – isomerase enzyme (3β-HSD). 3β-HSD also converts 17α-hydroxypregnenolone and dehydroepiandrosterone to 17α-hydroxyprogesteronene, and androstenedione, respectively (Gore-Langton & Armstrong, 1994). The enzyme dehydrogenates the A ring and creates a double ligand in the B ring of the substrate (Gore-Langton & Armstrong, 1994). The enzyme utilizes NAD + as a electron acceptor, and the reaction is essentially irreversible under physiological conditions (Gore-Langton & Armstrong, 1994).

P450 17α hydroxylase, C_{17-20}-lyase ($P450_{17OH}$)

Pregnenolone and progesterone are converted to 17α hydroxypregnenolone and 17α hydroxyprogesterone, by an hydroxylation reaction at the carbon 17 position (Le Goascogne *et al.*, 1995), such conversions are catalyzed by the $P450_{17OH}$ (Rodgers, 1990). Subsequently these can be cleaved between carbons 17 and 20, to be further converted into dehydroepiandrosterone and androstenedione, respectively (Youngblood *et al.*, 1991). $P450_{17OH}$ enzyme is the limiting step in androgen biosynthesis (Youngblood *et al.*, 1991) and is under gonadotropin control (Gore-Langton & Armstrong, 1994). In cattle, androstenedione is the principal aromatizable compound produced by theca interna cells (Fortune, 1986). The complementary

DNA for the $P450_{17OH}$ has been cloned in several species including cattle (Zuber *et al.*, 1986a), and $P450_{17OH}$ expression in this species is confined to theca interna cells of antral follicles (Voss *et al.*, 1993b; Soumano *et al.*, 1996).

17β-hydroxysteroid dehydrogenase (17β-HSD)

This enzyme can reversibly convert androstenedione to testosterone (catalyzed by the 17β-HSD type 3 enzyme localized in the testis) and estrone to estradiol-17β (catalyzed by the 17β-HSD type1 localized in granulosa cells), by dehydrogenation of their carbon 17 (Andersson *et al.*, 1997). The study of the regulation of its expression and activity in granulosa cells has received little attention (Tait *et al.*, 1989). However some studies have shown that in rats, expression of 17β-HSD increases with follicular diameter, and decreases during luteinization (Ghersevich *et al.*, 1994).

P450 aromatase ($P450_{arom}$)

The aromatase enzyme complex is composed of two polypeptides, aromatase cytochrome P450, and a flavoprotein, NADPH-cytochrome P450 reductase (Simpson *et al.*, 1994a). $P450_{arom}$ is found in the microsomal compartment of cells in which it is expressed and is responsible for binding C19 steroid substrate and catalyzing a series of reactions leading to formation of the phenolic A ring characteristic of estrogens (Simpson *et al.*, 1994a). Aromatase is specifically involved in the conversion of androstenedione to estrone and testosterone to estradiol (aromatization), whereas NADPH-cytochrome P450 reductase is an essential ubitiquous protein in the endoplasmic reticulum of most cell types and is responsible for transferring reduced equivalents from NADPH to any microsomal forms of cytochrome P450 with which it comes into contact (Simpson *et al.*, 1994a; Simpson *et al.*, 1997) .

The aromatase reaction apparently utilizes 3 moles of oxygen and 3 moles of NADPH for every mole of C19 steroid metabolized (Thompson & Siiteri, 1974). All three oxygen molecules are utilized in the oxidation of the C19 angular methyl group to formic acid, which occurs concomitantly with the aromatization of the A ring to give the phenolic structure characteristic of estrogens (Cole & Robinson, 1990).

Follicle growth and steroid biosynthesis are primarily under the control of pituitary gonadotropins, FSH and LH, thus the following section of this review will describe the action of gonadotropins on follicular cells.

CONTROL OF FOLLICULAR DEVELOPMENT

The gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are glycoprotein hormones composed of two dissimilar subunits (α and β subunits) associated by non-covalent bonds (Bousfield *et al.*, 1996). The α subunit is common to all gonadotropin hormones and is the product of a single gene, whereas the β subunit is specific for each gonadotropin, and is the product of specific single genes (Bousfield *et al.*, 1996).

Both gonadotropins are involved in the regulation of follicle growth and differentiation (Richards, 1994). Androgen secretion is under the control of LH, whereas estrogen secretion is under FSH control (Fortune & Armstrong, 1977). Their action on follicular cells is mediated by the cAMP/ protein kinase A (PKA) kinase pathway, although other possible pathways have been suggested, such as protein kinase C (PKC, Richards, 1994).

In ovarian follicles, FSH receptors (FSHr) are exclusively expressed on the cell surface of granulosa cells of developing follicles (Xu *et al.*, 1995a). In contrast, LH receptors are expressed on theca cells of antral follicles and in granulosa cells in advanced stages of follicular development (usually >8 mm diameter in cattle) (Lucy *et al.*, 1992). Gonadotropin receptors have a similar structure, composed of a large N-terminal extracellular domain with potential glycosylation sites, which is the hormone binding site (Xie *et al.*, 1990). They contain seven transmembrane regions forming 3 loops exposed to the cytoplasm and a short cytoplasmic tail (C-terminal, McFarland *et al.*, 1989;

Sprengel *et al.*, 1990). FSH and LH receptors are coupled to G proteins and activate the adenylyl cyclase second messenger system (Themmen *et al.*, 1994).

FSHr mRNA is first detected in early primary follicles in humans (Oktay *et al.*, 1997), sheep (Tisdall *et al.*, 1995), rats (Richards, 1980), cattle (Xu *et al.*, 1995a). However it is intriguing that preantral follicles can grow to the early antral stage in the absence of gonadotropins (Campbell *et al.*, 1995) as well as in FSH receptor deficient females (Dierich *et al.*, 1998). However, FSH increased the number of small preantral follicles in hypophysectomized mice (Wang & Greenwald, 1993) and the incorporation of H^3-thymidine into preantral follicles of hamsters (Roy & Greenwald, 1986). Further, FSH stimulates growth of preantral follicles in sheep (Cecconi *et al.*, 1999). In cattle undergoing superovulation with PMSG, which has both LH and FSH actions (Murphy and Martinuk, 1991), an increase in the number of preantral follicles was observed (Monniaux *et al.*, 1984). In rats (Spears *et al.*, 1998), FSH stimulated preantral follicles to grow and produce estradiol in vitro, suggesting that FSH may stimulate granulosa cell proliferation and differentiation at this stage of follicular development. LHr and 3β-HSD mRNA (Xu *et al.*, 1995a; Bao *et al.*, 1997b) as well as $P450_{scc}$, $P450_{17OH}$ protein in theca cells (Rodgers *et al.*, 1986) are first detected in secondary follicles in cattle, suggesting that these follicles are able to secrete androgens.

The most accepted model for follicular growth and steroidogenesis suggests that granulosa and theca cells are involved in the production of estradiol (two cell/two gonadotropin model; Fortune, 1986). In this model, granulosa cells contain FSH receptors (FSHr) and theca cells contain LH receptors (LHr) during earlier stages of development. Both cell types are presumed to have the cytochrome $P450_{scc}$ enzyme necessary for conversion of cholesterol to C-21 steroids (progestins). Binding of LH to its receptor on theca cells stimulates activity of the cytochrome $P450_{c17OH}$, enzyme (Campbell et al., 1998; Fortune, 1986. Campbell *et al.*, (1998) reported that follicular wall preparations containing granulosa and theca cells secreted greater quantities of androstenedione than did preparations of isolated theca cells, suggesting that progestins produced by granulosa cells are used as precursors for androgen production on theca cells. Granulosa cells did not produce androgens even in the presence of progestin precursors. Androgens are then metabolized to estradiol by the $P450_{arom}$ present in granulosa cells. Estradiol is secreted by follicles and act as a mitogen for granulosa cells in rats (Richards *et al.*, 1987). Estradiol and FSH induce synthesis of LH receptors on membranes of granulosa cells during later stages of follicular development, and, consequently, estradiol enhances its own secretion in rats (Richards, 1980).

Control of follicular recruitment

The mechanism that controls recruitment of antral follicles and determines which follicles are recruited is not known, but the signal that stimulates this process may be the slight elevation in plasma FSH concentrations that begins about 2.5 days before emergence of the new follicular wave (Adams *et al.*, 1992). In cattle there is a surge of FSH on the day of ovulation, which precedes the first follicular wave of the cycle (Dobson, 1978), and also there are slight elevations in FSH which precede the second and third follicular waves of the cycle (Adams *et al.*, 1992). Increases in FSH also precede the follicular waves in pregnant (Ginther *et al.*, 1989a) and in prepuberal animals (Adams *et al.*, 1994). Perturbations in these increases lead to concomitant changes in the pattern and /or number of recruited follicles; injections of inhibin rich follicular fluid delayed the first follicular wave of the cycle in cattle (Turzillo & Fortune, 1990), no follicular growth beyond 3-4 mm was observed when FSH was suppressed by inhibin (Lussier *et al.*, 1994) and the number of recruited follicles can be increased by exaggerating the height of the FSH surge (Gibbons *et al.*, 1997)

Nutrition is an important factor that alters follicular recruitment. Increased dietary intake was associated with an increased number of recruited follicles during the first follicular wave of the estrous cycle in heifers (Gutiérrez *et al.*, 1997c). This was independent of changes in circulating FSH and IGF-1 concentrations, but was associated with increased insulin concentrations. A similar effect was observed in cows supplemented with polysaturated fats

(Thomas *et al.*, 1997). Therefore, insulin may be one of the mediators of the effects of nutrition on follicular recruitment. Lactation is another factor that affects number of recruited follicles; this number is lower in lactating relative to non-lactating cows (Lucy *et al.*, 1992). This may be due to the very high nutritional requirements of lactation, which is characterised by a marked reduction in energy balance (Jolly *et al.*, 1995). The interaction between nutrition and reproduction is not well understood. Recently the protein leptin, a hormone product of adipose cells (Halaas *et al.*, 1995) has been postulated to be a key element (Chehab *et al.*, 1996). Evidence for a role of leptin in follicular recruitment comes from studies showing that leptin increased the number of follicles at all stages of follicular development in mice (Barash *et al.*, 1996).

Why only some follicles are recruited to continue their development is unknown. Maybe there are biochemical differences in the follicles at the time that they are recruited, such that some follicles are more sensitive to prewave elevations of FSH. For example, at the time of recruitment, the follicles of the recruited cohort are not all similar. In pigs, the smallest of the cohort have been shown to be the richest in EGF (Hsu *et al.*, 1987) and the most sensitive to the inhibitory effects of EGF on cell differentiation (Buck & Schomberg, 1988). Hence the stimulatory effect of FSH on cell differentiation (aromatase) may be more marked for the largest of the recruited follicles (Driancourt *et al.*, 1996).

The recruitment of a cohort of follicles 4-6 mm in diameter in cattle is associated with the simultaneous expression of $P450_{scc}$ and $P450_{arom}$ mRNA in granulosa cells of recruited follicles (Xu *et al.*, 1995b; Bao *et al.*, 1997a).

Induction of $P450_{scc}$ and $P450_{arom}$ mRNA expression in granulosa cells is likely stimulated by the transient increase in circulating FSH concentrations observed at the beginning of each follicular wave (Adams *et al.*, 1992; Hamilton *et al.*, 1995).

Recently, a GnRH-agonist model has been described whereby secretion of LH, but not FSH, is decreased over a 28 day period (Gong *et al.*, 1995). In this model, follicles grow to a size of 7 to 9 mm in diameter and persist, but further growth does not occur. If the GnRH-agonist treatment is extended for another 7 days, FSH is also depressed. The follicles of 7 to 9 mm in diameter regress and new follicular growth is arrested at 2 to 4 mm in diameter (no recruitment). These data suggest that recruitment and growth of follicles beyond 2-4 mm in diameter requires FSH. As follicles grow from 5 mm to 8 mm, granulosa cells express $P450_{arom}$ and $P450_{scc}$ mRNA, but not 3β-HSD mRNA (Xu *et al.*, 1995b; Bao *et al.*, 1997b), and theca cells express LHr, $P450_{scc}$, P450c17, 3β-HSD (Xu *et al.*, 1995b; Bao *et al.*, 1997b). This suggests that granulosa cells of follicles during recruitment start to metabolize cholesterol to pregnenolone and theca androgen to estradiol, but not pregnenolone to progesterone (Bao *et al.*, 1997b). To the contrary, $P450_{arom}$, $P450_{scc}$ and 3β-HSD mRNA were not detected in granulosa cells of follicles <4mm in diameter (Xu *et al.*, 1995b; Bao *et al.*, 1997b) indicating that granulosa cells of follicles <4mm are not able to convert androgens to estradiol or cholesterol to pregnenolone and subsequently to progesterone.

Control of follicular selection

The mechanism of follicular selection is poorly understood. Transient increases in FSH secretion precede the emergence of follicular waves in cattle (Webb *et al.*, 1992), and the subsequent decrease in circulating FSH concentrations is temporally associated with selection of the dominant follicle (Adams *et al.*, 1992). This point has been called deviation (Ginther *et al.*, 1996). Although it has been suggested that the acquisition of LH receptors on granulosa cells plays an important role both in the selection of the future dominant follicle (Spicer *et al.*, 1986; Xu *et al.*, 1995a; Jolly *et al.*, 1994; Bao *et al.*, 1997a), and in the expression of 3β-HSD mRNA (Bao et al., 1997), it has also been shown that follicle selection takes place before formation of LH receptors in granulosa cells occurs (Evans & Fortune, 1997a).

LHr and 3β-HSD mRNA expression are predominantly detected in one healthy follicle ≥ 8 mm in diameter per cow (Xu *et al.*, 1995a; Bao *et al.*, 1997b). Continued growth of dominant follicles is accompanied by an increase in expression of LHr and 3β-HSD mRNA in granulosa cells, which also express greater levels of mRNA for $P450_{scc}$ and $P450_{arom}$ than do their counterparts in subordinate follicles (Bao *et al.*, 1997a). Increases in expression of mRNA for FSHr, LHr, $P450_{scc}$, 3β-HSD, and $P450_{arom}$ in granulosa cells and, $P450_{17OH}$ and StAR can be found in thecal cells during selection of the dominant follicles, after the initiation of the first follicular wave (Xu *et al.*, 1995a; Bao *et al.*, 1997b; Bao *et al.*, 1998). This is the time that follicles produce increasing amounts of

estradiol (Evans *et al.*, 1997b). Regulation of LHr mRNA in granulosa cells during selection is not well understood in domestic animals. In rodents, induction of LHr in granulosa cells is dependent on the actions of FSH and estradiol (Richards, 1980; Segaloff *et al.*, 1990). Estradiol seems to enhance LHr mRNA expression in rat granulosa cells (Richards *et al.*, 1987).

Control of follicular dominance

The mechanism of follicular dominance has not been determined. It has been hypothesized that follicular dominance can be exerted directly or indirectly (Baker *et al.*, 1999). Direct dominance has been related to the secretion of a paracrine (intrafollicular) factor from the dominant follicle. However mechanisms of this sort cannot be involved in the cow, since the dominant follicle induces regression of subordinate follicles in both ovaries; such a factor would clearly have to be endocrine in nature (Fortune, 1994). Because follicular dominance occurs when FSH concentrations are decreased to basal levels, and removal of the dominant follicle 3 days after ovulation resulted in an immediate surge in FSH (Adams *et al.*, 1992), it is most likely that dominance is achieved indirectly, by means of some factor which has a negative feedback effect on FSH secretion (Price, 1991). Among the candidates are estradiol and other steroids secreted by the dominant and second largest subordinate follicle (Price, 1991). Other candidates are the proteinaceous components of follicular fluid, which include inhibin (produced by granulosa cells that directly reduces pituitary FSH secretion (Findlay, 1993) and follistatin (a high affinity, low capacity binding protein for

activin, produced by granulosa cells, Findlay, 1993). However it is not clear whether estradiol or other steroids produced by the largest and second largest subordinate follicles shortly after wave emergence are functionally related to FSH suppression (Bolt *et al.*, 1990; Bo *et al.*, 1993). A temporal relationship between an increase in estradiol in the vena cava and a decrease in FSH in the jugular vein has been reported (Evans *et al.*, 1997b). Nonetheless steroid-free bovine follicular fluid dramatically suppressed circulating concentrations of FSH and delayed or interrupted follicular development when administered systemically (Turzillo & Fortune, 1993; Wood *et al.*, 1993). Testosterone is not likely to be involved, as immunization against testosterone did not raise FSH concentrations in cattle (Price *et al.*, 1987). Recently it has been suggested that the decline in FSH was not attributable to endogenous progesterone and estradiol concentrations (Gibbons *et al.*, 1999).

Although inhibin can selectively control FSH concentrations in sheep (Martin *et al.*, 1988), and inhibin antiserum increases circulating FSH concentrations in cattle (Kaneko *et al.*, 1995), inhibin-free follicular fluid suppresses follicular growth without affecting FSH concentrations in cattle (Law *et al.*, 1992). Furthermore, injection of protein fraction of follicular fluid 3-6 days after ovulation prevented growth of the dominant follicle beyond 10 mm without altering FSH concentrations (Kastelic *et al.*, 1990b) suggesting that other components present in follicular fluid could suppress follicular growth independently of FSH concentrations. Recently, the presence of factors secreted by dominant follicles that inhibit follicular growth of subordinate follicles,

other than inhibin and steroids have been proposed (Armstrong & Webb, 1997). The nature of such factors remains to be elucidated, and there are more than 100 proteins in follicular fluid in cattle (Roche, 1996).

It is clear, however that dominant follicles are markedly less sensitive to decreased FSH concentrations than are subordinate follicles (Henderson *et al.*, 1987). Dominant follicles have a well developed vasculature (Moor & Seamark, 1986), thus increased blood supply, and consequently more FSH and LH may be distributed within an ovary to the dominant follicle (Brown & Driancourt, 1989). Increased blood supply may increase the availability of some factors which amplify FSH action on the cells of the dominant follicle. One candidate for such a positive autocrine action is IGF-I, which is present in high concentrations in large follicles (Echternkamp *et al.*, 1990). Increased IGF-I production, paralleled by increased receptivity of granulosa cells to IGF-I, stimulated $P450_{arom}$ activity in rats (Adashi *et al.*, 1985). Hence IGF-I, potentially by amplifying the action of FSH on aromatase activity and the number of LH receptors, may play a key role in the maintenance of dominance (Baker *et al.*, 1999).

An interesting point is that all growing follicles are capable of becoming dominant (Ginther *et al.*, 1996), as observed when FSH is used for superovulation early in the wave (Adams *et al.*, 1993). If all follicles of 5 mm in diameter except one are experimentally destroyed at the beginning of the wave, the remaining follicle becomes dominant (Gibbons *et al.*, 1997) and the second

subordinate follicle can assume dominance if the original dominant follicle is destroyed (Ko *et al.*, 1991).

Dominant follicles are characterised by their increased capacity to produce estradiol, which is related to increases in the expression of mRNA for gonadotropin receptors, steroidogenic enzymes, and StAR in theca cells (Xu *et al.*, 1995b; Bao *et al.*, 1997a; Bao *et al.*, 1998). Apparently a loss of functional dominance occurs in dominant follicles of the first follicular wave before an apparent decrease in size occurs. This is associated with a reduction in $P450_{scc}$, $P450_{c17OH}$, 3β-HSD, StAR, and FSHr mRNA levels as well as follicular fluid steroid hormones between day 4 and 6 of the follicular wave (Xu *et al.*, 1995b; Bao *et al.*, 1997a; Bao *et al.*, 1998). Expression of mRNA for LHr and $P450_{arom}$ remain elevated to day 6 before decreasing on day 8 of the wave (Xu *et al.*, 1995a). Similarly, Lucy *et al.*, (1992), reported that dominant follicles collected on day 5 and 8 of the bovine estrous cycle had similar $P450_{arom}$ activity, but concentrations of estradiol in follicular fluid were greater in dominant follicles collected on day 5 than those collected on day 8. Reduction in the ability of dominant follicles on day 6 to produce estradiol is believed to be due to insufficient androgen production in theca cells (Lucy *et al.*, 1992; Soumano *et al.*, 1996). It is possible that reduction in thecal cell androgen production is associated with atresia.

The dominant follicle continues to grow and change its gonadotropin dependency from FSH to LH, because at this stage of development, granulosa

cells have already developed LH receptors (Lucy *et al.*, 1992). However FSH remains critically important to the dominant follicle, even during the dominance phase, since experimental reduction of plasma FSH during that time in cattle is correlated with cessation of growth, and in some animals the demise of the dominant follicle (Turzillo *et al.*, 1993). If luteal regression occurs during the dominance phase, the follicle is exposed to additional hormonal signals that allow it to develop fully to the point of secreting enough estradiol to elicit the LH/FSH surge and ovulation. In cattle, the decline in plasma progesterone at luteal regression is followed by small increases in basal LH concentrations, and an increase in LH pulse frequency (Walters *et al.*, 1984).

The importance of the LH pulse frequency in the final stages of follicular development prior to the LH surge can be further demonstrated by artificially increasing LH pulse frequency to follicular phase levels. The dominant follicle continues to grow in a linear fashion, and plasma estradiol is elevated for much longer than normal (Sirois & Fortune, 1990; Stock & Fortune, 1993). Also, chronic treatment of cattle with a GnRH agonist suppresses the pulsatile secretion of LH, and the largest follicle does not grow beyond 7-9 mm, indicating the necessity of LH for continued follicular development (Gong *et al.*, 1995). Lactating cows on a low-energy diet had a lower LH pulse frequency, and the diameter of the largest follicle was less than in cows on a 100% energy diet (Grimard *et al.*, 1995).

In preovulatory follicles, before the LH surge, levels of mRNA for $P450_{scc}$ and 3β-HSD in granulosa cells increase, as do follicular fluid androstenedione

and estradiol-17β concentrations, suggesting that expression of mRNA for steroidogenic enzymes increases further in preovulatory follicles following luteal regression (Voss & Fortune, 1993a; Voss *et al.*, 1993b; Tian *et al.*, 1995). The expression of $P450_{c17OH}$ mRNA in theca cells, $P450_{arom}$ mRNA in granulosa cells, and $P450_{scc}$ in granulosa and theca cells decreases in preovulatory follicles after the LH surge. This decrease in steroidogenic enzyme mRNA is followed by decreases in follicular fluid androstenedione and estradiol concentrations and increases in progesterone concentrations (Voss *et al.*, 1993a)

Growth factors in follicular development

Although follicular development is primarily regulated by gonadotropins, it is also regulated by other factors (steroids and growth factors) of endocrine and paracrine origin (Monget & Monniaux, 1995).

Growth factors play an essential role in the regulation of cell proliferation and differentiation. The different growth factors have been classed in different families, based in their structure and biological activity : the epidermal growth factor (EGF) family, the fibroblast growth factor (FGF) family, the platelet – derived growth factor (PDGF) family, the insulin-like growth factor (IGF) family, the transforming growth factor β (TGF-β) family and the hemopoietic growth factors (cytokines). They are secreted from various types of cells and act locally in an autocrine, paracrine, juxtacrine or intracrine fashion (Monget *et al.*, 1995).

The IGFs are the most investigated of the ovarian growth factors. The IGF system is composed of two ligands (IGF-1 and IGF-II), two receptors (type I and II), six IGF-binding proteins, and specific IGFBP proteases (Jones & Clemmons, 1995). The type I receptor binds IGF-I with more affinity than IGF-II, and has a low affinity for insulin; they are structurally and functionally similar to the insulin receptor (Giudice, 1992). The type II receptor, or IGF-II mannose-6-phosphate (IGF-II-M6P) receptor, binds IGF-II but also IGF-I with very low affinity, but not insulin (Nissley & Kiess, 1991). The six IGF-binding proteins (IGFBPs) bind IGF-I and IGF-II with high affinity (Rechler, 1993). They are present in all biological fluids, and can be grouped as the small complex proteins (IGFBP 1,2,4,5 and 6) with a molecular weight range between 34 kDa and 35 kDa, and the large complex proteins (IGFBP-3) ranging between 42-44 kDa, which are the most predominant in serum. The IGFBPs potentiate or inhibit IGFs on target cells by sequestration (Monget *et al.*, 1995).

IGFs function as modulators of gonadotropin action at the cellular level and stimulate granulosa and theca cell proliferation and differentiation (Adashi *et al.*, 1992; Giudice, 1992). There is heterogeneity in the localization of IGF expression in the ovary between species. In rodents (Oliver *et al.*, 1989) and pigs, IGF-I is expressed mainly in granulosa cells of growing antral follicles (Hammond *et al.*, 1993), whereas IGF-II is limited to theca interna cells (Hernandez *et al.*, 1990; Yuan *et al.*, 1996). In contrast, in humans, IGF-I expression is confined to theca cells, whereas IGF-II is expressed in granulosa

cells of antral follicles (Zhou & Bondy, 1993). It appears that in pigs, FSH stimulates the synthesis of IGF-I by granulosa cells (Hatey *et al.*, 1995), but not in mice (Zhou *et al.*, 1997). In ruminants, however, the expression of IGF-I is controversial. IGF-I mRNA has been detected in ovine granulosa and theca cells (Leeuwenberg *et al.*, 1995). Whereas did not detected IGF-I in these tissues (Perks *et al.*, 1995). In cattle, it was found that both granulosa and theca cells contain IGF-I mRNA (Spicer *et al.*, 1993), although non-luteinized bovine granulosa cells did not produce IGF-I in serum free cultures (Gutiérrez *et al.*, 1997a). Expression of IGF-II mRNA has been detected in theca cells of bovine and sheep antral ovarian follicles (Armstrong *et al.*, 1997; Perks *et al.*, 1995).

It seems that IGF-I stimulates proliferation of granulosa cells from small antral follicles, whereas it stimulates differentiation of granulosa cells from large follicles in sheep (Monniaux & Pisselet, 1992)

The actions of IGF-1 on follicular cells are mediated by type I receptors (Adashi *et al.*, 1992) located in granulosa (Spicer *et al.*, 1994b) and theca cells (Bergh *et al.*, 1993). Some studies have shown that the number of IGF-1 receptors is similar among small, medium, and large porcine and sheep follicles (Hylka *et al.*, 1989; Monget *et al.*, 1989). In contrast, in cattle, granulosa cells from large follicles had higher numbers of IGF-I receptors than did cells from small follicles (Spicer *et al.*, 1994b) and expression of IGF-I receptors increased as follicles enlarge from preantral to antral size (Wandji *et al.*, 1992b). The hormonal regulation of granulosa cell IGF-I receptors is not completely understood. In rats, it has been shown that the expression of this receptor is

increased by estrogens and gonadotropins (Adashi *et al.*, 1992). In cattle, the number of IGF-I receptors in granulosa cells of small bovine follicles were increased by EGF, estradiol, and FSH, decreased by basic fibroblast growth factor (bFGF), and unaffected by LH and progesterone. In contrast, numbers of IGF-I receptors in granulosa cells of large follicles were unaffected by these treatments (Spicer *et al.*, 1994b).

The bioactivity of IGFs is controlled by their association with a family of specific IGF binding proteins (IGFBPs). Six distinct IGFBPs have been characterized, following either their purification or cDNA isolation and sequencing (Clemmons *et al.*, 1995). The pattern of distribution and expression of IGFBPs differs according to the diameter and degree of atresia in antral follicles. In sheep and cattle IGFBP-3 concentrations are higher and IGFBP-2 and IGFBP-4 are lower in large than in small healthy follicles (Monget *et al.*, 1993; Stewart *et al.*, 1996; de la Sota *et al.*, 1996). Conversely, IGFBP-2, IGFBP-5, and IGFBP-4 were higher in atretic than in healthy follicles, but IGF-I and IGF-II concentrations did not differ between follicular stages. (Monget *et al.*, 1993; Stewart *et al.*, 1996; de la Sota *et al.*, 1996). Thus, these results suggest that follicular development is accompanied by an increase in large complex IGFBP concentrations, whereas small complex IGFBP concentrations decrease.

In sheep and cattle, IGFBP-2 mRNA is confined to granulosa cells and IGFBP-4 and 5 mRNA was expressed in theca cells from large healthy follicles (Besnard *et al.*, 1996a; Armstrong *et al.*, 1998), and in cattle, immunoreactivity

for IGFBP2 was detected in granulosa cells and basement membranes of healthy antral follicles, and IGFBP-4 was found in both theca and granulosa cells (Armstrong *et al.*, 1998).

The IGFBPs are often found in association with the extracellular matrix (ECM) and cell membranes, controlling the activity of IGFs by sequestration, not only reducing IGF availability, but also providing an extracellular store of IGFs that can be targeted to specific cell populations by IGFBP-cell interactions (Ferry *et al.*, 1999). Information concerning IGFBP-6 is scarce but it has been suggested to be a relatively specific inhibitor of IGF-II actions (Bach, 1999)

The mechanisms that lead to changes in intrafollicular IGFBP concentrations in ovarian follicles are poorly understood. These may be a local paracrine processes, controlled by the actions of specific IGFBP proteases. In rats, it has been suggested that small molecular weight IGFBPs are degraded by specific intrafollicular proteases induced by FSH (Liu & Ling, 1993). In sheep, the presence of specific IGFBP-2 and IGFBP-4 proteases in ovine follicular fluid has been reported (Besnard *et al.*, 1996b). The nature of these proteases remains to be elucidated, but it has been suggested that a specific FSH-dependent IGFBP-5 endopeptidase exists in rat granulosa cells, possibly a metalloprotease (Resnick *et al.*, 1998). The inhibition of IGFBP expression by gonadotropins in follicular cells is postulated to occur by another mechanism. In pigs (Samaras *et al.*, 1993), sheep (Besnard *et al.*, 1996a) and cattle (Armstrong *et al.*, 1998) the decrease in IGFBP-2 concentrations in follicular fluid during follicle growth was shown to be due to a loss of expression of mRNA encoding

IGFBP-2 in granulosa cells in dominant follicles. In rats, IGFBP-4 and –5 decreased in FSH-stimulated granulosa cells *in vitro* (Liu *et al.*, 1993), and in humans the secretion of IGFBP-2 by luteinizing granulosa cells *in vitro* is decreased by hCG (Cataldo *et al.*, 1993). Thus in healthy growing follicles, gonadotropins would not only reduce the expression of small molecular weight IGFBPs, but also induce the secretion of a protease(s) which would degrade them.

It is hypothesised that IGFBP-4, produced by theca cells under the influence of LH, is transported in association with IGFs to the extracellular matrix surrounding granulosa cells, thus acting as a extracellular storage for IGF (Armstrong *et al.*, 1998). It has also been suggested that the IGF associated with IGFBP-4 can be accessed by granulosa cells through the activation/ production of specific IGFBP-4 proteases (Besnard *et al.*, 1996b). In contrast, IGFBP-2 produced by granulosa cells may play an inhibitory role. After binding IGF, the affinity of IGFBP-2 for heparin-like molecules in the ECM surrounding granulosa cells increases (Arai *et al.*, 1996). Hence IGFBP-2 could sequester free IGF from the vicinity of granulosa cells. Granulosa IGFBP-2 mRNA is inhibited by FSH resulting in the absence of IGFBP-2 mRNA and immunoreactive IGFBP-2 in dominant follicles (Armstrong *et al.*, 1998). This, combined with a corresponding increase in IGFBP-4 protease activity in large follicles (Mondschein *et al.*, 1991), may increase the biological availability of IGF-I and in turn increase the sensitivity of the granulosa cells to FSH, allowing

the follicle to maintain responsiveness to FSH during the period when systemic FSH concentrations decrease.

The TGF-β superfamily is composed of several members, including activins, inhibins and Mullerian inhibitory substance, growth differentiation factor-9 (GDF-9) as well as 4 growth factors (TGF-β1, TGF-β2, TGF-β3, TGF-β5; (Miller *et al.*, 1990), TGF-βs act through two different types of serine/theonine kinase receptors (type I receptor and type II receptor) (Monget *et al.*, 1995). In cows, TGF-βs have inhibitory effects on proliferation of granulosa and theca cells, while they enhance gonadotropin – stimulated steroidogenesis (Roberts & Skinner, 1991). TGF-βs are secreted in a latent form and bind to the ECM (Harpel *et al.*, 1992) and their activity appears to be dependent upon conversion to an active form; specific proteases may be involved in the release of the mature form of TGF-βs (Armstrong *et al.*, 1997).

At least twelve growth factors are known to form part of the FGF family (FGF-1 or aFGF, FGF-2 or bFGF, FGF-3 or int-2, FGF-4 or hst/ks, FGF-5, FGF-6, FGF-7 or keratinosite growth factor, FGF-8, FGF-9, FGF-10, FGF11 and FGF-12) (Goldfarb, 1996). Four different tyrosine kinase receptors have been described (Goldfarb, 1996). In the ovary, the most studied member of this family is FGF-2 (basic FGF), and is produced by bovine granulosa cells (Neufeld *et al.*, 1987), and specific binding sites has been found in granulosa cells of preantral

follicles (Wandji *et al.*, 1992a), suggesting a possible role for this growth factor in early stages of folliculogenesis. FGF-2 has also been shown to stimulate bovine theca cell proliferation (Spicer & Stewart, 1996). FGF-2 immunoreactivity has been detected in mouse follicles, and FGF-2 inhibits FSH-stimulated induction of LH receptor expression in granulosa cells and reduces the binding of IGF to theca cells (Wordinger *et al.*, 1993). FGFs are potent angiogenic factors, however their role in ovarian angiogenesis is not known (Redmer & Reynolds, 1996).

FGFs have a high affinity for heparan sulfate-like molecules in the ECM and plasma membranes, and it appears that, as for other growth factors, their association with the ECM is a key component in regulating their activity (Yayon *et al.*, 1991). During follicular growth and atresia there are considerable changes in the structure of the ECM as a result of tissue remodelling, and these changes may alter the bioactivity of FGFs (Song *et al.*, 1999).

EGF and TGF-α are mitogenic factors that act through a common receptor that has been detected in bovine and ovine granulosa cells (Armstrong *et al.*, 1997). TGF-α stimulates bovine theca and granulosa cell proliferation, while inhibiting gonadotropin-stimulated differentiation of cells (Roberts *et al.*, 1991). TGF-α has been detected in bovine theca cells from small antral follicles of 0.7-2.0 mm diameter (Lobb *et al.*, 1989). Using an ovarian autotransplant model, TGF-α has been shown to inhibit ovarian function in ewes (Campbell *et*

al., 1994), and to inhibit estradiol secretion in bovine granulosa cells (Rouillier *et al.*, 1997). These results suggest that TGF-α from thecal origin influences granulosa cell and differentiation via paracrine mechanisms.

Because the main objectives of this review are to describe how estradiol secretion and aromatase are regulated in the ovary, the following section describes several aspects of $P450_{arom}$ gene regulation and activity.

P450 AROMATASE GENE EXPRESSION AND REGULATION

The gonads and brain are the major sites in which aromatase expression occurs in most vertebrate species that have been examined (Simpson *et al.*, 1994a). In the brain, conversion of androgens into estrogens by aromatase is a key mechanism by which testosterone regulates many physiological and behavioral processes, including the activation of male sexual behavior, brain sexual differentiation and negative feedback effects of steroid hormones on gonadotropin secretion (Balthazart & Ball, 1998). A role for aromatase has been postulated for species in which sex determination is temperature-dependent, such as fishes and reptiles (Jeyasuria & Place, 1998). In humans and higher primates, aromatase expression occurs in placenta as well as in the peripheral tissues in the adult, including skin (Schweikert *et al.*, 1976), muscle and adipose tissue (Longcope *et al.*, 1978), bone (Sasano *et al.*, 1997b). Aromatase immunoreactivity has been found in coronary arteries in humans (Diano *et al.*,

1999). Aromatase expression in the placenta is not confined to humans but also is expressed in ungulate species such as cows (Furbass *et al.*, 1997), pigs (Conley *et al.*, 1997a) and horses (Marshall *et al.*, 1996). In cattle, there is no evidence of estrogen biosynthesis in adipose tissue, whereas in rodents neither adipose nor placenta have the ability to synthesize estrogens (Simpson *et al.*, 1994a). Aromatase activity has also been reported in Leydig cells of rats (Valladares & Payne, 1979), as well as in the preimplantation pig blastocyst (Perry *et al.*, 1973).

The physiological significance of estrogen biosynthesis in the placenta and adipose tissue of humans is unclear. The estrogen produced at each site can be quite tissue-specific. For example, the human ovary synthesizes primarily estradiol, whereas the human placenta synthesises estriol and adipose tissue synthesises estrone (Simpson *et al.*, 1994a). Apparently this is a reflection of the nature of the C19 steroid presented to the estrogen-synthesizing enzyme in each tissue site. Thus, in the case of adipose tissue, the principal source of substrate is circulating androstenedione produced by the adrenal cortex (Tchernof *et al.*, 1995). In the placenta, the principal substrate is 16α-hydroxydehydroisoandrosterone sulfate derived as a consequence of the combined activities of the fetal adrenal and liver. (Simpson *et al.*, 1994a). Although the physiological significance of the biosynthesis of estrogens in peripheral tissues is not understood, several pathological conditions have been

attributed to an excess of aromatase expression and estrogen secretion in humans (Simpson *et al.*, 1997)

Recently, aromatase knockout (ArKO) mice have been generated (Fisher *et al.*, 1998; Honda *et al.*, 1998). Although these animals grew to adulthood, female mice at 9 weeks of age displayed underdeveloped external genitalia and uteri (Fisher *et al.*, 1998; Honda *et al.*, 1998). The ovaries contained numerous follicles with abundant granulosa cells and evidence of antrum formation although no corpora lutea were present. Additionally, the stroma were hyperplastic with structures that appeared to be atretic follicles. Development of the mammary glands approximated that of a prepuberal female (Fisher *et al.*, 1998). The males of the same age displayed essentially normal internal anatomy but with enlargement of the male accesory sex glands due mainly to increased content of secreted material. The testes appeared normal and the males were fertile (Fisher *et al.*, 1998; Simpson, 1998), although sexual behavior was modified, showing significantly prolonged latencies to mount and decreased numbers of mounts in response to receptive stimulus females (Honda *et al.*, 1998). Whereas serum estradiol levels were at the limit of detection in ARKO mice, testosterone levels were elevated, as were the levels of follicle stimulating hormone and luteinizing hormone (Fisher *et al.*, 1998).

P450 aromatase gene structure

The $P450_{arom}$ gene (CYP19) has been cloned and characterized in humans (Means *et al.*, 1989; Harada *et al.*, 1990), rats (Hickey *et al.*, 1990), cattle (Hinshelwood *et al.*, 1993), pigs (Conley *et al.*, 1997a), fish (Shen *et al.*, 1994), chickens(McPhaul *et al.*, 1988).

The $P450_{arom}$ gene exists as a single-copy gene spanning at least 70 kb in humans and is larger than other members of the cytochrome P450 superfamily (Means *et al.*, 1989; Harada *et al.*, 1990). Although there is no evidence of additional Cyp19-related genes or isoforms in humans and cattle (Furbass *et al.*, 1997), related genes or isoforms have been detected in the porcine genome (Corbin *et al.*, 1995).

The aromatase promoter has been characterized in humans (Means *et al.*, 1989; Harada *et al.*, 1990) rodents (Yamada-Mouri *et al.*, 1996; Honda *et al.*, 1996), and cattle (Furbass *et al.*, 1997). In humans and rats, it consist of 10 exons, and at least 14 exons in cattle (Furbass *et al.*, 1997). The coding area of the gene is confined to exons 2-10. Exon I encodes the 5'-untranslated region, and is separated from exon 2 by an intron of more than 35 kb (Sasano & Harada, 1998; Furbass *et al.*, 1997). It has been shown that tissue-specific promoters made by alternative splicing regulate $P450_{arom}$ gene expression in humans (Simpson *et al.*, 1997), and cattle (Hinshelwood *et al.*, 1995; Furbass *et al.*, 1997), thus the expressed aromatase protein is subsequently identical regardless of splicing pattern (Harada *et al.*, 1993; Means *et al.*, 1991; Mahendroo *et al.*, 1991). Different nomenclature for these splice variants is

currently in use in humans (Simpson *et al.*, 1994a; Sasano *et al.*, 1998) and cattle (Furbass *et al.*, 1997). Major splice variants of exon 1 used as a promoter in aromatase gene expression are summarized as follows: exon 1a (or I.1) variant, is mainly present in placenta in humans and exon 1.1 and 1.3 in cattle. Exon 1b (or I.4) variant, is present in skin and adipose fibroblasts and fetal liver; exon 1c (or I.3) variant is present in the ovary in humans; and exon 1d (or PII) codes for the isoform in the testis or adrenal in both species where the expression of aromatase is low. Exon 1f occurs in brains of humans and exon 1.4 in cattle brain. (Simpson *et al.*, 1994a; Sasano *et al.*, 1998; Furbass *et al.*, 1997). This alternative splicing of the $P450_{arom}$ gene is also considered to play important roles not only in tissue-specific expression of aromatase but also in alteration of aromatase expression through development of neoplastic processes in breast cancer (Sasano *et al.*, 1998).

The size of the transcripts differs markedly among species. Whereas two transcripts of 3.4 and 2.9 kb are observed in all human tissues examined and three sizes of rat transcripts of 2.7, 2.2, and 1.7 kb are found (Mendelson & Simpson, 1987; Hickey *et al.*, 1990). It was reported that there is a single species of bovine transcript of 6kb (Hinshelwood *et al.*, 1993). In contrast, three transcripts of approximately 6.5, 3.4, and 1.8 kb have been reported in whole bovine follicles, and of these, only the 6.5 kb transcript is polyadenylated (Soumano *et al.*, 1996).

In cattle, it was suggested that in granulosa cells, the most important transcript is driven by the promoter region upstream from exon 2, P2 (The most

proximal promoter of the Cyp 19 gene) (Furbass *et al.*, 1997; Hinshelwood *et al.*, 1995). In cattle, there are two distinct transcriptional start sites in this region with two canonical TATA boxes at appropriate locations (Furbass *et al.*, 1997; Hinshelwood *et al.*, 1995). As no classical cAMP- responsive element has been identified, it was suggested that steroidogenic factor-1 (SF1) binding might also confer cAMP responsiveness on the bovine promoter 2 (Furbass *et al.*, 1997). The promoter adjacent to exon 1.1 also was suggested to have a role in granulosa cells (Furbass *et al.*, 1997).

In cattle, the $P450_{arom}$ gene encodes a protein of 503 amino acids and shares 84% identity with human $P450_{arom}$, and 77%, 81%, 73%, and 52% for rat, mouse, chicken, and trout $P450_{arom}$ (Hinshelwood *et al.*, 1993). In cattle, $P450_{arom}$ has been mapped to band q 2.6 of chromosome 10 (Goldammer *et al.*, 1994) Interestingly, the region of over 20 amino acids that is totally conserved in many $P450_{arom}$ isoforms has a single conservative change in the bovine compared to that in the human, which apparently markedly affects the cross-reactivity with a human antibody whose epitope covers this region (Hinshelwood *et al.*, 1993). Also an aromatase pseudogene (*CYP 19* ψ) has been found in cattle (Furbass & Vanselow, 1995).

Regulation of aromatase and estradiol secretion in the ovary

The pattern of estradiol secretion in ovarian follicles differs markedly between species. Whereas $P450_{arom}$ is expressed in granulosa cells of all mammalian species, the capacity to express $P450_{arom}$ and secrete estradiol in the corpora lutea is confined to certain species, including humans (Sasano & Suzuki, 1997a), rats (Hickey *et al.*, 1988), rabbits (Arioua *et al.*, 1997) horses (Albrecht & Daels, 1997) and pigs (Gregoraszczuk & Oblonczyk, 1996), although recently, it has been shown that luteinized pig granulosa cells do not express $P450_{arom}$ mRNA (Pescador *et al.*, 1999). In cattle, (Voss *et al.*, 1993b) and sheep (Murdoch, 1996) neither estradiol nor $P450_{arom}$ are produced by corpora lutea. In rats (Hickey *et al.*, 1988; Ishimura *et al.*, 1989), humans (Inkster & Brodie, 1991; Doody *et al.*, 1990; Sasano *et al.*, 1989) and cattle (Xu *et al.*, 1995b; Bao *et al.*, 1997a; Lautincik *et al.*, 1994) $P450_{arom}$ mRNA and protein are absent in small antral follicles before recruitment, appear in granulosa cells of recruited follicles and increase in dominant follicles. However, occasional $P450_{arom}$ expression in human (Inkster and Brodie, 1991) and pig (Gregoraszczuk *et al.*, 1998) follicular theca cells has been reported. The increase in $P450_{arom}$ mRNA levels is probably induced by FSH. In rats, increases in $P450_{arom}$ mRNA are induced with FSH in vivo (Fitzpatrick *et al.*, 1991). However there are no studies showing that FSH in vivo can induce $P450_{arom}$ mRNA in cattle.

Earlier studies on rodents demonstrated that the regulation of aromatase activity is controlled by FSH/LH via cAMP second messenger system (Richards, 1980; Hsueh *et al.*, 1987; Dorrington & Armstrong, 1979; Richards, 1980).

Whereas low physiological FSH concentration induce $P450_{arom}$ mRNA, exposure of preovulatory follicles to an ovulatory surge of hCG or LH in vivo caused a rapid decline in $P450_{arom}$ mRNA levels in rats (Hickey *et al.*, 1990) and cattle (Voss *et al.*, 1993b). Recent studies of rat preovulatory follicles have suggested that surge levels of gonadotropins work through multiple signal transduction pathways (Fitzpatrick *et al.*, 1997). Thus it is possible that the different effects of high and low doses of gonadotropins on bovine granulosa cells act through multiple intracellular mechanisms. It has also been shown that the downregulation of FSH receptors in bovine (Rouillier *et al.*, 1996) and porcine (Murphy & Dobias, 1999) granulosa cells occurs when these cells are exposed to high FSH concentrations.

Some of the molecular mechanisms by which $P450_{arom}$ is regulated by gonadotropins have been deciphered by the studies at the promoter level in the rat. FSH induces $P450_{arom}$ expression by acting on granulosa cells to stimulate adenylyl cyclase activity, thereby raising intracellular cAMP levels and increasing protein kinase A activity (Richards, 1994; Richards *et al.*, 1995). There are two regions, termed A and B, within the aromatase promoter that mediate cAMP-induced expression in rat and human granulosa cells granulosa and constitutive expression in R2C Leydig cells (Fitzpatrick & Richards, 1993; Lynch *et al.*, 1993; Fitzpatrick & Richards, 1994) and . Region A lies between -

90 and -66 bp from the start codon, and binds steroidogenic factor-1 (SF-1), an orphan receptor member of the steroid/thyroid hormone receptor superfamily (Lala *et al.*, 1992) The hexameric SF-1 binding site is present within a large number of steroidogenic genes (Lala *et al.*, 1992). SF-1 is expressed early during development and is believed to regulate not only sexual differentiation but also the expression of steroidogenic genes in the adult (Luo *et al.*, 1994). Targeted deletion of the SF-1 gene results in mice that lack gonads and adrenals and exhibit abnormal pituitary development (Luo *et al.*, 1994).

Region B lies at -161 and -138 bp and contains a cAMP-response element (CRE)-like sequence (Deutsch *et al.*, 1987; Montminy & Bilezikjian, 1987). This region has been shown to bind CRE-binding protein (CREB) along with two other factors designated X and Y (Fitzpatrick *et al.*, 1994). Recently, deletion studies showed that CREB is the major factor conferring functional activity of the CRE in region B, eliminating the possibility that the other two factors might provide redundant functions for CREB, and it has also been demonstrated that CREB and SF-1 interact in an additive manner to confer cAMP responsiveness in rat granulosa cells (Carlone & Richards, 1997a). Both FSH and forskolin induce increases in the phosphorylation of CREB (phosphoCREB) in granulosa cells (Carlone & Richards, 1997a). These increases in phospho CREB corresponded to an increased amount of A-kinase RII β subunit, thus reflecting an overall increase in total kinase activity (Adams *et al.*, 1991). CREB is phosphorylated (at serine 133) and activated by other

kinases, such as C-kinase or calmodulin kinase (Sheng *et al.*, 1991). Although SF-1 can be phosphorylated by the catalytic subunit of A-kinase (Zhang & Mellon, 1996) it is not certain that phosphorylation of SF-1 by A-kinase is required for its activation of the $P450_{arom}$ promoter (Carlone & Richards, 1997b).

Whereas aromatase mRNA is induced by FSH during the follicular phase of the estrous cycle, the LH surge downregulates $P450_{arom}$ mRNA levels, an effect dependent on de novo protein synthesis (Fitzpatrick *et al.*, 1997).

In rats, the inhibitory effect of LH surge was mimicked by GnRH and PMA (phorbol 12-myristate 13-acetate, a protein kinase C activator) *in vitro,* suggesting that the LH surge (via the A kinase pathway) interacts with the C-kinase pathway to inhibit transcription and or increase $P450_{arom}$ mRNA degradation (Fitzpatrick *et al.*, 1997). The molecular mechanisms by which the LH surge and PMA inhibit transcription of $P450_{arom}$ in granulosa cells of ovulatory follicles remain speculative. Perhaps levels of phosphoCREB may be limiting for the transcription of $P450_{arom}$ (Fitzpatrick *et al.*, 1997). However the half life of $P450_{arom}$ mRNA and protein are unknown, although it has been suggested that the rate of turnover of the protein exceeds that of the mRNA in rat preovulatory follicles (Hickey *et al.*, 1988).

Aromatase activity in rats (Dorrington *et al.*, 1975; Erickson & Hsueh, 1978; Fortune & Hilbert, 1986a; Fitzpatrick *et al.*, 1991), humans (Lobb *et al.*, 1998) and pigs (Picton *et al.*, 1999) and $P450_{arom}$ mRNA in rats (Fitzpatrick *et al.*, 1991) humans (Steinkampf *et al.*, 1987) and pigs (Picton *et al.*, 1999) are

maintained and induced in granulosa by high concentrations of both LH and FSH in vitro. However, in cattle, aromatase activity is not maintained (Henderson *et al.*, 1979; Skinner *et al.*, 1988; Luck *et al.*, 1990; Meidan *et al.*, 1990; Saumande, 1991; Spicer *et al.*, 1993). $P450_{arom}$ mRNA has not been detected in bovine (Voss *et al.*, 1993b) and pig (Pescador *et al.*, 1999) granulosa cells after 24 and 6 h of culture, respectively. This is because in these culture systems granulosa cells undergo spontaneous luteinization, and granulosa cells loss the capacity to produce estradiol.

Some studies have shown that several factors affect $P450_{arom}$ activity in cultured bovine granulosa cells, including location of cells within the follicles, size of follicles from which cells were harvested, duration of culture, incubator concentrations of O_2, serum supplements, and growth factors. Aspirated granulosa cells (luminal) produce more estradiol than scraped granulosa cells (mural) (Roberts *et al.*, 1994; Rouillier *et al.*, 1996; Rouillier *et al.*, 1998a). Granulosa cells from large follicles (≥ 8mm) produce more estradiol than cells from small follicles (2-7 mm) (Roberts *et al.*, 1994; Campbell *et al.*, 1996; Gutiérrez *et al.*, 1997b). Previous studies in vitro show that the addition of serum supplements caused luteinization in bovine granulosa cells (Gong *et al.*, 1994). Increased estradiol production in response to FSH was reported in bovine granulosa cells cultured in defined media after precoating the wells with fibronectin (Saumande, 1991) or with fetal calf serum (Wrathall & Knight, 1993) or in plastic wells designed to enhance cell attachment (Berndtson *et al.*, 1995a)

but in all these systems estradiol secretion could not be maintained over time in culture.

Few studies in bovine granulosa cells have shown a response of granulosa cells to FSH in terms of estradiol production. In some studies, low doses (1-2 ng/ml) of FSH stimulated estradiol secretion, whereas the highest doses of FSH inhibited estradiol secretion in differentiated granulosa cells (Saumande, 1991; Berndtson *et al.*, 1995b; Rouillier *et al.*, 1996). To the contrary, progesterone secretion was increased with higher doses of FSH (Saumande, 1991; Berndtson *et al.*, 1995b; Rouillier *et al.*, 1996) but even in the presence of these effective doses of FSH, estradiol secretion decreased with time in culture. In the cow, the reported concentrations of FSH are 0.5 -2 ng/ml in plasma and follicular fluid (Turzillo *et al.*, 1990; Gibbons *et al.*, 1999; Henderson *et al.*, 1982; Dieleman *et al.*, 1983; Fortune & Hansel, 1985), suggesting that physiological FSH concentrations are required to induce estradiol secretion in bovine granulosa cells in vitro.

Recently, a long term bovine granulosa cell culture system was developed by Gutiérrez *et al.*, (1997b) which not only demonstrated the stimulatory effect of low doses of FSH on estradiol secretion, but also permitted an increase in estradiol secretion with time of culture. Although aromatase activity was maintained with time of culture, no attempts were made to evaluate the regulation of $P450_{arom}$ gene expression in these studies.

Insulin and insulin like growth factor-I have been shown to stimulate aromatase activity of cultured granulosa cells. Estradiol secretion by bovine granulosa cells was enhanced by the presence of insulin in a dose dependent manner, even in the absence of FSH (Saumande, 1991; Gutiérrez *et al.*, 1997b). This implies that high concentrations of insulin may regulate estradiol production independent of gonadotropin stimulation. It is usually considered that the effects of doses of insulin higher than 50 ng/ml are mediated through IGF-I receptors (Kahn *et al.*, 1981). In general, concentrations of insulin (0.5-10 ng/ml) are equal to or lower in follicular fluid than in plasma (Spicer & Echternkamp, 1995). In cattle, insulin at physiological concentrations has been reported to be a more potent stimulator of estradiol production in granulosa cells in vitro than is IGF-I (Spicer *et al.*, 1993; Spicer *et al.*, 1994b; Gong *et al.*, 1994; Saumande, 1991).

The role of IGF-I in follicular growth is documented in cattle, as concentrations of IGF-I in follicular fluid correlate positively with follicular diameter (Echternkamp *et al.*, 1990). In vitro studies have shown stimulatory effects of insulin and IGF-I on granulosa cell proliferation and/or DNA synthesis (Spicer *et al.*, 1993), IGF-I being a more potent mitogen than insulin (Spicer *et al.*, 1995). Few studies have evaluated the dose response effect of IGF-II on granulosa cell proliferation, although IGF-II stimulates DNA synthesis in cultured porcine (Baranao & Hammond, 1984) and human (Di Blasio *et al.*, 1994) granulosa cells. Studies in cattle suggest that FSH and LH enhance the mitogenic effect of IGF-1 in granulosa cells from small (<5mm) but not large (>10 mm) follicles (Gong *et al.*, 1993).

The effects of insulin, IGF-I and IGF-II on estrogen production by granulosa cells seem to depend on the species studied. In rats and primates, insulin, IGF-I, and IGF-II can stimulate granulosa cell estradiol production in vitro in a rank order potency, suggestive of an effect mediated via type I IGF receptors (Adashi, 1998). However, in cattle, in vitro studies suggest that insulin appears to be a more potent stimulator of estradiol production than IGF-I (Spicer *et al.*, 1993; Spicer *et al.*, 1994b; Gong *et al.*, 1994; Saumande, 1991). IGF-I showed no effect on estradiol secretion by cultured bovine granulosa cells from small (Spicer et al., 1993; Gong et al., 1994) or large (>8mm) follicles (Meidan *et al.*, 1990). However (Gutiérrez *et al.*, 1997b) reported increased estradiol secretion by IGF-I in FSH-stimulated bovine granulosa cells. In vivo, insulin treatment during the superovulation of cattle increased follicular fluid estradiol concentrations fivefold in large follicles (Simpson *et al.*, 1994b).

Thus, in vitro and in vivo data in cattle collectively support the notion that insulin is a better stimulator of granulosa cell estradiol production than is IGF-I, but IGF-I is better stimulator of cell growth.

There are data suggesting that both inhibin and activin can regulate by paracrine and autocrine mechanisms follicular estrogen synthesis. In vitro studies in marmoset granulosa cells showed that $P450_{arom}$ activity induced by FSH is enhanced by the addition of activin, although inhibin or inhibin α subunit slightly suppressed FSH-inducible $P450_{arom}$ activity (Hillier & Miro, 1993). However, evidence from other studies suggests that inhibin might affect

aromatization indirectly by increasing thecal androgen (aromatase substate) production in humans (Hillier *et al.*, 1991) and cattle (Wrathall & Knight, 1995). An inhibitory action of activin on thecal cell androgen biosynthesis in these species was also reported (Hillier *et al.*, 1991; Wrathall *et al.*, 1995).

Several factors present in serum (Orly *et al.*, 1980) and follicular fluid (Rouillier *et al.*, 1998b) appear to inhibit estradiol secretion. Their nature remains to be elucidated. These may include epidermal growth factor (Mendelson *et al.*, 1986) and transforming growth factor α, which inhibited estradiol secretion in rats (Adashi & Reshick, 1986), ovine (Campbell *et al.*, 1996) and bovine (Rouillier *et al.*, 1997) granulosa cells. Another candidate is prolactin, which in rats shows inhibitory effects on $P450_{arom}$ activity and mRNA (Fortune *et al.*, 1986b; Krasnow *et al.*, 1990).

Various steroids including estradiol (Hsueh *et al.*, 1984), testosterone (Daniel & Armstrong, 1980) and dihydrotestosterone (Daniel *et al.*, 1980; Hillier & De Zwart, 1981) have been shown to augment FSH stimulated $P450_{arom}$ activity and mRNA in rats (Fitzpatrick *et al.*, 1991). Others, such as dexamethasone (Hsueh & Erickson, 1978; Schoonmaker & Erickson, 1983) and progesterone (Fortune & Vincent, 1983) inhibit aromatase activity in rats. The role of these steroids on estradiol secretion in bovine granulosa cells remains to be elucidated. Recently, it was shown that cortisol had little or no effect on basal, insulin or IGF-I induced estradiol production by granulosa cells from small or large bovine follicles (Spicer & Chamberlain, 1998a).

Cytokines appear to affect $P450_{arom}$ activity in less differentiated granulosa cells (i. e. those from small follicles) but not in the highly differentiated granulosa cells from large follicles. In cattle, estradiol production in granulosa cells from large follicles was not affected by interleukin (IL)-1 beta, tumor necrosis factor-alpha (TNF) alpha or interleukin (IL)-beta-2. In contrast, cytokines showed an inhibitory effect on estradiol secretion in cells from small follicles (Spicer & Alpizar, 1994a; Spicer, 1998). In contrast, interferon-beta (IFN beta), interferon gamma (IFN gamma) and bovine trophoblast protein-1 (bTP-1) inhibited estradiol production from cells of small follicles (Spicer *et al.*, 1994a). It had no effect on FSH-induced estradiol production from cells of large follicles, and TNF binding protein-I blocked the inhibitory effect of TNF-α on FSH-induced estradiol production by cells from small follicles (Spicer *et al.*, 1994a). IL-6 also showed inhibitory effects on estradiol secretion from granulosa cells of small follicles, but only elevated doses inhibited estradiol secretion from cells of large follicles. (Alpizar & Spicer, 1994). More recently, it was shown that leptin inhibited estradiol production by granulosa cells in cattle (Spicer & Francisco, 1998b), humans (Zachow & Magoffin, 1997) and rodents (Agarwal *et al.*, 1999).

HYPOTHESIS AND OBJECTIVES

The hypothesis of the present study was that bovine granulosa cells can be stimulated in vitro to express $P450_{arom}$ mRNA and maintain estradiol secretion in response to physiological FSH, IGF-I and insulin concentrations, and that $P450_{arom}$ mRNA and estradiol, secretion are regulated using different intracellular mechanisms.

The specific objectives of the present study were:

1. To measure $P450_{arom}$ mRNA abundance in bovine granulosa cells, and to test the effects of FSH on $P450_{arom}$ mRNA accumulation and estradiol secretion.

2. To evaluate the importance of FSH, IGF-1 and insulin in maintaining $P450_{arom}$ mRNA abundance and estradiol secretion in bovine granulosa cells in vitro.

3. To study the intracellular pathways utilized by FSH, IGF-1 and insulin to stimulate $P450_{arom}$ mRNA abundance and estradiol secretion in bovine granulosa cells in vitro.

ARTICLE 1

Effect of FSH on Steroid Secretion and Messenger Ribonucleic Acids Encoding Cytochromes P450 Aromatase and P450 Cholesterol Side Chain Cleavage in Bovine Granulosa Cells In Vitro[1]

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Running title: Aromatase in bovine granulosa cell cultures

ABSTRACT

We determined if (1) the previously observed induction of estradiol secretion in bovine granulosa cells cultured in serum-free conditions is associated with an increase in cytochrome P450 aromatase ($P450_{arom}$) mRNA abundance, and (2) $P450_{arom}$ mRNA levels are responsive to FSH in vitro. Granulosa cells from small (2-4 mm) follicles were cultured in serum-free medium. Estradiol secretion increased with time in culture, and was correlated with increased $P450_{arom}$ mRNA abundance. Progesterone secretion also increased with time in culture, but P450 cholesterol side-chain cleavage ($P450_{scc}$) mRNA abundance did not.

FSH stimulated estradiol secretion and $P450_{arom}$ mRNA abundance; the effect was linear for estradiol and quadratic for $P450_{arom}$ mRNA. Estradiol secretion and $P450_{arom}$ mRNA levels were correlated. FSH stimulated progesterone secretion and $P450_{ssc}$ mRNA abundance, although the minimum effective dose of FSH was lower for estradiol (0.1 ng/ml) than for progesterone (10 ng/ml) production. Insulin alone stimulated estradiol secretion and $P450_{arom}$ mRNA levels but not progesterone or $P450_{scc}$ mRNA abundance. We conclude that this cell culture system maintained both estradiol and $P450_{arom}$ mRNA abundance responsiveness to FSH and insulin, whereas $P450_{arom}$ mRNA abundance and progesterone secretion were responsive to FSH but not insulin.

INTRODUCTION

The raison d'être of an ovarian follicle is to release, at ovulation, a mature oocyte into the oviduct for fertilization. In spontaneously ovulating monovular species, the preovulatory follicle is responsible also for inducing estrus and the ovulatory LH surge, which it does by increased secretion of estradiol. Thus, the ability to synthesize estradiol is an essential characteristic of a healthy ovarian follicle.

Estradiol is synthesized in granulosa cells from thecal androgens, by two successive enzyme-catalyzed reactions [1, 2]. In cattle, androstenedione is the preferred substrate, which is converted to estrone by the cytochrome P450 aromatase complex ($P450_{arom}$). Estrone is then converted to estradiol by the enzyme 17b-hydroxysteroid dehydrogenase (17β-HSD). Measuring estradiol in follicular fluid allows a good estimation of the estrogenic capability of a follicle. Estradiol concentrations in small antral follicles are modest relative to large growing follicles [3]. Peak follicular estradiol concentrations are generally obtained during the growth phase of the dominant follicle, and estradiol concentrations decrease considerably as the follicle reaches its maximum diameter [4, 5]. The loss of estradiol synthetic capability is one of the early sings of follcle atresia [6, 7] or regression [4, 5, 8].

Studies in vivo have shown that $P450_{arom}$ mRNA abundance increases in small follicles during the emergence of a non-ovulatory follicle wave, then declines in all but the dominant follicle [9,10]. These data are consistent with the

above mentioned follicular fluid estradiol measurements. However, there are circumstances when $P450_{arom}$ mRNA levels and estradiol concentrations are not so closely associated. For example, significant decreases in follicular estradiol concentrations were observed before $P450_{arom}$ mRNA levels decreased [9], and during final preovulatory follicle maturation there is increased follicular estradiol content in the absence of increased $P450_{arom}$ mRNA levels [11]. Dissociations between $P450_{arom}$ mRNA, protein levels and aromatase activity have also been observed in rat gonadal tissues in vivo [12, 13], and it has also been reported that $P450_{arom}$ mRNA levels were not significantly correlated with aromatase activity in human breast tumors [14]. Thus it should not be assumed that mRNA levels reflect enzyme activity for aromatase.

A problem with many such in vivo studies is the very complexity of follicular steroidogenesis. Numerous enzymes are involved, and a change in the activity of any one may affect precursor supply to another. Additionally, steroid synthesis is affected by several tropic hormones and probably paracrine factors as well. The study of granulosa cell function under defined conditions in vitro would permit a detailed examination of the actions of specific hormones on steroidogenesis, without the above-mentioned complications. However, the study of estradiol secretion and P450arom expression in ruminant granulosa cells has not been feasible until recently, as estradiol production [15-18] and $P450_{arom}$ mRNA levels are either not detectable [19] or decline rapidly [20] in these cells in vitro. A culture system has now been developed by Webb and

colleagues, that permits the induction and maintenance of estradiol secretion in ovine [21] and bovine [22] granulosa cells in vitro.

The aim of the present study was to study $P450_{arom}$ mRNA abundance and estradiol secretion in bovine granulosa cells in vitro. We tested the hypotheses that FSH stimulates $P450_{arom}$ mRNA levels in non-luteinizing bovine granulosa cells, and that estradiol secretion and mRNA abundance are correlated in this culture system. As granulosa cells secrete progesterone as well as estradiol [1, 21, 22], we also determined the effect of FSH on cytochrome P450 cholesterol side-chain cleavage ($P450_{scc}$).

MATERIALS AND METHODS

Experimental design

To estimate the time course of steroidogenenic enzyme gene expression, granulosa cells were cultured for up to 8 days with 10 ng/ml bFSH (USDA-bFSH 17) and 100 ng insulin/ml. Cells were recovered on each of days 2, 4, 6 and 8 of culture. Steroids were measured in the medium collected at the same time as the cells, and this measurement thus represents steroid released during the 48-h period prior to day 2, day 4, day 6 and day 8, respectively. This experiment was performed 3 times.

To determine the effects of gonadotropin on steroidogenic enzyme gene expression and estradiol secretion, granulosa cells were cultured for 6 days in the absence or presence of 0.1, 1, 10 or 100 ng/ml bFSH. An additional

treatment was culture with 100 ng insulin /ml in the absence of FSH. This experiment was performed 4 times.

To obtain sufficient RNA to measure P450 $_{arom}$ mRNA, all cells from an entire plate were used for each treatment (time point or hormone dose). Cells of each plate were pooled for RNA and DNA extraction, and the medium from all wells of each plate was pooled. Conditioned medium was stored at –20 °C until assayed for estradiol and progesterone.

Cell culture

The cell culture system was based on that described by Campbell et al [21]. All materials were obtained from Gibco BRL Canada (Burlington, ON, Canada) unless otherwise stated.

Bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir and transported to the laboratory in M199 containing 25 mm Hepes, penicillin (100 iu/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml). Follicles were dissected free of surrounding tissue, and small follicles (2 - 4 mm diameter) were bisected into Dulbecco's PBS (without calcium and magnesium) at 37° C. Granulosa cells were recovered by passing the follicle walls repeatedly through a 1 ml disposable pipette. The follicle walls were allowed to sediment out under gravity, and the granulosa cell suspension was transferred to sterile centrifuge tubes. Cells were isolated by centrifugation at 800 x g for 5 min, and were washed 3 times in M199 containing 25 mm

Hepes, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Washed cells were resuspended in culture medium, and cell viability was estimated at 40 % by Trypan blue exclusion.

Cells were seeded into 24-well tissue culture plates (Falcon, Becton Dickinson and company, New Jersey) at a density of 10^6 viable cells in 1ml of a-MEM with l-glutamine containing sodium bicarbonate (10 mm), Hepes (20 mm), protease-free BSA (0.1 %), selenium (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (10-7 m), insulin (10 ng/ml), human recombinant IGF-1 (10 ng/ml), non-essential amino acid mix (1.1 mm), penicillin (100 iu/ml) and streptomycin (100 µg/ml). Cultures were maintained at 37° C in 5 % CO2 for up to 8 days, with 700 µl medium being replaced every 2 days.

Total RNA and DNA were extracted using Trizol (Gibco BRL) according to the manufacturer's instructions. Total DNA was quantified in duplicate by measuring fluorescence in the presence of Hoechst 33258 [23] and compared with a calf thymus DNA standard (Boehringer-Mannheim, PQ Canada).

Hybridization

The relative abundance of mRNA for each protein was determined by Northern hybridization [24]. Electrophoresis of 15 µg RNA was performed through a 1 % denaturing formaldehyde-agarose gel followed by overnight

capillary transfer onto a nylon membrane (Hybond-N; Amersham, Oakville, Ontario). Membranes were UV cross-linked in a commercial UV chamber (Bio Rad, Mississauga, Ontario) and incubated for 2 h in prehybridization solution, containing 10 % dextran sulfate, 5-strength saline-sodium phosphate-EDTE buffer (SSPE), 5-strength Denhardt's solution, 0.5% sodium duodecyl sulfate (SDS), and herring sperm DNA (200 mg/ml).

The bovine $P450_{arom}$ cDNA probe was prepared in our laboratory [25], and encompasses the entire heme-binding and I-helix regions. The probe is specific for estrogen-secreting tissues, and hybridizes to 3 bands of follicular total RNA at 6.5, 3.4 and 1.8 kb [25]. The bovine $P450_{scc}$ cDNA was a gift from Dr M.R. Waterman (Vanderbilt University School of Medicine, Nashville, TN), and is a 1.7 kb cDNA containing the complete coding sequence [26], which hybridizes to a single band of 2 kb [27].

Probes were labelled with [α-32P] dCTP by random primer extension using a kit (Boehringer Mannheim, Laval, Québec) to a specific activity of 1.5-3.0 x 109 dpm/mg, and purified by centrifugation through a minicolumn using Wizard PCR Preps DNA purification system (Promega, Montréal, Québec, Canada). Hybridization to the membranes was performed overnight at 65 °C. After hybridization, membranes were washed in 2 X SSPE-0.1 % SDS twice at room temperature (15 min each), and twice at 65 °C (15 min each). Membranes were then stripped and rehybridized to a labelled human 28 S ribosomal cDNA probe [37] for the standardization of RNA loading. The labelled membranes

were exposed to Kodak X-Omat film at -70 °C in the presence of an intensifying screen. Autoradiograms were scanned with a densitometer after 1 to 14 days exposure.

Steroid assays

Estradiol was measured in conditioned medium without extraction with the assay described by Bélanger et al [29], with modifications [5]. Inter- and intra-assay coefficients of variation were less than 12 %. Progesterone was measured as described [30] with inter- and intra-assay coefficients of variation of less than 10 %. The sensitivity of these assays was equivalent to 0.25 and 1 ng/ml medium for estradiol and progesterone, respectively.

Statistics

The density of hybridization signals was corrected for loading efficiency using hybridization to 28S ribosomal RNA. Steroid concentrations are expressed relative to total DNA content of the wells. Data were transformed to logarithms if they were not normally distributed (Shapiro-Wilk test). Analysis of variance (ANOVA) was used to test effects of FSH and of insulin treatments. To avoid potential confounding effects of culture replicate, effects of treatment were tested within replicate. Differences between groups were identified with the Tukey-Kramer HSD test. Correlations between steroid secretion and mRNA abundance were determined with the Pearson correlation coefficient. Analyses

were performed with JMP® software (SAS Institute, Cary, NC). The data are presented as means ± SEM.

RESULTS

Time course of steroid secretion and steroidogenic enzyme mRNA abundance.

Estradiol secretion from granulosa cells and the relative abundance of $P450_{arom}$ increased from day 2 to day 4 of culture ($P<0.01$; Fig 1), but did not change significantly thereafter. Estradiol secretion and $P450_{arom}$ mRNA abundance were correlated between days 2 and 6 of culture ($r=0.68$. $P<0.05$, $n=9$), but not so if the analysis included data from day 8 ($P>0.05$). As expected, the $P450_{arom}$ cDNA probe recognized three transcripts in freshly harvested granulosa cells, although one major band was detected in cultured cells. However, when signal strength is stronger, lower molecular weight bands can be detected (Fig 3).

Progesterone secretion increased with time of culture ($P<0.01$; Fig 2) and reached a maximum on day 8. Relative abundance of $P450_{scc}$ mRNA did not change significantly with time in culture ($P>0.05$; Fig 2), and progesterone secretion and $P450_{scc}$ RNA were not significantly correlated ($r=0.42$, $P>0.05$, $n=12$).

The mean DNA content of the cultures did not change with time in culture (4.9±1.1, 4.1±0.8, 5.1±0.5 and 3.4±1.3 μg/well, for days 2,4,6 and 8, respectively; $P>0.05$).

Effect of FSH on steroid secretion and steroidogenic enzyme mRNA abundance.

There were main effects of treatment on both estradiol secretion and relative $P450_{arom}$ RNA abundance ($P<0.01$), and no effect of culture replicate ($P>0.05$). For cells cultured with 10 ng insulin but without FSH, estradiol secretion was low, and stimulated by as little as 0.1ng bFSH/ml (Fig 3). The addition of 1ng bFSH significantly increased estradiol secretion above that observed with 0.1 ng/ml, although greater doses of FSH did not further affect estradiol secretion. There was a significant quadratic relationship between dose of FSH and estradiol secretion ($P<0.05$). Insulin alone at 100 ng/ml stimulated estradiol secretion compared to insulin alone at 10 ng/ml ($P<0.01$; Fig 3).

Relative $P450_{arom}$ RNA abundance was very low in the absence of FSH, and increased to a maximum in the presence of 1 ng FSH/ml ($P<0.05$; Fig 3). The relationship between FSH and relative $P450_{arom}$ RNA abundance was quadratic ($P<0.01$) and estradiol secretion and $P450_{arom}$ mRNA abundance were correlated ($r=0.57$, $P<0.05$). Insulin alone at 100 ng/ml stimulated $P450_{arom}$ RNA abundance relative to insulin alone at 10 ng/ml ($P<0.05$).

Progesterone secretion was not significantly stimulated by the addition of low doses of FSH (0.1 or 1 ng/ml) to cells cultured with 10 ng insulin (Fig 4), but was significantly stimulated with higher doses of FSH (10 and 100 ng/ml). Insulin alone at 100 ng/ml did not stimulate progesterone compared to insulin alone at 10 ng/ml.

Relative abundance of $P450_{scc}$ mRNA increased significantly with dose of FSH in a linear fashion ($r=0.88$, $P<0.001$; Fig 4), however, insulin alone at 100ng/ml did not stimulate $P450_{scc}$ mRNA abundance ($P>0.05$). Progesterone secretion and $P450_{scc}$ mRNA abundance were correlated ($r=0.63$, $P<0.001$).

The DNA content of the cultures was not affected by dose of FSH (7.2±0.6, 5.5±0.3, 6.7±0.5, 6.8±0.8 and 5.9±0.7 μg/well, for 0, 0.1, 1, 10 and 100 ng FSH/ml, respectively; $P>0.05$) or insulin (4,6±0.7 μg/well for 100 ng insulin/ ml).

DISCUSSION

This is the first report to show a) that $P450_{arom}$ mRNA levels in bovine granulosa cells increase during culture, and b) that $P450_{arom}$ mRNA abundance is modulated by FSH and by insulin. Previously, $P450_{arom}$ expression has been documented in bovine follicles in vivo [9,11,19,25]. However, bovine granulosa cells rapidly lose the ability to secrete estradiol in most cell culture systems, and $P450_{arom}$ mRNA has not been detected by Northern analysis [19]. Even in other culture systems that support limited FSH-responsive estradiol secretion [31],

P450arom mRNA has not been detected (L.A. Guilbault, J.G. Lussier, personal communication). Using ribonuclease protection assays, Berndtson et al [20] found that $P450_{arom}$ RNA levels decreased dramatically during the first 24 h of culture, and were not responsive to FSH. Thus our demonstration of increased $P450_{arom}$ RNA abundance in bovine granulosa cells during culture, and responsiveness of relative $P450_{arom}$ mRNA levels to FSH are novel findings.

The initial expression of $P450_{arom}$ mRNA in small (<4 mm) follicles in vitro in the present study was barely undetectable, which is in agreement with in situ hybridization studies in vivo [9,10]. In vivo, $P450_{arom}$ mRNA abundance increased between day 2 and day 4 after the emergence of a follicle wave, but did not increase further between day 4 and day 6 [9]; this temporal pattern of $P450_{arom}$ mRNA appearance is very similar to that observed in vitro (present study), and suggest that this is a good model for the study of follicle recruitment. It has been suggested [10] that the increase in $P450_{arom}$ mRNA abundance in medium sized follicles during emergence of the follicle wave is stimulated by the transient rise in plasma FSH concentrations that precedes wave emergence [32]. The present data are the first to demonstrate a direct effect of FSH on $P450_{arom}$ mRNA abundance in cattle, and thus support the role of FSH in this respect. It is interesting to note that physiological concentrations of FSH resulted in greatest stimulation of $P450_{arom}$ mRNA levels, whereas supraphysiological concentrations resulted in a somewhat muted response. Corresponding data are not available in vivo in cattle, although it is known that superovulation with FSH results in small follicles (3-5 mm) with similar $P450_{arom}$ mRNA content to non-

stimulated preovulatory follicles [25]. In rats, a biphasic response of ovarian $P450_{arom}$ mRNA levels to FSH was observed in vivo [33], but not in vitro, when increasing concentrations of FSH (generally above 100 ng/ml) further stimulated estradiol secretion and $P450_{arom}$ mRNA levels [33,34].

The mechanism of the biphasic response of $P450_{arom}$ RNA to FSH is not known, although it is clearly specific to $P450_{arom}$ RNA; no such effect was observed for $P450_{scc}$. Whereas estradiol secretion and $P450_{arom}$ mRNA levels are stimulated by FSH in rats, they are markedly inhibited by high levels of LH [33]. It has been proposed that FSH normally induces relatively small increases in intracellular cAMP, which stimulate steady-state $P450_{arom}$ mRNA levels, whereas LH induces much higher levels of cAMP accumulation, which in turn lead to luteinization and a reduction in steady-state $P450_{arom}$ mRNA levels [33]. Thus in the present experiment, 10 and 100 ng FSH/ml may have resulted in concentrations of intracellular cAMP that were sufficient to down-regulated $P450_{arom}$ mRNA levels. It has been suggested that high LH levels may also activate the protein kinase-C second messager pathway, leading to decreased $P450_{arom}$ mRNA levels [35]. As an alternative explanation, Rouillier et al [36] proposed that the biphasic response of estradiol secretion to FSH may be mediated through a down-regulation of granulosa cell FSH receptors. This is less likely to be valid in the present study, as the higher doses of FSH did not down-regulate $P450_{scc}$ mRNA abundance.

In contrast to the effects on $P450_{arom}$ mRNA levels, FSH did not have a biphasic effect on estradiol secretion at the dosages evaluated. Thus, we did not

observe the inhibition of estradiol secretion reported for high doses of FSH [18,22,31,36]. This discrepancy may be the result of the use of different preparations of FSH of biopotencies between laboratories. Another potential explanation includes the source of the cells. In previous studies, cells were obtained from large (>8 mm diameter) follicles [18,31,36], whereas the cells in this report were obtained from small (2-4 mm diameter). Cells from small follicles were also studied by Gutiérrez et al [22], and in the presence of insulin, FSH had not effect on estradiol secretion. As estradiol secretion did not decrease in concert with the decrease in $P450_{arom}$ mRNA abundance observed with high doses of FSH , it is possible that FSH is modulating aromatase activity at two levels-transcription and translation / enzyme activity.

The linear effect of FSH on progesterone secretion and $P450_{scc}$ mRNA abundance is similar to that previously observed in rat, pig and bovine [18,31,37,38] granulosa cell cultures. Interestingly, estradiol secretion was more sensitive to FSH compared to progesterone secretion; 1 ng FSH/ml induced near-maximum estradiol secretion, whereas this dose did not significantly affect progesterone secretion. This divergent effect of FSH on estradiol and progesterone secretion is similar to that observed for human granulosa cells though not as marked as that observed for human granulosa cells [39].

In the present study, increasing insulin concentrations form 10 to 100 ng/ml significantly increased estradiol secretion and $P450_{arom}$ RNA abundance, but had no effect on $P450_{scc}$ mRNA abundance or progesterone secretion. This is not fully in agreement with the literature, as it is generally considered that the

secretion of both estradiol and progesterone secretion from bovine granulosa cells is stimulated by insulin in vitro [reviewed in 40]. However, in many of these studies the cells are spontaneously luteinizing and/or estradiol secretion is not responsive to FSH [41-42]. In a study with FSH-responsive granulosa cell, insulin alone at 100ng/ml stimulated progesterone but not estradiol secretion [31]. In the first report of the culture system used herein for bovine granulosa cells, insulin stimulated estradiol secretion [22], although the effects of insulin on progesterone secretion were not reported. Thus these data suggest that there is a developmental switch in the role of insulin, from stimulation of estradiol secretion in non-luteinizing cells to that of progesterone in luteinizing cells.

We conclude from these data that the present cell culture technique permits the stimulation of $P450_{arom}$ RNA accumulation as well as estradiol secretion from granulosa cells from small bovine follicles. Both estradiol secretion and $P450_{arom}$ RNA abundance were responsive to FSH and insulin, whereas $P450_{scc}$ mRNA abundance and progesterone secretion were responsive to FSH but not insulin. This culture system may provide a good model for studying the mechanisms of follicle recruitment in vitro.

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Figure 1.

Estradiol production and $P450_{arom}$ RNA abundance for 10^6 granulosa cells cultured with 10 ng bFSH/ml and 100 ng insulin/ml for 2, 4, 6 or 8 days. Values are least-squares means ± SEM, and were obtained from 4 replicate experiments. Data for mRNA were corrected for loading (28 S rRNA) and expressed relative to values on Day 2 of culture; the upper panel shows a representative Northern blot for a positive control (C; a pool of freshly isolated granulosa cells) and after 2, 4, 6 and 8 days of culture.

Figure 1

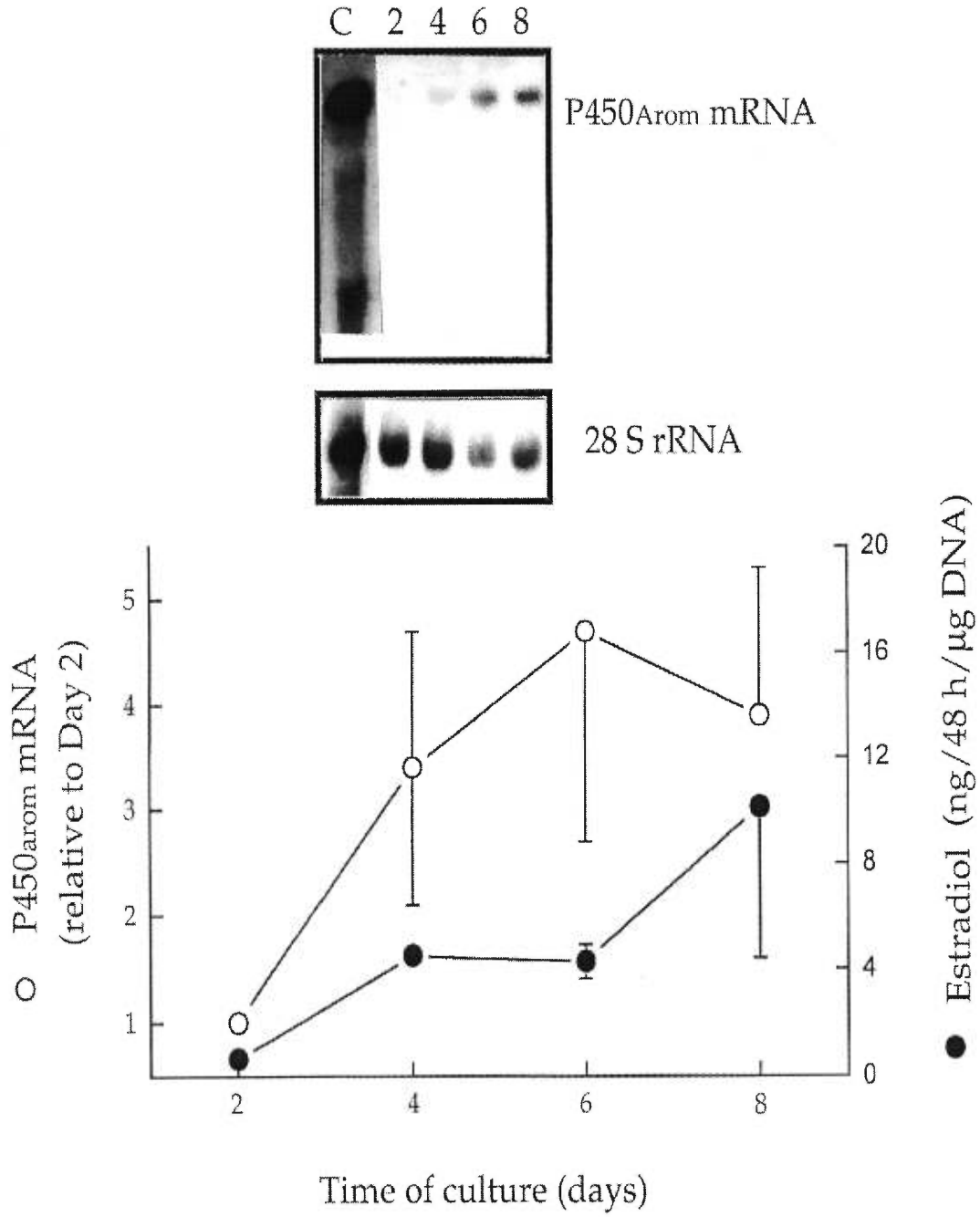

C 2 4 6 8
P450Arom mRNA
28 S rRNA
○ P450arom mRNA (relative to Day 2)
● Estradiol (ng/48 h/μg DNA)
Time of culture (days)

Figure 2.

Progesterone production and $P450_{scc}$ mRNA abundance for 10^6 granulosa cells cultured with 10 ng bFSH/ml and 100 ng insulin/ml for 2, 4, 6 or 8 days. Values are least-squares means ± SEM, and were obtained from 4 replicate experiments. Data for mRNA were corrected for loading (28 S rRNA) and expressed relative to values on Day 2 of culture; the upper panel shows a representative Northern blot for a positive control (C; a pool of freshly isolated granulosa cells) and after 2, 4, 6 and 8 days of culture.

Figure 2

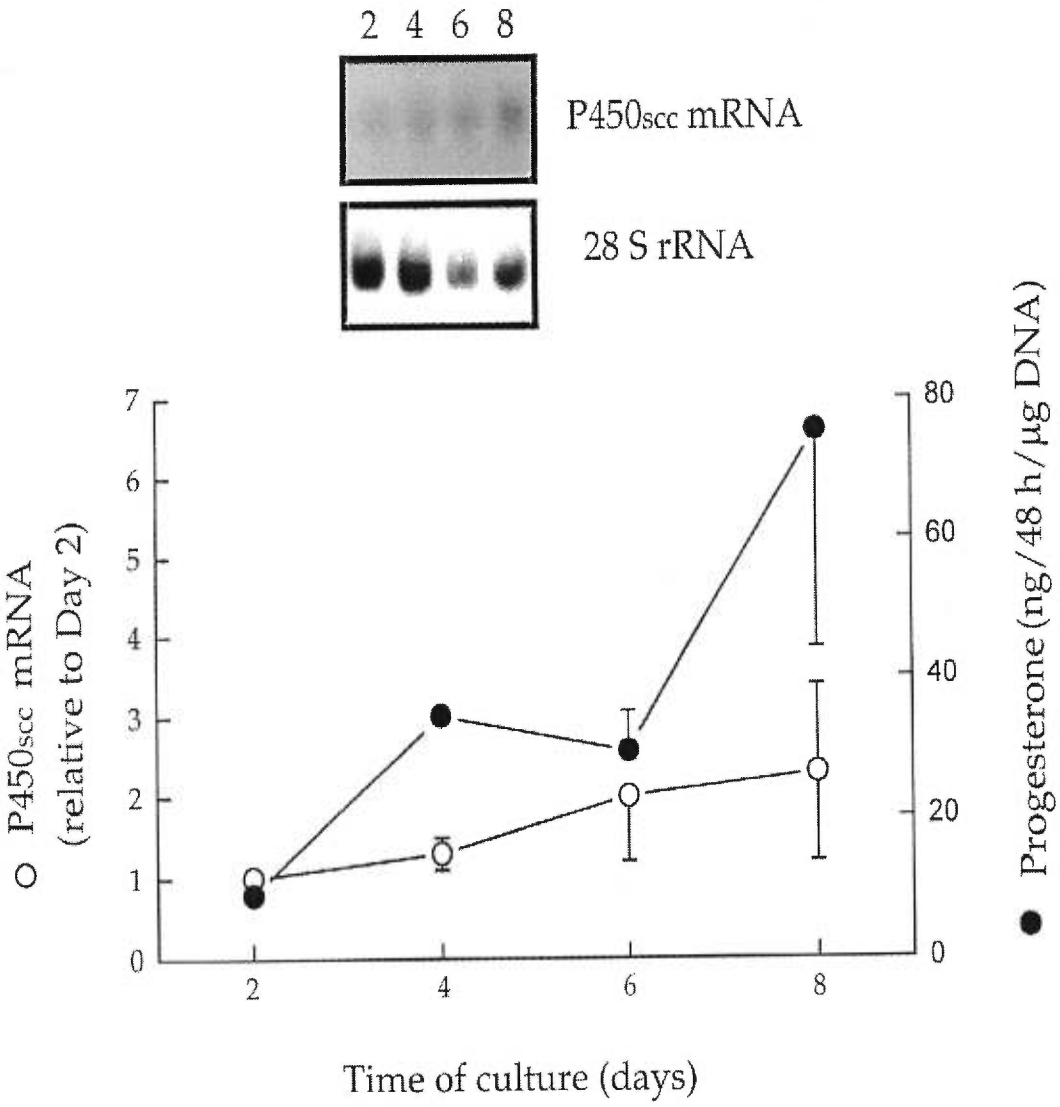

2 4 6 8
P450scc mRNA
28 S rRNA
○ P450scc mRNA (relative to Day 2)
● Progesterone (ng/48 h/μg DNA)
Time of culture (days)
0
1
2
3
4
5
6
7
0
20
40
60
80
2
4
6
8

Figure 3.

Effect of FSH and insulin on estradiol production and $P450_{arom}$ RNA abundance in cultured bovine granulosa cells. Cells were cultured with the indicated doses of FSH and insulin for 6 days in serum-free medium. Data (means ± SEM) were derived from 4 replicate experiments, and the upper panel demonstrates a representative Northern blot. Different superscripts denote significant effects of FSH, and asterisks denote effects of insulin, and were determined with the Tukey-Kramer HSD test ($P<0.05$).

Figure 3

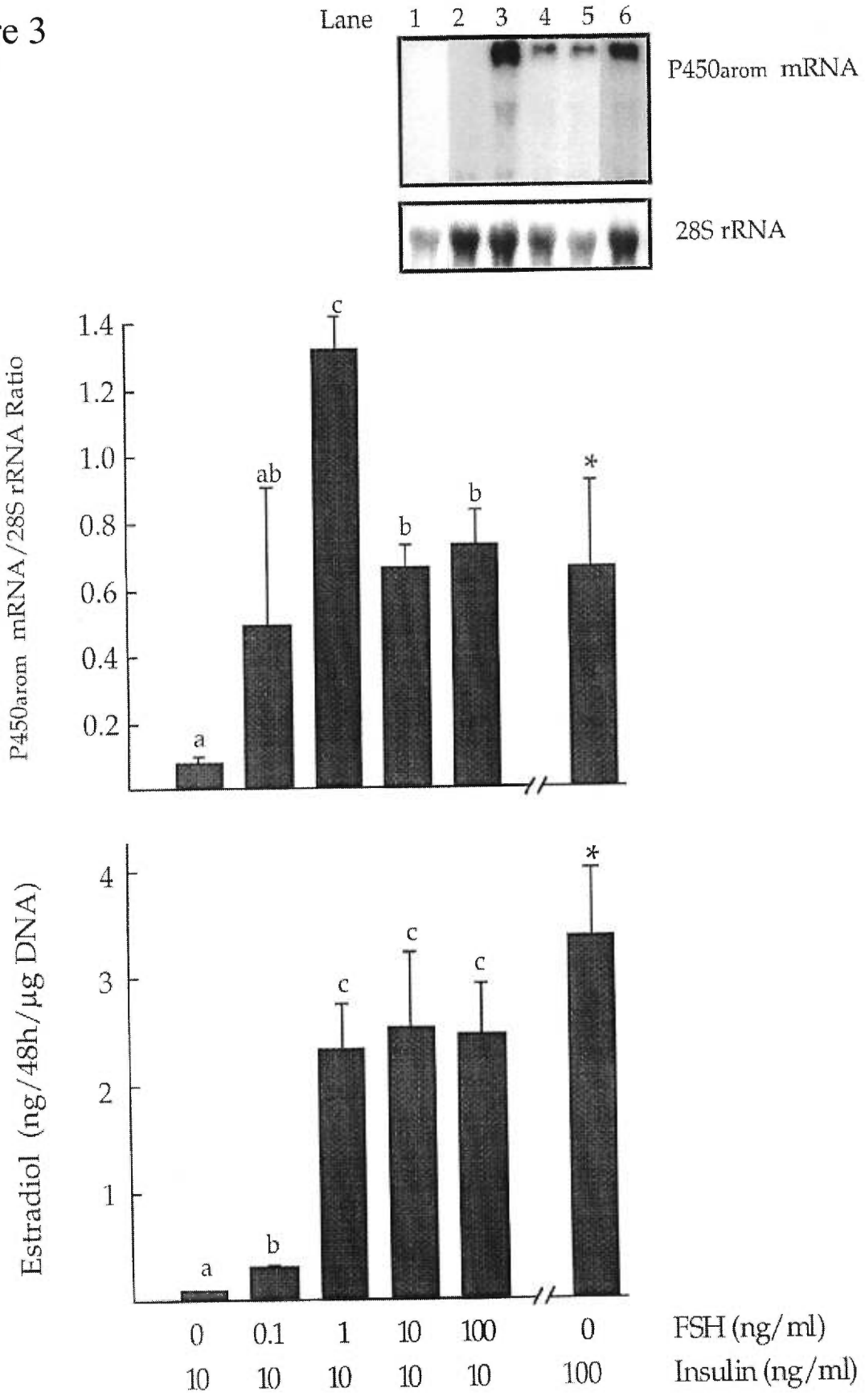
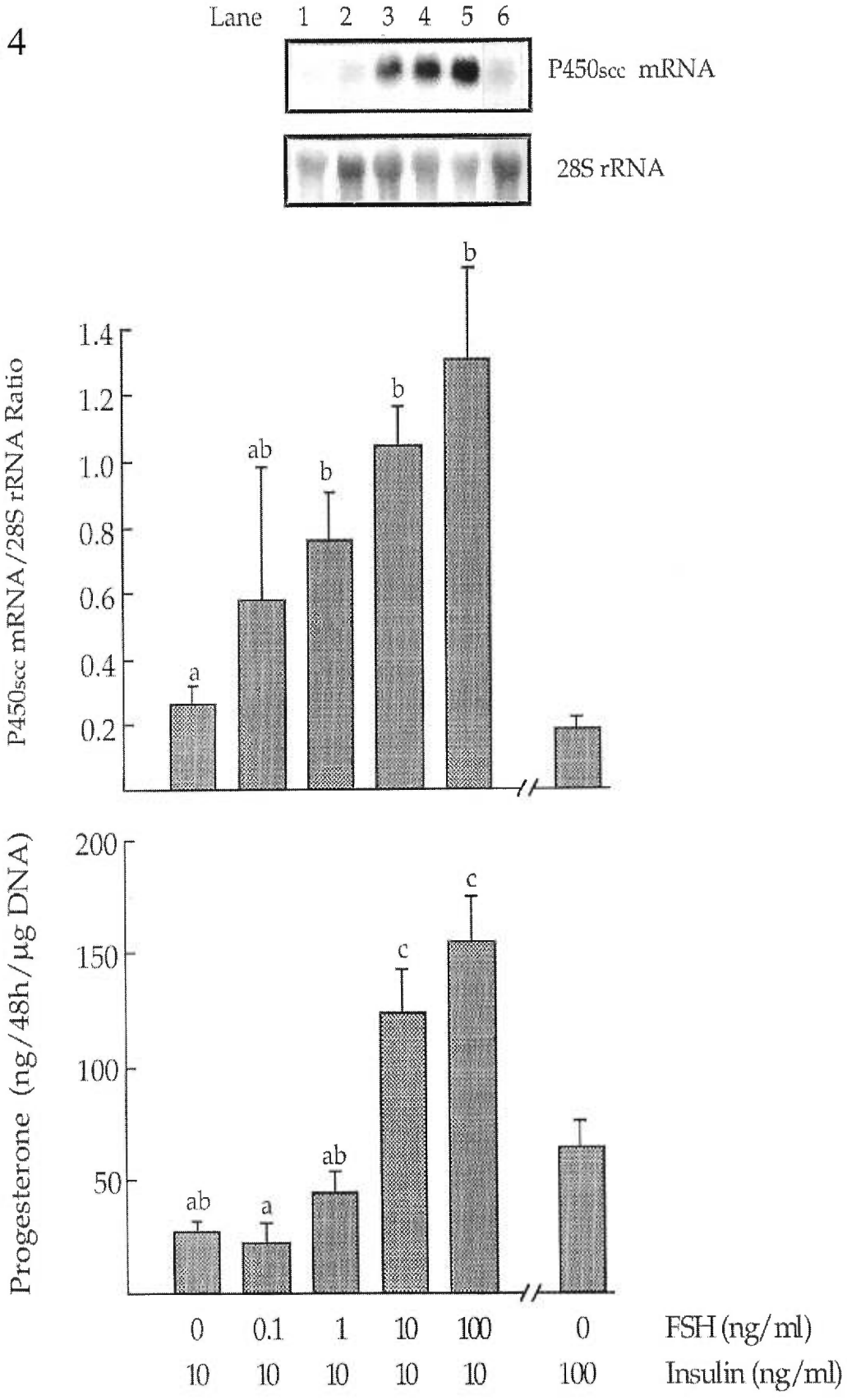

Lane 1 2 3 4 5 6
P450arom mRNA
28S rRNA
P450arom mRNA/28S rRNA Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
a
ab
c
b
b
*
Estradiol (ng/48h/μg DNA)
4
3
2
1
a
b
c
c
c
*
0 0.1 1 10 100 0
FSH (ng/ml)
10 10 10 10 10 100
Insulin (ng/ml)

Figure 4.

Effect of FSH and insulin on progesterone production and $P450_{scc}$ mRNA abundance in cultured bovine granulosa cells. Cells were cultured with the indicated doses of FSH and insulin for 6 days in serum-free medium. Data (means ± SEM) were derived from 4 replicate experiments, and the upper panel demonstrates a representative Northern blot. Differences between means (denoted by different superscripts) were determined with the Tukey-Kramer HSD test ($P<0.05$). There was no effect of insulin on progesterone secretion or on P450scc mRNA abundance ($p>0.05$).

Figure 4


Lane 1 2 3 4 5 6
P450scc mRNA
28S rRNA
P450scc mRNA/28S rRNA Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
a
ab
b
b
b
Progesterone (ng/48h/μg DNA)
200
150
100
50
ab
a
ab
c
c
0 0.1 1 10 100 0 FSH (ng/ml)
10 10 10 10 10 100 Insulin (ng/ml)

ARTICLE 2

Relative Importance of FSH, IGF-I and Insulin on Cytochrome $P450_{arom}$ mRNA Abundance and Estradiol Secretion in Cultured Bovine Granulosa Cells[1]

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Running title: Effect of FSH and insulin on $P450_{arom}$ mRNA.

ABSTRACT

The objective of the present study was to determine the relative importance of insulin, IGF-1 and FSH for the maintenance of $P450_{arom}$ mRNA levels and estradiol secretion. In the first experiment, granulosa cells were obtained from small (2-4 mm diameter) bovine follicles, and were cultured for four days with insulin (100 ng/ml) and IGF-I (10 ng/ml). At day four of culture, the medium was devoid of insulin and IGF-I, but containing FSH (0,1,10 or 100 ng/ml). Control cultures continued to receive insulin and IGF-I. In the second experiment, granulosa cells were cultured with insulin (10 ng/ml), IGF-I (10 ng/ml) and FSH 1 ng/ml). At day four of culture, FSH, insulin and IGF-I were withdrawn from the culture medium separately and in combination. In both experiments, spent medium and granulosa cells were recovered on day 7 of culture for estradiol assay and measurement of $P450_{arom}$ mRNA by Northern blot. The withdrawal of insulin and IGF-I significantly decreased $P450_{arom}$ mRNA and estradiol secretion to almost undetectable levels ($P<0.02$). The addition of FSH maintained estradiol secretion to levels similar to controls, but did not restore $P450_{arom}$ mRNA levels. The withdrawn of insulin and IGF-I did not affect progesterone or $P450_{scc}$ mRNA abundance, but increasing doses of FSH significantly increased progesterone secretion and $P450_{scc}$ mRNA abundance ($P<0.01$). The concentrations of progesterone and $P450_{SCC}$ mRNA abundance were correlated ($P<0.015$).

In experiment 2, the withdrawal of FSH significantly decreased $P450_{arom}$ mRNA and estradiol secretion ($P<0.05$). The withdrawal of insulin, IGF-I or both did not significantly alter estradiol secretion in the presence of FSH, whereas $P450_{arom}$ mRNA levels were decreased by insulin withdrawal, but not IGF-I ($P>0.05$). Progesterone secretion was not affected by the withdrawal of these hormones, whereas $P450_{scc}$ mRNA was reduced when FSH, or both insulin and IGF-I were withdrawn. Progesterone and $P450_{scc}$ mRNA were not correlated ($P <0.31$). We conclude that $P450_{arom}$ mRNA accumulation is critically dependent upon the presence of insulin, and that FSH can maintain estradiol secretion in the absence of detectable levels of $P450_{arom}$ mRNA.

INTRODUCTION

In mammals, the most important function of ovarian follicles is the production of an ovum which is released at ovulation [1]. The development of ovarian follicles in cattle is characterised by the recruitment of a group of small antral follicles, followed by the selection of one of them to continue growing, and finally selection, in which not only it continue growing but also induce death by atresia to their partners, the subordinate follicles [2]. Dominant follicles are characterised by their high capacity to secrete estradiol [3]. Thus the capacity to secrete estradiol is the most important feature of healthy ovarian follicles [4]. I t has been shown than in cattle, available IGF-I concentrations are higher in healthy estrogen-active follicles, than in atretic follicles [5, 6]. And in vitro studies have shown that IGF-I enhance the FSH capacity to produce estradiol in bovine

granulosa cells [7]. However other studies have shown as well that insulin is a better stimulator of IGF-I [8, 9].

Previoulsly we have shown that bovine granulosa cells secrete estradiol and express $P450_{arom}$ mRNA after 6 days of culture in serum free medium [10]. In that study, $P450_{arom}$ mRNA abundance and estradiol secretion were stimulated with low doses of FSH. We also showed that insulin alone (100 ng/ml) stimulates estradiol secretion and $P450_{arom}$ mRNA abundance. It is usually considered that the effects of doses of insulin higher than 50 ng/ml are mediated through IGF-1 receptors [11]. Thus, the objective of the present study was to determine first, if the induction of $P450_{arom}$ mRNA levels and estradiol secretion by insulin at 100 ng/ml and IGF-I at 10 ng/ml in bovine granulosa cells can be maintained by the addition of different doses of FSH alone, and second to determine the effect of the withdrawal of insulin, IGF-1 and FSH, used at physiological concentrations, on $P450_{arom}$ mRNA levels and estradiol secretion.

MATERIALS AND METHODS.

Cell culture

The cell culture system was based on that described previously [10,12]. All materials were obtained from Gibco BRL Canada (Burlington, ON, Canada) unless otherwise stated.

Bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir and transported to the laboratory in M199 containing 25 mm Hepes, penicillin (100 iu/ml), streptomycin (100 µg/ml) and

fungizone (1 µg/ml). Follicles were dissected free of surrounding tissue, and small follicles (2 - 4 mm diameter) were bisected into culture medium at 37 °C. Granulosa cells were recovered by passing the follicle walls repeatedly through a 1 ml disposable pipette. The follicle walls were allowed to sediment out under gravity, and the granulosa cell suspension was transferred to sterile centrifuge tubes. Cells were isolated by centrifugation at 800 x g for 5 min, and were washed 3 times in M199 containing 25 mm Hepes, penicillin (100 iu/ml) and streptomycin (100 µg/ml). Washed cells were resuspended in culture medium, and cell viability was estimated at 40 % by Trypan blue exclusion.

Cells were seeded into 24-well tissue culture plates (Falcon, Becton Dickinson and Company, New Jersey) at a density of 10^6 viable cells in 1ml of α-MEM with L-glutamine containing sodium bicarbonate (10 mm), Hepes (20 mm), protease-free BSA (0.1 %), selenium (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (10-7 M), insulin (100 or 10 ng/ml), human recombinant IGF-1 (10 ng/ml), non-essential amino acid mix (1.1 mM), penicillin (100 iu/ml) and streptomycin (100 µg/ml). Cultures were maintained at 37°C in 5 % CO_2 for 7 days, with 700 µl medium being replaced on days 2 and 4. bFSH (USDA-bFSH 17; biological potency 20 U/mg relative to oFSH-S1) was used at different doses.

Experiment 1

Bovine granulosa cells from small follicles (2-4 mm diameter) were cultured in the presence of IGF-I (10 ng/ml) and insulin (100 ng/ml), but without FSH. In the afternoon of day four of culture, IGF-1 and insulin were withdrawn by replacing 750 μl of culture medium devoid of IGF-1 and insulin but including 0, 1,10 or 100 ng/ml of FSH. On the morning of the following day (day 5), a second medium change was performed as described above, and the same treatments were added. Control cultures were maintained in IGF-1 and insulin supplemented medium. Granulosa cells were recovered on day 7 for the extraction of nucleic acids, and the culture medium frozen at –20°C for estradiol and progesterone assays.

Experiment 2

In this experiment granulosa cells were cultured for four days in the presence of insulin (10 ng/ml), IGF-1 (10 ng/ml) and FSH (1 ng/ml). At day 4 and 5 of culture, the hormones were withdrawn as in experiment 1, and the following treatments were added: **a)** Control treatment, containing insulin (10 ng/ml), IGF-1 (10 ng/ml) and FSH (1 ng/ml). **b)** insulin (10 ng/ml), IGF-1 (10 ng/ml) and FSH (0 ng/ml). **c)** insulin (0 ng/ml), IGF-1 (10 ng/ml) and FSH (1 ng/ml). **d)** insulin (10 ng/ml), IGF-1 (0 ng/ml) and FSH (1 ng/ml). **e)** insulin (0 ng/ml), IGF-1 (0 ng/ml) and FSH (1 ng/ml) Granulosa cells were recovered at day 7 of culture for extraction of nucleic acids, and medium stored at –20°C as above.

In both experiments, day 4 was chosen to withdraw the hormones, because granulosa cells from small follicles express abundant $P450_{arom}$ mRNA at this time. The medium was recovered at day 7 of culture to assure the maximum effect of the treatments.

The doses of insulin and IGF-1 were chosen based on the observations of the previous article that insulin alone at 100 ng/ml or 10 ng/ml plus 1 ng FSH /ml, in combination with IGF-1 at 10 ng/ml, were able to stimulate and maintain $P450_{arom}$ mRNA [10]. Furthermore such doses of insulin and IGF-1 correspond to the physiological concentrations found in follicular fluid in the cow [27]

Half the plate (12 wells) was used per treatment, and experiments were repeated 3 times. The same wells were used for extraction of total RNA and DNA using Trizol (Gibco BRL) according to the manufacturer's instructions. Total DNA per culture plate was quantified by measuring fluorescence in the presence of Hoechst 33258 [13] and compared with a calf thymus DNA standard (Boehringer-Mannheim, PQ Canada).

Hybridization

The relative abundance of mRNA was determined by Northern hybridization [10,14]. Electrophoresis of 15 μg RNA was performed through a 1 % denaturing formaldehyde-agarose gel followed by overnight capillary transfer onto a nylon membrane (Hybond-N; Amersham, Oakville, Ontario). Membranes were UV cross-linked in a commercial UV chamber (Bio Rad, Mississauga,

Ontario) and incubated for 2 h in prehybridization solution, containing 10 % dextran sulfate, 5-strength saline-sodium phosphate-EDTA buffer (SSPE), 5-strength Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and herring sperm DNA (200 mg/ml).

The bovine aromatase cDNA probe was prepared in our laboratory [15], and encompasses the entire heme-binding and I-helix regions. The probe is specific for estrogen-secreting tissues, and hybridizes to 3 bands of follicular total RNA at 6.5, 3.4 and 1.8 kb [15]. The bovine P450scc cDNA was a gift from Dr M.R. Waterman (Vanderbilt University School of Medicine, Nashville, TN), and is a 1.7 kb cDNA containing the complete coding sequence[17], which hybridizes to a single band of 2 kb in bovine follicles [16].

Probes were labeled with [α-32P]dCTP by random primer extension using a kit (Boehringer Mannheim, Laval, Québec) to a specific activity of 1.5-3.0 x 10^9 dpm/mg, and purified by centrifugation through a minicolumn using Wizard PCR Preps DNA purification system (Promega, Montréal, Québec, Canada). Hybridization to the membranes was performed overnight at 65°C. After hybridization, membranes were washed in 2 X SSPE-0.1 % SDS twice at room temperature (15 min each), and twice at 65 °C (15 min each). Membranes were hybridized to $P450_{arom}$ and $P450_{scc}$ probes sequentally, then stripped and rehybridized to a labeled human 28 S ribosomal cDNA probe [18] for the

standardization of RNA loading. The labeled membranes were exposed to Kodak X-Omat film at -70 °C in the presence of an intensifying screen. Autoradiograms were scanned with a densitometer after 1 to 14 days exposure.

Steroid assays

Estradiol was measured in conditioned medium without extraction with the assay described by [19], with modifications [10,20]. Inter- and intra-assay coefficients of variation were less than 12 %. Progesterone was measured as described [21], with inter- and intra-assay coefficients of variation of less than 10 %. The sensitivity of these assays was equivalent to 0.25 and 1 ng/ml medium for estradiol and progesterone, respectively.

Statistical analysis

The density of hybridization signals was corrected for loading efficiency using hybridization to 28S ribosomal RNA [10]. Steroid concentrations are expressed relative to total DNA content of the wells[10]. Data were transformed to logarithms if they were not normally distributed (Shapiro-Wilk test). Analysis of variance (ANOVA) was used to test effects of FSH and of insulin treatments. Culture replicate was included as an error term in the main effect model. Effects of treatment were tested. Differences between groups were identified with the Tukey-Kramer HSD test. Correlations between steroid secretion and mRNA

abundance were determined with the Pearson correlation coefficient. Analyses were performed with JMP® software (SAS Institute, Cary, NC). The data are presented as means ± SEM.

RESULTS

Experiment 1

Figure 1 depicts the effects of withdrawal of IGF-I and insulin from culture medium on $P450_{arom}$ mRNA abundance and estradiol secretion. Removal of both significantly ($P<0.02$) decreased estradiol secretion to almost undetectable levels. This effect was counteracted by the addition of FSH to the culture medium, which resulted in estradiol concentrations not significantly different from controls ($P>0.05$). Although there was a tendency to have higher concentrations of estradiol with 1ng FSH/ml than with the higher doses, this difference was not significant ($P>0.05$). In contrast to the estradiol data, $P450_{arom}$ mRNA was only detectable in control cells cultured with insulin and IGF-I; when insulin and IGF-I were withdrawn, even with addition of FSH, $P450_{arom}$ mRNA was undetectable by Northern blotting.

Figure 2 depicts the effects of withdrawal of IGF-1 and insulin from culture medium on $P450_{scc}$ mRNA abundance and progesterone secretion. The withdrawal of insulin and IGF-I did not significantly decrease progesterone secretion ($P> 0.05$), however, the addition of FSH significantly increase progesterone secretion ($P <0.01$). Contrary to $P450_{arom}$ mRNA abundance, $P450_{scc}$ mRNA levels were not affected by the withdrawal of insulin and IGF-I, and were significantly increased by the addition of 10 or 100 ng/ml FSH ($P< 0.01$). The concentrations of progesterone and the abundance of $P450_{scc}$ mRNA were correlated ($r=0.6$, $P< 0.05$ $n=15$).

Experiment 2

Figure 3 depicts the effect of the withdrawal of FSH, insulin, IGF-I or insulin and IGF-I, on $P450_{arom}$ mRNA and estradiol secretion in cultured bovine granulosa cells. The withdrawal of FSH alone significantly decreased estradiol secretion and $P450_{arom}$ mRNA abundance ($P<0.01$) compared to controls. The withdrawal of insulin, IGF-I or both did not significantly alter estradiol secretion in the presence of FSH, whereas $P450_{arom}$ mRNA levels were significantly decreased by the withdrawal of insulin or of insulin and IGF-I ($P<0.01$) but were not affected by the withdrawal of IGF-I alone ($P>0.05$). Overall, estradiol secretion and $P450_{arom}$ mRNA levels were not correlated ($r=0.5$, $P=0.06$, $n=15$).

Figure 4 depicts the effect of the withdrawal of FSH, insulin, IGF-I or insulin and IGF-I on $P450_{scc}$ mRNA and progesterone secretion in these cells. Progesterone secretion was not affected by the withdrawal of any of the components, however $P450_{scc}$ mRNA levels were significantly reduced by the withdrawal of FSH, and of both insulin and IGF-I. Overall, the concentrations of progesterone and the $P450_{scc}$ mRNA abundance were not correlated ($r=0.28$, $P>0.1$, $n=15$).

DISCUSSION

Our results demonstrate, that in bovine granulosa cells in vitro, $P450_{arom}$ gene expression is dependent upon the presence of both FSH and insulin at low concentrations. In cattle, the concentrations of FSH are 0.5 -2 ng/ml in plasma and follicular fluid [22-26] and concentrations of insulin oscillate between 0.5-10 ng/ml in plasma and follicular fluid [27]. We conclude in the present study that physiological concentrations of both hormones maintained $P450_{arom}$ mRNA levels in bovine granulosa cells.

In the absence of insulin, FSH could not alone maintain detectable $P450_{arom}$ mRNA. This is in contrast with data obtained with rat [28] and human [29] granulosa cells, in which $P450_{arom}$ mRNA levels could be induced by FSH in the absence of insulin and IGF-I. This may reflect differences between these species, or it may reflect differences in the culture conditions, although in all cases the culture medium was devoid of serum. Surprisingly, in contrast, estradiol secretion was maintained after insulin and IGF-I withdrawal by the presence of FSH alone. These data suggest that $P450_{arom}$ mRNA and estradiol secretion are differentially regulated in bovine granulosa cells. It is not known how FSH acts upon aromatase activity. In rats it has been shown that a decline in $P450_{arom}$ mRNA in differentiated granulosa cells as a consequence of the LH surge is not associated with a corresponding loss of $P450_{arom}$ activity [30]. This suggest that the half-life of the $P450_{arom}$ enzyme is much longer than that of its mRNA, thus FSH may decrease the decay of the $P450_{arom}$ protein in the absence of transcription. Alternatively, other components of estradiol synthesis

may be involved. The 17βHSD enzyme converts estrone, the product of the metabolism of androstenedione by $P450_{arom}$, to estradiol [32], and it has been shown that FSH stimulates 17βHSD expression and activity [31]. There are further data suggesting that 17βHSD is constitutively expressed in rat granulosa cells [33]. Thus if 17βHSD is affected remains to be elucidated.

$P450_{scc}$ mRNA and progesterone secretion were not affected by the withdrawal of insulin and IGF-I, and both were stimulated by increasing doses of FSH. This is in agreement with previous studies, in which FSH stimulated $P450_{scc}$ mRNA abundance in pig [34], rat [35], human [36] and bovine [10] granulosa cells. These data are also consistent with our previous observation that insulin alone did not stimulates progesterone secretion or $P450_{scc}$ mRNA accumulation in bovine granulosa cells [10].

From the present data, it is clear that FSH and insulin have different effects on $P450_{arom}$ and $P450_{scc}$ mRNAs levels. This agrees with previous data in vitro [10] and in vivo in cattle [57], and in vitro in pigs [34], suggesting that $P450_{arom}$ and $P450_{scc}$ mRNAs are differentially regulated.

We have previously shown that insulin alone at 100 ng/ml can increase $P450_{arom}$ mRNA accumulation [10] and the present data show this effect is reversible. As it is known that insulin at concentrations of 50 ng/ml acts through the IGF-I receptor [11], and that IGF-I is known for its gonadotropin-like action on the differentiation of granulosa cells [37], it is reasonable to conclude that the effect of insulin (at 100 ng/ml) is mediated via the IGF-I receptor. Insulin at 10

ng/ml may not act via the IGF-I receptor, and the withdrawal of IGF-I alone did not significantly affect $P450_{arom}$ mRNA abundance or estradiol secretion in cultured granulosa cells in the presence of insulin and FSH at physiological concentrations. These findings suggest that, in the presence of FSH and insulin, IGF-I is not essential for $P450_{arom}$ mRNA and estradiol biosynthesis in cattle. This conclusion agrees with previous studies with bovine granulosa cells [8, 9, 38, 39] which showed that insulin was a more potent stimulator of estradiol production than IGF-I. It is nevertheless in contrast to the growing body of evidence suggesting that the bioavailablility of IGF-I is important for follicle growth and estradiol production [40] in cattle. Several explanations could be advanced for the lack of an effect of IGF-I in the present work. First, insulin concentrations may be too high, such that IGF-I becomes important only when insulin concentrations are below 10 ng/ml. This is suggested by the difference in $P450_{arom}$ mRNA levels observed between cultures after withdarwal of insulin compared to cultures after withdrawal of both insulin and IGF-I. Second, granulosa cells may secrete IGF-binding proteins [41], so reducing the bioavailability of added IGF-I.

Thus, the present study suggests that both insulin and FSH, at physiological concentrations, are essential for $P450_{arom}$ gene expression and estradiol secretion in bovine granulosa cells. It is not well understood how insulin acts in concert with FSH to stimulate $P450_{arom}$ mRNA. It is well established that $P450_{arom}$ mRNA and activity in granulosa cells are stimulated by FSH via protein kinase A (PKA) in rats [30]. Data obtained from studies with IGF-I, which utilises

the same signalling pathway as insulin [42] indicate that insulin and IGF-I work through tyrosine phosphorylation of the substrate protein alpha IRS-1 but not through the PKA pathway [43]. It has been shown in pig [44] and rat [37] granulosa cells that FSH plus IGF-I significantly stimulates the PKA pathway by increasing intracellular levels of cAMP. The same effect was not seen with IGF-I alone. Furthermore, induction of $P450_{arom}$ activity in human granulosa cells by both FSH and IGF-I involved tyrosine phosphorylation [45] suggesting that IGF-I and insulin enhance FSH action on granulosa cells at a site distal to cAMP generation. However, an elevation of cAMP levels in mouse embryonic stem cells by insulin-related peptides has been shown [46]. In neuronal cells, IGF-I activates the nuclear transcription factor cAMP response element-binding protein (CREB) [47] which is indispensable for $P450_{arom}$ gene transcription in rat granulosa cells [30]. Clearly the second messenger pathways utilized by insulin to induce $P450_{arom}$ mRNA in granulosa cells need to be clarified.

Further evidence for the importance of insulin for estradiol secretion has been attained from diabetic, insulin-dependent pigs, in which ovarian follicles were macroscopically atretic, and concentrations of estradiol in follicular fluid were lower than in non-diabetic pigs [48, 49]. In humans, a pathological condition called polycystic ovarian syndrome (PCOS) is often associated with hyperandrogenism, hyperinsulinism and insulin resistance [50]. Insulin resistance involves a postreceptor defect in the insulin signal transduction [51] and results in low estradiol production despite high androgen concentrations [52]. Recently, it has ben shown that low estradiol secretion was correlated with

low $P450_{arom}$ mRNA levels in granulosa cells from insulin resistant humans [53]. In vivo studies in cattle have shown that an increase in dietary intake is followed by an increase in insulin concentrations, which is also followed by an increase in the number of recruited follicles [54, 55]. In cattle follicular recruitment has been associated with the expression of $P450_{arom}$mRNA [56]. These and our results indicate that insulin is essential for $P450_{arom}$ gene expression in a number of species.

In summary, we conclude that $P450_{arom}$ mRNA accumulation is dependent on both insulin and FSH at physiological concentrations, whereas estradiol secretion can be maintained by FSH in the absence of detectable quantities of mRNA. Thus we hypothesises that insulin and FSH can act to increase $P450_{arom}$ gene transcription and/or mRNA stability, whereas FSH can act to stabilize enzyme activity.

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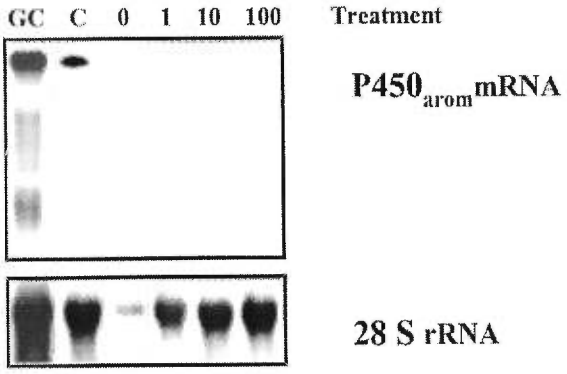
Figure 1.

Estradiol secretion and $P450_{arom}$ mRNA abundance in bovine granulosa cell cultures after withdrawal of IGF-I and insulin. Cells were cultured for 4 days with IGF-I (10 ng/ml) and insulin (100 ng/ml), then medium was replaced with IGF-I and insulin-free medium. Cells were supplemented with 0, 1, 10 or 100 ng/ml FSH for two additional days. Values are least-squares means ± SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and estradiol is expressed relative to total cell DNA. The upper panel shows a representative Northern blot, which includes total RNA from freshly harvested granulosa cells (GC). C represents a control treatments without FSH, insulin (100 ng/ml) and IGF-I (10 ng/ml)

Figure 1


GC C 0 1 10 100
Treatment
P450arom mRNA
28 S rRNA

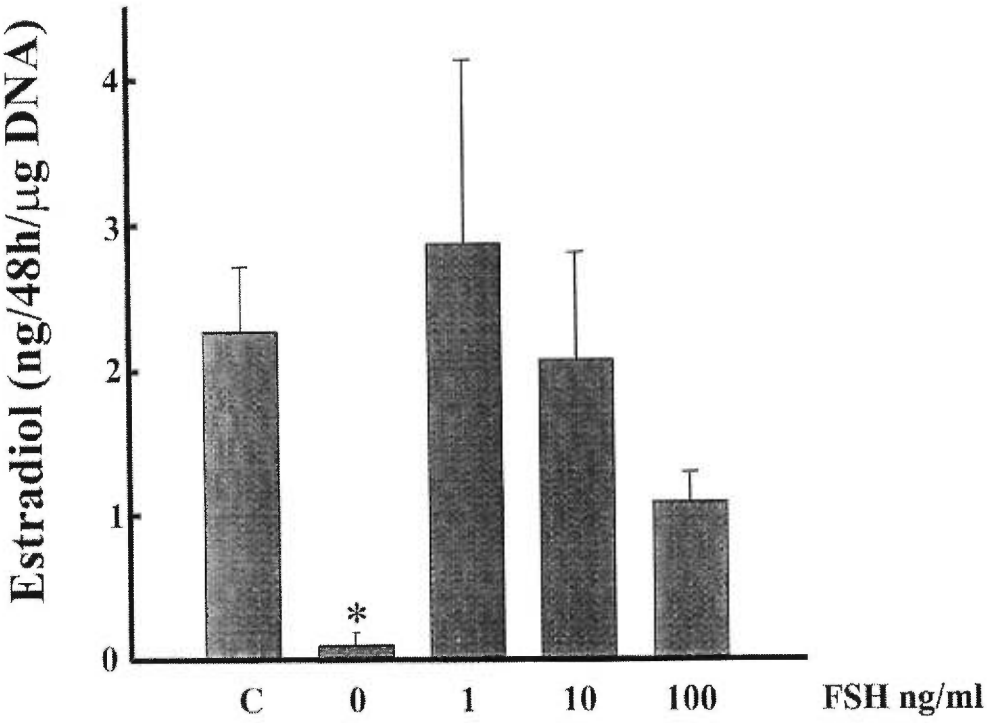

Estradiol (ng/48h/μg DNA)
0
1
2
3
4
*
C
0
1
10
100
FSH ng/ml

Figure 2.

Progesterone production and $P450_{scc}$ mRNA abundance in bovine granulosa cells after withdrawal of IGF-I and insulin Cells were cultured for 4 days with IGF-I (10 ng/ml) and insulin (100 ng/ml), then medium was replaced with IGF-I and insulin-free medium. Cells were supplemented with 0,1,10 or 100 ng/ml FSH for two additional days. Values are least-squares means ± SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and progesterone is expressed relative to total cell DNA. The upper panel shows a representative Northern blot.

Figure 2

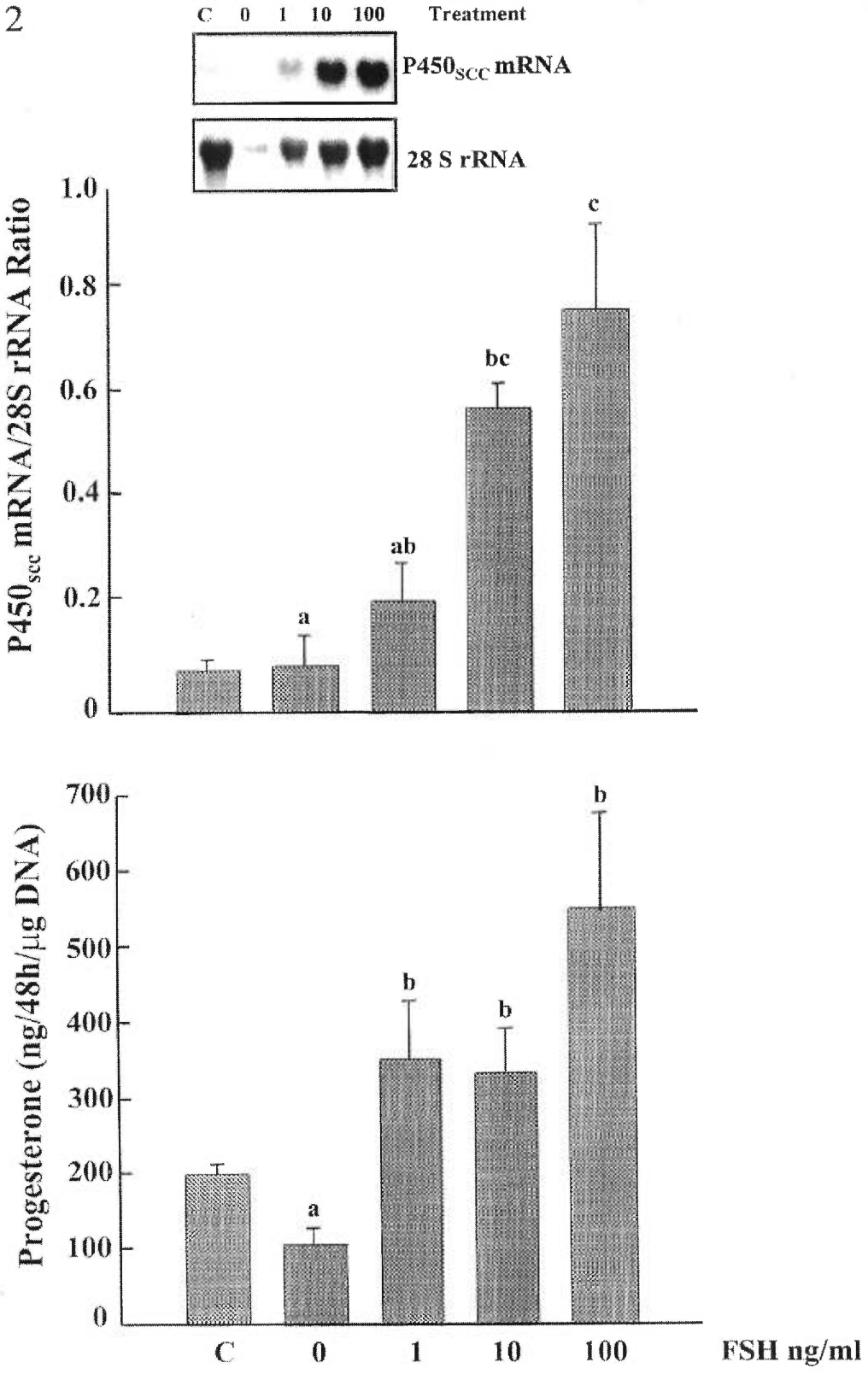

C 0 1 10 100 Treatment
P450SCC mRNA
28 S rRNA
P450scc mRNA/28S rRNA Ratio
1.0
0.8
0.6
0.4
0.2
0
a
ab
bc
c
Progesterone (ng/48h/μg DNA)
700
600
500
400
300
200
100
0
a
b
b
b
C 0 1 10 100 FSH ng/ml

Figure 3.

Estradiol secretion and $P450_{arom}$ mRNA abundance in bovine granulosa cells after selective hormone withdrawal. Cells were cultured for 4 days with insulin, IGF-I and FSH (10 ng/ml, 10 ng/ml and 1 ng/ml, respectively). Then incubated for a further 3 days with the indicated concentrations of each hormone. Values are least-squares means ± SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and estradiol is expressed relative to total cell DNA. The upper panel shows a representative Northern blot.

Figure 3

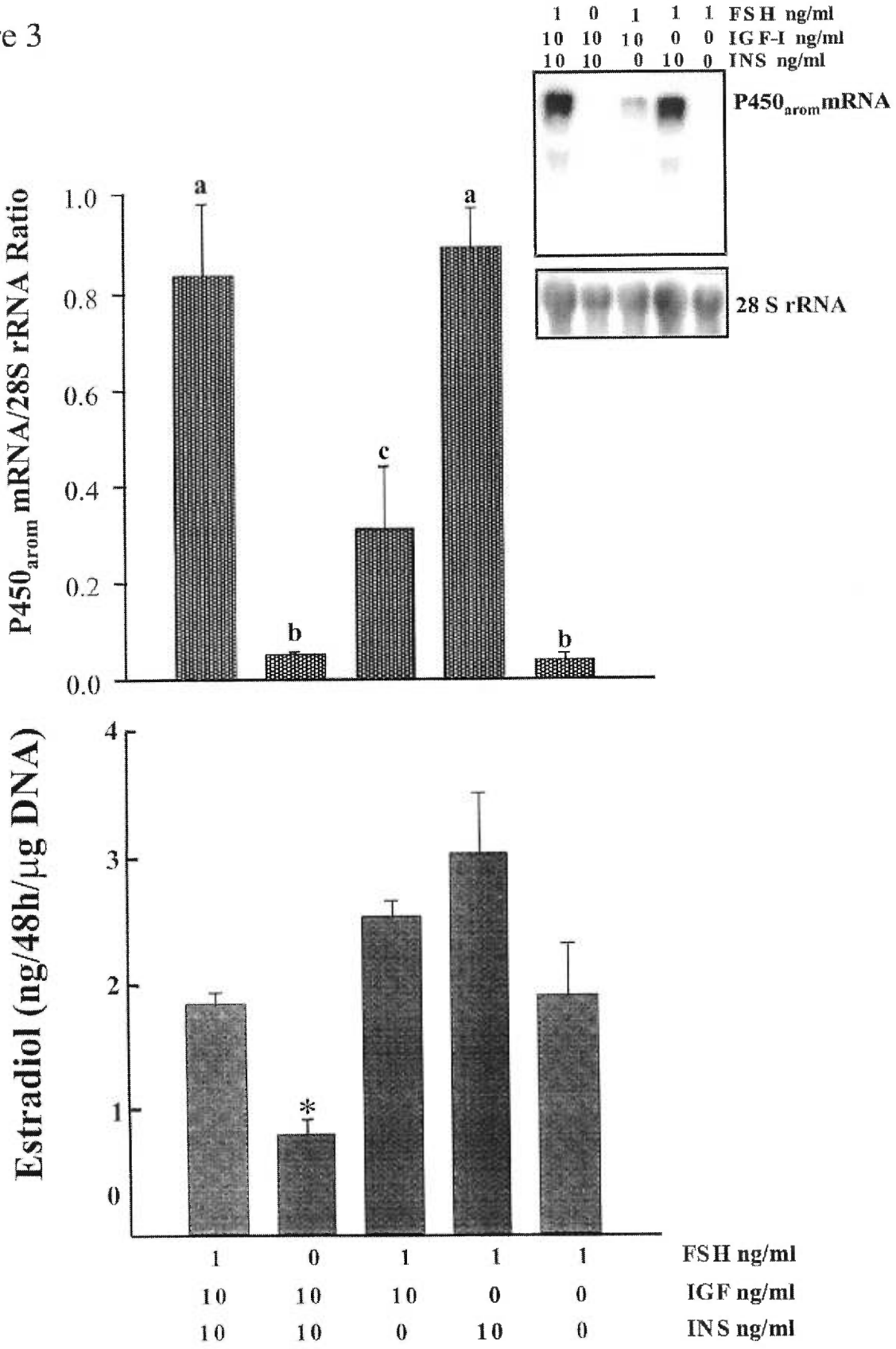
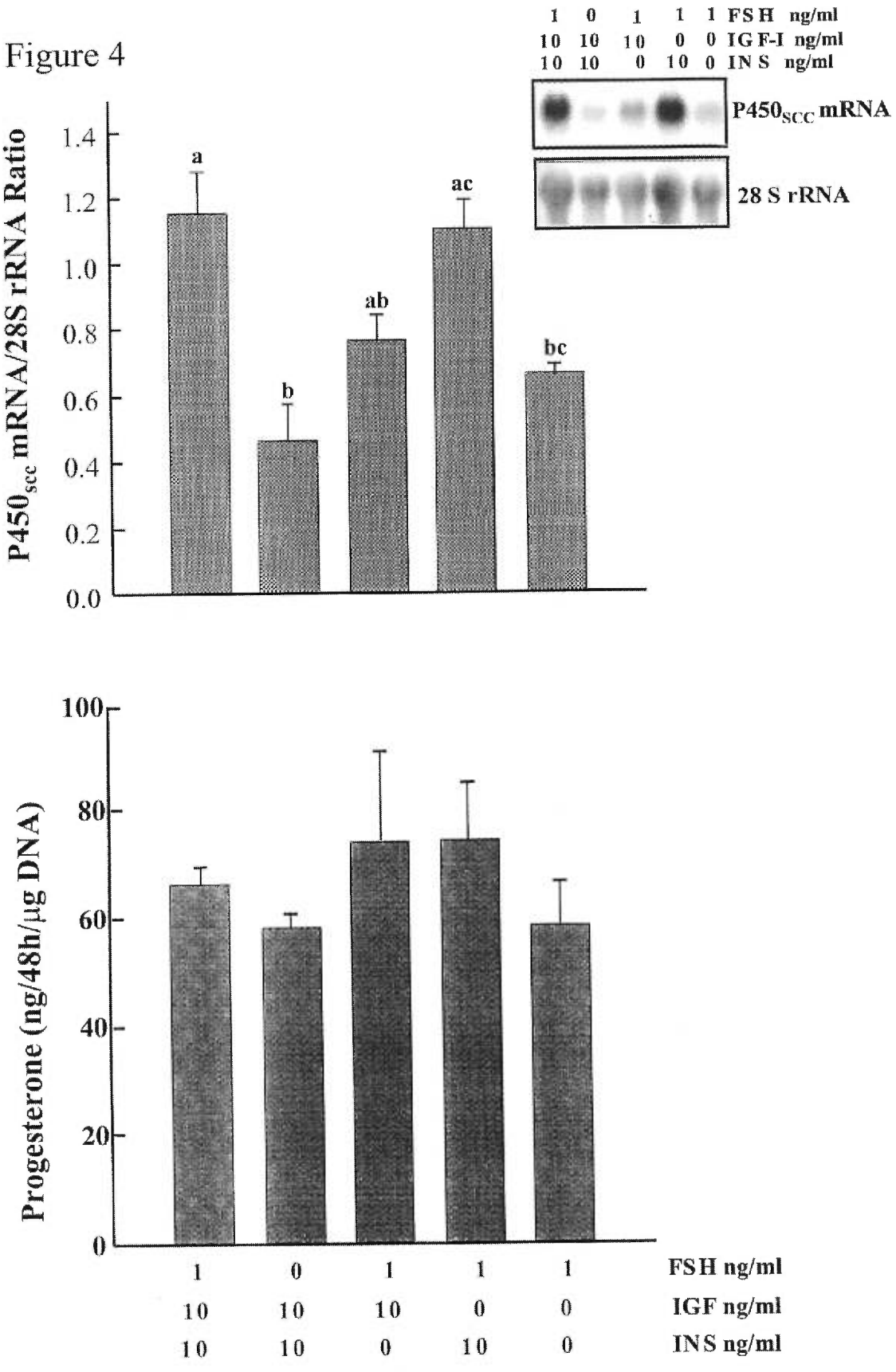

1 0 1 1 1 FSH ng/ml
10 10 10 0 0 IGF-I ng/ml
10 10 0 10 0 INS ng/ml
P450arom mRNA
28 S rRNA
P450arom mRNA/28S rRNA Ratio
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
a
b
Estradiol (ng/48h/μg DNA)
4
3
2
1
0
*
1 0 1 1 1 FSH ng/ml
10 10 10 0 0 IGF ng/ml
10 10 0 10 0 INS ng/ml

Figure 4.

Progesterone secretion and $P450_{scc}$ mRNA abundance in bovine granulosa cells after selective hormone withdrawal. Cells were cultured for 4 days with insulin, IGF-I and FSH (10 ng/ml, 10 ng/ml and 1 ng/ml, respectively). Then incubated for a further 3 days with the indicated concentrations of each hormone. Values are least-squares means ± SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and progesterone is expressed relative to total cell DNA. The upper panel shows a representative Northern blot.

Figure 4


1 0 1 1 1 FSH ng/ml
10 10 10 0 0 IGF-I ng/ml
10 10 0 10 0 INS ng/ml
P450scc mRNA
28 S rRNA
P450scc mRNA/28S rRNA Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
ab
ac
bc
Progesterone (ng/48h/μg DNA)
100
80
60
40
20
0
1 0 1 1 1 FSH ng/ml
10 10 10 0 0 IGF ng/ml
10 10 0 10 0 INS ng/ml

ARTICLE 3

Second Messenger Pathways used by FSH and Insulin in the Stimulation of Cytochrome $P450_{arom}$mRNA Abundance and Estradiol Secretion in Bovine Granulosa Cells in Vitro.[1]

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Running title: Second messengers in granulosa cells

ABSTRACT

The objective of the present study was to determine the main intracellular pathways utilized by insulin alone and insulin with FSH to induce $P450_{arom}$mRNA and estradiol secretion. In the first experiment bovine granulosa cells from small follicles (2-4 mm in diameter) were cultured in the presence of insulin (100 ng/ml). In the second experiment, granulosa cells from the same size follicles, were cultured with insulin (10 ng/ml) and FSH (1 ng/ml). In both experiments, the following specific second messenger inhibitors were added on day 4 of culture: LY-294002 (a specific inhibitor of phosphatidylinositol 3-kinase PI3K; 20μM), GF 109203X (a specific protein kinase C inhibitor; 3μM), H-89 (a specific protein kinase A inhibitor;10μM), and PD-98059 a specific inhibitor of MEK (MAP kinase kinase) activation; 50mM). Granulosa cells were recovered at day 6 of culture. In the first experiment, PI3 kinase and PKC inhibitors significantly decreased insulin-stimulated $P450_{arom}$ mRNA levels and estradiol secretion ($P<0.001$). In the second experiment, the PKA inhibitor significantly decreased FSH-stimulated $P450_{arom}$ mRNA abundance, whereas estradiol secretion was significantly inhibited by PI3K, PKC and PKA inhibitors. Inhibition of MEK pathway significantly increased $P450_{arom}$ mRNA abundance, but did not affect estradiol secretion. $P450_{scc}$ mRNA levels and progesterone secretion were not affected by any inhibitor in either experiments. We conclude that $P450_{arom}$ mRNA is regulated by insulin and FSH by two different intracellular

mechanisms, whereas estradiol secretion is exclusively regulated by PI3K and PKC pathways.

INTRODUCTION

The regulation of ovarian follicular development involves a series of biochemical processes leading to differentiation of follicular cells to increase their capacity to produce steroids [1] Estradiol is the principal steroid produced by granulosa cells before their terminal differentiation is induced by the LH surge [2]. Estradiol is synthesized in granulosa cells from thecal androgens, by two successive enzyme-catalyzed reactions [3, 4]. In cattle, androstenedione is the preferred substrate, which is converted to estrone by the cytochrome $P450_{arom}$ complex. Estrone is then converted to estradiol by the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD). The expression of $P450_{arom}$ mRNA, the microsomal enzyme responsible of estradiol production in granulosa cells, is universally known to be induced by FSH via cAMP/PKA intracellular pathway [5]. In vivo and in vitro studies in rat [6] and in vitro in human [7], pig [8] and bovine [9] granulosa cells have been shown that FSH induces $P450_{arom}$ mRNA abundance. In cattle, low FSH concentrations induce estradiol secretion in cultured granulosa cells [9-12]. Furthermore, many other factors have been shown to interact with FSH to enhance estradiol secretion in cattle. One of these is IGF-I for which bioavailability is important to in vivo [13, 14] and in vitro responses [12]. It has been shown that insuline can also enhance the FSH stimuli to produce estradiol [10, 12, 15-17] in bovine granulosa cells. In vivo, insulin treatment during the superovulation of cattle increases follicular fluid estradiol concentrations in large follicles [18]. Insulin and IGF-I cause tyrosine

phosphorylation of the substrate protein alpha IRS-1, which is mediated through PKC and mitogen-activated protein kinase (MAPK) pathways but not through the PKA pathway [19, 20]. Previously, we have shown that bovine granulosa cells express and maintain $P450_{arom}$ mRNA in vitro. Such expression can be induced in two ways, first by culturing granulosa cells in the presence of insulin at 100ng/ml and IGF-I (10 ng/ml), with no FSH added, second $P450_{arom}$ mRNA is induced when granulosa cells are cultured in the presence of low IGF-I (10 ng/ml) insulin (10 ng/ml) and FSH (1 ng/ml) concentrations [9]. We have shown that the withdrawal of IGF-I from the culture medium had no effect $P450_{arom}$ mRNA levels, when insulin and FSH were present at physiological concentrations [9]. Thereby, in cattle, the presence of FSH and insulin in the culture medium are key regulatory elements in the regulation of $P450_{arom}$ mRNA in granulosa cells. Two questions emerged from these studies. First how is insulin alone able to induce $P450_{arom}$ mRNA levels in the absence of FSH? Second, are the combination of FSH plus insulin at low concentrations acting through the same intracellular pathway as insulin alone to induce $P450_{arom}$ mRNA ? Thus, the aim of the present study was to investigate which intracellular pathways are used by FSH and insulin to increase $P450_{arom}$ mRNA levels in bovine granulosa cells.

MATERIALS AND METHODS

Cell culture

The cell culture system was based on that described previously [9,21]. All materials were obtained from Gibco BRL Canada (Burlington, ON, Canada) unless otherwise stated.

Bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir and transported to the laboratory in M199 containing 25 mm Hepes, penicillin (100 iu/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml). Follicles were dissected free of surrounding tissue, and small follicles (2 - 4 mm diameter) were bisected into culture medium at 37 °C. Granulosa cells were recovered by passing the follicle walls repeatedly through a 1 ml disposable pipette. The follicle walls were allowed to sediment out under gravity, and the granulosa cell suspension was transferred to sterile centrifuge tubes. Cells were isolated by centrifugation at 800 x g for 5 min, and were washed 3 times in M199 containing 25 mm Hepes, penicillin (100 iu/ml) and streptomycin (100 µg/ml). Washed cells were resuspended in culture medium, and cell viability was estimated at 40 % by Trypan blue exclusion.

Cells were seeded into 24-well tissue culture plates (Falcon, Dickinson and company, New Jersey) at a density of 10^6 viable cells in 1ml of a-MEM with l-glutamine containing sodium bicarbonate (10 mm), Hepes (20 mm), protease-free BSA (0.1 %), selenium (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (10-7 m), human recombinant IGF-1 (10 ng/ml), non-essential amino acid mix

(1.1 mm), penicillin (100 iu/ml) and streptomycin (100 µg/ml). cells were stimulated with insulin plus FSH as outlined below. Cultures were maintained at 37°C in 5 % CO_2 for up to 6 days, with 700 µl medium being replaced every 2 days.

Treatments

Cells were cultured under conditions that promoted insulin or FSH-stimulated $P450_{arom}$mRNA abundance and estradiol secretion. For insulin-stimulated estrogen biosynthesis, cells were cultured for six days in the presence of insulin (100 ng/ml) without FSH. For FSH-stimulated $P450_{arom}$mRNA abundance and estrogen biosynthesis, cells were cultured with insulin at 10 ng/ml and with FSH (USDA bFSH-17; 1 ng/ml). Under these conditions, the withdrawal of FSH causes a loss of $P450_{arom}$mRNA and estradiol secretion [9].

In both experiments, at day four of culture, specific second messenger inhibitors were added for two days. The inhibitors were: 20µM LY-294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K) ; 3µM GF 109203X, a specific protein kinase C inhibitor (PKC). 10µM H-89 a specific protein kinase A inhibitor (PKA). 50mM PD-98059, a specific inhibitor of MEK (MAP kinase kinase) activation (MEK). Controls were cultured with and without DMSO (the no not vehicle of inhibitors, 0.1 %). Doses of the inhibitors were based on those

used by other groups [63, 64, 65]. All inhibitors were added directly to each well in a total volume of 5 μl/well. The inhibitors were obtained from Biomol Research laboratories, Inc. (U.S.A.).

Conditioned medium was stored at -20 °C until assayed for estradiol and progesterone. Half of each culture plate (12 wells) was used per treatment per replicate, and experiments were repeated 3 times. Same wells were used for extraction of total RNA and DNA using Trizol (Gibco BRL) according to the manufacturer's instructions. Total DNA was quantified by measuring fluorescence in the presence of Hoechst 33258 [22] and compared with a calf thymus DNA standard (Boehringer-Mannheim, PQ Canada).

Hybridization

The relative abundance of mRNA for each protein was determined by Northern hybridization [9,23]. Electrophoresis of 15 mg RNA was performed through a 1 % denaturing formaldehyde-agarose gel followed by overnight capillary transfer onto a nylon membrane (Hybond-N; Amersham, Oakville, Ontario). Membranes were UV cross-linked in a commercial UV chamber (Bio Rad, Mississauga, Ontario) and incubated for 2 h in prehybridization solution, containing 10 % dextran sulfate, 5-strength saline-sodium phosphate-EDTA buffer (SSPE), 5-strength Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and herring sperm DNA (200 mg/ml).

The bovine aromatase cDNA probe was prepared in our laboratory [24], and encompasses the entire heme-binding and I-helix regions. The probe is specific for estrogen-secreting tissues, and hybridizes to 3 bands of follicular total RNA at 6.5, 3.4 and 1.8 kb [24]. The bovine P450scc cDNA was a gift from Dr M.R. Waterman (Vanderbilt University School of Medicine, Nashville, TN), and is a 1.7 kb cDNA containing the complete coding sequence [25], which hybridizes to a single band of 2 kb in bovine follicles [26].

Probes were labeled with [a-32P]dCTP by random primer extension using a kit (Boehringer Mannheim, Laval, Québec) to a specific activity of 1.5-3.0 x 10_9 dpm/mg, and purified by centrifugation through a minicolumn using Wizard PCR Preps DNA purification system (Promega, Montréal, Québec, Canada). Hybridization to the membranes was performed overnight at 65°C. After hybridization, membranes were washed in 2 X SSPE-0.1 % SDS twice at room temperature (15 min each), and twice at 65°C (15 min each). Membranes were then stripped and rehybridized to a labeled human 28 S ribosomal cDNA probe [27] for the standardization of RNA loading. The labeled membranes were exposed to Kodak X-Omat film at -70°C in the presence of an intensifying screen. Autoradiograms were scanned with a densitometer after 1 to 14 days exposure.

Steroid assays

Estradiol was measured in conditioned medium without extraction with the assay described elsewhere [28], with modifications [29]. Inter- and intra-

assay coefficients of variation were less than 12 %. Progesterone was measured as described **Erreur! Signet non défini.**, with inter- and intra-assay coefficients of variation of less than 10 %. The sensitivity of these assays was equivalent to 0.25 and 1 ng/ml medium for estradiol and progesterone, respectively.

Statistical analysis

The density of hybridization signals was corrected for loading efficiency using hybridization to 28S ribosomal RNA [9]. Steroid concentrations are expressed relative to total DNA content of the wells. Data were transformed to logarithms if they were not normally distributed (Shapiro-Wilk test). Analysis of variance (ANOVA) was used to test effects of FSH and of insulin treatments. To avoid potential confounding effects of culture replicate, effects of treatment were tested within replicate. Differences between groups were identified with the Tukey-Kramer HSD test. Correlations between steroid secretion and mRNA abundance were determined with the Pearson correlation coefficient. Analyses were performed with JMP® software (SAS Institute, Cary, NC). The data are presented as means ± SEM.

RESULTS

Insulin-stimulated cells

Figure 1 shows the effect of specific second messenger inhibitors on insulin-stimulated $P450_{arom}$ mRNA abundance and estradiol secretion in cultured

bovine granulosa cells. The addition of DMSO alone had no effect on $P450_{arom}$ mRNA or estradiol secretion. The addition of PI3K and PKC inhibitors significantly decreased $P450_{arom}$mRNA abundance and estradiol secretion ($P<0.001$), whereas the addition of the PKA inhibitor had no effect. However, the addition of the inhibitor of MEK significantly increased $P450_{arom}$ mRNA abundance ($P<0.05$), although it was without effect on estradiol secretion. There was a linear correlation between $P450_{arom}$ mRNA and estradiol secretion ($r=0.65$; $P<0.01$ $n=12$).

Figure 2 depicts the effect of specific second messenger inhibitors on insulin-stimulated $P450_{scc}$ mRNA abundance and progesterone secretion in cultured bovine granulosa cells. $P450_{scc}$ mRNA abundance and progesterone secretion were not affected by the addition of DMSO or any of the specific inhibitors; $P450_{scc}$ mRNA abundance and progesterone secretion were not correlated ($r=0.08$; $P>0.73$ $n=12$).

FSH-stimulated cells

Figure 3 shows the effect of specific second messenger inhibitors on FSH-stimulated $P450_{arom}$ mRNA abundance and estradiol secretion in cultured bovine granulosa cells. The addition of the PKA inhibitor decreased $P450_{arom}$ mRNA abundance and estradiol secretion ($P<0.01$). Whereas the PI3K and PKC inhibitors had no significant effect on $P450_{arom}$ mRNA abundance, estradiol secretion was significantly inhibited by these two treatments. As was seen in the

first experiment, the MEK kinase inhibitor significantly increased $P450_{arom}$ mRNA levels, but was without effect on estradiol secretion. Overall, estradiol secretion and $P450_{arom}$ mRNA levels were not correlated ($r= -0.05$, $P>0.10$, $n=12$).

Figure 4 depicts the effect of the second messenger inhibitors on $P450_{scc}$ mRNA abundance and progesterone secretion in these cells. As in the first experiment, $P450_{scc}$ mRNA abundance and progesterone secretion were not affected by the inhibitors, and these two variables were not correlated ($r= -0.24$, $P>0.10$, $n=12$).

DISCUSSION

Our results strongly support the hypothesis that $P450_{arom}$ gene expression and estradiol secretion in bovine granulosa cells in vitro can be stimulated by insulin and FSH by alternative intracellular pathways. The inhibition of PI3K and PKC signalling pathway was followed by decreased $P450_{arom}$ mRNA abundance and estradiol secretion when granulosa cells were stimulated with insulin, whereas the inhibition of PKA did not affect $P450_{arom}$ mRNA abundance and estradiol secretion under these conditions. Insulin may stimulate estrogen biosynthesis through insulin binding and receptor autophosphorylation, followed by tyrosine phosphorylation of a protein termed insulin receptor substrate 1 (IRS-1) [20]. This results in the activation of PI3K and phospholipase C, with subsequent formation of phosphatidylinositol-3 phosphate (Insp3), which releases Ca^{+} from the calcium-sequestering

compartments [31]. Insulin receptor stimulation also results in the activation of protein kinase C by diacylglycerol (DAG) [32]. Other evidence of the tyrosine kinase signalling pathway in estradiol synthesis comes from studies with IGF-I in humans [33] and TGFα in humans [34] and pigs [62]. Nevertheless there are contradictory data showing that TGFα inhibits estradiol secretion in rat [35], ovine [21] and human [36] granulosa cells. Collectively these data suggest the existence of multiple signalling pathways to induce $P450_{arom}$ mRNA and estradiol secretion.

Insulin may also activates MAPK activity, as it was observed in Chinese hamster ovary cells [37]. Insulin is known to increas levels of cAMP in mouse embryonic stem cells [38]. However, present data clearly show that, in bovine granulosa cells, insulin-dependent stimulation of estradiol biosynthesis is independent of PKA activation.

Contrary to insulin (and IGF-I), it is well established that FSH stimulates $P450_{arom}$ gene expression and estradiol secretion via the cAMP/PKA pathway [2]. The present data are entirely in agreement with this, as the inhibition of PKA pathway significantly decreased FSH-stimulated $P450_{arom}$ mRNA abundance and estradiol secretion. Interestingly, when FSH-stimulated cells where cultured with inhibitors of PI3K and PKC, there was a significant decrease in estradiol secretion but not $P450_{arom}$ mRNA abundance, suggesting that estradiol secretion is regulated by the PKC pathway, at a site downstream of translation. This is in contrast with studies showing that phorbol 12-myristate 13-acetate

(PMA; a phorbol ester that activates protein kinase C), reduced estradiol secretion from FSH/cAMP stimulated pig granulosa cells from small and medium sized follicles [39], although the same compound showed no effect on estradiol secretion in another study of the same species [40]. Furthermore, $P450_{arom}$ activity was inhibited by prolactin in rat granulosa cells through activation of the PKC pathway [41]. The calcium ionophore A23187 (which mimics PIP3) and PMA, synergistically increased estradiol production in goldfish ovarian follicles [42]. Likewise, estradiol secretion was increased, decreased, and unchanged in pig granulosa cells from large, small and medium follicles, respectively, under basal or FSH-treated cultured pig granulosa cells by the treatment with A23187 and TPA [43]. We utilized granulosa cells from small follicles, but at day four of culture, bovine granulosa cells are already differentiated under these culture conditions [12]. Thus it is possible that $P450_{arom}$ mRNA estradiol secretion may come under the stimulatory control of regulators of protein kinase C at an advanced stage of granulosa cell differentiation. Alternatively, these could be species differences in the action of IP3K and protein kinase C on estradiol secretion. Another explanation is that intermediates of androgen metabolism may be affected by the PKA pathway, such as 17βHSD [44]. It has been shown that A23187 and TPA increased 17βHSD type 1 expression in JET-3 choriocarcinoma cells [45]. Although other studies of ovarian cells suggest 17βHSD type 1 is constitutively expressed [46]. It is unlikely that the effects of PI3K and PKC inhibitors are non-specific, as none of the treatments applied in

the present study affected $P450_{scc}$ mRNA accumulation or progesterone secretion. The weak signal observed on 28S rRNA on insulin-stimulated cells can create confusions, it is not possible that the inhibitors for PI3K and PKC cause a reduction on 28S rRNA, because it was not observed in FSH-stimulated cells.

Finally, PI3K and PKC pathway may be involved in an FSH-dependent protection of $P450_{arom}$ protein degradation or maintenance of catalytic activity.

Interestingly, the specific inhibitor of MEK, significantly increased $P450_{arom}$ mRNA of both insulin- and FSH-stimulated cells in the present studies. Insulin binds to a transmembrane receptor, ligand binding causes activation of the intrinsic receptor tyrosine kinase, which results in autophosphorylation of the insulin receptor and subsequent phosphorylation of numerous substrates within the cell (19). Of these substrates, those involved in signal transduction through activation of MAP kinases have received much attention [47-50]. This pathway is initiated by activation of Ras. Activated Ras in turn phosphorylates Raf-1 [51]. Once activated, Raf-1 phosphorylates another kinase, known as MAP kinase kinase or MEK (MAP or ERK kinase). Once activated it serves as a specific activator of MAP kinase or extracellular signal-related kinases [52]. MAP kinase then transduces signals to the nucleous by phosphorylating transcription factors that include the product of oncogenes [48].

Recently, it has been shown that FSH, via cAMP, activates MAPKs in pig [53] and rat [54, 55] immature granulosa cells. However, the authors did not

measure steroids. It is possible that the activation of MAP kinases are important in early stages of follicular development, as the growth factor EGF, which expression is confined to preantral and early antral follicles [56]. EGF is a potent stimulator of MAPKs in granulosa cells [57]. EGF has been shown to inhibit estradiol secretion in vivo [58] and in vitro [59], possible by inducing MAPKs activity. It has been also suggested that the activation of MAPKs are related to granulosa cell luteinization [60]. Furthermore, MAPKs have been implicated in the regulation of cytoskeletal elements [61], and cytoskeleton plays an important role in the transport of cholesterol and granulosa cell luteinization [61]. Thus the inhibition of the MAPKs may act through preventing the induction of luteinization, increasing $P450_{arom}$ mRNA abundance by a mechanism that requires further elucidation.

In summary, $P450_{arom}$ mRNA abundance can be induced by FSH and insulin by alternative intracellular pathways, whereas estradiol secretion appears to be regulated by PKC pathway.

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Figure 1

$P450_{arom}$mRNA abundance and estradiol secretion in insulin-stimulated bovine granulosa cells. Cells were cultured for 6 days with insulin (100 ng/ml) and IGF-1 (10 ng/ml). At day 4 of culture specific inhibitors were added to the cultures. The treatments were: Control, DMSO, and the inhibitors for PI3K, PKC, PKA and MEK. Values are least-squares means ±SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and estradiol is expressed relative to total cell DNA. The upper panel shows a representative Northern blot.

FIGURE 1

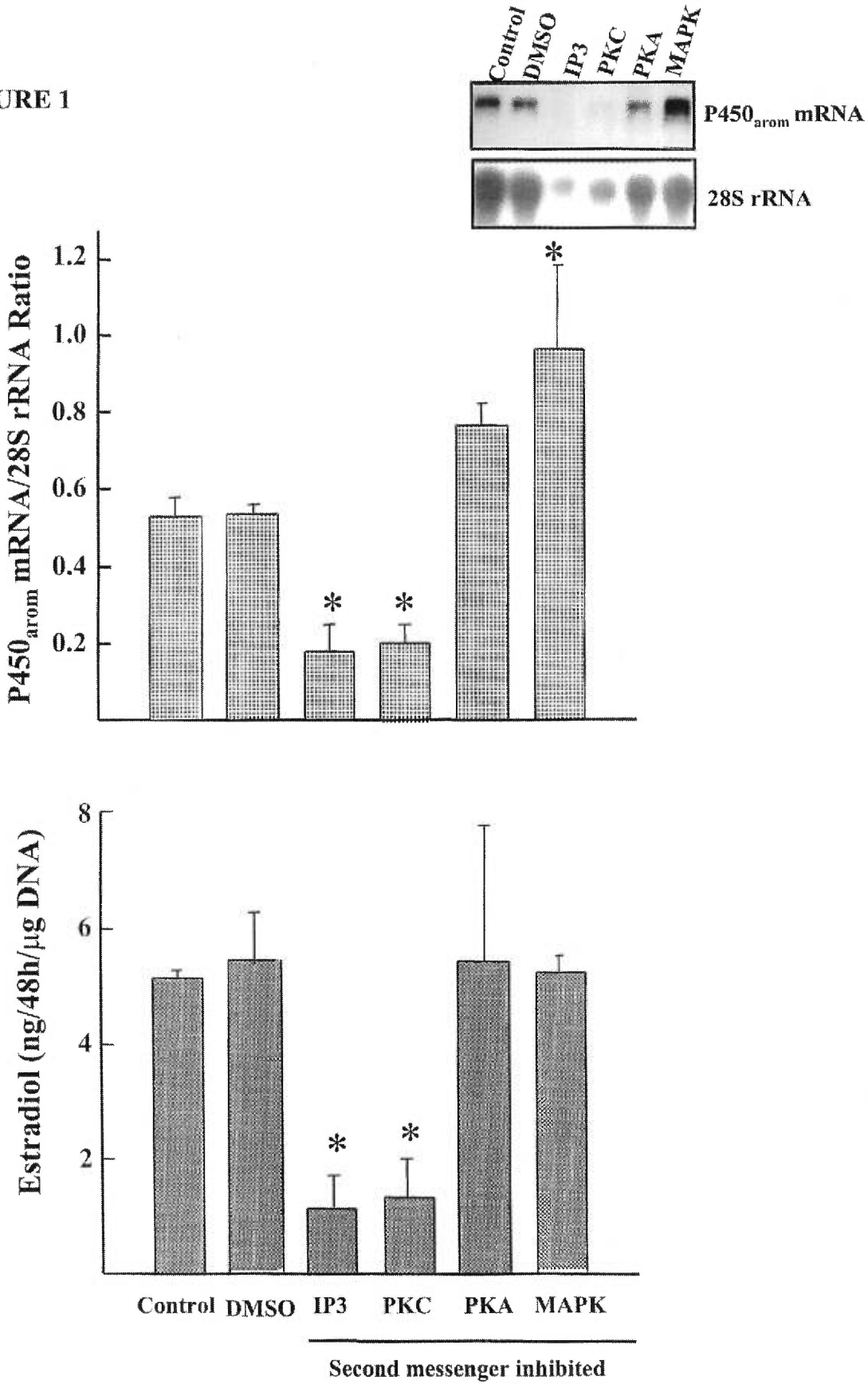

Control
DMSO
IP3
PKC
PKA
MAPK
P450arom mRNA
28S rRNA
P450arom mRNA/28S rRNA Ratio
1.2
1.0
0.8
0.6
0.4
0.2
*
Estradiol (ng/48h/μg DNA)
8
6
4
2
Control
DMSO
IP3
PKC
PKA
MAPK
Second messenger inhibited

Figure 2

$P450_{scc}$ mRNA abundance and progesterone secretion in insulin-stimulated bovine granulosa cells. Cells were cultured for 6 days with insulin (100 ng/ml) and IGF-1 (10 ng/ml). At day 4 of culture specific inhibitors were added to the cultures. The treatments were: Control, DMSO, and the inhibitors for PI3K, PKC, PKA and MEK. Values are least-squares means ±SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and progesterone is expressed relative to total cell DNA. The upper panel shows a representative Northern blot.

FIGURE 2

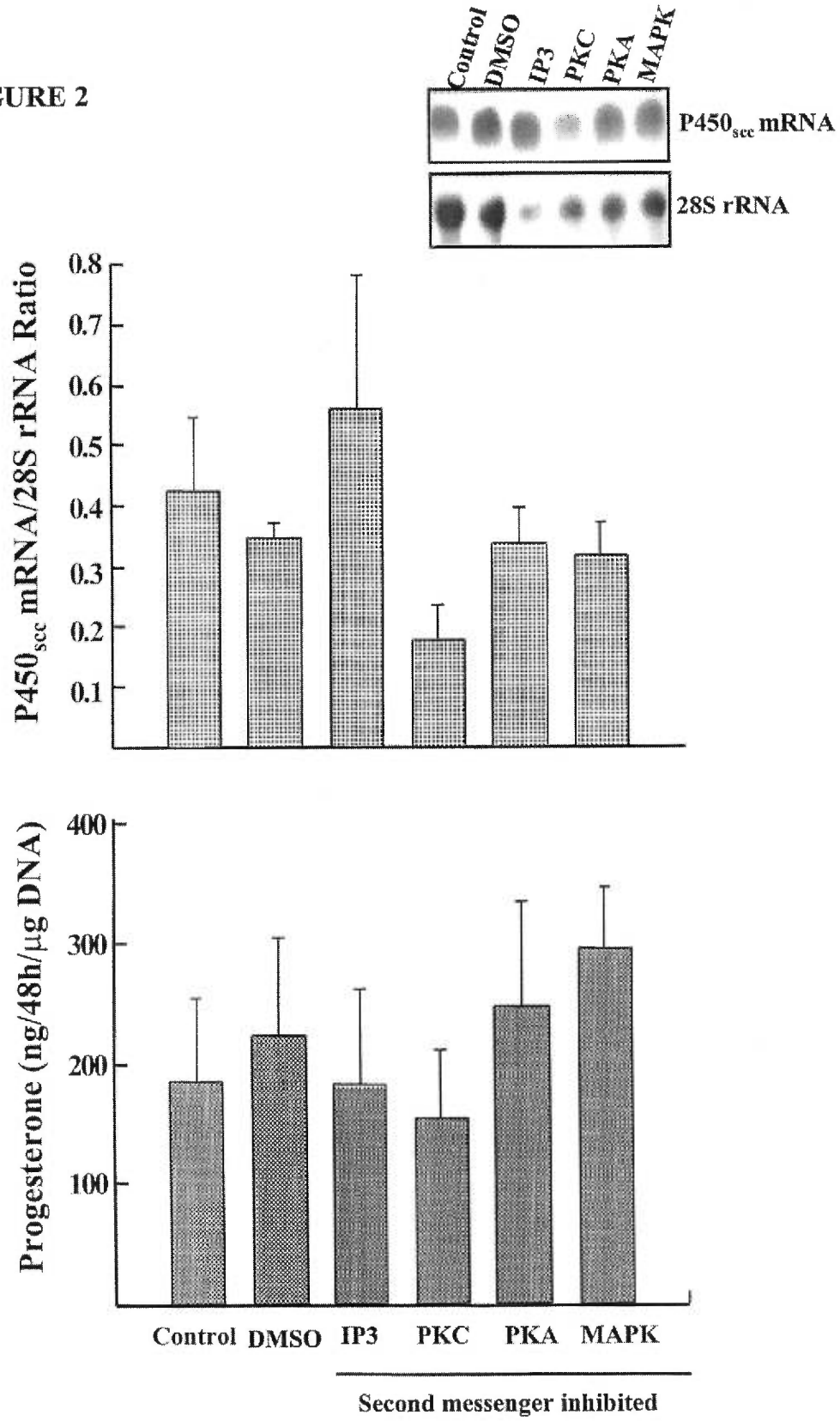

Control
DMSO
IP3
PKC
PKA
MAPK
P450scc mRNA
28S rRNA
P450scc mRNA/28S rRNA Ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Progesterone (ng/48h/μg DNA)
400
300
200
100
Control
DMSO
IP3
PKC
PKA
MAPK
Second messenger inhibited

Figure 3

$P450_{arom}$mRNA abundance and estradiol secretion in FSH-stimulated bovine granulosa cells. Cells were cultured for 6 days with FSH (1ng/ml), insulin (10 ng/ml) and IGF-1 (10 ng/ml). At day 4 of culture specific inhibitors were added to the cultures. The treatments were: Control, DMSO, and the inhibitors for PI3K, PKC, PKA and MEK. Values are least-squares means ±SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and estradiol is expressed relative to total cell DNA. The upper panel shows a representative Northern blot.

FIGURE 3

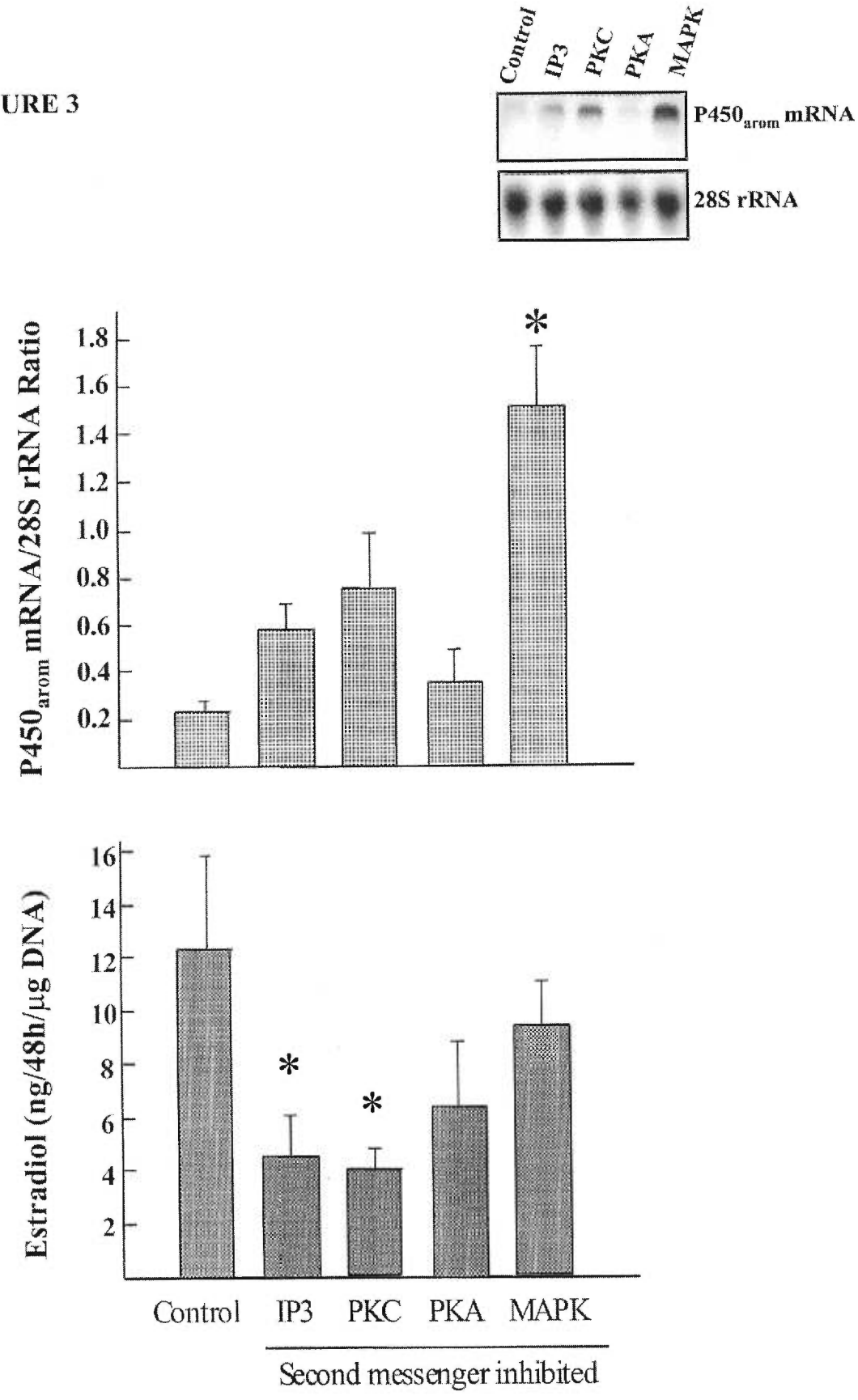
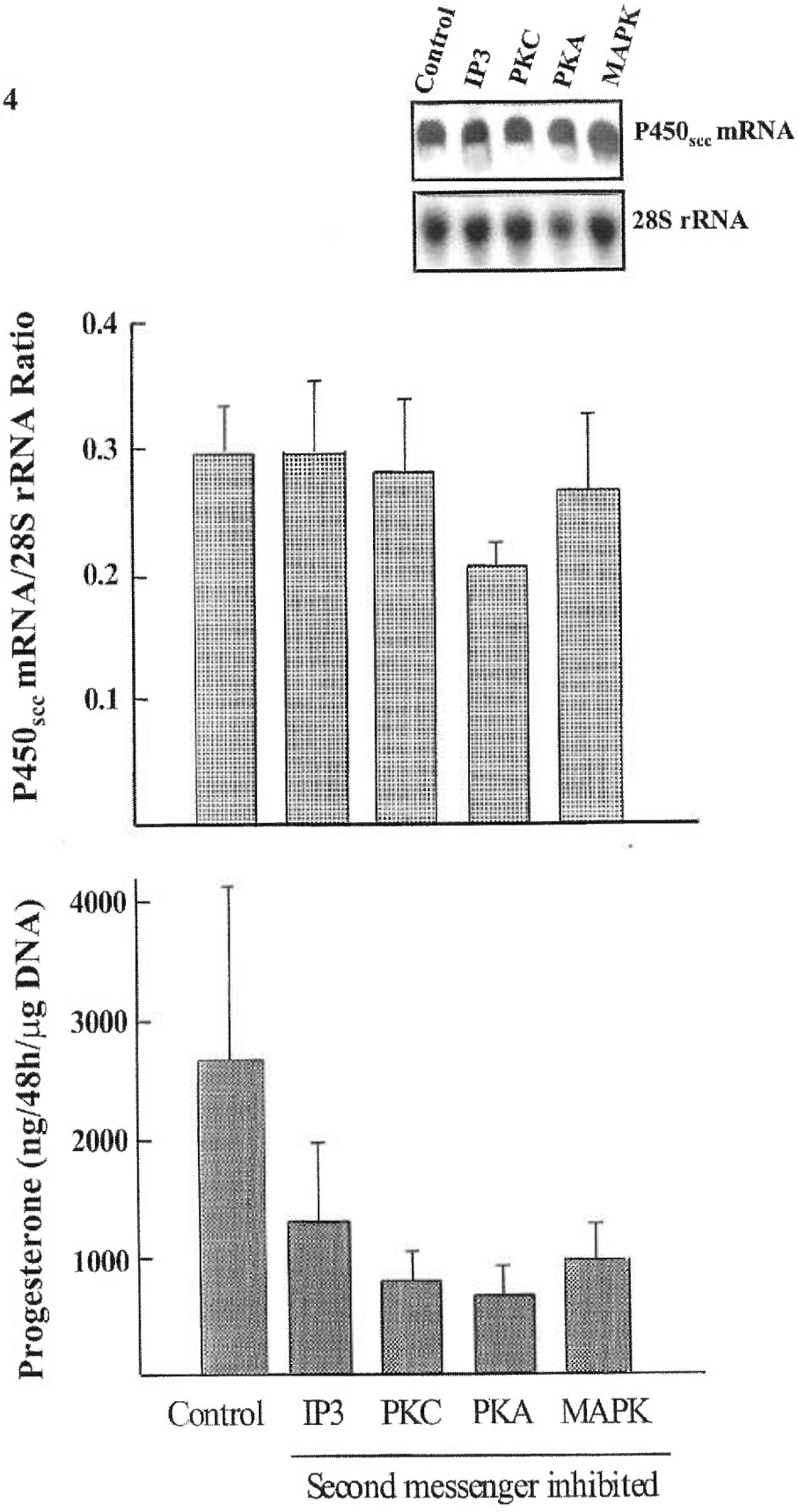

Control
IP3
PKC
PKA
MAPK
P450arom mRNA
28S rRNA
P450arom mRNA/28S rRNA Ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
Estradiol (ng/48h/μg DNA)
16
14
12
10
8
6
4
2
*
*
Control
IP3
PKC
PKA
MAPK
Second messenger inhibited

Figure 4

$P450_{scc}$ mRNA abundance and progesterone secretion in FSH-stimulated bovine granulosa cells. Cells were cultured for 6 days with FSH (1ng/ml), insulin (10 ng/ml) and IGF-1 (10 ng/ml). At day 4 of culture specific inhibitors were added to the cultures. The treatments were: Control, DMSO, and the inhibitors for PI3K, PKC, PKA and MEK. Values are least-squares means ±SEM, and were obtained from 3 replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and progesterone is expressed relative to total cell DNA. The upper panel shows a representative Northern blot.

FIGURE 4


Control
IP3
PKC
PKA
MAPK
P450scc mRNA
28S rRNA
P450scc mRNA/28S rRNA Ratio
0.4
0.3
0.2
0.1
Progesterone (ng/48h/μg DNA)
4000
3000
2000
1000
Control
IP3
PKC
PKA
MAPK
Second messenger inhibited

GENERAL DISCUSSION

Ovarian follicular development involves a series of events through which granulosa cells differentiate with an enhanced capacity to produce estradiol (Richards, 1994). Estradiol is the principal steroid secreted by the follicle before luteinization occurs (Bigelow & Fortune, 1998). In cattle, once luteinization is induced by the LH surge, the estradiol secreting granulosa phenotype shifts to a progesterone secreting granulosa phenotype (Voss *et al.*, 1993b). In vitro, bovine granulosa cells undergo spontaneous luteinization, characterized by a decrease in estradiol and an increase in progesterone secretion, and loss of response to FSH (Henderson *et al.*, 1979; Spicer *et al.*, 1993). The recent development of a long term serum-free cell culture system in which estradiol secretion can be maintained and induced in response to FSH (Gutiérrez *et al.*, 1997b) provides a valuable model for the study of granulosa cell differentiation, before the luteinization process takes place (Smith *et al.*, 1994). Our adaptation of this culture system, provides the first information concerning the hormonal regulation of $P450_{arom}$ mRNA in granulosa cells in cattle. This study shows that in cattle, $P450_{arom}$ mRNA abundance can be induced and maintained in FSH- and insulin-stimulated cells using two different intracellular pathways, whereas estradiol secretion was maintained by a common intracellular pathway.

This is the first report showing that $P450_{arom}$ mRNA levels can be induced and maintained by physiological tropic hormone concentrations, whereas previous laboratories have failed to detect $P450_{arom}$ mRNA transcripts in bovine granulosa cells after 24 h of culture (Voss *et al.*, 1993b). In the present study $P450_{arom}$ mRNA abundance was increased in granulosa cells from small follicles cultured for 48h, the time required to induce $P450_{arom}$ mRNA in immature rat granulosa cells (Fitzpatrick *et al.*, 1991). In vivo, 48 h after follicular recruitment is the time required to detect $P450_{arom}$ mRNA levels in bovine granulosa cells (Bao *et al.*, 1997a). Furthermore, bovine granulosa cells responded to low physiological FSH concentrations in terms of $P450_{arom}$ mRNA abundance, as it has been shown for estradiol production (Saumande, 1991; Berndtson *et al.*, 1995a; Gutiérrez *et al.*, 1997b; Rouillier *et al.*, 1996). Thus the present cell culture system is a good model to study the various aspects of bovine granulosa cell differentiation. The stimulatory effect of FSH on $P450_{arom}$ mRNA was mediated through the PKA pathway, as the inhibition of this pathway, significantly decreased $P450_{arom}$ mRNA abundance, which is in agreement with previous studies on rat granulosa cells (Fitzpatrick *et al.*, 1997). An interesting finding is that insulin, at physiological concentrations, is necessary to induce $P450_{arom}$ mRNA abundance in the presence of FSH. In this study, FSH alone was unable to maintain $P450_{arom}$ mRNA abundance, this is in contrast with studies showing that FSH alone induced $P450_{arom}$ mRNA abundance in human (Steinkampf *et al.*, 1987) and rat (Fitzpatrick *et al.*, 1991) granulosa cells, suggesting the existence of species differences in the control of

$P450_{arom}$ gene transcription in granulosa cells. The fact that FSH alone could not stimulate $P450_{arom}$ mRNA is very interesting. In cattle, one of the principal problems in the embryo transfer industry is the high variability in the response to the treatments for superovulation. Superovulation increases the number recruited follicles (Soumano et al., 1996), and recruitment has been associated with the initiation of expression of $P450_{arom}$ mRNA (Bao et al., 1997a). Because in the present study, FSH alone could not induce and maintain $P450_{arom}$ mRNA abundance, in vivo, it is possible that superovulation with FSH is not enough to induce $P450_{arom}$ mRNA abundance and to increase the number of recruited follicles, thus it can be explain in part the variability in the response to FSH preparations used in superovulation in cattle. Estradiol secretion was maintained with FSH alone, and in the absence of detectable $P450_{arom}$ mRNA transcripts, through an intracellular mechanism mediated at least in part by PI3K and PKC, but not PKA pathway. It has already been shown that FSH acts through tyrosine kinase signalling to stimulate estradiol production in luteinized human granulosa cells (Costrici *et al.*, 1995). Alternatively, the half life of the $P450_{arom}$ enzyme may be longer than the corresponding mRNA, or intermediate components of estradiol secretion may be affected, such as 17β-HSD (Ghersevich *et al.*, 1994). However this remain to be elucidated.

A second intracellular mechanism by insulin was used to induce $P450_{arom}$ mRNA was used, as inhibition of IP3K and PKC, inhibited mRNA and estradiol production. This is consistent with previous studies in which estradiol secretion

was induced by cAMP/PKA-independent mechanisms by IGF-I in humans (Costrici *et al.*, 1994) and by TGFα in pigs (Gangrade *et al.*, 1991). If insulin acts through its own receptor to increase of $P450_{arom}$ mRNA abundance is unknown. Insulin at this concentration (100 ng/ml) acts through IGF-I receptors (Kahn *et al.*, 1981). However, the present study showed that IGF-I, contrary to insulin was not as effective as insulin at (10 ng/ml) to maintain $P450_{arom}$ mRNA abundance in the presence of FSH. This is in agreement with previous studies showing that insulin is a better stimulator of estradiol secretion that IGF-I in cultured bovine granulosa cells (Spicer *et al.*, 1993; Spicer *et al.*, 1994b). Our findings question the role of IGF-I in stimulation of estradiol secretion in cattle, although IGF-I is more abundant in healthy estrogenic large follicles than in small or atretic follicles in vivo (Echternkamp *et al.*, 1994; de la Sota *et al.*, 1996), and IGF-I in vitro, enhanced the stimulatory effect of FSH to induce estradiol secretion in rats (Adashi, 1998).

Of particular importance was the effect produced by the inhibition of MEK, in both FSH and insulin-stimulated cells. In both, $P450_{arom}$ mRNA abundance was increased above control levels, suggesting that there are inhibitors of $P450_{arom}$ mRNA, which are stimulated by the activation of MAPKs. The nature of such factors remains to be elucidated.

The results of this thesis, provide the first information concerning the regulation of $P450_{arom}$ gene expression in granulosa cells in cattle. This information is of valuable importance in the field of ovarian physiology, and can

increase our understanding of the complex mechanisms of follicular development in domestic ruminants, and lead to improved tools for the regulation of fertility in these species.

Future directions

Several questions remain with the results of the present study. It would be interesting to know the half life of the $P450_{arom}$ mRNA and protein, to understand better the effects of FSH on mRNA and protein stability, as it was observed in the present study, the presence of FSH alone could maintain estradiol secretion in the absence of $P450_{arom}$ mRNA levels, suggesting that the half live of the $P450_{arom}$ protein is longer than $P450_{arom}$ mRNA. Hypothesis that remains to be elucidated. Another interesting direction is to address the importance of FSH and insulin in the expression and maintenance of intermediary elements of estradiol secretion, such as 17β-HSD, to evaluate the true rate-limiting step for estradiol biosynthesis in granulosa cells.

The observation that the inhibition of IP3K and PKC in insulin-stimulated cells decrease $P450_{arom}$ mRNA abundance, is limiting by the sometimes non-specific nature of these compounds. Further studies should investigate the effect of specific activators of PKC (phorbol esters) and IP3K on $P450_{arom}$ mRNA and estradiol secretion. The measurements of IP3K and PKC activities, or calcium release and its correlation with $P450_{arom}$ mRNA and estradiol secretion will be of

interest to understand their precise and specific role in granulosa cell estradiol synthesis.

Finally, further studies are necessary to understand the role of MAPKs in the regulation of $P450_{arom}$ mRNA gene expression by the fact that in the present study, the inhibition of such kinases increased $P450_{arom}$ mRNA levels. It is possilble that they action on $P450_{arom}$ mRNA is specific, however, a nonspecific effect, for example on proteins which have a negative effect on $P450_{arom}$ mRNA They specific action on $P450_{arom}$ mRNA remains to be elucidated.

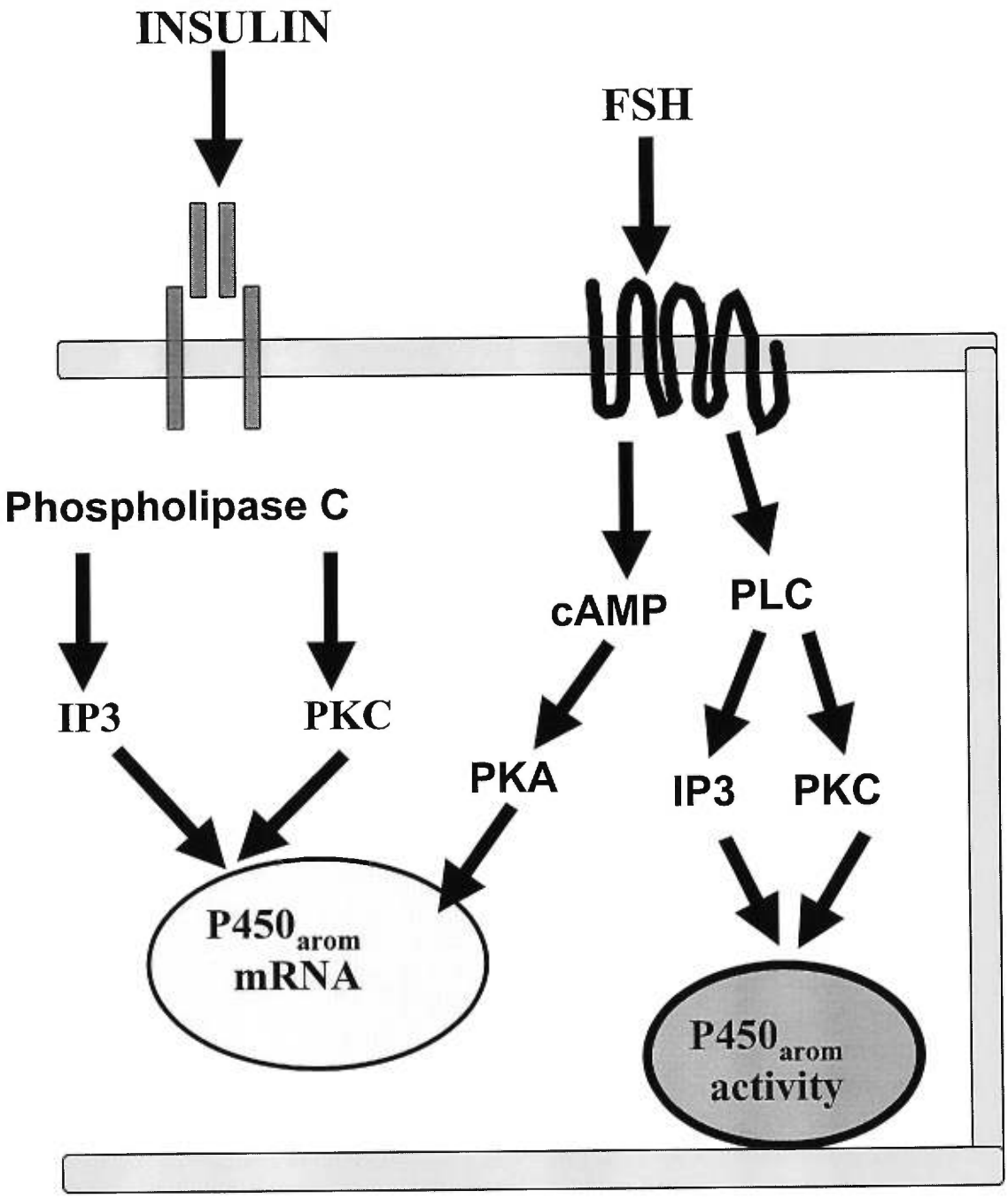


Figure 3. Regulation of $P450_{arom}$mRNA and estradiol secretion in bovine granulosa cells.

GENERAL CONCLUSION

The present work describes the first studies on the expression of $P450_{arom}$ mRNA in cultured bovine granulosa cells. Our results show that in cattle $P450_{arom}$ mRNA abundance can be induced by FSH and insulin at physiological concentrations (FSH-stimulated cells), or by insulin alone at higher concentrations (100 ng/ml; insulin-stimulated cells). In FSH-stimulated cells $P450_{arom}$ mRNA abundance is incrased through the classic cAMP/PKA pathway, whereas in insulin-stimulated cells $P450_{arom}$ mRNA is induced through a phospholipase C-mediated pathway dependent upon activation of IP3K and PKC. In both FSH and insulin-stimulated-cells, $P450_{arom}$ mRNA is under repressive regulation by MAPKs. Contrary to $P450_{arom}$ mRNA, the control of estradiol secretion is also mediated via a IP3K and PKC intracellular pathway (Figure 3).

We accept the hypothesis that bovine granulosa cells can be stimulated in vitro to express $P450_{arom}$ mRNA and maintain estradiol secretion in response to physiological FSH and insulin concentrations, and that $P450_{arom}$ mRNA and estradiol secretion are regulated using different intracellular mechanisms.

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