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Molecular control of prostaglandin G/H synthase-2 expression in bovine preovulatory follicles

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SOMMAIRE

Les prostaglandines jouent un rôle important dans l'ovulation chez les mammifères. La prostaglandine G/H synthéthase (PGHS), dont deux isoformes, la PGHS-1 et la PGHS-2, ont été caractérisées, est la première enzyme limitante de la biosynthèse des prostaglandines à partir de l'acide arachidonique. L'induction sélective de la PGHS-2 par la gonadotrophine chorionique humaine (hCG) dans les follicules pré-ovulatoires a été démontrée chez plusieurs espèces. L'induction de la PGHS-2 survient rapidement chez le rat, soit 2 à 4 h après un traitement à la hCG, mais est tardive chez la vache (18 h post-hCG) et la jument (30-33 h post-hCG). Il est intéressant de noter que l'intervalle entre l'induction de la PGHS-2 et l'ovulation est similaire d'une espèce à l'autre, soit environ 10 heures. Il semblerait donc que la commande de l'induction de la PGHS-2 soit spécifique et détermine la chronologie ovulatoire chez les mammifères. Cependant on ne connaît pas les différences au regard de l'expression de la PGHS-2 chez les espèces à ovulation rapide par opposition aux espèces à ovulation tardive. L'objectif général de mon projet était de caractériser la régulation de la PGHS-2 dans les follicules pré-ovulatoires bovins.

Le premier sous-objectif était de déterminer si l'induction de la PGHS-2 dans les follicules pré-ovulatoires bovins est un événement physiologique déclenché par un pic endogène de l'hormone lutéinisante (LH). Nos résultats ont démontré que le transcrit PGHS-2 (4,0 kilobases) et la protéine (74 000 M_r)

étaient respectivement absent, présent en faible quantité et présents en grande quantité chez les follicules pré-ovulatoires isolés en début d'oestrus, 18 h après le début de l'oestrus (16.0 ± 1.2 h après le pic de LH) et 24 h après le début de l'oestrus (20 ± 0 h après le pic de LH). Les analyses de type Western et immunohistochimiques ont révélé que la protéine PGHS-2 était induite de manière sélective dans les cellules de la granulosa et que l'immunoréactivité à la PGHS-2 était plus prononcée autour du noyau des cellules de la granulosa. Les concentrations de PGE_2 et de $PGF_{2\alpha}$ dans le liquide folliculaire ont augmenté de manière significative ($p < 0,01$) entre 0 et 24 h après le début de l'oestrus, soit respectivement de 2.8 ± 0.2 à 87.9 ± 30.9 ng/ml et de 0,05 ± 0.02 à 68.9 ± 23.6 ng/ml. Cette étude a donc démontré que l'induction de la PGHS-2 dans les follicules pré-ovulatoires bovins est un événement physiologique qui survient après le pic endogène de LH.

Le deuxième sous-objectif de ce projet était de caractériser l'expression de la PGHS-2 dans les follicules bovins pendant la surovulation et de voir si elle correspondait à des changements dans la stéroïdogenèse folliculaire et la production de prostaglandines et à certaines caractéristiques morphologiques du complexe cumulus-oocyte. La PGHS-2 n'a été détectée dans aucun des follicules isolés 0 h post-hCG (n = 119) ou des petits follicules (< 8 mm) isolés entre 0 et 24 h post-hCG (n = 27 follicules). Cependant, à 18 h post-hCG, 12.3% des follicules moyens (8 à 10 mm) et 43.7% des gros follicules (≥ 10 mm) étaient positifs pour la PGHS-2. À 24 h, ces pourcentages ont respectivement atteint 45.9% et 91.0% chez les follicules moyens et les gros

follicules ($p < 0,05$). Les concentrations de PGE_2 et de $PGF_{2\alpha}$ dans le liquide folliculaire étaient faibles chez les follicules isolés à 0 h. Elles ont augmenté seulement chez les follicules PGHS-2 positifs isolés 24 post-hCG ($p < 0.05$). Les concentrations de progestérone et d'oestradiol-17ß à 0 h étaient respectivement de 28.2 ± 5.8 et 291.8 ± 13.0 ng/ml. Vingt-quatre heures après l'administration d'hCG, nous avons observé un recul de l'oestradiol–17ß au profit d'une dominance par la progestérone (lutéinisation) seulement chez les follicules PGHS-2 positifs. L'expansion du complexe cumulus-oocyte a également été observée 24 h post-hCG seulement chez les follicules PGHS-2 positifs. Par conséquent, chez la vache, l'induction de la PGHS-2 pendant la surovulation est tributaire de la taille du follicule: l'enzyme est exprimée seulement chez les follicules de plus de 8 mm au moment de l'ovulation. L'absence de l'expression de la PGHS-2 dans les follicules supérieurs à 8 mm a été associée à l'absence de lutéinisation folliculaire et d'expansion du cumulus. L'expression de la PGHS-2 pourrait donc être utilisée comme marqueur des follicules destinés à l'ovulation dans un contexte de surovulation. La surovulation constitue une approche intéressante pour isoler de grandes quantités de cellules de la granulosa à des fins d'étude *in vitro* sur la régulation de la PGHS-2.

Le sous-objectif final de mon projet était d'élucider certains des mécanismes moléculaires en jeu dans l'induction tardive de la PGHS-2 dans les follicules bovins avant l'ovulation. Pour ce faire, un modèle *in vitro* de culture primaire de cellules de la granulosa bovines a été élaboré. La chronologie et le

déroulement de l'induction de la PGHS-2 *in vitro* à l'aide de la forskoline étaient similaires à ce qu'on observe *in vivo* à la suite d'un traitement aux gonadotrophines: cela confirme la validité de notre modèle. Des dosages de l'activité promotrice ont été effectués dans des cultures de cellules de granulosa bovines transfectées de manière transitoire avec des constructions du promoteur de la PGHS-2 fusionné à un gène rapporteur, la luciférase. L'analyse des mutants de délétion a révélé que la région située entre –147 et –2 (+1 = site de départ de transcription) joue un rôle central dans l'induction sous dépendance de la forsksoline du promoteur de la PGHS-2 dans les cellules de la granulosa bovines. La mutagenèse dirigée a démontré que l'élément de la boîte E compris dans la région -147/-2 est nécessaire à l'activité promotrice de base ou induite par la forsksoline et qu'une mutation de l'élément C/EBP provoque une diminution de 29% de l'activité promotrice. Les analyses de retard de migration sur gel (EMSA) à l'aide du fragment –147/-2 du promoteur et des extraits nucléaires de cellules de la granulosa isolés 0, 18 ou 20 h post-hCG a révélé la présence de nombreux complexes qui interagissaient avec des éléments de la boîte E et du C/EBP. Les " supershifts EMSA " et les analyses de type Western ont révélé que les facteurs de transcription USF-1 et USF-2 interagissaient avec la boîte E alors que les facteurs C/EBPα et C/EBPß se liaient à l'élément C/EBP. Il est intéressant de noter que l'isoforme USF-2 tronqué à son extrémité amino terminale et donc dépourvue de son domaine de transactivation a été détectée dans les extraits nucléaires de cellules de granulosa isolés 0 h post-hCG. Par contre, elle n'était pas présente dans les

extraits isolés 18 ou 20 h post-hCG. Ces nouvelles observations nous permettent de postuler que la présence de l'USF tronqué jouerait un rôle dans la répression de l'expression de la PGHS-2 chez les espèces où le processus ovulatoire est long.

Globalement, les études réalisées au cours de mon projet démontrent que l'induction de la PGHS-2 dans les folliculaires pré-ovulatoires est un événement physiologique durant le cycle oestral et tributaire de la taille du follicule dans le contexte d'une surovulation. L'identification d'une nouvelle interaction protéine/ADN dans le promoteur proximal PGHS-2 bovin permet de postuler une nouvelle hypothèse de travail en ce qui a trait à la commande moléculaire du retard de l'induction de la PGHS-2 chez les espèces qui présentent un long processus ovulatoire. Le rôle potentiel de répresseur de la protéine USF tronquée dans l'activité du gène de la PGHS-2 nécessitera d'autres études.

SUMMARY

Prostaglandins play an important role in the ovulatory process in mammals. Prostaglandin G/H synthase (PGHS) is the first rate-limiting enzyme in the biosynthesis of prostaglandins from arachidonic acid, and two isoforms of PGHS, PGHS-1 and PGHS-2, have been characterized. The selective induction of PGHS-2 by human chorionic gonadotropin (hCG) in preovulatory follicles has been demonstrated in several species. The induction of PGHS-2 is rapid in rats (2-4 h post-hCG), but is delayed in cows (18 h post-hCG) and mares (30-33 h post-hCG). Interestingly, the time interval from PGHS-2 induction to ovulation appears constant across species (about 10 h), suggesting that the species-specific control of PGHS-2 induction serves as a determinant of the mammalian ovulatory clock. However, the molecular control of PGHS-2 expression in species with a short *versus* a long ovulatory process is unknown. The general objective of my project was to characterize the regulation of PGHS-2 in bovine preovulatory follicles.

The first specific goal of my project was to establish whether the induction of PGHS-2 in bovine preovulatory follicles was a physiological event triggered by the endogenous surge of luteinizing hormone (LH). Results showed that PGHS-2 transcript (4.0 kilobases) and protein (74,000 M_r) were absent in preovulatory follicles isolated at the onset of estrus, low in follicles obtained 18 h after the onset of estrus (16.0 ± 1.2 h post-LH surge), and markedly induced 24 h after onset of the estrus (20 ± 0 h post-LH surge). Immunoblot and immunohistochemical analyses revealed that PGHS-2 protein

was selectively induced in granulosa cells, and that PGHS-2 immunoreactivity was more pronounced around the granulosa cell nuclei. Follicular fluid concentrations of PGE_2 and $PGF_{2\alpha}$ increased significantly ($p < 0.01$) between 0 and 24 h after the onset of estrus (from 2.8 ± 0.2 to 87.9 ± 30.9 ng/ml for PGE_2; from 0.05 ± 0.02 to 68.9 ± 23.6 ng/ml for $PGF_{2\alpha}$, respectively). Thus, this study demonstrated that induction of PGHS-2 in bovine preovulatory follicles is a physiological event that occurs after the endogenous LH surge.

The second specific goal of my project was to characterize the expression of PGHS-2 in bovine follicles during superovulation, and relate it to changes in follicular steroidogenesis, prostaglandin production, and cumulus oocyte complex morphology. Results showed that PGHS-2 was not detected in any follicles isolated at 0 h post-hCG (n = 119) or in small follicles (< 8 mm) isolated between 0 and 24 post-hCG (n = 27 follicles). However, 12.3% of medium (8 to < 10 mm) and 43.7% of large (≥ 10 mm) follicles were PGHS-2 positive at 18 h post-hCG, and these percentages rose at 24 h to 45.9% and 91.0% in medium and large follicles, respectively ($p < 0.05$). Follicular fluid concentrations of PGE_2 and $PGF_{2\alpha}$ were low in follicles isolated at 0 h, and increased only in PGHS-2 positive follicles isolated 24 h post-hCG ($p < 0.05$). Concentrations of progesterone and estradiol-17β at 0 h were 28.2 ± 5.8 and 291.8 ± 13.0 ng/ml, respectively, and a shift from estradiol-17β to progesterone dominance (luteinization) occurred at 24 h post-hCG only in PGHS-2 positive follicles. Expansion of the cumulus oocyte complex was also detected at 24 h post-hCG only in PGHS-2 positive follicles. Therefore, induction of PGHS-2

during superovulation in cows is follicle size-dependent, with the enzyme being expressed only in follicle of ovulatory size (> 8 mm). Lack of PGHS-2 expression in follicles greater than 8 mm was associated with an absence of follicular luteinization and cumulus expansion. Thus, it is proposed that PGHS-2 expression can be used as a marker for follicular commitment to ovulation during superovulatory treatment. Also, superovulation offers a valuable approach to isolate large amounts of granulosa cells for *in vitro* studies of PGHS-2 regulation.

The final goal of my project was to elucidate some of the molecular mechanisms involved in the delayed induction of PGHS-2 in bovine follicles prior to ovulation. To achieve this goal, an *in vitro* model of primary cultures of bovine granulosa cells was established. The time-course induction of PGHS-2 *in vitro* by forskolin paralleled that observed *in vivo* following gonadotropin treatment, indicating the suitability of the *in vitro* model. Promoter activity assays were performed using cultures of bovine granulosa cells transiently transfected with PGHS-2 promoter constructs fused into a luciferase reporter vector. Deletion mutant analyses revealed that the promoter region between –147 and –2 (+1 = transcription start site) plays a central role in forskolin-dependent induction of the PGHS-2 promoter in bovine granulosa cells. Site-directed mutagenesis showed that an E-box element within –147/-2 is absolutely required for basal and forskolin-induced promoter activity, and that disruption of the C/EBP element decreased promoter activity by 29%. Electrophoretic mobility shift assays (EMSA), using the -147/-2 promoter

fragment and nuclear extracts of granulosa cells isolated at 0, 18, and 20 h post-hCG, revealed the present of multiple complexes interacting with E-box and C/EBP elements. Supershift EMSAs and Western blots indicated that upstream stimulatory factor-1 (USF-1) and USF-2 interacted with the E-box; whereas C/EBPα and C/EBPβ bound to the C/EBP element. Interestingly, a putative N-terminus truncated USF-2 isoform, which is believed to lack the transactivation domain, was detected in the nuclear extracts of granulosa cells isolated 0 h post-hCG, but not present at 18 and 20 h post-hCG. This novel finding allows us to hypothesize that the presence of N-terminus truncated USF is involved in the repression of PGHS-2 expression in species with a long ovulatory process.

Collectively, studies from my project demonstrate that induction of PGHS-2 in preovulatory follicles is a physiological event, and occurs in a follicle size-dependent manner during superovulatory treatment. The identification of novel protein/DNA interactions in the bovine proximal PGHS-2 promoter provides a new and interesting working hypothesis regarding the molecular control of delayed PGHS-2 induction in species with a long ovulatory process. The putative repressor role of N-terminus truncated USF proteins in the activation of PGHS-2 gene requires further investigation.

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ABBREVIATIONS

AA	amino acid
AP-1/-2	acute protein-1/-2
ATF	activation transcriptional factor
cAMP	cyclic 3',5'-adenosine monosphate
cDNA	complementary deoxyribonucleic acid
C/EBP	CAAT enchancer binding protein
CL	corpus luteum
COX	cyclooxygenase
COC	cumulus oocyte complex
CREB	cAMP response element-binding protein
CRE	cAMP response element
DNA	deoxyribonucleic acid
E-box	CACGTG sequence or c-Myc binding site
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
EP	prostaglandin E receptor
FCS	fetal calf serum
FP	prostaglandin F receptor
FSK	forskolin
FSH	follicle-stimulating hormone
GRE	glucocorticoid ressponse element

h	hour
hCG	human chorionic gonadotropn
HETE	hydroxy eicosatetraenoic acid
IL	interleukin
IgG	immunoglobin G
kb	kilobase
kDa	kilodalton
LH	luteinizine hormone
LUC	luciferase
M	mole
mRNA	messenger ribonucleic acid
NF-IL6	nuclear factor of IL-6
NF-kB	nuclear factor kB
ng	nanogram
NSAIDs	non-steroidal anti-inflammatory drugs
OVX	ovariectomy
PEA-3	polyoma enchancer A
pg	picogram
PGE	prostaglandin E
PGF2α	prostaglandin F2 alpha
PGFS	prostaglandin F synthase
PGHS	prostaglandin G/H synthase
PGI	prostaglandin I

PGT	prostaglandin transporter
PLA2	phospholipase A2
PMA	phorbol 12-myristate 13-acetate
PKA	protein kinase A
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
RT-PCR	reverse transcriptase polymerase chain reaction
SP-1	stimulated protein-1
TATA box	TATAAA sequence
TXA2	thromboxane A2
μg	microgram
μl	microliter
USF	upstream stimulation factor
UTR	untranslated region
XRE	xenobiotic response element

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1. INTRODUCTION

Mammalian ovulation is a complex process, and includes a series of biochemical and morphological changes that are triggered by the surge of luteinizing hormone (LH) (LeMaire, 1989). One of the most dramatic changes associated with ovulation is the rapid increase of prostaglandins in preovulatory follicles. The regulation of prostaglandin synthesis during ovulation has been extensively studied since 1972 (Murdoch et al., 1993). The precise mechanisms of prostaglandin actions during mammalian ovulation are still not fully understood.

Prostaglandins are well known as potent mediators of various biological processes, such as reproductive functions (ovulation, implantation, luteolysis and parturition), inflammatory reaction, immune response, glomerular filtration and bone development (DeWitt and Smith, 1995; Herschman, 1996; Williams and DuBois, 1996). Prostaglandin G/H synthase (PGHS, also known as cyclooxygenase, COX) is the first rate-limiting enzyme in the biosynthetic pathway of all prostanoids from arachidonic acid. There are two PGHS isoforms, called PGHS-1 and PGHS-2 (or COX-1 and COX-2), which are encoded by separated genes (Williams and DuBois, 1996). The two isoforms share common structural features, and have very similar kinetic properties (Herschman, 1994; Williams and DuBois, 1996). Both enzymes are the target of nonsteroidal anti-inflammatory drugs (NSAIDS), and inhibitors specific for each isoform have been developed (Kalgutkar et al., 1998).

PGHS-1 is generally considered as a constitutive enzyme; prostagandins produced from PGHS-1 are thought to be involved in maintaining the homeostasis of physiological responses, the so-called housekeeping functions. In contrast to PGHS-1, PGHS-2 is an inducible enzyme, and is normally absent from tissues or cells, but can be induced in different types of

cells by a variety of agonists, including growth factors, tumor promoters, cytokines and hormones (Williams and DuBois, 1996). It is believed that PGHS-2 synthesizes prostaglandins on the nuclear envelop and these products function at the level of the nucleus during cell replication or differentiation (DeWitt and Smith, 1995).

The discovery of the inducible PGHS isoform (PGHS-2) led to re-evaluate the roles and regulation of prostaglandins in follicles prior to ovulation. Induction of PGHS-2 by human chorionic gonadotropin (hCG) in preovulatory follicles has been examined in rats (Sirois et al., 1992), cattle (Sirois, 1994; Tsai et al., 1996) and horses (Sirois and Dore, 1997; Boerboom and Sirois, 1998). Results have shown that induction of PGHS-2 in preovulatory follicles is granulosa cell-specific, and time- and gonadotropin-dependent (Sirois et al., 1992; Sirois, 1994; Tsai et al., 1996). A striking difference between species is that expression of PGHS-2 is delayed in animals with a long ovulatory process. However, the time interval from induction to ovulation appears to be constant (10 h) (Sirois and Dore, 1997). Richards (1997) proposed that PGHS-2 could function as an alarm to sound the mammalian ovulatory clock. However, how the induction of PGHS-2 is precisely timed in different species with a short *versus* a long ovulatory process is largely unknown. In contrast to PGHS-2, expression of PGHS-1 in follicular wall does not change throughout the process of ovulation, suggesting that the increased prostaglandin levels in follicular fluid are due to the induction of PGHS-2.

Gene targeting studies have revealed that mice lacking the PGHS-2 gene are infertile because of ovulatory failure and other impaired reproductive processes (Dinchuk et al., 1995; Lim et al., 1997). These results further support the concept that prostaglandins, at least in mice, are essential for a normal ovulation. However, the precise prostaglandin subtype involved in the

process of follicular rupture remains unclear. It seems that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is not involved in the ovulation because mice lacking PGF receptor gene have normal ovulation and fertilization functions (Sugimoto et al., 1997). Mice lacking each of the four prostaglandin E receptor (EP) subtypes have been generated recently (Nguyen et al., 1997; Ushikubi et al., 1998). Results showed that subtypes EP_1-, EP_2- and EP_3-deficient mice were fertile (Ushikubi et al., 1998), whereas the fertility of EP_4-deficient mice needs to be examined (Nguyen et al., 1997). Studies on adipocyte differentiation revealed that prostaglandin J_2 (PGJ_2) and its derivatives can function as efficacious activators of peroxisome proliferator activated receptors (PPARs) (Kliewer et al., 1995; Lehmann et al., 1995). PPAR are members of the nuclear receptor subfamily of ligand-activated transcription factors (Kliewer et al., 1995). To date, three mammalian PPAR subtypes, PPARα, β and γ have been identified. Two isoforms of PPAR γ cDNA have been isolated and partially characterized in cattle, and PPARγ was shown to present in bovine ovary (Sundvold et al., 1997). Whether or not PPARs are regulated in preovulatory follicles remains unknown.

There is no information on the regulation of PGHS-2 in follicles during superovulation in cows, but prostaglandin levels were shown to be increased in the follicular fluids (Downs and Longo, 1982; Algire et al., 1992). Superovulation is an important procedure to increase the number of offspring of genetically valuable cows. However, variability in the number of ovulations and embryos is a major drawback in the application of commercial embryo transfer in cattle (Moor et al., 1984; Armstrong, 1993). One adverse effect of current superovulation treatment is the development of a high proportion (up to 20%) of follicles greater than 8 mm that fail to ovulate (Laurincik et al., 1993;

Pruwantara et al., 1994). The reason for the lack of ovulation in these ovulatory size follicles are not clear.

The molecular mechanisms involved in the regulation of PGHS-2 have drawn much attention in recent years. In particular, the *trans*-activation of PGHS-2 gene has been extensively studied in different cell systems. The promoter of the PGHS-2 gene contains various consensus *cis*-acting regulatory elements, including, CAAT/enchancer binding protein (C/EBP), nuclear factor κ B (NFκB), polyoma enchancer (PEA-3), acute protein-1 (AP-1), AP-2, stimulated protein-1 (SP-1), GATA box, cAMP response element (CRE), c-Myc binding site or E-box, and the xenobiotic response element (XRE) (Fletcher et al., 1992; Sirois and Richards, 1993; Xie et al., 1994; Inoue et al., 1995; Xie and Herschman, 1995; Morris and Richards, 1996; Williams and DuBois, 1996). Several *cis*-elements, including the binding site for NFκB, CAAT box (C/EBP), ATF/CRE and E-box, were shown to be functionally important for the activation of PGHS-2 promoter in various cell types (Sirois and Richards, 1993; Xie et al., 1994; Inoue et al., 1995; Xie and Herschman, 1995; Morris and Richards, 1996).

Studies in rats have revealed that the E-box is essential for the basal and forskolin-induced activities of the PGHS-2 promoter in granulosa cells (Morris and Richards, 1996). The upstream stimulatory factor (USF) was shown to bind to the E-box region, but the binding did not change before and after hCG (Morris and Richards, 1996). Furthermore, the level of USF protein was not regulated in whole extracts of granulosa cells of preovulatory follicles isolated before or after hCG treatment (Morris and Richards, 1996). Therefore, the precise role of USF in the regulation of PGHS-2 in granulosa cells of preovulatory follicles needs further study. It is known that USF has two isoforms and interacts with its target DNA as a dimer. Differential dimerizations

(homo- or heterodimer) binding to target DNA could have inverse functions (repressor or activator) (Ghosh et al., 1997). Therefore, it is possible that differential dimerizations of USF proteins is involved in the transcriptional regulation of PGHS-2 gene in rat granulosa cells in response to the LH surge.

Based on above considerations, the general objective of my project was to study the induction of PGHS-2 in bovine follicles under physiological conditions and during superovulatory treatment, and to establish an *in vitro* model to explore the molecular mechanisms involved in the delayed *trans*-activation of the PGHS-2 gene in this species.

2. LITERATURE REVIEW

2.1. Characteristics of bovine follicular development

2.1.1. Follicular dynamics

The number of primordial follicles in mammalian ovaries is fixed near the time of birth (Erickson, 1966). In cattle, there are about 150,000 primordial follicles present in the ovary at birth, and the number of follicles decreases to 3,000 by 15-20 years of age (Erickson, 1966). Primordial follicles continuously enter the growing pool of follicles in the ovaries (Peters et al., 1975), and more than 99% of the follicles in the growing pool undergoes atresia (Ireland and Roche, 1983). The factors that initiate the growth of resting primordial follicles remain unknown. However, it has been known that the growth of follicles is controlled by gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Richards, 1994).

The further development of growing follicles in cattle is characterized by a continuous turnover in follicular waves, each of which involves a sequential process of follicle recruitment, selection, and dominance (Figure 1) (Sirois and Fortune, 1988; Ginther et al., 1989; Fortune, 1994). Studies by ultrasonography have shown that two- or three-waves of follicular development are present during each bovine estrous cycle (Sirois and Fortune, 1988; Ginther et al., 1989; Fortune, 1994). Follicular waves begin around Days 2, 9, and 16 of the estrous cycle in heifers with three waves of follicular development, and around Days 2 and 11 in heifers with two waves (Sirois and Fortune, 1988). Each wave of follicular development is characterized by a simultaneous emergence of a group of follicles (5-7 follicles, large than 5 mm), from which one is selected to continue its growth and becomes the dominant follicle, while others (subordinate follicles) undergo atresia. The first dominant follicle

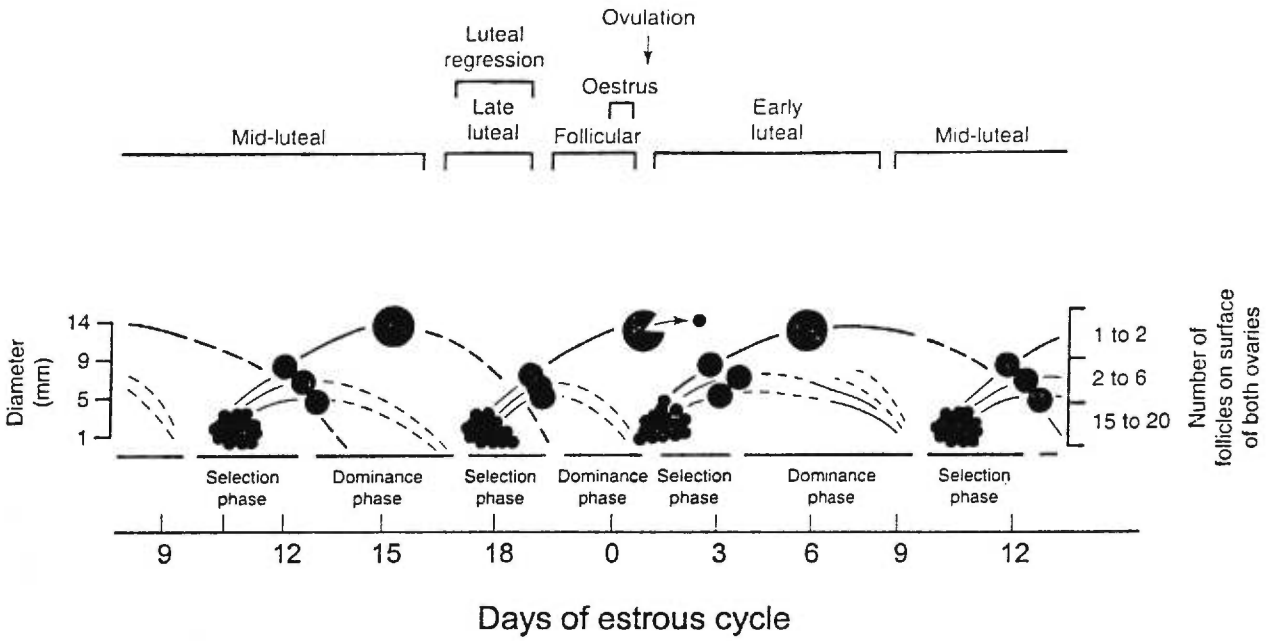

Figure 1. Waves of follicular development during the bovine estrous cycle. Cohorts of follicles arise, and while one is selected to become dominant, the others become atretic. In the early and mid-luteal phase, the dominant follicle also regresses, but in the cohort that develops in the late luteal phase, the dominant follicle ovulates (Modified from Roche, 1996).

normally reaches a maximum size of 10-15 mm in diameter and remains dominant for 3-6 days until it becomes atretic and regresses. Then, this dominant follicle is replaced by another dominant follicle grown from the subsequent wave of follicles. If luteal regression occurs during the growth phase or early period of dominance, the dominant follicle will continue to develop to preovulatory size (up to 20 mm) and will eventually ovulate in response to the LH surge (Fortune, 1994).

Mechanisms controlling the turnover of follicular waves are very complex. Follicle-stimulating hormone is the major regulator of emergence of a follicular wave (Fortune, 1994; Ginther et al., 1996). In cattle, not only does a secondary surge of FSH on the day of ovulation precede the first follicular wave of the cycle, but also slight elevations in FSH have been shown to precede the second and the third follicular waves of the cycle (Fortune, 1994). Factors controlling increasing and decreasing concentrations of circulating FSH during a FSH surge have not been defined (Ginther et al., 1996). However, abolishing the secondary surge of FSH by injections of follicular fluid containing inhibin prevents the recruitment of the ovulatory cohort of the next cycle in rats (Grady et al., 1982), and delays the first follicular wave of the cycle in cattle (Turzillo and Fortune, 1990), suggesting that the proteinaceous components of follicular fluid have a striking inhibitory effect on FSH and follicle growth. The ability of follicles to suppress FSH is gained when they reach 3-5 mm (Gibbons et al., 1999). Ovarian steroids (progesterone and estradiol) and inhibin were shown to be involved in the regulation of follicular waves (Ireland and Roche, 1983; Campbell et al., 1995). However, a more recent study has demonstrated that the presence of endogenous progesterone does not affect FSH patterns or follicular development, and that the circulating estradiol do not suppress FSH (Gibbons et al., 1999).

2.1.2. Acquisition of gonadotropin receptors

Follicle-stimulating hormone (FSH) and LH are the primary hormones necessary for folliculogenesis. The actions of gonadotropins on follicular growth and development are through specific FSH receptor (FSHr) and LH receptor (LHr) located on the follicular cell surface. In mammals, FSH receptor is exclusively localized in the granulosa cells of follicles; whereas LHr is initially present on theca cells and then induced in the granulosa cells by the actions of FSH and steroids during maturation of the preovulatory follicle (Richards, 1994).

In cattle, expression of FSHr mRNA is first detected in granulosa cells shortly after the time follicles leave the primordial pool (Peters et al., 1975). This is similar to results from studies in rats (Richards et al., 1980; Camp et al., 1991) and human (Yamoto et al., 1992) in which FSHr or its mRNA are present on granulosa cells of small preantral follicles. Although FSH is required for supporting follicular growth beyond the early antral stage, levels of FSH binding and FSHr mRNA do not vary with follicular size (Peters et al., 1975; Ireland and Roche, 1983; Spicer et al., 1986). The expression of LHr in theca cells starts approximately at the time of antrum formation, and the level of LHr mRNA increases with the growth of early antral follicles (Peters et al., 1975; Bao et al., 1997). Luteinizing hormone receptor mRNA is expressed in bovine granulosa cells only after healthy follicles reach 9 mm (Peters et al., 1975). Expression of LHr in granulosa cells may be critical to the establishment and maintenance of follicular dominance.

Regulation of LHr expression in granulosa cells has not been characterized in domestic animals. In rodents, induction of LHr in granulosa cells is dependent on the actions of FSH and estradiol-17β (Richards et al., 1980; Segaloff et al., 1990). Genes encoding FSHr and LHr have been cloned

and partially characterized in rats (Richards, 1994). There are no homologies between promoters of these two genes. This may account for the cell-specific expression of the FSHr in granulosa cells and the LHr in theca cells of developing follicles, as well as for the induction of LHr in granulosa cells by FSH and steroids (Segaloff et al., 1990; Piquette et al., 1991).

2.1.3. Steroidogenesis

All steroid hormones are derived from cholesterol. In ovary, three forms of cytochrome P450 (cholesterol side-chain cleavage enzyme, 17α-hydroxylase and aromatase) and two hydroxysteroid dehydrogenases (3β-HSD and 17β-HSD) are the main enzymes involved in the biosynthesis of various ovarian steroids (Figure 2) (Omura and Morohashi, 1995). The cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) carries out the initial and rate-limiting step in the production of all steroid hormones (Omura and Morohashi, 1995). Recent studies have revealed that the steroidogenic acute regulatory protein (StAR) plays an essential role in steroidogenesis by controlling the transport of cholesterol into mitochondria (Stocco and Clark, 1996). In this regard, StAR is also considered as rate-limiting protein for steroidogenesis (Stocco and Clark, 1996; Stocco, 1997). The regulation of steroidogenic enzymes is primarily controlled through the cAMP-dependent pathway (Sugawara et al., 1997) and, as expected, cAMP-response element (CRE) is present in each P450 gene promoter (Omura and Morohashi, 1995). Several putative *trans*-acting factors have been shown to be involved in the cell- and tissue-specific expression of P450 genes, one of which, known as steroidogenic factor-1 (SF-1), has been well-characterized and is believed to play a central role in the expression of steroidogenic genes (Sugawara et al., 1997).

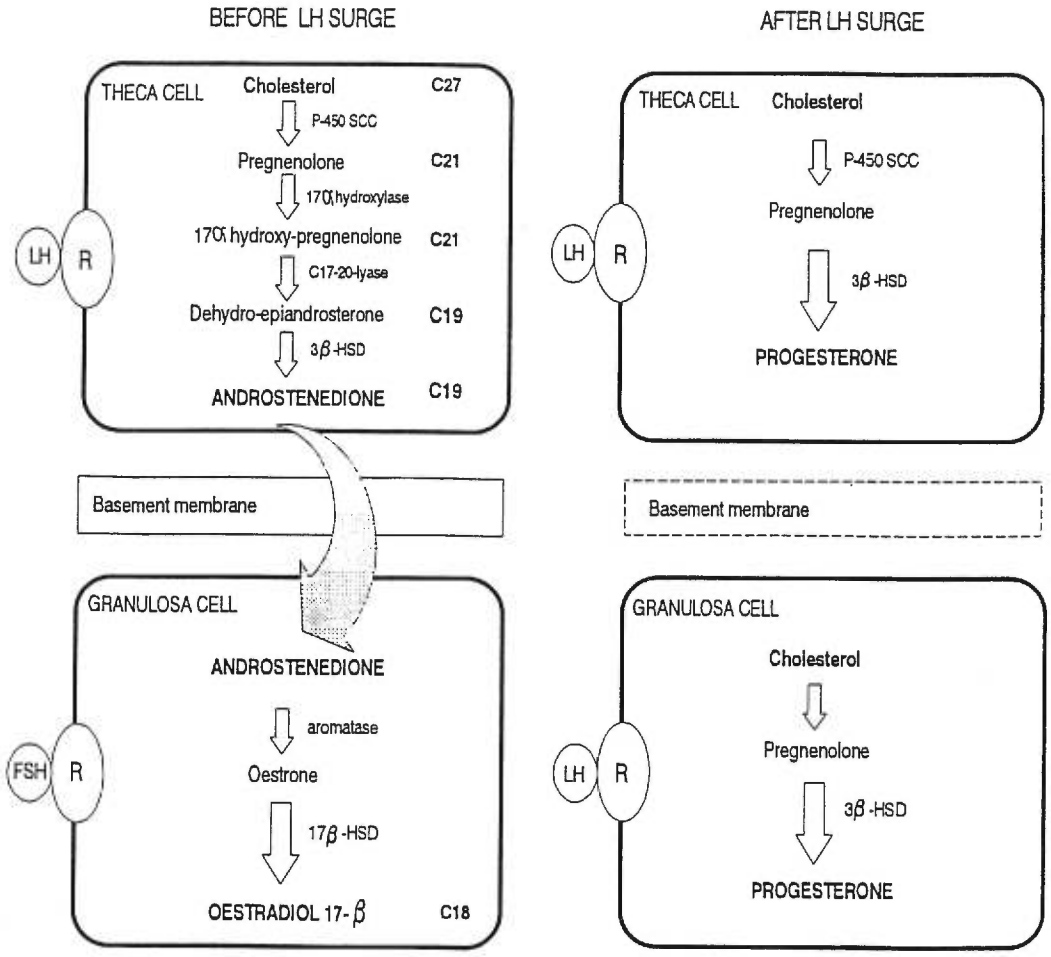

Figure 2. Principal pathways of steroid hormone biosynthesis in bovine ovary. Ovary steroids consist of three major types : estrogens (C-18), androgens (C-19), and progestogens (C-21). Estadiol-17β is the major steroid synthesized in granulosa cells before LH surge, and androstenedione derived from theca cells is the main precursor for estrogens synthesis in bovine follicle. After LH surge, ovarian cells synthesize mainly progesterone (Taken from Dieleman et al., 1993).

The production of steroid hormones in mammalian follicles depends on the availability of specific steroidogenic enzymes (Strauss et al., 1981). In bovine follicles, P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD) are detected in both granulosa and thecal cells (Bao et al., 1997; Bao and Garverick, 1998). P450 17α-hydroxylase (P45017α) and StAR are only localized to thecal cells, whereas P450 aromatase (P450arom) is exclusively localized to granulosa cells. Expression of P45017α in thecal cells, P450arom in granulosa cells, and P450scc and 3β-HSD in theca and granulosa cells decreases dramatically in preovulatory follicles following the LH surge (Voss and Fortune, 1993).

Steroidogenesis in bovine preovulatory follicles supports the two-cell, two-gonadotropin model of follicular estrogen synthesis initially proposed in rats (Hsueh et al., 1984; Erickson et al., 1985). Cholesterol is converted to pregnenolone by P450scc in mitochondria, and then into progesterone, androgens and estrogens by a series of enzymes residing in the endoplasmic reticulum. In theca cells of bovine preovulatory follicles, the conversion of pregnenolone to dehydroepiandrosterone (DHEA) is catalyzed by P45017α and conversion of DHEA to androstenedione by 3β-HSD (Lacroix et al., 1974). Granulosa cells of bovine preovulatory follicles can not synthesize androgens *de novo*, and the androgens are supplied by theca cells and then converted into estradiol by P450arom (Rodgers et al., 1986; Voss and Fortune, 1993). Androgen synthesis in theca cells is regulated through the LHr, StAR, P450scc, P45017α and 3β-HSD (Bao and Garverick, 1998).

2.2. Ovulation

2.2.1. Changes in preovulatory follicles

It is well known that LH, perhaps with some contribution from FSH, is the physiological trigger for the ovulatory process (LeMaire, 1989). The effects of LH on follicular maturation are mainly mediated *via* the protein kinase A pathway (LeMaire, 1989). The changes induced in the preovulatory follicle include luteinization of granulosa cells, resumption of oocyte maturation, synthesis of prostaglandins/leukotrienes, and activation of proteolytic enzymes. Another dramatic change induced by LH is a rapid switch in granulosa cells from the highly proliferative stage to the nonproliferative, terminally differentiated state (Robker and Richards, 1998a). The mechanisms involved in this switch, as well as their control by hormones, have been examined recently in rats (Robker and Richards, 1998a; Robker and Richards, 1998b), and results clearly show that the LH surge regulates acutely cyclin D2 and p27Kip1 in an inverse manner in preovulatory follicles (Robker and Richards, 1998a).

2.2.2. Inflammatory-like changes

The major biochemical events of an inflammatory reaction includes vasodilatation, hyperemia, exudation, edema, collagenolysis, cell proliferation and tissue remodeling (Gallin et al., 1992). The concept that ovulation can be compared with an inflammatory reaction was proposed nearly 20 years ago (Espey, 1980). Since then, several agents commonly associated with the inflammation cascade have been studied in preovulatory follicles. Vasoactive agents such as bradykinin, histamine, and platelet-activating factor (PAF) have been examined in preovulatory follicles and were shown to be regulated by gonadotropins (Espey, 1994).

The arachidonate cascade is initially activated by phospholipases and subsequently by prostaglandin synthases and lipoxygenases during the

inflammatory reaction (Espey, 1980). Similarly, prostaglandins are significantly increased in follicular fluid prior to ovulation, and have been considered as potential mediators of ovulation.

A number of other factors involved in inflammation have been also evaluated as putative mediators of the ovulatory process. They include interleukins, tumor necrosis factor, and the mitogenic growth factors such as fibroblast growth factor and epidermal growth factor (Espey, 1994). In addition, increasing evidence has shown that white blood cells may play an important role in the regulation of ovulation (Gerdes et al., 1992; Brannstrom et al., 1994; Brannstrom et al., 1994; Sirotkin and Luck, 1995). Furthermore, recent studies have documented that the free radical, nitric oxide (NO), is involved in the process of ovulation in experimental animals (Jablonka-Shariff and Olson, 1997; Yamauchi et al., 1997).

2.2.3. Superovulation in cattle

Equine chorionic gonadotropin (eCG, formly called pregnant mare serum gonadotropin, PMSG) and FSH are commonly used for induction of superovulation in cattle (Armstrong, 1993). Exogenous gonodotropins are given during the luteal phase, approximately between Days 8-12 of the estrous cycle, with FSH (30-50 mg) given in a total of 8 injections over 4 days, or eCG (2000-3000 I.U) administered as a single injection (Hahn, 1992). Three days after the first injection with gonadotropin, one or two injections of prostaglandin $F_{2\alpha}$ or its analogue are given at an interval of 12 h. Animals show estrus 36-48 h after the first injection of prostaglandin. Although this superovulation protocol is routinely used in practice, a highly individual variability is often observed (Armstrong, 1993).

A major problem associated with superovulation in cattle is the large variability in the ovulation rate and yield of viable embryos (Hahn, 1992; Webb et al., 1994). Attempts to reduce the variability through improved treatments and better control of environmental conditions have resulted in little advances (Hahn, 1992). Although exogenous gonadotropin treatment can induce superovulation, the mechanisms that control the growth of follicles and the number of ovulation are not fully understood (Webb et al., 1994).

The endocrinology of the superovulatory follicles has been extensively studied (Fortune and Hansel, 1985; Laurincik et al., 1993; Assey et al., 1994). It has been shown that estradiol and especially progesterone concentrations of potentially ovulatory follicles in superovulatory heifers are lower than in comparable follicles of unstimulated animals (Fortune and Hansel, 1985). The administration of exogenous gonadotropins interferes with the natural regulation of folliculogenesis, resulting in a shortening of the follicular phase (Assey et al., 1994). The time available for oocytes to complete the prematurational changes is also significantly reduced during superovulation in cows (Assey et al., 1994). Furthermore, a high proportion of ovulatory size follicles (> 8 mm) do not undergo ovulation, and eventually undergo atretic or become cystic (Laurincik et al., 1993; Pruwantara et al., 1994). A better understanding of these drawbacks may help us improve the efficacy of superovulatory treatment.

2.2.4. Prostaglandins and ovulation

The role of prostaglandins in ovulation was first demonstrated in rats and rabbit by a number of researchers (Armstrong and Grinwich, 1972; Grinwich et al., 1972; O'Grady et al., 1972; Orczyk and Behrman, 1972), and since then indomethacin and other NSAIDs, well-known inhibitors of

prostaglandin synthesis, have been shown to block ovulation in several species (Murdoch et al., 1993). Levels of prostaglandins are markedly increased in preovulatory follicles shortly before ovulation (Ainsworth et al., 1975; Bauminger and Lindner, 1975; LeMaire et al., 1975). Increases in intrafollicular prostaglandins are restricted to follicles destined to ovulate (Ainsworth et al., 1990). In pigs, the inhibition of ovulation by indomethacin has no effect on other ovarian responses, such as oocyte maturation, luteinization and steroidogenesis (Ainsworth et al., 1979). Furthermore, blockage of ovulation with indomethacin could be reversed by the administration either PGE or PGF (Orczyk and Behrman, 1972; Tsafriri et al., 1972; Sato et al., 1974; Downey and Ainsworth, 1980). However, in rats, indomethacin not only suppresses the morphological changes normally occurring within the follicular wall during preovulatory development, but also blocks germinal vesicle breakdown (GVBD) of oocytes in two-thirds of follicles examined (Downs and Longo, 1982). Therefore, the roles of prostaglandins during ovulation may differ between species.

Recent studies have demonstrated that disruption of PGHS-2 gene, which encodes a key enzyme in the prostaglandin biosynthetic pathway, prevents animals from normal ovulation and fertilization (Dinchuk et al., 1995; Lim et al., 1997). Therefore, gonadotropin-dependent induction of prostaglandin synthesis in preovulatory follicles is, at least in mice, required for ovulation. However, the exact mechanism by which prostaglandins are involved in follicular rupture remains unknown. A number of possible mechanisms have been proposed, including an action on ovarian muscle fibre contractility (Gimeno et al., 1975; Wright et al., 1976), the stimulation of enzymatic breakdown of the follicular wall (Reich et al., 1985; Reich et al., 1991) and the promotion of a highly localized inflammatory-like reaction at the

apex of the follicle (Espey, 1980; Espey, 1994). Since the crucial event in follicular rupture appears to be the weakening of the follicular wall, prostaglandins are believed to be involved in the activation of proteolytic enzymes (LeMaire, 1989). Studies in rats have indicated that eicosanoids play an essential role in gonadotropin-dependent collagenase production in follicular wall prior to ovulation (Reich et al., 1991).

The precise type(s) of prostaglandins required for ovulation remains unclear. Early studies have shown that $PGF_{2\alpha}$ appears more effective in restoring indomethacin-blocked ovulation in rabbits (Diaz-Infante et al., 1974; Hamada et al., 1977) and in pigs (Downey and Ainsworth, 1980). However, in mice, PGE_2 was more potent than $PGF_{2\alpha}$ in restoring ovulation (Saksena et al., 1974). Gene targeting studies have documented that mice lacking the gene encoding the $PGF_{2\alpha}$ receptor have normal ovulation but compromised parturition (Sugimoto et al., 1997), suggesting that $PGF_{2\alpha}$ is not required for ovulation in this species. No direct evidence has indicated that PGE_2 is necessary for ovulation. Mice with disrupted PGE_2 receptor subtypes -1,-2 and -3 were fertile (Ushikubi et al., 1998), and prostaglandin I_2 receptor deficient mice have apparently normal reproductive functions (Murata et al., 1997), indicating that PGE_2 and PGI_2 might not be essential for ovulation. Interestingly, other prostaglandins, such as PGJ_2 and PGA_2, were shown to bind and activate a nuclear receptor, the peroxisome proliferator-activated receptor (PPAR)r (Forman et al., 1995; Kliewer et al., 1995). Whether or not PGJ_2 and PGA_2 are involved in the regulation of follicular rupture-related genes remains to be studied.

2.3. Prostaglandins

2.3.1. History of prostaglandins

The discovery of prostaglandins should be credited to two European scientists, Goldblatt in England and Von Euler in Sweden (Goldblatt, 1933; Von Euler, 1934). However, it was Von Euler (1935) who established for the first time that the active factor present in accessory sexual glands of sheep and human was a lipid soluble acid that belongs to a completely new group of natural substances. Believing that the substance was a product of the prostate gland, Von Euler (Von Euler, 1935; Von Euler, 1936) named this factor "prostaglandin". Although it was subsequently found that the seminal vesicles were the source of the prostaglandins in seminal plasma (Eliasson, 1959), the name prostaglandin has become firmly established.

After a gap of more than 10 years, the work on the identification of prostaglandins commenced in 1949 by the Swedish chemist Sune Bergström who confirmed Von Euler's findings that the biological activity of human seminal fluid extract was due to a new group of highly active lipid unsaturated hydroxy fatty acids (Eliasson, 1959). Finally, Bergström and Sjövall purified two biologically active compounds, PGE_1 and $PGF_{1\alpha}$ in crystalline form from sheep seminal vesicular gland in 1957 (Bergström and Sjövall, 1957), and then a vast field of chemical, biological and clinical importance opened up.

2.3.2. Structure and nomenclature

All prostaglandins are oxygenated polyunsaturated 20-carbon fatty acids containing a cyclopentane ring and two side-chains. They can be considered as derivatives of a parent structure called prostanoic acid, and are termed collectively as prostanoids (Ninnemann, 1988). Each prostaglandin is designated by a letter A-J, indicating nature and position of substituents on the cyclopentane ring (Figure 3). For example, prostaglandin E (PGE) and F series differ only by a single chemical substitution at the C-9 position, these simple

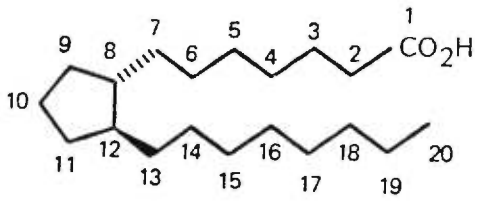

Figure 3. Structure of prostanoic acid (Taken from Ninnemann, 1988).

substitutions profoundly affect their biological activities. The subscript numerals 1, 2 and 3 in each series denotes the number of double bonds in the side chains of the cyclopentane ring. The subscripts α and β is added in PGF series to specify the spatial orientation of the C-9 hydroxyl group. All natural members of the PGF family have an α-orientation at the C-9 position. PGFβ with a C-9 β configuration are produced only under the experiment condition (Ninnemann, 1988).

2.3.3. Biosynthesis of eicosanoids

'Eicosanoids' is a collective name for 20-carbon unsaturated lipids derived from arachidonic acid, or similar polyunsaturated fatty acid precursors, via the cyclooxygenase or lipoxygenase pathways. This group of compounds includes prostaglandins, prostacyclins, thromboxanes, leukotrienes, lipoxins and various hydroxy- and hydroperoxy-fatty acids (Slater and McDonald-Gibson, 1987).

Arachidonic acid, which is derived from linoleic acid, is the dominant precursor of eicosanoids. It is stored primarily in an esterified state within the phospholipid bilayer backbone of cellular membranes (Figure 4) (Ninnemann, 1988). The initial, rate-limiting step in the eicosanoid cascade is the enzymatic release of unesterified fatty acid from the plasma membrane. This is catalyzed by phospholipases A2, C, or D (Smith, 1992). In blood platelets, arachidonate is principally derived from phosphatidylinositol *via* the phospholipase C pathway

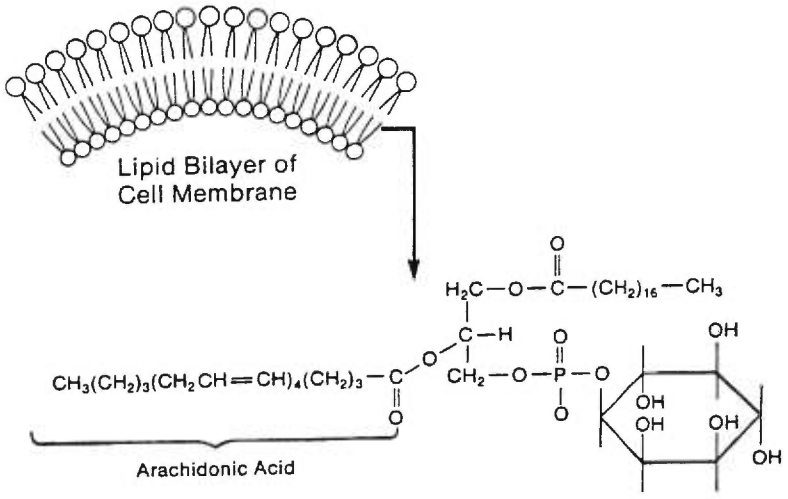

Figure 4. Diagram of the membrane lipid bilayer, consisting of polar groups (choline, inositol, etc.) on the outside, with nonpolar, long-chain fatty acids (esterified at the C-1 and C-2 positions) on the inside. The structure of one membrane lipid, phosphatidylinositol, which serves as a source of arachidonic acids, is also shown (Ninnemann, 1988).

(Neufeld and Majerus, 1983). In other cells, most of the arachidonate probably arises directly or indirectly from phosphatidylinositol and/or phosphatidylcholine *via* the action of phospholipases A2 (PLA2) (Chilton et al., 1989; Dennis et al., 1991). Several PLA2s have been characterized, including cytosolic PLA2 and secretory PLA2 (Smith, 1992).

Following its liberation from cell membranes, the arachidonic acid can be metabolized *via* the cyclooxygenase pathway or *via* the lipoxygenase pathway (Smith and Marnett, 1991; Smith, 1992). Figure 5 displays an overview of arachidonic acid metabolism. Prostaglandins, prostacyclin and thromboxanes are produced *via* the cyclooxygenase pathway. First, arachidonic acid is converted into the cyclic endoperoxides, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by PGHS. Two PGHS isoforms have been identified, and further discussions about these isoenzymes will be given later. PGHS is a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activities (Smith and Marnett, 1991). The cyclooxygenase component of this enzyme introduces two molecules of oxygen into arachidonate to yield PGG_2, while the peroxidase activity reduces PGG_2 to PGH_2. The metabolism of PGH_2 into biologically active prostanoids is controlled by a set of specific synthases called PGD synthase, PGE synthase and PGF synthase (Urade et al., 1995). Formation of prostacyclin (PGI) and thromboxane A_2 (TXA_2) from PGH_2 occurs through the actions of PGI synthase and TXA_2 synthase respectively (Smith and Marnett, 1991).

The second primary pathway of arachidonic acid metabolism is through the lipoxygenase (Smith and Marnett, 1991). There are three major lipoxygenases named for their ability to insert molecular oxygen at a specific carbon of arachidonic acid. The cellular distribution of lipoxygenases is more restricted than that of PGHS. For example, leukocytes and mast cells are rich

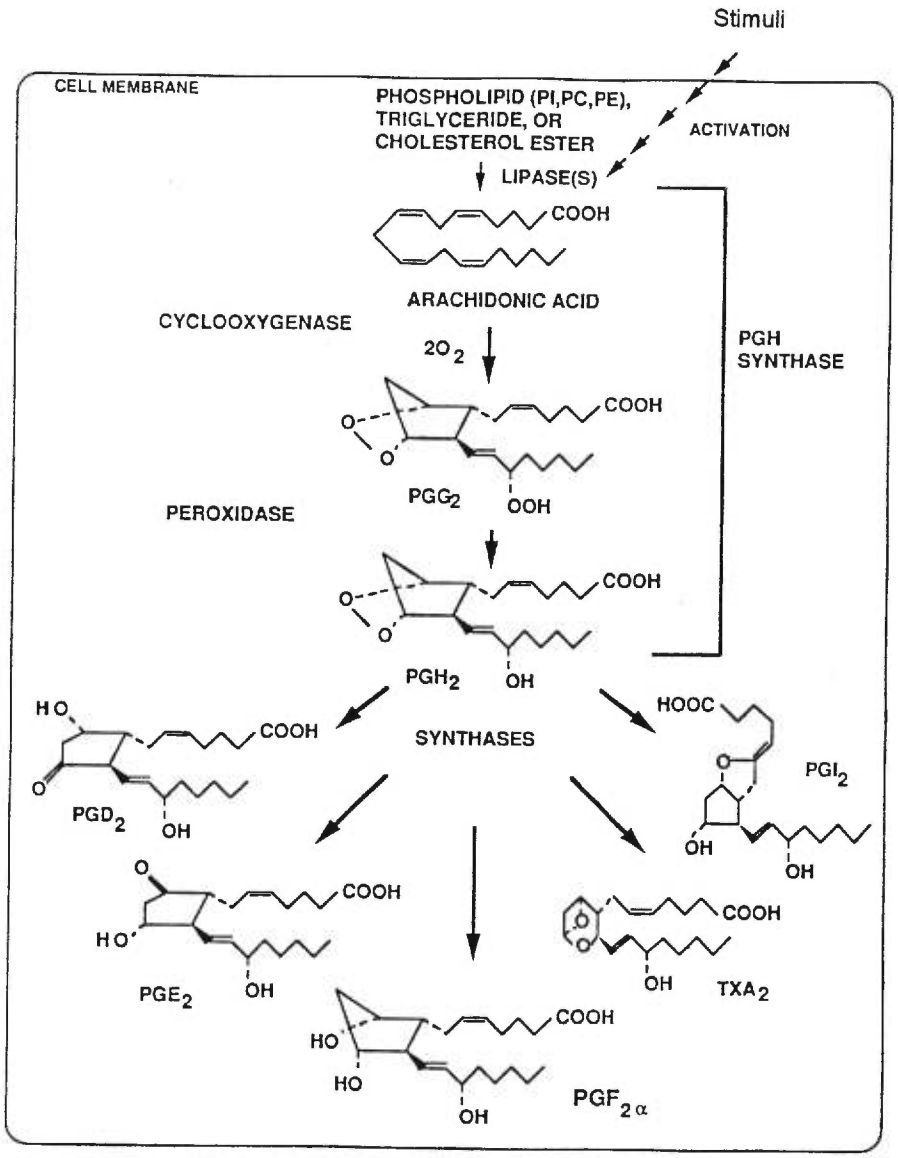

Figure 5. The biosynthetic pathway of prostaglandins. Stimuli include hormones, growth factors, cytokines (Taken from Smith, 1992).

in 5-lipoxygenase, whereas the 12- and 15-lipoxygenases are present more in platelets and respiratory tissues (Murdoch et al., 1993).

Leukotrienes (LTs) originate from the 5-lipoxygenase pathway. Arachidonic acid is converted into the hydroxy eicosatetraenoic acids (HETEs) by either 12- or 15- lipoxygenase. Lipoxins are majorly derived from 15-lipoxygenase pathway, and they may also result from sequential action of 5-, 12- or 15-lipoxygenases (Sigal, 1991).

2.3.4. Mechanisms of prostaglandin actions

Prostaglandins are widely distributed in mammalian tissues. Unlike many other biologically active substances, prostaglandins are formed immediately prior to release and are not stored in the body. Once a prostaglandin is formed, it exits the cell, probably via carrier-mediated transport (Cozzini and Dawson, 1977). Recent studies have demonstrated that the metabolism of prostaglandins is mediated by prostaglandin transporter (PGT) (Kanai et al., 1995). The gene encoding for PGT has been cloned in rats and humans (Schuster, 1998), but the exact role of PGT in prostaglandin metabolism remains largely unknown.

Classically, prostaglandins are considered as local hormones, they function in an autocrine or paracrine fashions, and mediate their effects though membrane guanine nucleotide binding regulatory (G) protein-coupled receptors such as PGF receptor and PGE_2 receptors (EP-1, -2, -3, -4) (Coleman et al., 1994). However, recent studies have documented that prostaglandins such as PGJ_2 and PGA_2 can function as intranuclear messengers, and they can directly bind and activate the peroxisome proliferator-activated receptor PPARγ family (Forman et al., 1995). PPARγ is widely expressed in adipocytes and is a

critical regulator of adiptocyte differentiation (Kliewer et al., 1995; Lehmann et al., 1995; Yu et al., 1995), and is currently under intense study.

2.3.5. Metabolism of prostaglandins

The half-life of prostaglandins in the blood stream is very short, and generally less than 1 min (Samuelsson et al., 1975). Endogenous concentrations of prostaglandins in the peripheral plasma do not exceed 2 pg/ml (Slater and McDonald-Gibson, 1987). Prostaglandins and thromboxanes are rapidly inactivated through metabolism which occurs mainly in lungs, liver and kidneys, or at the site of production (Samuelsson, 1970). For example, over 90% of PGE and PGF are metabolized during one circulation through the lungs and liver (Karim and Rao, 1975). PGE_2 and $PGF_{2\alpha}$ are rapidly metabolized to the biologically inactive 15-keto-13,14-dihydro metabolite, and several other metabolic processes degrade the molecule further into a large number of products, which are finally excreted into urine (Slater and McDonald-Gibson, 1987).

2.4. Prostaglandin receptors

2.4.1. General comments

The presence of specific prostaglandin receptors was first recognized pharmacologically in 1982 (Kennedy et al., 1982). Molecular cloning of prostaglandin receptors began in the early 1990s. The first prostaglandin receptor cloned was thromboxane A_2 receptor (TP) (Hirata et al., 1991). Subsequently, receptors for PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 have been cloned (Sugimoto et al., 1992; Funk et al., 1993; Honda et al., 1993; Watabe et al., 1993; Kunapuli et al., 1994). Four receptor subtypes specific for PGE_2 (EP_1, EP_2, EP_3 and EP_4) have been characterized and shown to be derived from

separate genes (Pierce et al., 1995). In addition, alternative mRNA splice variants have been identified for the EP3 receptor (Namba et al., 1993) and for TP receptor (Hirata et al., 1991). All these prostaglandin receptors have seven transmembrane domains, and are coupled to G-proteins linked to either adenylate cyclase or the phospholipase C-inositol triphosphate pathway. However, exception always exists in nature, PGJ_2 and its derivatives do not interact with membrane receptors, but interact with nuclear receptors that directly act on genome to regulate gene expression (Kliewer et al., 1995; Lehmann et al., 1995).

The overall homology among prostaglandin receptors is relative low, but the region encoding the seventh transmembrane domain is highly conserved (Coleman et al., 1994).

2.4.2. PGD2 receptors

Prostaglandin D_2 is involved in diverse physiological functions such as thermoregulation, sleep induction and sedation, release of pituitary gonadotropins, smooth muscle relaxation and contraction, and vision (Gerashchenko et al., 1998). Prostaglandin D2 receptors (DP) are distributed largely in blood platelets, vascular smooth muscle, the central nervous system and eye tissues, but they are also found in gastrointestinal, uterine, airway smooth muscle and epididymis (Coleman et al., 1994; Oida et al., 1997; Gerashchenko et al., 1998). Responses mediated by DP receptors are majorly inhibitory such as inhibition of platelet aggregation, relaxation of smooth muscle and inhibition of autonomic neurotransmitter release (Coleman et al., 1994). However, DP receptors are also involved excitatory events in some afferent sensory nerves, where they can induce pain or hyperalgesia (Horiguchi et al., 1986).

The cDNA for the DP receptor has been cloned in mouse (Boie et al., 1995) and human (Hirata et al., 1994). The transcript is about 3.5 Kb, and Northern blot analysis showed a very limited tissue distribution, with mRNA detectable only in retina and small intestine in humans (Boie et al., 1995). In mice, DP receptor was expressed abundantly in the ileum, lung, stomach, and uterus (Hirata et al., 1994). The human DP receptor consists of 359 amino acid residues, with a predicted molecular mass of 40,276 (Hirata et al., 1994). The deduced amino acid sequence of the human DP, when compared with all other members of the prostanoid receptor family, shows the highest degree of identity with the human IP and EP_2 receptors, followed by the EP_4 receptor (Boie et al., 1995).

2.4.3. PGE_2 receptors

Prostaglandin E_2 mediates many biological functions in the cardiovascular, pulmonary, and immune system (Coleman et al., 1994). PGE_2 can bind to and activate a set of functionally distinct cell surface receptors, EP_1-EP_4, which are classified on the basis of their responses to various PGE_2 agonists and antagonists (Coleman et al., 1994). EP_1 is coupled to calcium mobilization, both EP_2 and EP_4 are coupled to the stimulation of adenylyl cyclase, whereas EP_3 is coupled to the inhibition of adenylyl cyclase (Negishi et al., 1996).

Studies have shown that the EP_1, EP_3, and EP_4 genes are expressed in the periimplantation mouse uterus in a spatio-temporal manner, suggesting compartmentalized actions of PGE_2 during this period (Yang et al., 1997). Further studies showed that EP_2 messenger RNA (mRNA) is expressed exclusively in the luminal epithelium primarily on day 4 (the day of implantation) and day 5 (early implantation) of pregnancy, suggesting that EP_2 could be a

potential mediator of PGE_2 actions in regulating luminal epithelial differentiation and serve as a marker for uterine receptivity for implantation (Lim and Dey, 1997).

Mice lacking each of these receptors (EP_1-EP_4) have been generated by homologous recombination (Segi et al., 1998; Ushikubi et al., 1998). EP_1-, EP_2- and EP_3-deficient mice appeared fertile (Ushikubi et al., 1998). However, mice lacking the EP_3 receptor failed to show a febrile response to PGE_2 and to IL-1β or LPS, indicating that PGE_2 mediates fever generation by acting at the EP_3 receptor (Ushikubi et al., 1998). Loss of the EP_4 receptor was not lethal in utero, but most EP_4 (-/-) neonates became pale and lethargic approximately 24 h after birth and died within 72 h. Less than 5% of the EP_4 (-/-) mice survived and grew normally more than a year. Histological examination revealed that the ductus arteriosus in dead neonates remained open, suggesting that the EP_4 receptor is essential in neonatal adaptation of the circulatory system (Segi et al., 1998; Ushikubi et al., 1998).

2.4.4. Prostaglandin F receptors

Prostaglandin $F_{2\alpha}$ receptors (FP) have been found in a variety of different tissues such as the corpus luteum, kidney, heart, stomach, ocular tissue, uterus and lung (Coleman et al., 1994; Sugimoto et al., 1994). Expression of FP receptors is abundant in the corpus luteum, where they mediate luteolysis (Sugimoto et al., 1994). The presence of FP receptors in ocular tissue has important pharmacological values because $PGF_{2\alpha}$ and its analogues were proven to be effective in reducing intraocular pressure and in the treatment of glaucoma (Coleman et al., 1994).

Expression of FP receptors in the corpus luteum has been detected during the luteal phase of the estrous cycle, and during the early and mid-

pregnancy in cattle (Sakamoto et al., 1995). FP receptor genes are up-regulated in granulosa cells and luteal cells in response to gonadotropins (Tsai et al., 1996; Narko et al., 1997). The expression of FP gene in luteal cells of the corpus luteum is induced by gonadotropins, probably by acting through a cAMP-mediated pathway (Sugatani et al., 1996). Studies in myometrium have shown that expression of FP is up-regulated during periimplantation and then down-regulated during pregnancy (Matsumoto et al., 1997; Yang et al., 1997).

The cDNAs for FP receptors in the cow, mouse, rat, sheep and human have been isolated (Coleman et al., 1994). A recent study has identified a novel isoform of FP receptor in sheep, this isoform (FPb) is identical to the original one (FPa) throughout the seven-transmembrane domains, but diverges nine amino acids in the carboxyl terminus (Pierce et al., 1997). The physiological significance of these two isoforms has not been clarified.

Mice lacking FP receptor developed normally but were unable to deliver normal fetuses at term. These FP-deficient mice showed no abnormality in the estrous cycle, ovulation, fertilization, or implantation, but they did not respond to exogenous oxytocin because of the lack of induction of oxytocin receptor in uterus (Sugimoto et al., 1997).

2.4.5. Prostacyclin receptor

Prostacyclin is primarily synthesized by the vascular endothelium and it plays an important inhibitory role in the local control of vascular tone and platelet aggregation (Coleman et al., 1994). Preliminary studies have shown that human uterine tissue can synthesize prostacyclin (Chegini and Rao, 1988), and IP-receptors may present in the non-pregnant human myometrium (Senior et al., 1992). The IP receptor-deficient mice were generated, and shown to have normal reproductive functions (Murata et al., 1997).

2.4.6. Thromboxane receptor

Thromboxane A_2 is a biologically potent arachidonate metabolite (Smith, 1992). It induces platelet aggregation and smooth muscle contraction, and may promote mitogenesis and apoptosis of other cells (Smith, 1992). The action of thromboxane A_2 on cells is mediated *via* Thromboxane receptor (TP). In many tissues, TP receptors are the counterpart of IP receptors (Coleman et al., 1994). TP receptors are widely distributed in vascular smooth muscle and platelets, where they invariably mediate excitatory activities such as vasoconstriction and platelet aggregation (Coleman et al., 1994). They are also present in myofibroblasts, mesangial cells and epithelium of the gastrointestinal tract (Coleman and Sheldrick, 1989). Recent studies have shown that TP receptors are expressed in human myometrium (Senchyna and Crankshaw, 1996) and bovine corpus leteum (Lei et al., 1992), suggesting that they may be involved in reproductive processes.

The complementary DNA of TP receptor has been cloned in human (Hirata et al., 1991) and mouse (Namba et al., 1992). The human and mouse TP receptors consist of 343 and 341 amino acids, respectively, and are 76% identical (Coleman et al., 1994). The gene is about 15 kb containing three exons and two introns.

2.5. Prostaglandin G/H synthase

2.5.1. Identification of PGHSs

Prostaglandin G/H synthase was originally purified from ovine (Hemler and Lands, 1976; Van der Ouderaa et al., 1977) and bovine (Miyamoto et al., 1976) seminal vesicles more than 20 years ago. The cDNA coding for this PGHS isoform was subsequently cloned in sheep (DeWitt and Smith, 1988),

mice (DeWitt et al., 1990) and humans (Funk et al., 1991), and shown to correspond to a 2.8 kb mRNA (DeWitt and Smith, 1988; Merlie et al., 1988). Since PGHS was present in relatively comparable levels in many tissues, it was originally thought that the conversion of arachidonate into PGH_2 was not a regulatory step. However, Raz et al. (1988) showed that ligand treatment of fibroblasts or monocytes caused a rise in PGHS activity and in PGHS protein synthesis. However, mRNA levels were not changed by ligand or hormone treatment. These results led to the assumption that different pools, a constitutive and an agonist-inducible PGHS, may exist. Interestingly, Rosen et al. (1989) demonstrated that the induction of a 4.0-kilobase message (not the 2.8-kilobase PGHS transcript) was likely involved in prostaglandin synthesis in mitogen-treated sheep mucosa cells. Also, immunological studies suggested the presence of two PGHS proteins (69,000 Mr and 72,000 Mr) in the rat ovary (Wong and Richards, 1991).

In the early 1990s, Xie et al. (1991) cloned a chicken fibroblast cDNA encoding a protein with 57% identity to ovine PGHS. During the same year, Kujubu et al. (1991) reported the cloning of a novel cDNA (called TIS 10) from cultures of murine fibroblasts stimulated with forskolin and phorbol esters. Upon sequencing, it was showed that TIS 10 gene was highly homologous to the chicken PGHS-related cDNA (Kujubu et al., 1991). In 1992, Sirois and Richards reported the purification and partially characterization of PGHS isoform (72,000 Mr) induced in granulosa cells of rat preovulatory follicles by hCG. The N-terminal sequence of the inducible rat PGHS was distinct from the ovine isoform characterized thus far, but highly homologous to the deduced amino acid sequence derived from the mouse and chicken PGHS-related cDNA. Thus, the combined evidences from cloning of the cDNA and purification of the rat protein suggests the presence of a novel isoform of

PGHS. The isoform originally purified from seminal vesicle is now referred as PGHS-1 (or COX-1), whereas the isoform identified in the early 90's is named as PGHS-2 (COX-2).

2.5.2. Characteristics of PGHS-1 and PGHS-2

2.5.2.1. Structures and functions of PGHS proteins

The mature forms of PGHS-1 and -2 have 576 and 587 amino acids, respectively (Williams and DuBois, 1996). The primary sequences of these two enzymes are 60% identical within a species, and 80-85% identical for a given isoform across species. The active enzymes of both PGHS-1 and -2 exist as a head-to-tail homodimer with a heme group (Picot et al., 1994). Each subunit consists of three domains: a small N-terminal domain composed of an epidermal growth factor (EGF) module, a membrane-binding domain containing four short helices, and a large, globular C-terminal catalytic domain which contains the cyclooxygenase and peroxidase active sites (Picot et al., 1994).

The molecular weights calculated from the amino acid sequences for PGHS-1 and -2 are 65 KDa and 67 kDa, respectively (DeWitt and Smith, 1988). However, actual migration bands on SDS-PAGE for PGHS-1 and -2 are 68-72 kDa (Miyamoto et al., 1976) and 72-74 kDa (Sirois and Richards, 1992; Otto et al., 1993), respectively. The differences are attributable to the presence of different N-linked glycosylations (Otto et al., 1993).

Differences in the primary structures between PGHS-1 and -2 include the signal peptides, the membrane-binding domains, and the amino- and caboxyl-termini. Particularly, the N-terminus is slightly truncated in PGHS-2 compared with PGHS-1, and the C-terminus of PGHS-2 has an 18-amino-acid insert that is absent in PGHS-1 (Williams and DuBois, 1996). Studies have

shown that deletion of the 18 amino acid cassette has no effects on cyclooxygenase activity and subcellular distribution (Regier et al., 1995).

All PGHSs have a PTEL (Pro-Thr-Glu-Leu), STEL (Ser-Thr-Glu-Leu) or SAEL (Ser-Ala-Glu-Leu) sequence at their caboxyl termini depending on isoform and species. Since both PGHS-1 and -2 are found on the lumen side of the endoplasmic reticulum (ER), it was anticipated that P/STEL sequence would serve as ER retention signals for these enzymes. Early studies have indicated that modification or deletion of this sequence resulted in less active mutant enzymes, but surprisingly showed that there was no effect on subcellular targeting (Regier et al., 1995; Ren et al., 1995; Ren et al., 1995). However, one study indicates that C-terminal mutants of PGHS-1 are more concentrated in the Golgi (Song and Smith, 1996).

The crystal structures of PGHS-1 and -2 have been revealed (Picot et al., 1994; Kurumbail et al., 1996). Comparison of the crystal structures of murine PGHS-2 and ovine PGHS-1 indicates that their overall structures are highly conserved (Luong et al., 1996). The major difference between the two isoforms is the substrate access channel (Kurumbail et al., 1996; Luong et al., 1996), which allows to develop specific drugs to inactivate PGHS-2 (Kalgutkar et al., 1998).

2.5.2.2. Structures of PGHS genes

PGHS-1 and -2 are encoded by separated genes located to different chromosomes (Williams and DuBois, 1996). The human PGHS-1 is located on chromosome 9 (Funk et al., 1991), whereas PGHS-2 is located on chromosome 1 (Jones et al., 1993). The genomic structure of PGHS-1 consists of 11 exons and 10 introns and spans 22.5 kb (Yokoyama and Tanabe, 1989; Kraemer et al., 1992). However, the PGHS-2 gene is much smaller and is only about 8 kb

in size; it has 10 exons and 9 introns, and the first two exons of PGHS-1 are condensed to form a single exon in PGHS-2 (Figure 6) (Williams and DuBois, 1996). The difference in size between PGHS-1 and PGHS-2 is due principally to the presence of smaller introns in the PGHS-2 gene (Williams and DuBois, 1996). Interestingly, the locations of intron/exon boundaries are highly conserved between the two isoforms (Smith and Marnett, 1991).

The size of the of PGHS-1 and -2 transcripts are 2.8 and 4.4 kb (Williams and DuBois, 1996), respectively. The 3'-untranslated region (UTR) of PGHS-2 contains many copies of the Shaw-Kamens sequence (Shaw et al., 1986), which is often present in immediate early response genes and is believed to be involved in mRNA degradation (Kosaka et al., 1994).

2.5.2.3. Inactivation and inhibition of PGHSs

There are two different inactivation processes for PGHSs: (1) auto-inactivation of cyclooxygenase (the 'suicide' reaction); and (2) peroxide-dependent inactivation of cyclooxygenase and peroxidase activities (Smith and Marnett, 1991). The rate of 'suicide' inactivation is directly related to the rate of cyclooxygenase catalysis, but is relatively independent of the presence of newly formed product (Smith et al., 1990). The exact mechanism of 'suicide' inactivation remains unclear, but it has been suggested that the tyrosyl radicals formed during PGHS catalysis are involved (Hsi et al., 1994). Both the cyclooxygenase and peroxidase activities are inactivated when the enzyme is incubated with hydroperoxides (Smith et al., 1990). This type of inactivation

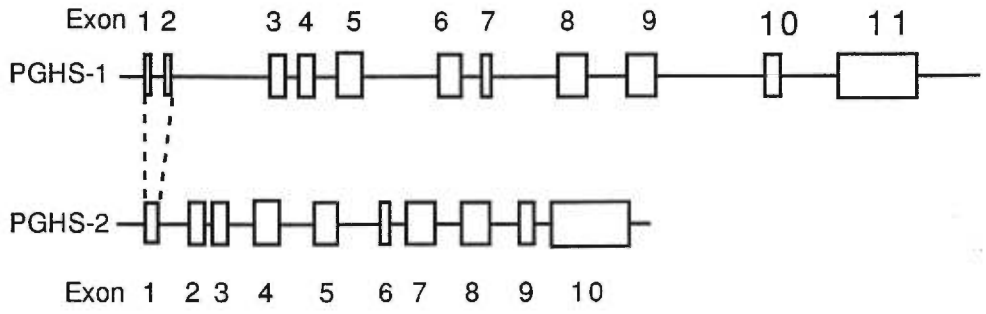

Figure 6. Human prostaglandin G/H synthase-1 and -2 genomic structure (Taken from Williams et al., 1996 with minor modification).

may involve the modification of the heme group associated with PGHSs (Smith et al., 1990).

The activities of PGHSs can be inhibited by most NSAIDs, which compete with arachidonate for binding to the cyclooxygenase active site, and thus block its activity (Smith et al., 1990). However, NSAIDs have no effect on the peroxidase activity (Williams and DuBois, 1996). NSAIDs can be grouped into three types (Kurumbail et al., 1996). Aspirin, the only known example of the first group, irreversibly inactivates both PGHS-1 and -2 by acetylating an active serine site (Vane, 1971; DeWitt et al., 1990). The second group consists of reversible, competitive inhibitors of both PGHS isoforms, these drugs compete with substrates for the cyclooxygenase active site (Kurumbail et al., 1996). The third group of inhibitors can cause a slow, time-dependent inhibition of PGHSs (Kulmacz and Lands, 1985), such as indomethacin and Flurbiprofen.

The inhibitory effect of aspirin on PGHS-1 and PGHS-2 is different. PGHS-1 is completely inhibited by aspirin through acetylation of serine-530, whereas PGHS-2 converts arachidonic acid to 15-R-HETE after aspirin treatment, thus functioning as a 15-lipoxygenase (Williams and DuBois, 1996). In general, the beneficial effects of NSAIDs come from their ability to block PGHS-2 involved in inflammation, pain, fever, and probably in cancer and Alzheimer's disease (Pennisi, 1998). Efforts to find specific PGHS-2 inhibitors heated up during last few years. Recently, Kalgutkar et al. (1998) have designed aspirin-like drugs that selectively inactivate PGHS-2 and that have no deleterious gastrointestinal or hematological side effects.

2.6. Regulation of PGHSs

2.6.1. Differential expression

The most obvious difference between PGHS-1 and -2 is their distinct pattern of expression. PGHS-1 is constitutively expressed in most mammalian tissues, and performs house-keeping functions, including the regulation of renal water and sodium reabsorption, gastroprotection, and vascular homeostasis (DeWitt and Smith, 1995). In contrast, PGHS-2 is normally undetected in most tissues but can be expressed transiently in selected cells following stimulation by cytokines, growth factors, hormones, or tumor promoters (Williams and DuBois, 1996). Also, expression of PGHS-2 is often related to pathological conditions, and some nuclear events such as cell differentiation and replication (Herschman, 1996).

2.6.2. Differential localization

One study showed no apparent difference in the subcellular localization of PGHS-1 and PGHS-2 (Regier et al., 1995). However, by using quantitative confocal fluorescence imaging microscopy and histofluorescence staining, Morita et al. (1995) reported that both PGHS-1 and PGHS-2 are located in the endoplasmic reticulum and nuclear envelop, whereas PGHS-2 is preferentially associated with the nuclear envelop. Based on this result, it was proposed that differential compartment of these two isoforms may serve, at least in part, to separate the activities of PGHS-1 and -2 within the cell (Morita et al., 1995). Surprisingly, a more recent study using 3T3 cells, human monocytes, and human umbilical vein endothelial cells (HUVECs) has revealed that both isoforms are present in similar proportions in the endoplasmic reticulum and the outer and inner nuclear membrane, and that the localization of these two isoenzymes and the nature of the products are the same for both PGHS-1 and -2 (Spencer et al., 1998).

A number of reports have suggested that PGHS-1 and -2 utilize different sources of arachidonic acid and perhaps different phospholipase systems to mobilize arachidonate (Murakami et al., 1994; Reddy and Herschman, 1994). PGHS-1 was proposed to utilize exogenous arachidonic acid, while PGHS-2 would preferentially utilize endogenous arachidonic acid. The physiological significance of this difference remains unclear.

2.6.3. Regulation of PGHS-1

PGHS-1 is expressed constitutively in mammalian cells and tissues, and relatively high levels of this isoform are detected in some differentiated cells, including platelets, renal collecting tubules, macrophages, and endothelial cells (DeWitt et al., 1983; Smith, 1986). Studies *In vitro* have demonstrated that the promonocytic cells, THP-1 and U973, increase their PGHS-1 expression during their differentiation into a macrophage phenotype (Hoff et al., 1993; Smith et al., 1993). Also, platelets and renal collecting tubules only express PGHS-1 but not PGHS-2 in response to an appropriate hormonal stimulus (Patrignani et al., 1995). Expression of PGHS-1 can be enchanced in some types of cells by serum, cytokines, or growth factors (Wu, 1995). The expression of PGHS-1 in endothelial cells was stimulated approximately two fold over the basal level by phorbol 12-myristate 13-acetate (PMA) and interleukin-1β (Xu et al., 1996). Recent studies confirmed that the promoter activity is increased in HUVECs after PMA stimulation (Xu et al., 1997). It should be mentioned that expression of PGHS-1 is not essential for mammalian development, since PGHS-1 knockout mice were shown to develop and mature normally (Langenbach et al., 1995).

2.6.4. Regulation of PGHS-2

In contrast to PGHS-1, PGHS-2 is only expressed in few tissues under normal physiological conditions, including the dendrites and cell bodies of neurons in the brain (O' Neill and Ford-Hutchinson, 1993; Breder et al., 1995), and the macula densa of the juxtaglomerular apparatus in the kidney (Harris et al., 1994). Interestingly, PGHS-2 is also constitutively expressed in transformed cells and some cancer/tumor cells. In fact, the first PGHS-2 cDNA was cloned from *Rous sarcoma* virus-transformed chicken embryo fibroblasts (Simmons et al., 1989). Overexpression of PGHS-2 has been observed in colon carcinomas (Eberhart et al., 1994; Kargman et al., 1995) and during the formation of colorectal adenomas (Oshima et al., 1996). The exact causal link between the activity of PGHS-2 and carcinogenesis remains uncertain. Elevated expression of PGHS-2 is believed to inhibit apoptosis (Tsujii and DuBois, 1995; Tsujii et al., 1997), and *v-src*-transformed chicken embryo fibroblasts, which express high level of PGHS-2, were shown to undergo programmed cell death when treated with NSAIDs (Lu et al., 1995).

Regulation of PGHS-2 expression has been extensively studied during last five years. Expression of PGHS-2 can be induced in a wide range of cell types including fibroblasts, endothelial cells, monocyte/macrophages, epithelial cells (DuBois et al., 1994; Muller-Decker et al., 1995), mesothelial cells (Topley et al., 1994), osteoblasts (Pilbeam et al., 1993), mesangial cells (Kester et al., 1994), mast cells (Murakami et al., 1994), granulosa cells (Sirois et al., 1992), uterine stromal and epithelial cells (Jacobs et al., 1994; Asselin et al., 1997; Xiao et al., 1998), amnion cells (Hirst et al., 1995), vascular smooth muscle cells, chondrocytes (Miller et al., 1998), embryos (Day 8-17) (Charpigny et al., 1997), and neurons in the central nervous (Herschman et al., 1995; Dubois et al., 1998). The expression of PGHS-2 is stimulated by a variety of biological stimuli, including growth factors, cytokines, oncogenes, phorbol esters,

carcinogens and overexpression of protein kinase C (PKC) (Williams and DuBois, 1996; Kelley et al., 1997; Miller et al., 1997). PGHS-2 is believed to be an early-response gene in most cell type where its induction is rapid (within 30 min) (Williams and DuBois, 1996). However, PGHS-2 is not an early-response gene in some cell types in which induction is delayed, including in monocyte/macrophages (O'Sullivan et al., 1992), mast cells (Reddy and Herschman, 1994), and granulosa cells (Sirois and Dore, 1997).

2.6.5. Transcriptional regulation of PGHS-1 and PGHS-2

2.6.5.1. *Trans*-activation of PGHS-1

Comparisons between PGHS-1 and PGHS-2 promoters reveal little similarity. There are numerous consensus response regulatory elements (*cis*-elements) present in the 5'-flanking regions of each genes, which are summarized in Table 1. PGHS-1 has multiple transcription start sites, do not possess a TATA box or a CAAT box, and is GC-rich (Williams and DuBois, 1996). These features are commonly shared by constitutively expressed housekeeping genes (Williams and DuBois, 1996). The promoter of PGHS-1 gene contains several putative *cis*-elements such as AP-1, AP-2, SP-1, GATA-box, PEA-3, and putative glucocorticoid response element (GRE) (Wang et al., 1993). In contrast, more putative *cis*-elements are present in PGHS-2 promoter, including NF-IL6 or C/EBPβ, NF-κB, PEA-3, AP-1, AP-2, SP-1, GATA-box, CRE, c-Myc binding site or E-box, and XRE (Fletcher et al., 1992; Sirois et al., 1993; Xie et al., 1993; Inoue et al., 1995; Xie and Herschman, 1995; Morris and Richards, 1996).

Transient transfection studies have demonstrated that the promoter activity of PGHS-1 was very weak (Wang et al., 1993), and was not changed by lipopolysaccharide (LPS) and phorbol ester in vascular endothelial cells (Inoue

Table I. Regulation of PGHS-1 and PGHS-2 expression

Parameter	PGHS-1	PGHS-2
Regulation	usually constitutive	inducible
Range of induced gene expression	2-4 Fold	10-80 Fold
Rate of gene activation	24 hrs	within 30 min, but delayed in ovarian cells
Transcriptional control elements	AP1, AP2, SP1 GRE, GATA, SP1 PEA-3,	NFkB, CRE C/EBP, PEA-3, AP1, AP2, ATF CAAT-box, E-box
Effect of glucocortioids types	little or none	inhibitory effect in most cell stimulatory in amnion cells

et al., 1995). To further identify the elements involved in the constitutive expression of the PGHS-1 gene, a series of 5'-deletion mutants of PGHS-1 promoter were ligated into luciferase expression vector and transiently transfected in HUVECs (Xu et al., 1997). Results have clearly indicated that binding of Sp1, or its related proteins, to two Sp1 sites in the PGHS-1 promoter activates the basal gene transcription (Xu et al., 1997).

2.6.5.2. *Trans*-activation of PGHS-2

Trans-activation of the PGHS-2 gene is more complex and appears differentially regulated depending on the cell type and agonist involved (Sirois et al., 1993; Xie et al., 1994; Inoue et al., 1995; Xie and Herschman, 1995; Yamamoto et al., 1995; Morris and Richards, 1996). Multiple signaling pathways have been linked to stimulation of PGHS-2 gene transcription, including the protein kinase A pathway (Kujubu et al., 1991; Morris and Richards, 1993), protein kinase C pathway (Hla and Maciag, 1991; Kujubu et al., 1991; DeWitt and Meade, 1993), tyrosine kinase pathway (Kester et al., 1994), and through viral transformation (*v-src*) (Evett et al., 1993). Inflammatory cytokines such as IL-1 (Feng et al., 1993; Jones et al., 1993; Crofford et al., 1994; Narko et al., 1997), and interferon-τ (Asselin et al., 1997; Xiao et al., 1998) also induce PGHS-2, but the signaling pathways utilized by these effectors are not fully understood.

The 5'-flanking regulatory regions of the human (Hla and Neilson, 1992; Kosaka et al., 1994), mouse (Fletcher et al., 1992), chicken (Xie et al., 1993), rat (Sirois and Richards, 1993), bovine (Antaya et al., 1997) and equine (Boerboom and Sirois, 1998) PGHS-2 genes have been isolated, and partially characterized. Deletion analyses of the PGHS-2 promoter have identified specific regions involved in the *trans*-activation of PGHS-2 genes, including the

regions between -195/32 in the rats (Sirois and Richards, 1993), -140/-132 and -124/-52 in humans (Inoue et al., 1994; Inoue et al., 1995), and -371/+2 , -186/-131 and -80/+32 in mice (Fletcher et al., 1992; Xie et al., 1994; Xie and Herschman, 1995).

Early studies in rats showed that the CAAT box in the context of an incomplete proximal PGHS-2 promoter confers cAMP induced reporter gene activity in granulosa cells (Sirois et al., 1993). However, more recent site-directed mutagenesis experiments done with the full proximal promoter region revealed that binding to the CAAT box was not required for promoter activation in granulosa cells (Morris et al., 1996). This result is further supported by gene targeting study, in which induction of PGHS-2 is not compromised in C/EBPβ knockout female mice (Sterneck et al., 1997). However, the down-regulation of PGHS-2 gene is impaired in these knockout female mice (Sterneck et al., 1997). Therefore, C/EBPβ may play an important role in the repression of PGHS-2 gene after its activation. Morris et al. (1996) reported that the E-box is absolutely required for the basal and gonadotropin-dependent induction of PGHS-2 in rat granulosa cells, and mutation of the E-box completely abolished promoter activities (Morris and Richards, 1996). Electrophoretic mobility shift assays (EMSA) have shown that a basic helix-loop-helix transcription factor, the upstream stimulatory factor (USF), binds to E-box region (Morris and Richards, 1996). However, the level of USF protein remained constant and was not regulated in the whole extracts of granulosa cells post-hCG (Morris and Richards, 1996).

In mice, elements necessary for the transcriptional activation of the PGHS-2 gene by platelet-derived growth factor (PDGF), serum and growth factors are located within the first 371 nucleotides upstream of the cap site (Fletcher et al., 1992). Mutational analyses of the NFκB and C/EBP elements

have shown that both sites were important for the tumor necrosis factor-induction of mouse PGHS-2 promoter activity in osteoblastic cells (Yamamoto et al., 1995). However, a CRE element is responsible for the v-*src* induced PGHS-2 activity in NIH 3T3 fibroblast cells (Xie et al., 1994). In the human, several elements such as NFκB, C/EBP and CRE were shown to be involved in the *trans*-activation of PGHS-2 gene in different cell types (Inoue et al., 1994; Inoue et al., 1995). A recent study has demonstrated that the AP-1 is also involved in the activation of human PGHS-2 promoter in human SK-GT-4 cells (Zhang et al., 1998) and chondrocytes (Miller et al., 1998). Collectively, studies clearly indicate that the transcriptional activation of the PGHS-2 gene is not only cell type-dependent, but also agonist-dependent.

2.7. PGHS and reproduction

2.7.1. Ovulation

Expression of PGHS in preovulatory follicles in response to the LH surge was first studied in rats by several groups more than 10 years ago (Curry et al., 1987; Hedin et al., 1987; Huslig et al., 1987). Immunoblot analyses demonstrated that the PGHS enzyme is transiently induced in granulosa cells after the administration of hCG (Hedin et al., 1987; Wong et al., 1989). Further studies identified two distinct variants of PGHS (69,000 Mr and 72,000 Mr) using two anti-PGHS polyclonal antibodies (Wong and Richards, 1991). The 72,000 Mr variant was transiently induced in granulosa cells post-hCG, whereas the 69,000 Mr variant was constitutively expressed in thecal cells (Wong and Richards, 1991). The inducible PGHS variant was then purified from rat preovulatory follicles (Sirois and Richards, 1992), and is now known as PGHS-2, whereas the constitutive isoform is PGHS-1.

The availability of cDNAs and antibodies specific for PGHS-1 and PGHS-2 allowed to re-examine the expression of PGHS in rat preovulatory follicles (Sirois et al., 1992). Results clearly documented that both PGHS-2 mRNA and protein are transiently and rapidly induced (2-4 h post-hCG) in granulosa cells of rat preovulatory follicles after hCG administration (Sirois et al., 1992). Studies *in vitro* have also demonstrated that PGHS-2 can be induced by LH, FSH, forskolin, IL-6β, and gonadotropin-releasing hormone in granulosa cells of rat, cow and women (Sirois et al., 1992; Wong and Richards, 1992; Morris and Richards, 1993; Tsai et al., 1996; Liu et al., 1997; Narko et al., 1997). Studies have shown that multiple signaling pathways are involved in the regulation of PGHS-2 in granulosa cells (Morris and Richards, 1993). Protein kinase A (PKA) appears to play a primary role in PGHS-2 induction in granulosa cells, but protein kinase C and tyrosine kinase are also involved (Morris and Richards, 1993). Comparative studies have further revealed that PGHS-2 is also induced in bovine and equine preovulatory follicles post-hCG (Sirois, 1994; Sirois and Dore, 1997), suggesting that induction of PGHS-2 prior to ovulation is conserved across species. However, a marked difference in the time-course of PGHS-2 induction was observed between rats, cows and mares. The induction of PGHS-2 is rapid in rats (2-4 h post-hCG), but delayed in cows (18 h post-hCG) and in mares (30-33 h post-hCG) (Sirois, 1994; Tsai et al., 1996; Sirois and Dore, 1997; Boerboom and Sirois, 1998). These results suggest that the molecular mechanisms involved in the regulation of PGHS-2 gene are different between species with a short (rat) and a long (cow, mare) ovulatory process. Interestingly, the time interval from PGHS-2 induction to ovulation appears constant (about 10 h, Figure 7), suggesting that induction of PGHS-2 could be involved in dictating the species-specific length of the ovulatory process (Sirois and Dore, 1997).

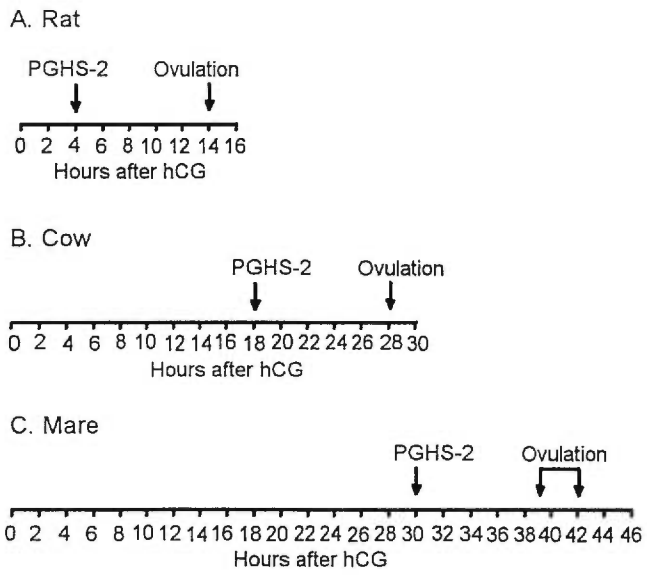

Figure 7. Relationship among PGHS-2 induction, time of ovulation, and length of the ovulatory process in rats, cows, and mares (Taken from Sirois et al., 1997).

Gene targeting studies have demonstrated that PGHS-2 (-/-) female mice were infertile and this was originally attributed to ovulation failure (Dinchuk et al., 1995). Reexamination of these PGHS-2 deficient mice revealed multiple defects during the female reproductive process, including failures during ovulation, fertilization, implantation, and decidualization (Lim et al., 1997).

2.7.2. Implantation

As early as in 1970s, prostaglandins were shown to play a role in the initiation of blastocyst implantation in rats (Lau et al., 1973; Kennedy, 1977; Hoffman et al., 1978). The involvement of prostaglandins is suggested by their high concentrations at the implantation sites, the inhibitory effect of NSAIDs on implantations, and the reversal of this inhibition by co-administration of prostaglandins. The first striking sign for the initiation of implantation is increased endometrial vascular permeability at the site of blastocyst apposition (Psychoyos, 1973). This is followed by extensive proliferation and differentiation of uterine stromal cells into decidual cells, in which prostaglandins are thought to be involved (Psychoyos et al., 1995; Chakraborty et al., 1996).

Among various prostaglandins, PGE_2 has been considered as a primary candidate involved in implantation and decidualization in rodents (Kennedy, 1985;Tawfik et al., 1987). PGE_2 can bind to and activate a set of functionally distinct cell surface PGE receptors, including EP_1, EP_2, EP_3 and EP_4 (Coleman et al., 1994). Studied have shown the EP_1, EP_3 and EP_4 genes are expressed in the mouse uterus during the periimplantation period (Yang et al., 1997). Expression of EP_3 in a subpopulation of stromal cells at the mesometrial side, and of EP_4 in the epithelium and stroma on days 3-5 of pregnancy,

suggested that activation of these receptors by PGE_2 could be important for preparation of the uterus for implantation. Also, expression of these receptors during the postimplantation period (Days 5-8) suggested their roles during the decidualization process (Yang et al., 1997).

Expression of PGHS-2 was localized to the uterine stroma in regions of mouse blastocyst attachment (Jacobs et al., 1994). In mice, studies have revealed that PGHS-1 is present before implantation in uterine epithelial cells, and becomes undetectable by the time of the attachment (Chakraborty et al., 1996). However, PGHS-2 is induced in the luminal epithelium and underlying stromal cells solely at the sites of blastocyst attachment reaction (Chakraborty et al., 1996). A recent study in mink has also shown that PGHS-2 is present in uterine epithelium, stroma and necks of endometrial glands at the site of implantation (Song et al., 1998). Wild-type blastocysts transferred into uteri of PGHS-2 deficient mice fail to implant, which further confirmed the definitive role of PGHS-2 during the implantation process (Lim et al., 1997).

Studies have demonstrated that the embryo can also expressed PGHS-2 (Charpigny et al., 1997). PGHS-2 is highly expressed in the trophoblast cells of ovine embryo during the implantation period (Charpigny et al., 1997). Previous results showed that both the endometrium and embryo could produce prostaglandins, but the endometrium rather than the embryo was identified as the major source of prostaglandins involved in implantation (Snabes and Harper, 1984). Embryo-derived prostaglandins have been considered to be responsible for the fluid accumulation and hatching, and trophoblast elongation (Snabes and Harper, 1984).

In general, uterine events during the implantation period are regulated primarily by the coordinated and cell-specific effects of estrogen and progesterone (Yang et al., 1997). However, there is no direct steroid response

element within the PGHS-2 promoter, and that *trans*-activation of PGHS-2 gene may involve growth factors or cytokines. Preliminary studies have indicated that epithelial growth factor (EGF) induces the expression of PGHS-2 in rat uterine stromal cells (Bany and Kennedy, 1997), and that cytokines such as interleukin-1β and interferon-τ can regulate PGHS-2 expression in uterine cells *in vitro* (Kennard et al., 1995; Xiao et al., 1998).

2.7.4. Parturition

Prostaglandins are important regulators in the process of parturition (Thorburn et al., 1972; Challis et al., 1976; Wimsatt et al., 1993; Rice et al., 1995; Hirst et al., 1995; Slater et al., 1995; Challis et al., 1997; Gibb, 1998]. Studies in sheep showed that PGHS-2 increased gradually in the cotyledons from 120-139 days gestation, with the highest expression observed at term (Wimsatt et al., 1993). However, expression of PGHS-2 was not detected in the amnion or allantochorion (Wimsatt et al., 1993). In contrast, PGHS-2 mRNA is present in human amnion; its level is elevated after the onset of labor (Hirst et al., 1995). Prostaglandin levels are also increased in amniotic fluid prior to myometrial contractions (Mitchell et al., 1995; Gibb, 1998), and this increase in prostaglandins is due to the induction of PGHS-2, but not PGHS-1 (Slater et al., 1995). Other studies have further shown that induction of PGHS-2 around the time of parturition occurs principally in fetal portion of placenta rather than maternal portion (Gibb and Sun, 1996; Challis et al., 1997).

Interestingly, studies *in vitro* have demonstrated that glucocorticoids can stimulate the expression of PGHS-2 in human amnion (Zakar et al., 1995; Economopoulos et al., 1996), which contrasts with their inhibitory action on PGHS-2 expression in other tissues (Herschman et al., 1995). It has been proposed that elevated glucocorticoids in fetal membrane are responsible for

increasing PGHS-2 expression and PGE_2 export in amnion *in vivo* (Challis et al., 1997; Gibb, 1998).

Gene targeting studies have demonstrated that mice with disrupted PGHS-1 or mice lacking PGF receptor have a compromised labor (Langenbach et al., 1995; Sugimoto et al., 1997), suggesting that PGHS-1, but not PGHS-2, plays an important role in the parturition.

2.7.4. Luteolysis

It has been well established that $PGF_{2\alpha}$ is essential for initiation of luteolysis in many species. In ruminants, uterine $PGF_{2\alpha}$ reaches the ovary by diffusion from the uterine vein to the ovarian artery, the so-called countercurrent mechanism (McCracken et al., 1972). Hysterectomy, inhibition of prostaglandin synthesis with NSAIDs, or immunization against $PGF_{2\alpha}$ were shown to prevent luteolysis (Scaramuzzi and Baird, 1976; Lewis and Warren, 1977; Fairclough et al., 1981). However, a recent study in cows has demonstrated that PGHS-2 is induced within corpus luteum by exogenous $PGF_{2\alpha}$, suggesting that an the intraluteal source of $PGF_{2\alpha}$ may be also involved in the process of luteolysis (Tsai and Wiltbank, 1997).

In ruminants, luteolysis $PGF_{2\alpha}$ is secreted in a series of high-amplitude, short-period pulses in response to peaks of luteal oxytocin (Zarco et al., 1988; Flint et al., 1992). However, the precise mechanism by which $PGF_{2\alpha}$ release is controlled during the estrous cycle is not fully understood. A recent study in sheep has demonstrated that expression of PGHS-1 does not change during the estrous cycle and pregnancy (Charpigny et al., 1997). In contrast, PGHS-2 is highly and transiently expressed in the endometrium between days 12 and 15 of the estrous cycle (Charpigny et al., 1997). The marked cyclic changes of

PGHS-2 in the endometrium is primarily controlled by ovarian steroids because PGHS-2 was not expressed after ovariectomy (Charpigny et al., 1997).

Studies *in vitro* have shown that progesterone increases, but estradiol decreases the production of $PGF_{2\alpha}$ in bovine epithelial cells (Goff, 1993; Asselin et al., 1996; Asselin et al., 1997; Xiao et al., 1998). However, steroid hormones had no effect on prostaglandin production in stromal cells (Asselin et al., 1996; Xiao et al., 1998). The amount of $PGF_{2\alpha}$ production in epithelial cells was greater than that of PGE_2, and the opposite was true in stromal cells (Asselin et al., 1996). Studies also showed that oxytoxin stimulated the production of $PGF_{2\alpha}$ and PGE_2 in epithelial but not stromal cells (Asselin et al., 1996), and the stimulation of $PGF_{2\alpha}$ production by oxytocin is *via* the protein kinase C effector pathway (LaFrance and Goff, 1990).

In contrast to ruminants, the luteolysins in primates and rabbits do not come from the uterus, because hysterectomy does not prolong the lifespan of the corpus luteum in these species (Neill et al., 1969; Beling et al., 1970; Lytton and Poyser, 1982). It has been postulated that intraluteal production of $PGF_{2\alpha}$ may be involved in initiation of luteolysis (Michael et al., 1994). However, a recent study has documented that mice lacking PGF receptors in their corpora lutea have estrous cycles of normal length (Sugimoto et al., 1997), thus challenging the concept that uterine or ovarian PGF2α is luteolytic in rodents.

2.8. Prostaglandin D, E, and F synthases

2.8.1. PGD synthase

Prostaglandin D synthase catalyzes the isomerization of PGH_2 to PGD_2 (Urade et al., 1995). Three distinct PGD synthases have been identified, including (1) glutathione (GSH)-independent PGD synthase, originally identified as β-trace in the cerebrospinal fluid (Clausen, 1961), also known as brain-type

PGD synthase (Shimizu et al., 1979; Urade et al., 1985); (2) GSH-dependent PGD synthase, also called spleen-type PGD synthase (Christ-Hazelhof and Nugteren, 1979; Urade et al., 1987); and (3) GSH-S-transferase (Ujihara et al., 1988).

GSH-independent PGD synthase is mainly localized in epithelia or near the blood-tissue barriers (Hoffmann et al., 1996). Similar to other PG synthases, it is found in the rough endoplasmic reticulum and the nuclear membrane; however, it is also detected in biological fluids such as in the cerebrospinal fluid, seminal fluid, and amniotic fluid (Ito et al., 1989).

The complementary DNA for GSH-independent PGD synthase has been isolated in several species (Urade et al., 1995). A homology search has revealed that GSH-independent PGD synthase is a new member of the lipocalin family, which is composed of secretory proteins that bind and transport small lipophilic molecules (Tanaka et al., 1997). There is increasing evidence suggesting that GSH-independent PGD synthase may play important role in the reproductive system. A recent study has demonstrated that GSH-independent PGD synthase is an important molecule in testicular and epididymal function and that it is likely involved in spermatogenesis and sperm maturation (Sorrentino et al., 1998).

GSH-dependent PGD synthase was first purified from rat spleen (Christ-Hazelhof and Nugteren, 1979), and it is considered as a member of the GSH-S-transferase family, although the molecular cloning of cDNA for this enzyme has not been reported.

2.8.2. PGE synthase

Although PGE_2 is synthesized by diverse cells and tissues, little is known about PGE synthase. Because GSH-S-transferase can catalyze the

conversion of PGH2 to PGE2, it may function as PGE synthase *in vivo* (Urade et al., 1995). A recent study has shown that PGE synthase activity in kidney requires glutathione (GSH) (Watanabe et al., 1997). In contrast, this enzyme activity in the heart, spleen, and uterine microsomes did not require GSH for its catalytic activity, suggesting that two different types of PGE synthases, GSH-dependent and GSH-independent enzymes, are present in microsomal fractions of rat tissues (Watanabe et al., 1997). The molecular cloning of this enzyme has not been reported.

2.8.3. PGF synthase

Three biosynthetic pathway of $PGF_{2\alpha}$ have been postulated, including (1) 9,11-endoperoxide reduction of PGH2, (2) 9-ketoreduction of PGE2, and (3) 11-ketoreduction of PGD2 (Urade et al., 1995).

Two groups identified independently the enzymatic 11-ketoreduction of PGD2 in rabbit liver (Wong, 1981) and in rat lung (Watanabe, 1981). This enzyme was then purified from bovine lung (Watanabe et al., 1985), and then named PGF synthase (Urade et al., 1995).

Two isoforms of PGF synthase have been identified by cDNA cloning, a lung form (Watanabe et al., 1985) and a liver form (Chen et al., 1992). The two isoforms differ only eleven nucleotides, and it is still unclear if they are derived from distinct genes (Urade et al., 1995). Sequence analyses have revealed that bovine lung PGF synthase is 65% identical to human liver aldehyde reductase in amino acid level, and is 77% identical to ρ-crystallin from the eye lens of frogs, suggesting that PGF synthase belongs to the aldo-keto reductase family (Urade et al., 1995).

Little information is available about the regulation of PGF synthase in reproduction system. Immunoblot analysis with anti-bovine PGF synthase

antiserum demonstrated that a 36-kDa protein band corresponding to PGF synthase increased in the uterus during diestrus and late pregnancy, suggesting that $PGF_{2\alpha}$ production may change during the estrous cycle and increase at term (Unezaki et al., 1996). A recent study *in vitro* has shown that interferon-τ decreases PGFS mRNA in both stromal and epithelial cells, and this was associated with the increase in $PGE_2/PGF_{2\alpha}$ ratio (Xiao et al., 1998).

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

The general hypothesis of my Ph.D. thesis was that the preovulatory rise in gonadotropins induces the expression of PGHS-2 gene in granulosa cells of bovine preovulatory follicles, and that unique molecular mechanisms are responsible for the delayed *trans*-activation of PGHS-2 in this species.

3.2. Objectives

The project consisted of three specific objectives:

A. To establish that the induction of PGHS-2 in bovine preovulatory follicles is a physiological event triggered by the endogenous LH surge.

B. To characterize the expression of PGHS-2 in bovine follicles during superovulation, and relate it to changes in follicular steroidogenesis prostaglandin production and cumulus-oocyte complex morphology.

C. To elucidate some of the molecular mechanisms involved in the delayed induction of PGHS-2 in granulosa cells of bovine preovulatory follicles.

4. ARTICLE ONE

Prostaglandin G/H synthase-2 is expressed in bovine preovulatory follicles after the endogenous surge of luteinizing hormone

rostaglandin G/H Synthase-2 Is Expressed in Bovine Preovulatory Follicles after ıe Endogenous Surge of Luteinizing Hormone[1]

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BSTRACT

To determine whether prostaglandin G/H synthase-2 (PGHS- induction occurs under physiological conditions, heifers were /ariectomized and preovulatory follicles were isolated 0 (n = , 18 (n = 4), and 24 h (n = 4) after the onset of estrus. The me of the LH surge was determined in blood samples collected /ery 4 h pre- and postestrus. Preparations of follicle wall and olated theca interna and granulosa cells were analyzed for GHS-2 protein and mRNA by immunoblot and Northern blot, spectively. Immunohistochemistry was used to document the situ localization of PGHS-2 protein, and follicular fluid con- ntrations of prostaglandin (PG) E_2 and $PGF_{2\alpha}$ were determined monitor changes in PG synthetic activities. Results showed ıat PGHS-2 mRNA (4.0 kilobases) and protein (74 000 M_r) ere absent in preovulatory follicles isolated at the onset of strus, low in follicles obtained 18 h after estrus (16.0 ± 1.2 h ost-LH surge), and markedly induced 24 h postestrus (20 ± 0 post-LH surge). Immunoblot and immunohistochemical anal- ses revealed that PGHS-2 protein was selectively induced in anulosa cells. Follicular fluid concentrations of PGE_2 and $GF_{2\alpha}$ increased significantly ($p < 0.01$) between 0 and 24 h ter estrus (from 2.8 ± 0.2 to 87.9 ± 30.9 ng/ml for PGE_2; om 0.05 ± 0.02 to 68.9 ± 23.6 ng/ml for $PGF_{2\alpha}$). Collectively, ıese results clearly demonstrate that the induction of PGHS-2 bovine preovulatory follicles is a physiological event that oc- urs after the endogenous LH surge.

NTRODUCTION

Prostaglandins are important mediators of a variety of iological processes such as inflammation, immune re- ponses, mitogenesis, ovulation, parturition, and carcino- enesis [1–4]. Prostaglandin G/H synthase (PGHS) is the rst rate-limiting enzyme in the conversion of arachidonic cid to prostaglandins and other prostanoids [1–4]. The en- yme was purified 20 yr ago from ovine and bovine seminal esicles [5, 6]. The existence of two distinct PGHS iso- orms, referred to as PGHS-1 and PGHS-2 (or cyclooxy- enase-1 and -2), has been clearly documented. The two soforms are derived from distinct genes located on differ- nt chromosomes and encoding different-sized mRNAs [4, –10]. Despite these differences, PGHS-1 and PGHS-2 pro- eins are relatively similar in size (about 70 000 M_r). Also, ll important structural and catalytic domains are highly onserved between the two isoforms, and a 60% identity is bserved in the amino acid sequence [11, 12]. However, the egulation of PGHS-1 and PGHS-2 expression differs markedly. PGHS-1 is expressed constitutively in a variety of mammalian tissues and is believed to function as a housekeeping gene involved in the synthesis of PG necessary for normal physiological functions [3, 4]. In contrast, while PGHS-2 is undetectable in most mammalian tissues, it is dramatically induced by a wide variety of extracellular and intracellular stimuli such as growth factors, cytokines, tumor promoter, and gonadotropins [3, 4, 10, 13, 14]. Differences in the primary structure of PGHS-1 and PGHS-2 promoters likely explain distinct patterns of gene expression [4]. Recent gene-targeting studies suggest that the control of prostaglandin synthesis during inflammatory processes is not under the control of a single PGHS isoform [15, 16].

The ovulatory process has been compared to an inflammatory reaction [17]. Levels of prostaglandins are dramatically increased in preovulatory follicles of a variety of animal species preceding follicular rupture [18]. This rise in prostaglandin synthesis results from a gonadotropin-dependent and cell type-specific induction of PGHS [19, 20]. Recent studies have documented that high levels of gonadotropins cause a rapid and transient induction of PGHS-2, but not of PGHS-1, mRNA and protein in granulosa cells of rat preovulatory follicles in vivo [21–23]. A similar induction was observed in vitro in cultures of rat granulosa cells and intact preovulatory follicles stimulated with LH, FSH, forskolin, and GnRH [22, 24, 25]. Functional analyses of the rat PGHS-2 promoter in granulosa cells have identified a CAAT box and an E box as putative modulators of PGHS-2 gene expression by gonadotropins [26, 27].

Little is known about the control of prostaglandin synthesis in large monoovulatory species. As in the rat, the regulation of PGHS-2 in bovine follicles has been reported only in models of induced ovulation, and not under physiological conditions. With a model using hCG, PGHS-2 was shown to be induced in bovine preovulatory follicles in vivo [28]. Interestingly, a striking difference was observed in the time course of PGHS-2 induction in rat (4 h post-hCG) versus bovine (18 h post-hCG) preovulatory follicles. However, the interval of time from PGHS-2 induction to ovulation appeared highly conserved in both species (about 10 h) [28]. These results suggested that differences in the regulation of PGHS-2 gene expression could play a key role in defining the species-specific length of the ovulatory process. With a different model, Tsai et al. [29] recently reported the induction of PGHS-2 mRNA in bovine preovulatory follicles 24 h after the administration of GnRH in vivo. However, since GnRH itself was shown to induce PGHS-2 in rat preovulatory follicles in vitro [22, 24], and to induce ovulation in hypophysectomized rats and in perfused rabbit and rat ovaries in vitro [30–32], it is unclear whether the induction of PGHS-2 in bovine follicles after the administration of GnRH in vivo resulted from a direct ovarian effect or not. The objective of this study was to demonstrate that the induction of PGHS-2 in bovine preo-

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vulatory follicles is a physiological event triggered by the natural endogenous LH surge. Therefore, the regulation of PHGS-2 mRNA, protein, and activity was characterized in preovulatory follicles of heifers that had not received exogenous gonadotropins or GnRH.

MATERIALS AND METHODS

Materials

Diethyldithiocarbamic acid (DEDTC), octyl β-D-glucopyronoside (octyl glucoside), diaminobenzidine tetrahydrochloride, and diethyl ether were purchased from Sigma Chemical Company (St. Louis, MO); Lutalyse from Upjohn (Kalamazoo, MI); APL (hCG) from Ayerst Laboratories (Montreal, PQ, Canada); ^{125}I-protein A and Biotrans nylon membranes (0.2 μm) from ICN Pharmaceuticals (Montreal, PQ, Canada); [α-^{32}P]dCTP, [^{3}H]prostaglandin E_2, and [^{3}H]prostaglandin (PG) $F_{2\alpha}$ from DuPont NEN Research Products (Mississauga, ON, Canada); PGE_2 and $PGF_{2\alpha}$ antibodies from Advanced Magnetics Inc. (Cambridge, MA); [^{125}I]progesterone from Amersham (Oakville, ON, Canada); [^{125}I]estradiol-17β and antibody from Inter Medico (Willowdale, ON, Canada); nitrocellulose membranes (0.45 μm) from Schleicher & Schuell (Kneene, NH); Rainbow molecular weight markers from Amersham (Arlington Heights, IL); QuikHyb from Stratagene Cloning Systems (La Jolla, CA); RNA ladder (0.24–9.5 kilobases) from Gibco BRL Life Technologies (Gaithersburg, MD); Prime-a-Gene labeling system from Promega (Madison, WI); Vectastain ABC kit from Vector Laboratories (Burlingame, CA); Kodak film X-OMAT AR from Eastman Kodak Company (Rochester, NY); PBS from Gibco (Grand Island, NY); and Bio-Rad Protein Assay and electrophoretic reagents from Bio-Rad Laboratories (Richmond, CA).

Animals and Experimental Design

To obtain a relatively homogenous population of bovine preovulatory follicles, Holstein heifers (2–3 yr old) were injected on Day 7 of the estrous cycle (Day 0 = day of estrus) with 25 mg $PGF_{2\alpha}$ (Lutalyse) to induce luteolysis. In this model, the developing dominant follicle present on Day 7 consistently becomes a preovulatory follicle when luteal regression is induced [28, 33–35]. Animals were observed every 4 h after the administration of $PGF_{2\alpha}$ to determine the onset of standing estrus. The ovary bearing the preovulatory follicle was removed by ovariectomy via colpotomy 0 (group 1, n = 4), 18 (group 2, n = 4), and 24 h (group 3, n = 4) after the onset of estrus, as described previously [28]. The ovary was immediately transferred into ice-cold PBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Prior to ovariectomy, ovarian follicular development was monitored daily by transrectal ultrasonography with a 5-MHz probe starting on Day 3 of cycle to identify the ovary bearing the developing preovulatory follicle [28]. Animals were bled every 12 h between Days 3 and 7, and every 4 h between the injection of $PGF_{2\alpha}$ and the ovariectomy, to characterize changes in progesterone concentrations and to determine the time of the LH surge. Blood samples (10 ml) were collected from the coccygeal vein into Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged at 4°C for 10 min; the extracted plasma was stored at −20°C. All animal procedures were approved by the Institutional Animal Care and Use Committee (Comité de déontologie animale) of the Université de Montréal.

Isolation of Bovine Preovulatory Follicles

The preovulatory follicle was dissected from the ovary with a scalpel, and the follicular fluid was aspirated and stored at −70°C until assayed for progesterone, estradiol-17β, PGE_2, and $PGF_{2\alpha}$. The preovulatory follicle was cut into four pieces: one was fixed in formalin and embedded in paraffin, and three pieces were used to obtain preparations of follicle wall (theca interna with attached granulosa cells) and isolated theca interna and granulosa cells as previously described [28]. Samples were stored at −70°C.

Cell Extracts and Immunoblot Analysis

Solubilized cell extracts were prepared as previously described [28], with minor modifications. Briefly, tissues were homogenized on ice in 700 μl TED homogenization buffer (50 mM Tris, 10 mM EDTA, 1 mM DEDTC, pH 8.0) supplemented with 2 mM octyl glucoside and centrifuged at 30 000 × *g* for 1 h at 4°C. The crude pellets containing membranes, nuclei, and mitochondria were sonicated (5 sec/cycle; 4 cycles) in 250 μl TED sonication buffer (20 mM Tris, 50 mM EDTA, 0.1 mM DEDTC, pH 8.0) containing 32 mM octyl glucoside. The sonicates were centrifuged at 13 000 × *g* for 25 min at 4°C. The supernatants (solubilized cell extracts) were stored at −70°C until immunoblot analysis. The protein concentration was determined by the method of Bradford (Bio-Rad Protein Assay) [36].

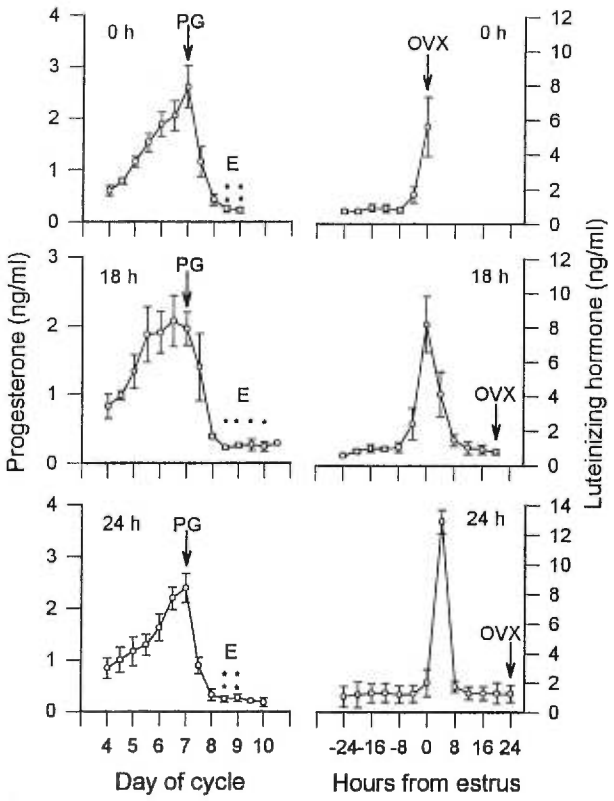
Proteins (50 μg) of cell extracts were resolved by one-dimensional SDS-PAGE and electrophoretically transferred onto nitrocellulose filters. Filters were incubated with anti-PGHS antibody 9181, and ^{125}I-labeled protein A was used to visualize immunopositive proteins as previously described [28]. Antibody 9181 had been shown earlier to recognize both bovine PGHS-1 and PGHS-2 [28]. Filters were exposed to x-ray film at −70°C. The relative intensity of PGHS-2 signal was quantified by densitometric analysis (Collage Analysis; Fotodyne Inc., Hartland, WI).

RNA Isolation and Northern Analysis

Cytoplasmic RNA was isolated from follicle wall with an extraction buffer containing 1% Nonidet P-40 (Sigma) and was quantified by measurement of its absorbance at 260 nm, as previously described [22]. For Northern analysis, RNA samples (20 μg) were denatured at 55°C for 15 min in 45% formamide and 5.4% formaldehyde, separated by electrophoresis in a 1% agarose gel, and transferred onto a nylon membrane as described previously [22, 28]. The blot was prehybridized and hybridized to a ^{32}P-labeled cDNA probe encoding mouse PGHS-2 using the QuikHyb solution (Stratagene). After the radioactivity was stripped with 0.1% saline-sodium citrate (SSC)-0.1% SDS for 30 min at 100°C, the same blot was hybridized with the rat elongation factor Tu cDNA as a control gene for RNA loading [37]. Each probe was labeled with the Promega DNA labeling kit by using [α-^{32}P]dCTP to a final specific activity greater than 1×10^8 cpm/μg DNA. The relative intensity of signal was quantified by densitometry (Collage Analysis; Fotodyne Inc.).

Immunohistochemistry

Immunohistochemical staining for PGHS was performed using the Vectastain avidin:biotin complex (ABC kit; Vector Laboratories), as previously described [38]. Briefly, pieces of follicles were fixed in formalin and embedded in



1. Plasma concentrations of progesterone (left graphs) and LH (right ihs) in heifers ovariectomized 0 (top graphs), 18 (middle graphs), and ı (bottom graphs) after the onset of estrus. In left graphs, arrows in- te the time of PG injection, and asterisks indicate onset of estrus (E) ıdividual animals. In right graphs, results are presented according to et of estrus (0 h), and arrows indicate the time of ovariectomies (OVX). ılts are shown as means ± SEM (n = 4 animals per time point).

affin, and 3-μm-thick sections were deparaffinized ɔugh graded alcohol series. Endogenous peroxidase was ɛnched by incubating the slides in 0.3% hydrogen per- de in methanol for 30 min. After rinsing in PBS for 15 ı, sections were incubated with diluted normal goat se- ı for 20 min at room temperature. Primary anti-PGHS ibody 9181 (diluted at 1:100) was applied, and sections ɛ incubated overnight at 4°C. Control sections were in- ated with PBS only or with anti-PGHS antibody 8223 : selectively identifies PGHS-1 [21, 23, 28]. After rins- in PBS for 10 min, a biotinylated goat anti-rabbit an- ɔdy (1:222 dilution) was applied, and sections were in- ated for 45 min at room temperature. Sections were ɜhed in PBS for 10 min and then incubated with the dlin DH-biotinylated horseradish peroxidase H reagents ctastain ABC kit) for 45 min at room temperature. After 0-min PBS wash, the reaction was revealed using diaminobenzidine tetrahydrochloride as the peroxidase substrate. Sections were counterstained with Gill's hematoxylin stain and mounted.

Hormone Assays

Concentrations of progesterone and estradiol-17β were measured by specific RIAs in nonextracted aliquots of plasma and follicular fluid, as previously described [39, 40]. The sensitivity of the progesterone assay was 3.12 pg/assay tube, and the intra- and interassay coefficients of variation were 10.6% and 9.9%, respectively. The sensitivity of the estradiol assay was 0.16 pg/assay tube, and the intraassay coefficient of variation was 7.9% (all samples were analyzed in a single assay).

To determine the concentrations of PGE_2 and $PGF_{2\alpha}$ in follicular fluid, samples were extracted with diethyl ether and assayed by specific RIAs as previously described [28]. Since the $PGF_{2\alpha}$ antibody cross-reacts 100% with $PGF_{1\alpha}$, prostaglandins measured with the assay were referred to as total PGF. Recovery rates for PGE_2 and $PGF_{2\alpha}$ were 81.7% and 89.3%, respectively. All samples were analyzed in a single assay, and intrassay coefficients of variation for PGE_2 and $PGF_{2\alpha}$ were 5.9% and 5.1%, respectively.

Concentrations of LH in plasma samples were determined by a specific RIA as previously described [41]. Bovine LH (USDA-bLH-B-5) was used as standard and ovine LH (NIDDK-oLH-I-2; Baltimore, MD) for radioiodination. The first antibody was a rabbit anti-ovine LH (NIDDK anti-oLH-1). The sensitivity of the assay was 0.06 ng/assay tube, and the intra- and interassay coefficients of variation were 14.5% and 15.2%, respectively.

Statistical Analysis

Results are presented as means ± SEM. Owing to the heterogeneous variance in follicular fluid steroid and prostaglandin concentrations, logarithmic transformations were performed prior to analysis. One-way ANOVA was used to test the effect of time of ovariectomy on concentrations of steroid hormones and prostaglandins in follicular fluid, on the interval between prostaglandin injection and the LH surge (peak value), and on the interval between the LH surge (peak value) and the ovariectomy. When ANOVAs indicated significant differences ($p < 0.05$), Scheffé's test was used to compare individual means. All statistical analyses were performed according to the Statistical Analysis System (SAS Institute Inc., Cary, NY).

RESULTS

Plasma and Follicular Concentrations of Hormones

To define the endocrine environment prior to the ovariectomy, progesterone concentrations in plasma were char-

LE 1. Relationship between time of induced luteolysis (PG), onset of estrus, LH surge, and ovariectomy (ovx).

tment p*	No. of heifers	Interval of time (h)		
		PG-estrus†	PG-LH surge	LH-ovx
	4	49.5 ± 1.5[a] (48–54)	—‡	—‡
	4	58.3 ± 5.3[a] (44.5–68.5)	60.3 ± 5.3[b] (48–68.5)	16 ± 1.2[c] (14–18)
	4	49.6 ± 3.9[a] (40–58.5)	53.6 ± 3.9[b] (44–62.5)	20 ± 0[d] (20)

ɛfined as the interval of time from onset of estrus to ovariectomy.
fined as the interval of time from PG injection to onset of estrus.
ludes two heifers that had not initiated an LH surge at the time of the surgery (see text).
Data with different superscripts within the same column differ significantly ($p < 0.05$; mean ± SEM).

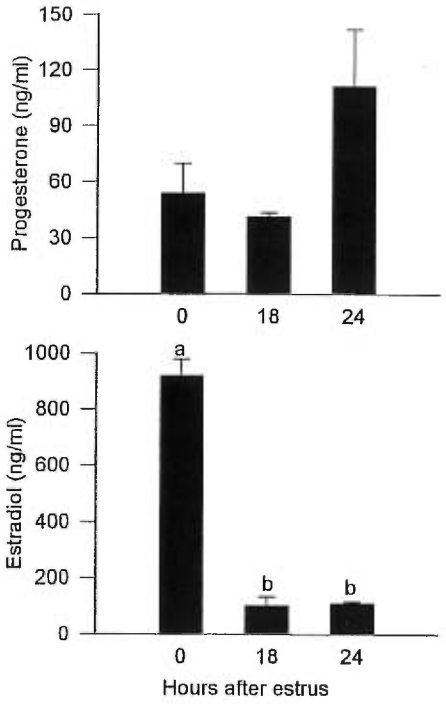


FIG. 2. Concentrations of progesterone (top) and estradiol-17β (bottom) in follicular fluid of bovine preovulatory follicles isolated 0, 18, and 24 h after the onset of estrus. Bars with different superscripts are significantly different ($p < 0.05$). Results are shown as means ± SEM (n = 4 follicles per time point).

acterized before and after prostaglandin injection, and the time of the LH surge was determined. Results show that plasma progesterone concentrations increased between Days 4 and 7 (Day 0 = estrus) in all three groups (Fig. 1, left graphs). After induction of luteolysis on Day 7, progesterone levels dropped to an average of 0.22 ± 0.03 ng/ml at the time of ovariectomy (Fig. 1, left graphs). No significant differences were observed among groups in the interval of time from prostaglandin injection to the onset of estrus (Table 1). An LH surge was observed in 2 of 4, 4 of 4, and 4 of 4 heifers ovariectomized 0, 18, and 24 h after the onset of estrus, respectively (Fig. 1, right graphs). Intervals of time between the LH surge and the ovariectomies in the last two groups were 16.0 ± 1.2 and 20 ± 0 h ($p < 0.05$), respectively (Table 1).

Follicular fluid concentrations of progesterone and estradiol-17β were measured to determine the developmental stages of preovulatory follicles at the time of ovariectomy. Results show that follicles isolated at the onset of estrus had relatively low levels of progesterone (54.1 ± 15.5 ng/ml; Fig. 2, top graph) and high levels of estradiol (920 ± 57.6 ng/ml; Fig. 2, bottom graph). Progesterone concentrations tended to increase in follicles collected 24 h after the onset of estrus (111.2 ± 30.9 ng/ml; $p = 0.07$), while a marked and significant decrease in estradiol concentrations was observed in follicles isolated 18 h (101.9 ± 35.6 ng/ml; $p < 0.05$) and 24 h (109.8 ± 7.0 ng/ml; $p < 0.05$) after estrus (Fig. 2). These results showed that follicles isolated at 18 and 24 h postestrus had responded to the endogenous LH surge.

Expression of PGHS-2 in Bovine Preovulatory Follicles

To determine whether PGHS-2 was expressed following the endogenous LH surge, cellular extracts were prepared

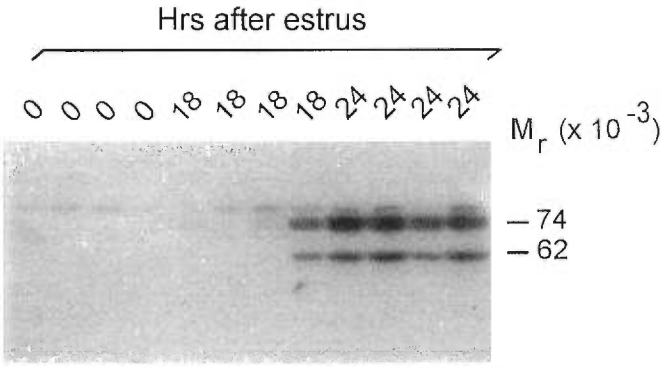


FIG. 3. Time-dependent expression of PGHS-2 protein in bovine preovulatory follicles. Cellular extracts were prepared from preovulatory follicles isolated 0, 18, and 24 h after the onset of estrus, and proteins (50 μg/lane) were analyzed by one-dimensional SDS-PAGE and immunoblotting techniques using anti-PGHS antibody 9181. Results from extracts of four follicles for each time point are shown. Markers on the right indicate migration of intact bovine PGHS-2 (74 000 M_r band) and a putative proteolytic fragment (62 000 M_r band). The filter was exposed to film at −70°C for 14 h.

from preovulatory follicles isolated 0, 18, and 24 h after the onset of estrus (equivalent in this study to 0, 16, and 20 h postestrus; Table 1). Extracted proteins were resolved by one-dimensional SDS-PAGE and analyzed by Western blots using anti-PGHS antibody 9181. Results show no immunoreactive PGHS-2 protein in follicles isolated at the onset of estrus (0 h), a weak but detectable signal in three of four follicles isolated at 18 h postestrus, and a strongly immunoreactive signal in all four follicles isolated 24 h after the onset of estrus (Fig. 3). A 400-fold increase was observed in the relative abundance of PGHS-2 protein at 24 h versus 0 h postestrus. Immunoreactive bovine PGHS-2 appeared as a 74 000 M_r band (intact form) and a 62 000 M_r band (proteolytic fragment).

Cellular Localization of PGHS-2 in Bovine Preovulatory Follicles

To determine the cellular localization of follicular PGHS-2 expression, isolated preparations of theca interna

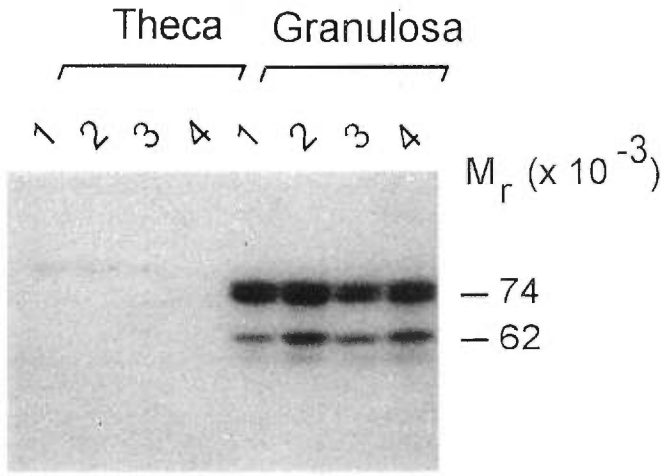
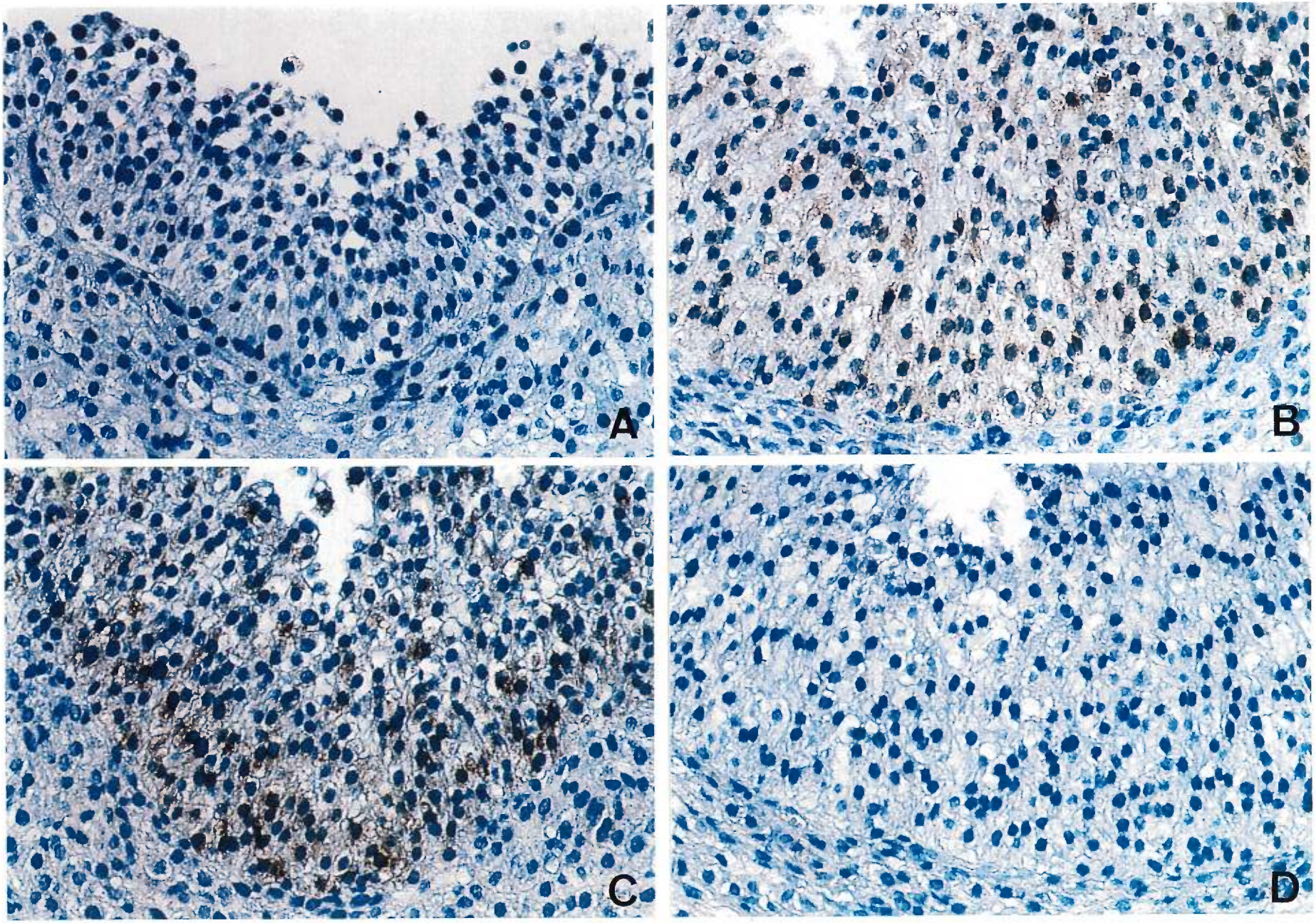


FIG. 4. Cell-dependent expression of PGHS-2 in bovine preovulatory follicles. Preparations of granulosa cells and theca interna were obtained from four preovulatory follicles isolated 24 h after the onset of estrus. Cellular extracts were prepared and proteins (50 μg/lane) were analyzed by one-dimensional SDS-PAGE and immunoblotting techniques. Markers on the right indicate migration of intact bovine PGHS-2 (74 000 M_r band) and a putative proteolytic fragment (62 000 M_r band). The filter was exposed to film at −70°C for 13 h.

24 h after the onset of estrus. Immunostaining results with anti-PGHS antibody 9101 show the absence of PGHS-2 positive granulated cells in a pre[illegible] but a marked staining in granulosa cells of two follicles isolated 24 h after the onset of estrus (**B, C**). No staining was present when antibody 8223 (selective for PGHS-1) was used (**D**). ×400 (reproduced at 95%).



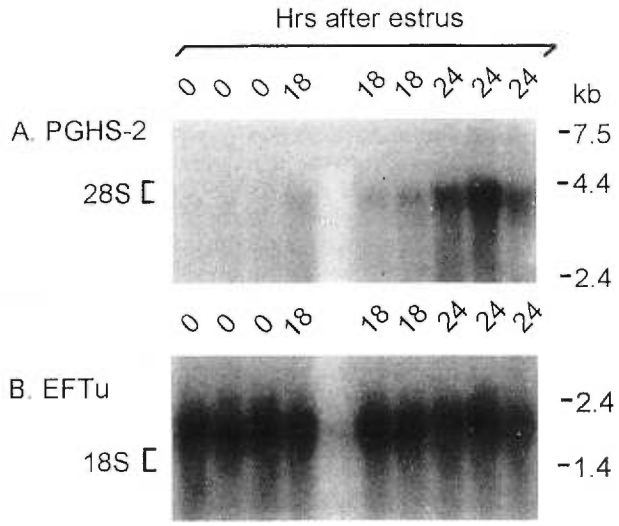


FIG. 6. Time-dependent induction of PGHS-2 mRNA in bovine preovulatory follicles. Total RNA was extracted from preovulatory follicles and analyzed by Northern blots using a ^{32}P-labeled cDNA encoding the mouse PGHS-2 (**A**). The blot was stripped of radioactivity and rehybridized with the rat elongation factor Tu (EFTu) as a control gene for RNA loading (**B**). Brackets on the left show the migration of 28S and 18S ribosomal bands, and markers on the right indicate migration of RNA standards. Filters **A** and **B** were exposed to film at −70°C for 18 and 11 h, respectively.

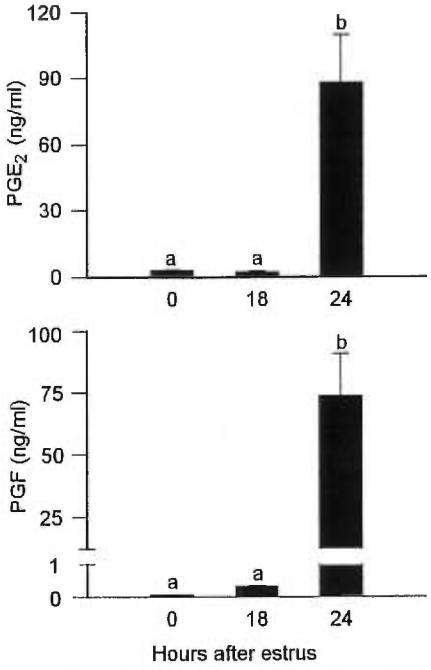


FIG. 7. Concentrations of PGE_2 (top) and $PGF_{2\alpha}$ (bottom) in follicular fluid of bovine preovulatory follicles. Bars with different superscripts are significantly different ($p < 0.05$). Results are shown as means ± SEM (n = 4 follicles per time point).

and granulosa cells were obtained from four follicles collected 24 h after the onset of estrus, and extracts were analyzed by SDS-PAGE and Western blots. Results clearly indicated the presence of PGHS-2 in granulosa cells (Fig. 4). In contrast, PGHS-2-immunoreactive proteins were not detected in extracts of theca interna.

To further study the cell-specific localization of PGHS-2, immunohistochemistry was performed on formalin-fixed sections of bovine preovulatory follicles isolated 0 and 24 h after the onset of estrus. Results showed no PGHS-2 staining in follicles isolated at the onset of estrus (Fig. 5A). In contrast, a distinct positive signal was observed in granulosa cells of follicles isolated 24 h after the onset of estrus (Fig. 5, B and C). The staining was particularly intense around the nucleus. No PGHS-2 immunoreactivity was detected when adjacent sections of follicles isolated 24 h postestrus were first incubated with PBS alone (data not shown) or with a PGHS-1-selective antibody (Fig. 5D).

Time-Dependent Expression of PGHS-2 mRNA in Preovulatory Follicles

To determine whether the expression of PGHS-2 protein after the endogenous LH surge was associated with the induction of specific transcripts, RNA was extracted from follicles and analyzed by Northern blots. Results show no PGHS-2 transcript in follicles isolated at the onset of estrus (0 h), a faint but detectable signal in follicles isolated 18 h postestrus, and a stronger signal in follicles isolated 24 h after onset of estrus (Fig. 6). A 79-fold increase was observed in the relative abundance of PGHS-2 mRNA at 24 h versus 0 h postestrus.

Concentrations of Prostaglandins in Follicular Fluids

To document whether the induction of PGHS-2 mRNA and protein in preovulatory follicles after the endogenous LH surge resulted in increased prostaglandin synthetic activities, concentrations of PGE_2 and $PGF_{2\alpha}$ were determined in follicular fluid of bovine preovulatory follicles. Figure 7 shows that while prostaglandin concentrations remained basal in follicles isolated 0 and 18 h postestrus, a significant increase in levels of PGE_2 and $PGF_{2\alpha}$ was observed in follicles obtained 24 h after the onset of estrus (87.9 ± 30.9 and 68.9 ± 23.6 ng/ml for PGE_2 and $PGF_{2\alpha}$ concentrations, respectively).

DISCUSSION

Our results demonstrate for the first time that the induction of PGHS-2 in preovulatory follicles is a physiological event that occurs after the natural endogenous LH surge. In previous studies, demonstrations of PGHS-2 induction in rat and bovine preovulatory follicles in vivo had been shown only in models of induced ovulation using exogenous hCG [19–23, 28] and GnRH [29]. It could be argued that the hCG induction of PGHS-2 was not physiological considering the heterologous nature of the agonist. In the GnRH study, the results could also be interpreted in the context that the decapeptide itself is known to directly induce PGHS-2 in vitro in rat preovulatory follicles [22, 24], and to cause ovulation in hypophysectomized rats and in perfused rabbit and rat ovaries [30–32]. Therefore, it was not clear whether the induction of PGHS-2 mRNA in bovine follicles after the administration of GnRH in vivo resulted from a direct ovarian effect or not. By omitting the use of exogenous gonadotropins or GnRH, the present study clearly establishes that PGHS-2 is induced in bovine preovulatory follicles after the natural endogenous surge of LH, that the induction is granulosa cell-specific, and that it is associated with increased follicular prostaglandin synthetic activities prior to ovulation.

Estrus in cattle lasts about 14–18 h, and the LH surge occurs within the first 6–8 h [42]. Ovulation is observed 12 to 16 h after the end of estrus, giving an interval of time between the onset of estrus and ovulation of about 30 h

. Since the precise time of the LH surge could only be ɪrmined retrospectively in this study, animals were mon-ɛd frequently, and the onset of estrus was used as the rence point for the isolation of preovulatory follicles ɔre and after the LH surge. Retrospective analyses of ulating levels of LH and follicular fluid concentrations teroids and prostaglandins clearly showed that all pre-latory follicles were indeed at their predicted develop-ıtal stage. In heifers ovariectomized at the onset of es-, two had initiated an LH surge while the other two e ovariectomized prior to the surge. However, all four .cles had elevated concentrations of estradiol character-: of a nonluteinized preovulatory phenotype. The LH ge was observed in all heifers ovariectomized 18 h and h after the onset of estrus, and the marked decline in icular concentrations of estradiol clearly indicated that preovulatory follicles had initiated luteinization. The ɛnce of a significant increase in follicular fluid concen-ons of progesterone at these time points, which corre-ıded to 16 h and 20 h post-LH surge, compares with ılts obtained in the hCG study in which the marked in-ıse in follicular fluid levels of progesterone was ob-ɣed only 24 and 26 h postgonadotropin [28].

The immunohistochemical results in this study clearly ɛnd the granulosa cell-specific nature of follicular HS-2. Moreover, intense PGHS-2 staining around gran-ɜa cell nuclei suggests its preferential subcellular local-ion. Morita et al. [43] have recently shown differing acellular location for PGHS-1 and PGHS-2 in fibro-ɜts and endothelial cells, with PGHS-2 being predomi-tly concentrated in the nuclear envelope.

One striking feature of PGHS-2 induction in bovine ɔvulatory follicles is its distinct time course. Results ob-ed in this study suggest that PGHS-2 is first expressed 18 h post-LH surge, which is in agreement with the time rse observed with hCG [28]. However, this is in sharp trast with the more rapid induction (2–4 h post-hCG) ɛrved in rat preovulatory follicles, and likely relates to erences in the species-specific length of the ovulatory cess [22, 28]. The relatively delayed induction of HS-2 by gonadotropins in bovine preovulatory follicles ers also from the very rapid induction (within 30 min) sed by tetradecanoyl phorbol acetate, forskolin, and se-ı in mouse 3T3 fibroblasts [12, 44]. While PGHS-2 is sidered an immediate-early response gene in these cells, ɛr studies have shown that protein synthesis is required PGHS-2 gene expression in granulosa cells [45] and efore PGHS-2 is not a primary response gene in ovarian s.

The precise role of prostaglandin synthesis during the latory process remains to be established. Reich et al.] have proposed that eicosanoids are required for the ıction of collagenolytic activity needed for follicular ture. The necessary role of PGHS-2 expression during ovulatory process was also recently underscored in a e-targeting study [15] in which infertility was observed emale, but not male, PGHS-2 null mice, despite nu-ous matings. The infertility was associated with the vir- absence of corpora lutea in the presence of normal icular development and suggested an impaired ovula-ɣ process.

The molecular mechanisms involved in the hormonal ılation of PGHS-2 expression in granulosa cells have n extensively studied in the rat [25–27, 47]. Multiple ıaling pathways such as protein kinase A, protein kinase and tyrosine kinase pathways, but not the calmodulin kinase pathway, are involved in the agonist induction of PGHS-2 in rat granulosa cells in vitro [25, 47]. The isolation of the rat PGHS-2 promoter [26, 27] and studies of its regulation in granulosa cells have shown that the upstream regulatory factor binds to an E-box region located between −67 and −39 base pairs from the transcriptional start site and that it plays a critical role in the transcriptional activation of the promoter by gonadotropins [27]. It remains unclear whether the delayed induction of PGHS-2 expression observed in species with a long ovulatory process relates to differences in signaling pathways and/or in the assembly of the transcriptional machinery. The isolation of the bovine PGHS-2 gene and the functional characterization of its promoter should help unravel the molecular basis for the species-specific regulation of PGHS-2 in granulosa cells.

In summary, the present study documents that the induction of PGHS-2 in bovine preovulatory follicles is a physiological event that is triggered by the endogenous LH surge, that the induction is time dependent and granulosa cell specific, and that it is associated with increased follicular prostaglandin synthetic activities prior to ovulation.

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5. ARTICLE TWO

Follicle size-dependent induction of prostaglandin G/H synthase-2 during superovulation in cattle

Follicle Size-Dependent Induction of Prostaglandin G/H Synthase-2 during Superovulation in Cattle[1]

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ABSTRACT

Under physiological conditions, prostaglandin G/H synthase-2 (PGHS-2) is induced in bovine preovulatory follicles by the endogenous surge of gonadotropins. To characterize the pattern of follicular PGHS-2 expression during superovulation in cattle, heifers were treated with exogenous FSH and ovulation was induced with hCG. Animals were ovariectomized 0, 18, and 24 h post-hCG, and extracts of follicles ≥ 6 mm were analyzed by Western blotting. Follicular fluid concentrations of prostaglandin (PG) E_2, $PGF_{2\alpha}$, progesterone, and estradiol-17β were determined by RIAs, and the morphology of the cumulus oocyte complex was examined. Results showed that PGHS-2 protein was absent in all follicles isolated at 0 h post-hCG (n = 119) and in small follicles (6 to < 8 mm) isolated between 0 and 24 h post-hCG (n = 27 follicles). In contrast, 12.3% of medium (8 to < 10 mm) and 43.7% of large (≥ 10 mm) follicles were PGHS-2-positive at 18 h post-hCG, and these percentages rose at 24 h to 45.9% and 91.0% in medium and large follicles, respectively ($p < 0.05$). Follicular fluid concentrations of PGE_2 and $PGF_{2\alpha}$ were low in follicles isolated at 0 h and increased only in PGHS-2-positive follicles isolated 24 h post-hCG ($p < 0.05$). Concentrations of progesterone and estradiol-17β at 0 h were 28.2 ± 5.8 and 291.8 ± 13.0 ng/ml, respectively, and a shift from estradiol-17β to progesterone dominance (luteinization) occurred at 24 h post-hCG only in PGHS-2-positive follicles. Also, expansion of the cumulus oocyte complex was detected at 24 h post-hCG only in PGHS-2-positive follicles. Lack of PGHS-2 induction in follicles of ovulatory size (> 8 mm) was associated with an apparent failure to respond to hCG (absence of luteinization and cumulus expansion). Collectively, these results demonstrate the presence of a time- and follicle size-dependent induction of PGHS-2 in bovine follicles during superovulatory treatment and suggest that PGHS-2 expression can be used as a marker for follicular commitment to ovulation during ovarian hyperstimulation protocols.

INTRODUCTION

Prostaglandin G/H synthase (PGHS), also known as cyclooxygenase (COX), is a key rate-limiting enzyme in the prostaglandin biosynthetic pathway, as it catalyzes the conversion of arachidonic acid into prostaglandin H_2 (PGH_2), a common precursor for all prostaglandins, prostacyclins, and thromboxanes [1–3]. Two genes encoding distinct PGHS isoforms, referred to as PGHS-1 and PGHS-2, have been characterized [4–6]. The PGHS-1 and -2 proteins are about 60% identical at the amino acid level, but differences in their patterns of expression and regulation suggest that the two isoforms could serve different biological functions [4, 5].

Regulation of PGHS enzyme expression has been demonstrated during various reproductive functions, including early embryonic development and implantation [7–10], parturition [11–13], and luteolysis [14, 15]. The process of ovulation is another reproductive event during which the synthesis of prostaglandins is required [16, 17]. The marked increase in prostaglandin synthesis in rat follicles before ovulation is related to the induction of PGHS, and more specifically PGHS-2 [18–21]. The induction is rapid (2–4 h post-hCG), occurs only in granulosa cells, and is caused by high (ovulatory) levels of gonadotropins. Comparative studies in cattle and horses showed that the selective induction of PGHS-2 in granulosa cells is a molecular mechanism conserved in mono-ovulatory species with a long ovulatory process [22–25]. The time course of PGHS-2 induction in bovine (18 h post-hCG) and equine (30 h post-hCG) preovulatory follicles is delayed as compared to the rapid induction observed in rats (2–4 h post-hCG). However, the interval from PGHS-2 induction to follicular rupture is remarkably conserved (~10 h) in all species, suggesting that PGHS-2 expression could serve as an alarm that controls the mammalian ovulatory clock [26].

The administration of exogenous gonadotropins in cattle is used to override the intrinsic mechanisms of follicular recruitment and selection, and to induce multiple ovulations [27–29]. Follicular rupture during superovulatory treatments was shown to occur almost exclusively in follicles that have reached a diameter of 8 mm [30]. However, despite increased understanding of the control of bovine follicular development and the production of purified gonadotropin preparations, the outcome of current superovulation treatments remains unpredictable [28, 31]. One undesirable effect of these protocols is the development of a proportion (up to 20%) of follicles greater than 8 mm that fail to ovulate and that eventually undergo atresia or become cystic [30, 32]. Understanding the development of such follicles during ovarian hyperstimulation has been hampered by the inability to distinguish within a population of follicles those committed to ovulation from those not committed. With the recent demonstration of a gonadotropin- and time-dependent induction of PGHS-2 in bovine preovulatory follicles during the estrous cycle [22, 23, 25], we hypothesized that expression of PGHS-2 could be used during superovulatory protocols as a marker of follicular commitment to ovulation. Therefore, the objective of this study was to characterize the pattern of expression of PGHS-2 in bovine superovulatory follicles and relate it to changes in follicular steroidogenesis, prostaglandin production, and cumulus-oocyte complex morphology.

MATERIALS AND METHODS

Materials

Diethyl ether, octyl β-D-glucopyronoside (octyl glucoside), and diethyldithiocarbamic acid were purchased from

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a (St. Louis, MO); Lutalyse from Upjohn (Kalamazoo, Folltropin-V from Vetrepharm Canada Inc. (London, Canada); APL (hCG) from Ayerst Laboratories ıtreal, PQ, Canada); ^{125}I-Protein A and Biotrans nylon branes (0.2 μm) from ICN Pharmaceuticals (Montreal, Canada); prostaglandin E_2 (PGE_2) and prostaglandin ($PGF_{2\alpha}$) antibodies from Advanced Magnetics Inc. ıbridge, MA); ^{125}I-progesterone from Amersham (Oak- ON, Canada); [^{3}H]PGE_2 and [^{3}H]$PGF_{2\alpha}$ from DuPont Research Products (Mississauga, ON, Canada); ^{125}I-diol-17β and antibody from Inter Medico (Willowdale, Canada); nitrocellulose membranes (0.45 μm) from :icher & Schuell (Keene, NH); rainbow molecular ht markers from Amersham (Arlington Heights, IL); from Gibco Bethesda Research Laboratories Life nologies Inc. (Gaithersburg, MD); Bio-Rad protein as- nd electrophoretic reagents from Bio-Rad Laboratories ımond, CA); and Kodak film X-OMAT AR from East- Kodak Company (Rochester, NY).

ıals, Superovulatory Protocol, and Ovariectomy

velve Holstein heifers (2–3 yr old) were subjected to ndard superovulatory regimen initiated between Days d 10 of the estrous cycle. The treatment consisted of a of 320 mg FSH (Folltropin-V) given in 8 decreasing tions 12 h apart over a period of 4 days [33]. Luteal :ssion was induced by injecting i.m. 25 mg $PGF_{2\alpha}$ (Lu- e) at the fifth and sixth FSH injection. To precisely :ol the time of ovulation, hCG (2500 IU) was admin- :d i.v. 36 h after induction of luteolysis. Ovaries were :cted by ovariectomy via colpotomy at 0 (n = 4), 18 : 4), and 24 h (n = 4) after hCG. The ovaries were :ferred immediately into ice-cold PBS supplemented penicillin (50 U/ml)-streptomycin (50 μg/ml), and :ported to the laboratory. All animal procedures were oved by the Comité de déontologie animale of the 'ersité de Montréal.

ıtion of Follicles

ll follicles ≥ 6 mm were carefully dissected from the ›unding ovarian tissue with a scalpel and were mea- d before being cut with fine scissors. The morphology ıe cumulus oocyte complex (absence or presence of ınsion) was examined under a dissecting microscope, the follicular fluid was recovered and stored at −70°C assayed for progesterone, estradiol-17β, PGE_2, and $_{2\alpha}$. The theca externa and other surrounding tissues : dissected away from the theca interna using fine for- , as previously described [22, 25]. The resulting theca na with attached granulosa cells was subsequently re- :d to as a follicle wall preparation. All samples were :d at −70°C until used for preparation of cell extracts.

ular Extracts and Western Blotting

olubilized cell extracts were prepared from pieces of cle wall as previously described [22, 25] and were :d at −70°C. The protein concentration was determined he method of Bradford [34] using the Bio-Rad protein y kit.

rotein extracts (50 μg) were resolved by one-dimen- al SDS-PAGE and electrophoretically transferred onto ›cellulose membranes as previously described [22, 25]. nbranes were incubated 18 h at 4°C with affinity-puri- polyclonal antibody 9181 raised in rabbits against ovine PGHS [20, 21]. The specificity of antibody 9181 has been characterized in rats [20, 21], sheep [11], cattle [22, 25], and horses [24], with the antibody recognizing both PGHS-1 and PGHS-2 in all species tested. ^{125}I-Labeled protein A (1×10^6 cpm/ml Tris-buffered saline with 2% milk) was used to visualize immunoreactive proteins. Filters were exposed to x-ray film at −70°C.

Hormone Assays

Nonextracted aliquots of follicular fluid were assayed for progesterone and estradiol-17β by specific RIAs, as previously described [25, 35, 36]. The sensitivities of the progesterone and estradiol-17β assays were 3.12 and 0.16 pg/assay tube, respectively. The intra- and interassay coefficients of variations for progesterone were 11.9% and 8.0%, respectively, and were 9.6% and 6.4%, respectively, for estradiol-17β.

Samples of follicular fluid were acidified to pH 3.0 with 1 N HCl, extracted with diethyl ether, and assayed for PGE_2 and $PGF_{2\alpha}$ by specific RIAs, as previously described [22, 25]. The recovery rates for PGE_2 and $PGF_{2\alpha}$ were 82% and 89%, respectively. The intra- and interassay coefficients of variations for PGE_2 were 10.7% and 10.6%, respectively, and were 10.1% and 7.3% for $PGF_{2\alpha}$, respectively.

Statistical Analyses and Follicle Size Categories

One-way ANOVA was used to test the effect of time after hCG on concentrations of progesterone, estradiol-17β, PGE_2, and $PGF_{2\alpha}$. Scheffé's test was used to compare individual means when ANOVA indicated significant differences ($p < 0.05$). Data were transformed to logarithms before analysis when heterogeneity of variance was observed with the Bartlett test. To test the effect of time after hCG on follicular expression of PGHS-2 and cumulus oocyte complex expansion, follicles were grouped into three size categories, including small (6 to < 8 mm), medium (8 to < 10 mm) and large follicles (≥ 10 mm). Results were analyzed by one-way ANOVA followed by the Tukey-Kramer HSD test for comparison of multiple means. Data were transformed arcsines before analysis when heterogeneity of variance was observed with the Bartlett test.

RESULTS

Time- and Follicle Size-Dependent Expression of PGHS-2

Figure 1 shows the expression of PGHS enzymes in animals ovariectomized 0, 18, or 24 h post-hCG. No PGHS-2 protein was detected in a group of 18 follicles of 7–12.5 mm isolated at 0 h post-hCG (Fig. 1A). However, a faint but detectable PGHS-1 signal (70 kDa) comigrating with the bovine PGHS-1 standard was observed in several follicles. Although the nature of the higher band detected with the anti-PGHS antibody in all follicle extracts remains unknown, its presence has previously been reported in cattle [22] and rats [37]. Immunoreactive PGHS-2 protein was detected in 5 of 16 follicles isolated 18 h post-hCG, and in 12 of 15 follicles isolated at 24 h (Fig. 1, B and C). The enzyme appeared as a 72-kDa band (intact protein), and a smaller proteolytic fragment of 62 kDa [22, 25]. The relative abundance of the PGHS-2 signal at 24 h appeared higher than at 18 h post-hCG.

A clear time- and follicle size-dependent expression of PGHS-2 was observed when results from all animals were examined (Fig. 2). PGHS-2 expression was never observed

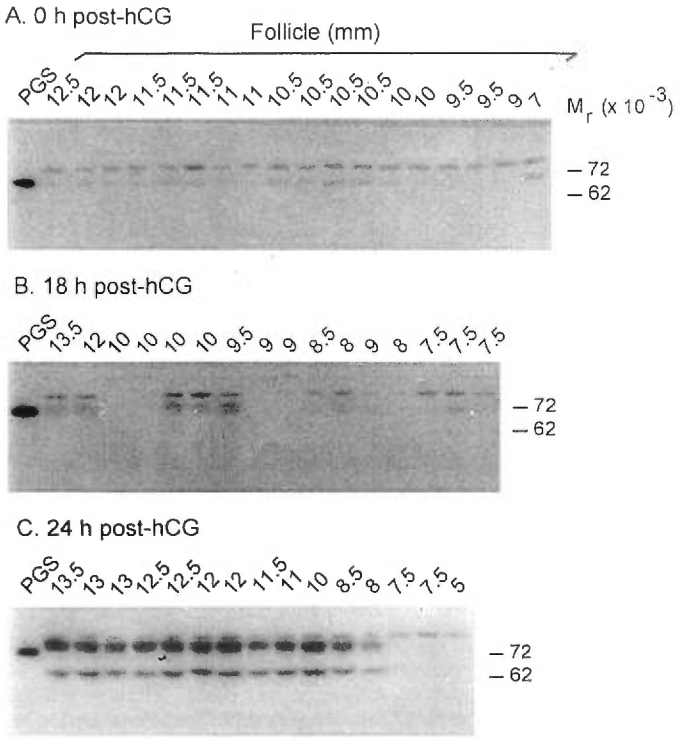


FIG. 1. Immunoblot analysis of PGHS-2 protein in bovine follicles during superovulatory treatment. Protein extracts (50 μg/lane) prepared from bovine follicles were analyzed by one-dimensional SDS-PAGE and immunoblotting techniques using an affinity-purified anti-PGHS antibody. Results from superovulated heifers ovariectomized at 0, 18, and 24 h post-hCG are shown in **A–C**, respectively. Markers on the right indicate migration of intact bovine PGHS-2 (72 000-*M*, band) and a putative proteolytic fragment (62 000-*M*, band). An extract of bovine platelets was used as a PGHS-1 standard in each blot (PGS, 20 μg/lane) [22]. Filters were exposed to film at −70°C for 14 h.

in small follicles, whether they were isolated 0 (n = 14 follicles), 18 (n = 7), or 24 h (n = 6) post-hCG (Fig. 2, upper panel). Also, no PGHS-2 protein was detected in medium (n = 41) or large (n = 64) follicles isolated at 0 h post-hCG (Fig. 2). In contrast, an average 12.3% and 45.9% of medium follicles per animal were PGHS-2-positive at 18 and 24 h post-hCG, respectively ($p < 0.05$; total of n = 27 and 19 follicles examined at 18 and 24 h, respectively). The proportion of large PGHS-2-positive follicles increased with time, with an average of 43.7% and 91.0% large follicles per animal being positive at 18 and 24 h post-hCG, respectively ($p < 0.05$; total of n = 39 follicles examined at both 18 and 24 h).

Follicular Fluid Concentrations of PGs

Follicular fluid concentrations of PGE_2 and $PGF_{2\alpha}$ were analyzed in relation to PGHS-2 expression (Fig. 3). All follicles isolated 0 h post-hCG were PGHS-2-negative and contained low concentrations of PGE_2 (0.47 ± 0.07 ng/ml) and $PGF_{2\alpha}$ (0.07 ± 0.03 ng/ml). No significant changes in prostaglandin concentrations were observed at 18 h post-hCG, whether follicles were PGHS-2-negative or -positive (Fig. 3). However, follicular fluid concentrations of PGE_2 and $PGF_{2\alpha}$ increased in PGHS-2-positive follicles isolated 24 h post-hCG, rising to 13.15 ± 1.60 ng/ml and 4.99 ± 0.79 ng/ml, respectively ($p < 0.05$). Concentrations of prostaglandins in PGHS-2-negative follicles isolated 24 h post-hCG were not significantly different from those in follicles isolated at 0 or 18 h ($p > 0.05$).

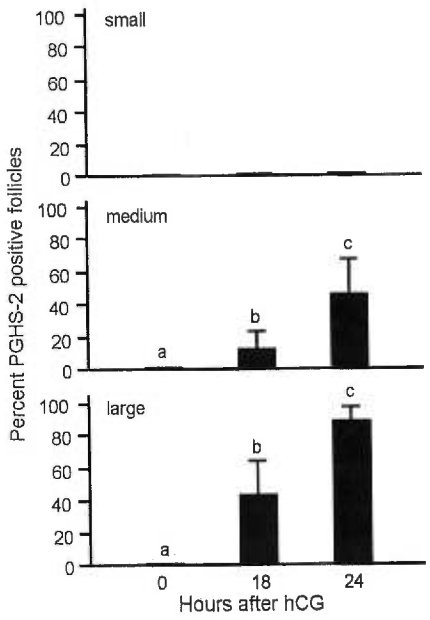


FIG. 2. Follicle size- and time-dependent expression of PGHS-2 in bovine follicles during superovulatory treatment. Bovine follicles (≥ 6 mm) were isolated at 0, 18, and 24 h post-hCG, and were analyzed by Western blotting. Follicles were defined as PGHS-2-negative or PGHS-2-positive on the basis of the absence or presence of a 72 000-*M*, band on Western blots, and were grouped into three size categories, including small (upper panel, n = 27 follicles), medium (middle panel, n = 87 follicles), and large follicles (lower panel, n = 142 follicles). Results are shown as percentage of PGHS-2-positive follicles per animal per size category and time point post-hCG (mean ± SEM). Bars with different superscripts are significantly different ($p < 0.05$).

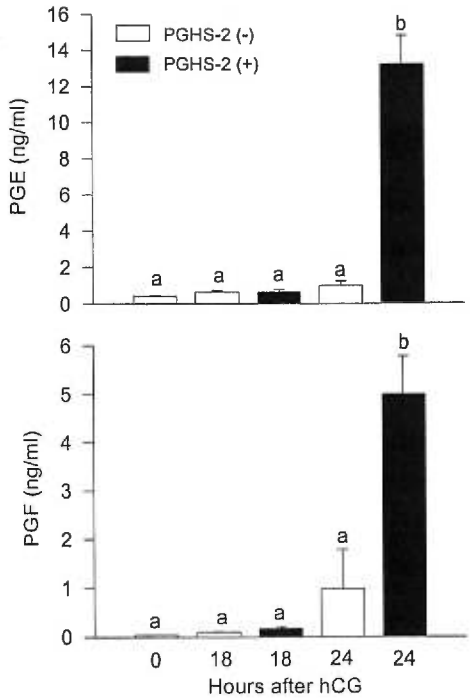
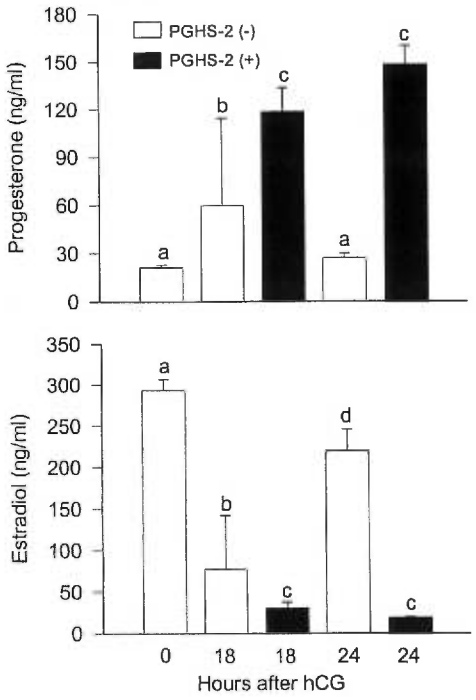


FIG. 3. Follicular fluid concentrations of PGE_2 and $PGF_{2\alpha}$ in bovine follicles during superovulatory treatment. Bovine follicles (≥ 6 mm) were isolated at 0, 18, and 24 h post-hCG, and concentrations of PGE_2 (top panel) and $PGF_{2\alpha}$ (bottom panel) in follicular fluid were analyzed in relation to follicular PGHS-2 expression (negative [−] or positive [+]). Note that only PGHS-2-negative follicles were present at 0 h post-hCG. Results are shown as means ± SEM, and bars with different superscripts are significantly different ($p < 0.05$).



1. Follicular fluid concentrations of progesterone and estradiol-17β vine follicles during superovulatory treatment. Bovine follicles (≥ 6 were isolated at 0, 18, and 24 h post-hCG, and concentrations of sterone (top panel) and estradiol-17β (bottom panel) were analyzed ation to follicular PGHS-2 expression (− or +). Note that only 5-2-negative follicles were present at 0 h post-hCG. Results are n as means ± SEM, and bars with different superscripts are signifi- different ($p < 0.05$).

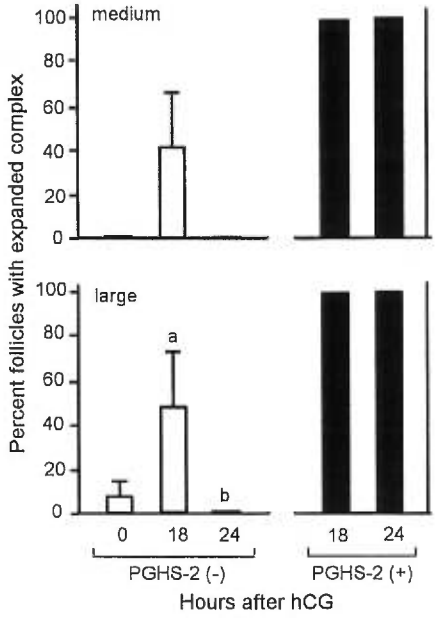


FIG. 5. Morphology of the cumulus oocyte complex in bovine follicles during superovulatory treatment. The morphology of the cumulus-oocyte complex in small (not shown), medium (upper panel), and large follicles (lower panel) isolated at 0, 18, and 24 h post-hCG was analyzed in relation to follicular PGHS-2 expression (negative [−] or positive [+]). Results are shown as percentage of follicles with an expanded cumulus per animal per time point after hCG in PGHS-2-negative (left panels) and -positive follicles (right panels). Bars with different superscripts are significantly different ($p < 0.05$). Note that no follicles were PGHS-2-positive at 0 h post-hCG and, therefore, only results from PGHS-2-positive follicles at 18 and 24 h post-hCG are presented.

cular Fluid Concentrations of Progesterone and idiol-17β

ollicular fluid concentrations of progesterone and estra-17β were analyzed in relation to follicular PGHS-2 ession (Fig. 4). Results showed that follicles isolated 0 ost-hCG (all PGHS-2-negative) were estrogen-domi-, with relatively low progesterone (28.2 ± 5.8 ng/ml) high estradiol-17β (291.8 ± 13.0 ng/ml) levels (Fig. After hCG treatment, PGHS-2-negative and -positive cles had distinct steroidogenic capacities. At 24 h post-, PGHS-2-negative follicles were still estrogen-domi-, with relatively high estradiol-17β (220.0 ± 25.8 ng/ and low progesterone (27.1 ± 2.8 ng/ml). In contrast, IS-2-positive follicles at 24 h post-hCG had undergone nization as evidenced by a marked decrease in estra-17β (18.4 ± 1.6 ng/ml, $p < 0.0001$) and increase in gesterone levels (163.3 ± 18.9 ng/ml, $p < 0.0001$). At 1 post-hCG, PGHS-2-positive follicles were also lutein-, and concentrations of both steroids were not signifi-ly different from those at 24 h post-hCG (Fig. 4). How-; in contrast to results observed in PGHS-2-negative cles at 24 h, PGHS-2-negative follicles at 18 h post-showed evidence of luteinization (Fig. 4).

phology of the Cumulus-Oocyte Complex

Cumulus expansion remained undetected in almost all ll follicles isolated between 0 and 24 h post-hCG (n = follicles), except for one PGHS-2-negative follicle iso-d 18 h post-hCG. Cumulus expansion was rare or absent nedium and large PGHS-2-negative follicles isolated 0 24 h post-hCG (Fig. 5, left panels). However, an average of 43.7% of medium and 48.3% of large PGHS-2-negative follicles isolated 18 h post-hCG had an expanded cumulus (Fig. 5, left panels).

A distinct pattern of cumulus expansion was observed in medium and large PGHS-2-positive follicles. No medium or large follicles isolated at 0 h were PGHS-2-positive. However, cumulus expansion was observed in all medium (n = 20 follicles) and large (n = 44) PGHS-2-positive follicles isolated 18 and 24 h post-hCG (Fig. 5).

DISCUSSION

The pattern of expression of PGHS-2 in bovine follicles during superovulatory treatment clearly shows that the enzyme is induced in a time- and follicle size-dependent manner. The time course of PGHS-2 induction in bovine superovulatory follicles appears to be similar to that observed in the preovulatory follicle of a normal estrous cycle (18 h post-hCG) [22, 25]. A diameter of 8 mm is an important developmental stage for bovine follicles, because induction of PGHS-2 occurred only in follicles that had reached this size. The gonadotropin-dependent expression of PGHS-2 at this developmental stage is probably related to the coincident expression of LH receptors in granulosa cells [38, 39]. A similar developmental- and gonadotropin-dependent induction of PGHS-2 has been reported in rats, a multiovulatory species in which PGHS-2 expression is restricted to large preovulatory follicles exposed to high levels of gonadotropins [18, 20, 21].

Exogenous hCG was administered at the end of a standard superovulatory protocol to precisely control the induction of the ovulatory process. The observed effect of hCG on PGHS-2 induction most likely mimics that nor-

mally caused by the endogenous LH surge. Previous studies have shown that both gonadotropins (hCG and LH) cause a similar pattern of PGHS-2 induction during the normal estrous cycle [22, 25]. The expression of PGHS-2 in superovulatory follicles at 24 h post-hCG was associated with an increase in prostaglandin synthetic activities, but this association was not observed at 18 h post-hCG. A similar pattern is observed in equine preovulatory follicles in which induction of PGHS-2 and an increase in follicular prostaglandins are first detected at 30 and 36 h, respectively [24]. Lower expression of PGHS-2 at early time points and the relatively large volume of follicular fluid (dilution effect) could be responsible, at least in part, for the interval observed in both species.

Prostaglandins have been linked to ovulation for more than 25 yr [16, 40–43]. Recent gene targeting studies in mice have provided convincing evidence for an obligatory role of prostaglandin synthesis during the ovulatory process [44, 45]. PGHS-2-deficient female mice were shown to be infertile because of lack of ovulation. Absence of ovulation of follicles smaller than 8 mm reported during superovulation in cattle [30] may result, at least in part, from the inability of gonadotropins to induce PGHS-2 in these small follicles. Similarly, the absence of PGHS-2 expression in a proportion of follicles larger than 8 mm at 24 h post-hCG could represent one of the mechanisms involved in the development of large anovulatory follicles during superovulation treatment. The incidence of PGHS-2-negative follicles larger than 8 mm at 24 h post-hCG in this study (24%; n = 14 of 58 follicles) is very similar to the incidence of anovulatory follicles detected by ultrasonography during superovulatory treatment [30].

One important finding of this study is the identification of PGHS-2 as a marker to differentiate subpopulations of superovulatory follicles with distinct steroidogenic capacities. At 24 h post-hCG, PGHS-2-positive follicles were clearly luteinized, with high progesterone and low estradiol, whereas PGHS-2-negative follicles were not luteinized and had low progesterone and high estradiol in follicular fluid. Interestingly, the presence of high estradiol in PGHS-2-negative follicles indicates that they were not atretic [46, 47]. Differences in LH receptor numbers and/or activation of intracellular signalling pathways are potential causes for the lack of hCG responsiveness. In previous studies, the unintentional grouping of these two subpopulations of follicles is presumably responsible for the marked heterogeneity reported in follicular fluid concentrations of steroids during superovulation in cattle [48, 49]. Similarly, the high variability in concentrations of progesterone and estradiol in PGHS-2-negative follicles isolated at 18 h post-hCG in this study was probably caused by the inadvertent pooling of different subpopulations of follicles (luteinized and unluteinized). However, the problem at this earlier time point is that induction of follicular PGHS-2 is not completed. Since follicular luteinization occurs before PGHS-2 induction in bovine follicles [22, 25, 50], we predict that the population of PGHS-2-negative follicles at 18 h post-hCG contained some follicles that had luteinized but had not yet expressed PGHS-2. Therefore, PGHS-2 should be considered as a good differentiating marker just before ovulation.

In addition to its effects on ovulation and luteinization, the preovulatory gonadotropin surge is the physiological trigger for resumption of oocyte maturation. The structural changes of the bovine cumulus oocyte complex during its final maturation in vivo have been well characterized in superovulated and unstimulated animals [51–54]. The present study shows that PGHS-2 expression is closely related to the morphology of the complex. All PGHS-2-positive follicles contained an expanded cumulus, whereas the vast majority of small, medium, and large PGHS-2-negative follicles isolated between 0 and 24 h post-hCG had a compact unexpanded complex. The only time at which cumulus expansion was observed in a considerable number of PGHS-2-negative follicles was at 18 h post-hCG. Considering that cumulus expansion precedes PGHS-2 induction [25, 52], we predict that some PGHS-2-negative follicles with expanded cumulus at 18 h would have become positive shortly thereafter. At 24 h post-hCG, all medium and large PGHS-2-negative follicles contained an unexpanded cumulus, indicating that the entire follicle unit remained unresponsive to hCG.

In summary, induction of PGHS-2 in bovine follicles during superovulatory treatment is time- and size-dependent, and results support our initial hypothesis that PGHS-2 is a molecular marker for follicular commitment to ovulation. The expression of PGHS-2 was consistently associated with other signs of terminal follicular differentiation, including luteinization and cumulus oocyte expansion. In contrast, lack of PGHS-2 induction in follicles of ovulatory size (> 8 mm) was linked to an apparent failure to respond to the gonadotropin preovulatory signal (hCG), since no concomitant changes in follicular steroidogenesis and cumulus morphology were observed. Because these latter follicles are likely to remain anovulatory, they should become the focus of future investigations as they represent a known drawback of current superovulatory protocols in cattle.

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6. ARTICLE THREE

The delayed activation of the prostaglandin G/H synthase-2 promoter in bovine granulosa cells is associated with a downregulation of a truncated USF

The Delayed Activation of the Prostaglandin G/H Synthase-2 Promoter in Bovine Granulosa Cells is associated with a downregulation of a truncated USF*

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ABSTRACT

Prostaglandins play an important role during the ovulatory process of mammals. Induction of prostaglandin G/H synthase-2 (PGHS-2) by gonadotropins in preovulatory follicles is obligatory for the ovulatory process. However, there is a marked difference in the time-course of PGHS-2 induction in species with a short (rats) *versus* a long ovulatory process (cows, mares). To elucidate the molecular mechanisms involved in the delayed induction of PGHS-2 in species with a long ovulatory process, the bovine PGHS-2 gene and its putative promoter were cloned and characterized, and an *in vitro* model was developed to study the *trans*-activation of PGHS-2 promoter in granulosa cells. Results showed that the bovine PGHS-2 gene is composed of 10 exons and 9 introns and spans 7,638 bp. The complete promoter, and a series of 5'-deletion mutants were fused into a luciferase reporter vector, and transfected into primary cultures of bovine granulosa cells. When the full-length promoter (-1573/-2 PGHS.LUC) was transfected, a time-dependent induction of reporter gene activity was observed, with maximal levels observed at 36 h post-forskolin. Deletion analyses identified that a DNA fragment extending from -147 to -2 confers full promoter activity in response to forskolin, indicating this region plays a critical role in the *trans*-activation of PGHS-2 gene. Several consensus *cis*-elements were identified within this region, including E-box, ATF/CRE, C/EBP and AP2. Site-directed mutagenesis studies showed that the E-box is absolutely required for basal and forskolin-induced PGHS-2 promoter activities, but not sufficient for the full promoter activity. Disruption of CAAT-box decreased promoter activity by 29%, whereas point-mutation within ATF/CRE element had no effect on forskolin-induced promoter activity. Electrophoretic mobility shift assays (EMSAs) revealed that DNA/protein interactions within the region -147/-2 were regulated by human chorionic gonadotropin (hCG). Four

DNA-protein complexes were located to the E-box region in 0 h granulosa cell nuclear extracts, whereas only one complex was present in 18 h extracts. Antibodies anti-USF-1/-2 identified that both USF-1 and USF-2 bound to E-box, and that a putative N-terminus truncated USF-2 bound to E-box only in 0 h nuclear extracts of granulosa cells. The C/EBPβ protein was shown to bind to CAAT-box at 0 h, with a slight decrease at 18 h. Western blots indicated that a putative N-terminus truncated USF-2 was present in nuclear extracts of granulosa cells isolated at 0 h, but absent in extracts from 18 and 20 h post-hCG. Collectively, this study demonstrates that the promoter region -147/-2 plays a central role in basal and forskolin-dependent PGHS-2 promoter activities in bovine granulosa cells, and that a putative N-terminus truncated USF is potentially involved in the delayed induction of PGHS-2 gene in species with a long ovulatory process.

INTRODUCTION

Prostaglandin G/H synthase (PGHS, also known as COX) is the first rate-limiting enzyme in the biosynthetic pathway of prostaglandins from arachidonic acid (Smith, 1992; Williams and DuBois, 1996; Jouzeau et al., 1997). PGHS carries two sequential enzymatic functions, a cyclooxygenase reaction responsible for the conversion of arachidonic acid to PGG_2, and a peroxidase reaction involved in the conversion of PGG_2 to PGH_2 (Smith, 1992). PGH_2 is the common precursor for the synthesis of all prostaglandins, prostacyclins and thromboxanes (Smith et al., 1992; Smith and DeWitt, 1995). Two isoforms of PGHS have been characterized. The first isoform, designated PGHS-1 or COX-1, was isolated 20 years ago from ovine and bovine seminal vesicles (Miyamoto et al., 1976; Van der Ouderaa et al., 1977), whereas the second isoform, named PGHS-2 or COX-2, was cloned from chicken and mouse fibroblasts and purified from rat granulosa cells in the early 90s (Kujubu et al., 1991; Xie et al., 1991; Sirois and Richards, 1992). The two isoforms share similarities at the protein level: they are approximately the same size (600-604 amino acids) and have conserved structural and functional domains. However, they are derived from separated genes encoding different-size mRNAs (Williams and DuBois, 1996). Most importantly, the two isoforms differ markedly in their expression and regulation. PGHS-1 is present in a variety of tissues and is often referred to as the constitutive isoform (DeWitt and Smith, 1995; Williams and DuBois, 1996). In contrast, PGHS-2 is undetectable in most tissues but can be induced by several agonists, and is generally referred to as the inducible form (DeWitt and Smith, 1995; Herschman et al., 1995; Williams and DuBois, 1996). Gene-targeting experiments have underscored the distinct physiological roles of each isoform, and suggested that they are

both implicated in inflammation (Dinchuk et al., 1995; Langenbach et al., 1995; Morham et al., 1995).

Prostaglandins are key mediators of the ovulatory process, and several studies have shown that PGHS-2 is selectively induced by gonadotropins in granulosa cells prior to ovulation (Sirois et al., 1992; Sirois, 1994; Tsai et al., 1996; Liu et al., 1997; Sirois and Dore, 1997). The obligatory role of PGHS-2 expression during the ovulatory process was underscored in female PGHS-2 deficient mice, which proved to be infertile because of ovulation failure and other impaired reproductive processes (Lim et al., 1997). Interestingly, comparative studies using human chorionic gonadotropin (hCG) to induce ovulation identified a marked difference in the time-course of PGHS-2 induction in species with a short *versus* a long ovulatory process (Sirois, 1994; Sirois and Dore, 1997). In rats, a species with a short ovulatory process (12-14 h post-hCG), PGHS-2 induction is very rapid and occurs within 2-4 h post-hCG (Sirois et al., 1992). In contrast, induction of PGHS-2 in species with a long ovulatory process like cows (28 h post-hCG) and mares (39-42 h post-hCG) is remarkably delayed and occurs only at 18 h and 30 h post-hCG, respectively (Sirois, 1994; Sirois and Dore, 1997; Boerboom and Sirois, 1998). This marked difference among species in the control of PGHS-2 gene expression was proposed to be involved in the control of the mammalian ovulatory clock (Richards, 1997); however its molecular basis remains unknown.

The isolation and characterization of the mouse (Fletcher et al., 1992), rat (Sirois and Richards, 1993), and human PGHS-2 promoter (Inoue et al., 1994) have provided some of the molecular mechanisms involved in PGHS-2 transcriptional activation. Consensus *cis*-acting elements such as NF-kappa B, NF-IL6 (C/EBP), ATF/CRE and E-box were shown to play important roles in the induction of PGHS-2 transcription (Sirois et al., 1993; Inoue et al., 1994; Xie

and Herschman, 1995; Morris and Richards, 1996). However, the relative importance of each element varies greatly depending on the type of cells and agonists involved (Xie et al., 1994; Inoue et al., 1995; Morris and Richards, 1996). Thus far, the regulation of the PGHS-2 promoter in granulosa cells has been studied only in a species with a short ovulatory process, the rat (Sirois et al., 1993; Sirois and Richards, 1993; Morris and Richards, 1996). Deletion and site-directed mutant analyses of the rat promoter revealed that the proximal region (first 200 bp upstream of the transcription start site) plays a central role in cAMP-dependent regulation of the promoter (Sirois and Richards, 1993; Morris and Richards, 1996). An E-box located at -140/-132 and a C/EBP element located at -55/-50 were identified as putative modulators of PGHS-2 promoter activity (Sirois et al., 1993; Morris and Richards, 1996). The E-box was shown to be essential for activation of the PGHS-2 gene in granulosa cells, and the upstream stimulatory protein (USF) was identified as one transcription factor binding to this element (Morris and Richards, 1996). However, no regulation of USF proteins was observed in response to hCG, thus leaving the mechanisms of gonadotropin-dependent *trans*-activation unclear. Also, the C/EBP element, initially proposed to be involved in PGHS-2 induction (Sirois et al., 1993), was recently proved not be implicated when site-directed mutagenesis studies were performed in the context of the complete proximal promoter (Morris and Richards, 1996).

Although progress has been achieved in our understanding of the rapid activation of PGHS-2 gene expression in rat granulosa cells, there has been no attempt to unravel the molecular basis for the delayed induction of PGHS-2 in species with a long ovulatory process. Such studies are important as they will likely be relevant to humans, a species with a long ovulatory process (36 h post-hCG) (Yen and Jaffe, 1986). Therefore, the general objective of the

present study was to develop a model to study the molecular mechanisms involved in the delayed PGHS-2 induction in species with a long ovulatory process. The specific objectives were to clone and characterize the bovine PGHS-2 gene and promoter, to study the regulation in primary cultures of bovine granulosa cells, and to characterize the hormonal control of the bovine PGHS-2 promoter *in vitro*.

EXPERIMENTAL PROCEDURES

Materials. [α-^{32}P]dCTP, [α-^{32}P]dATP, [γ-^{32}P]ATP and [^{35}S]ATP were obtained from Mandel Scientific NEN Life Science Products (Mississauga, Ontario). QuikHyb hybridization solution and a bovine genomic library were purchased from Stratagene Cloning Systems (La Jolla, CA). An additional bovine genomic library was purchased from ClonTech (Bio/Can Scientific, Mississauga, Ontario). TRIzol total RNA isolation reagent, 1 kb DNA ladder, synthetic oligonucleotides, Lipofectamine and culture media were purchased from Gibco Bethesda Research Laboratories Life Technologies Inc (Gaithersburg, MD). Tissue culture plates were obtained from Corning-Costar (Fisher Scientific, Montreal, Quebec). Fetal bovine serum was obtained from HyClone Laboratories (Logan, Utah). RNAsin, Prime-a-Gene labeling system, DNA 5'-End Labeling System, AMV reverse transcriptase, Dual-Luciferase Reporter Assay and plasmids pGEM3Zf(-), pRL-SV40, pGL3-Basic and pGL3-control were purchased from Promega (Madison, WI). Electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA). Vent DNA polymerase was obtained from New England Biolabs (Beverly, MA), while Taq polymerase, T4 polynucleotide kinase, all sequencing reagents were purchased from Pharmacia LKB Biotechnology (Piscataway,NJ).

Isolation of bovine PGHS-2 gene and promoter. A first bovine genomic library (Stratagene) was screened following the manufacturer's protocol with a 5'-1.2 kb *Eco RI* fragment of the mouse PGHS-2 cDNA probe (Simmons D.L, 1992) that was random prime labeled with [α-^{32}P]deoxy-CTP using the Prime-a-Gene labeling system (Promega). Out of 900,000 phage plaques screened, six positive clones were identified, purified and characterized by Southern blot analyses. DNA fragments were subcloned into pGEM 3Zf(-) and sequenced by the dideoxy method (Sanger et al., 1977). While the six clones varied in size from 12-18 kb, none contained the region upstream of intron 4. To clone the promoter and the 5'-region of the bovine PGHS-2 gene, a second bovine genomic library (ClonTech) was screened with the mouse PGHS-2 cDNA probe. From 100,000 phage plaques screened, one positive clone of 15 kb was isolated and shown to contain the complete bovine PGHS-2 gene as well as about 4 kb of 5'-flanking DNA. The entire gene (10 exons and 9 introns) and 1650 bp of the putative promoter were characterized by DNA sequencing.

Primer extension analysis. The transcription initiation site of the bovine PGHS-2 gene was determined by primer extension analysis, as previously described (Sirois and Richards, 1993; Boerboom and Sirois, 1998). The reaction used total RNA extracted from preovulatory follicles isolated 0 or 24 h after hCG treatment as previously described (Boerboom and Sirois, 1998), and a 24-mer antisense oligonucleotide 5'-GAGGGCGGTGCGGAGTTCCGGGCG-3' designed from the bovine PGHS-2 cDNA and located 35-73 bp downstream of the transcription initiation site identified in human PGHS-2 (Kosaka et al., 1994; Tazawa et al., 1994). The extension product was analyzed by electrophoresis on a 6% polyacrylamide/7M urea gel, and the site of transcription initiation was determined by comparison with an adjacent

sequencing reaction that used the same oligonucleotide as primer and a PGHS-2 genomic clone that contained this region as template.

Semiquantitative analysis and RT-PCR. For analysis of PGHS-2 mRNA in primary cultures of granulosa cells, total RNA was extracted with TriZol (Life Technologies) from granulosa cells incubated for 0, 6, 12, 24, 36 and 48 h in the presence of forskolin (10 μM). RT reactions were performed using 5 μg RNA, 20 μM of an oligo poly dT_{15} primers and 10 U of AMV reverse transcriptase. For amplication of PGHS-2, sense (primer 1, Table 1) and antisense (primer 2, Table 1) primers specific to bovine PGHS-2 were designed to produce a 1.1 kb fragment. As an internal control, a set of sense and anti-sense primers (primers 3 and 4, Table 1) were designed to amplify a fragment of about 0.85 kb of a constitutively expressed transcript coding for bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Tsai et al., 1996). PCR reactions were performed using 1 μl of Taq polymerase, 50 pmol of each primer, 5 mM dNTPs and the following cycling conditions: 32 cycles of 30-sec denaturation at 95°C, 30-sec annealing at 48°C, and 1 min elongation at 72°C. A final extension at 72 C was performed for 5 min after the last cycle, and PCR products were analyzed on a 1% agarose gel.

Immnunoblot analysis. Bovine granulosa cells were collected after 0, 6, 12, 24, 36, 48 and 72 h of culture in the presence of forskolin (10 μM), and solubilized cell extracts were prepared as previously described (Sirois et al., 1992; Liu et al., 1997). Protein concentration was determined by the method of Bradford (Bio-Rad Protein Assay). Samples (50 μg of proteins) were resolved by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretically transferred to nitrocellulose filters, and incubated polyclonal anti-PGHS antibody 9181 and [^{125}I]Protein A, as described (Sirois et al., 1992; Liu et al., 1997). Filters were exposed to film at -70 °C.

Immunoblot analyses were also performed using nuclear extracts prepared from granulosa cells isolated 0, 18 and 20 h post-hCG (see below). Filters were incubated with the different polyclonal antibodies raised against USF-1, USF-2 (including two antibodies directed either against the NH_2- or COOH-terminus), C/EBPα, C/EBPß, and C/EBPδ (Santa Cruz). Each antiserum was used at a 1:100 dilution.

Prostaglandin E_2 radioimmunoassays. Media was collected from primary cultures of bovine granulosa cells incubated for 0, 12, 24, 36, 48 and 72 h in the absence or presence of forskolin (10 μM), and was assayed for PGE_2 using a specific radioimmunoassay (Xiao et al., 1998).

Deletion and point mutants of the bovine PGHS-2 promoter. Five bovine PGHS-2 promoter fragments, including -1573/-2, -491/-2, -324/-2, -126/-2 and -87/-2 (where +1 = transcription initiation site), were generated by PCR amplification using five specific sense primers (oligonucleotides 5, 6, 7, 8, 9 respectively, Table 1) and one common antisense primer (oligonucleotide 10, Table 1). In addition, a sixth promoter fragment was produced by the annealing of two complementary oligonucleotides spanning the DNA region from -38 to -2 (oligonucleotides 11 and 12, Table 1). Each fragment was inserted upstream of the firefly luciferase reporter gene in the vector pGL3-Basic (Promega) to generate the chimeric constructs -1573/-2PGHS.LUC, -491/-2PGHS.LUC, -324/-2PGHS.LUC, -126/-2PGHS.LUC, -87/-2PGHS.LUC and -38/-2PGHS.LUC. Prior to transfections, the integrity of each construct was confirmed by DNA sequencing.

Four site-directed mutants were generated using the -147/-2 PGHS-2 promoter as a template, including mutations within the E-box (-147/-2PGHSE-BOX.LUC), C/EBP (-147/-2PGHSC/EBP.LUC), ATF/CRE (-147/-2PGHSATF.LUC) and AP2 sites (-147/-2PGHSAP2.LUC). Mutants were

produced by inverse PCR (Silver and Keerikatte, 1989) using primers described in Table 1. After migration on 1% agarose gel, PCR products were cut with *Eco RI* and *XhoI* to release the -147/-2 mutant fragment and ligated into pGL3-Basic, and mutations were confirmed by DNA sequencing (Sanger et al., 1977).

Cell culture, transient transfections and promoter activity assays. Paired bovine ovaries bearing a newly formed corpus luteum and a follicle of 8 to 12 mm in diameter were obtained from a slaughterhouse, and were transported on ice to the laboratory. The follicle, which is the putative dominant follicle of the first wave of the estrous cycle (Sirois and Fortune, 1988), was dissected from the ovary and granulosa cells were obtained as previously described (Liu and Sirois, 1998). Cells were washed twice with MEM supplemented with 2% fetal bovine serum (FBS), and then seeded in 24-well plates at a density of 2-5 X 10^5 cells per 0.5 ml MEM supplemented with 2% FBS, insulin (1 μg/ml), transferin (5 μg/ml), penicillin (100 U/ml)-streptomycin (100 μg/ml). Cultures were incubated at 37 °C in a humidified incubator gassed with 95% air and 5% CO_2. When cultures reached 70-80% confluency after 4-5 days, cells were assigned to a specific experiment (regulation of PGHS-2 mRNA or protein *in vitro*, or transient transfections).

Cultures of bovine granulosa cells were transiently transfected with 90 fmoles/well of bovine PGHS-2 promoter-luciferase construct (PGHS-LUC) using 2 μl Lipofectamine (Gibco) in 0.3 ml of serum/antibiotic-free MEM, and following the manufacturer's protocol. Co-transfection with the SV40 *Renilla* luciferase control vector (pRL-SV40; Promega) was performed to normalize results. A ratio of experimental (PGHS-LUC) to control vector (pRL-SV40) of 10:1 was used. After 3 h of transfection, cells were incubated in 0.5 ml fresh MEM supplemented with 2% FBS in the absence of presence of forskolin (10 μM) for variable intervals of time. At the end of the culture period, cell lysates were

prepared, and firefly and *Renilla* luciferase activities were determined using the Promega Dual Luciferase Assay System and a Lumat luminometer (Promega). Each experiment was performed in triplicates and repeated three times.

Granulosa cell nuclear extracts and electrophoretic mobility shift assays. Bovine preovulatory follicles were obtained from superovulated holstein heifers ovariectomized 0, 18 and 20 h after hCG, according to a model previously characterized (Liu and Sirois, 1998). Granulosa cells were isolated and nuclear extracts were prepared as described (Sirois et al., 1993). Protein concentration in each extract was determined by the method of Bradford (Bio-Rad Protein Assay). Electrophoretic mobility shift assays (EMSAs) were performed as described with minor modifications (Sirois et al., 1993; Sirois and Richards, 1993). Briefly, extracts of nuclear proteins (1.5 μg/reaction) were incubated with 25,000-50,000 cpm of end-labelled DNA fragment (-147/-2) and 5 μg poly [dI/dC] in a final volume of 20 μl in buffer containing 100 mM KCl, 15 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$ and 12 % glycerol. When antibodies were used, the nuclear extract was first preincubated for 20 min with the antiserum prior to the addition of other reagents. Binding reactions were resolved by 5% acrylamide/0.5X TBE gel electrophoresis.

RESULTS

Induction of PGHS-2 in primary cultures of bovine granulosa cells. To determine the suitability of a *in vitro* model to study the delayed induction of PGHS-2 in bovine preovulatory follicles, the regulation of PGHS-2 was characterized in primary cultures of bovine granulosa cells stimulated with forskolin (10 μM). Results showed that no PGHS-2 mRNA was detected by RT-PCR after 0 and 6 h of forskolin stimulation, but became detectable at 12 h,

and peaked at 24 h (Fig. 1A). Immunoblot analysis revealed a marked induction of PGHS-2 protein in granulosa cells between 12 and 24 h of forskolin stimulation, followed by a progressive loss in protein expression between 24 and 72 h (Fig. 1B). To determine if the time- and forskolin-dependent induction of PGHS-2 mRNA and protein was associated with changes in prostaglandin synthetic activities, PGE_2 concentrations were measured in culture media. Figure 1C shows no difference in PGE_2 levels in granulosa cells cultured in the absence of forskolin (ANOVA, $P > 0.05$). However, PGE_2 concentrations were significantly higher in cultures stimulated for 24 to 72 h with forskolin, as compared to controls, and most of the stimulatory effect was observed between 12 and 36 h (Fig. 1C). Thus, these findings indicate that the model *in vitro* largely replicates the delayed induction of PGHS-2 observed in bovine follicles *in vivo*.

Cloning and characterization of the bovine PGHS-2 gene and promoter. The complete primary structure of the PGHS-2 gene was determined from clones obtained from two bovine genomic libraries. Results showed that the gene consists of 7,638 bp, and has 10 exons and 9 introns (Fig. 2A). Exon size ranges from 84 bp (exon 6) to 1975 bp (exon 10), while intron size varies from 111 bp (intron 6) to 1052 bp (intron 1). The exon/intron boundaries were confirmed by comparisons with the bovine PGHS-2 cDNA (Antaya et al., 1997) (GeneBank accession number AF031698) , and each splice site agreed with the consensus donor/acceptor (GT/AG) sequence (Fig. 2B). Primer extension analysis was used to identify the transcription initiation site. Figure 2C shows that a 57 nucleotide extension product was produced when the primer was hybridized to a sample known to contain PGHS-2 mRNA (preovulatory follicle isolated 24 h post-hCG). The transcription start site was identified at a guanidine residue.

A 1.6 kb DNA fragment located immediately upstream of the bovine PGHS-2 transcription start site was isolated and sequenced (Fig. 3). Several consensus *cis*-acting elements were identified within the first 600 bp upstream of the cap site, including PEA3, E-box, ATF/CRE, C/EBPß, SP-1, AP-2, AP-1 and NF-kappa B elements (Fig. 4). However, no consensus TATA box motif was present within the putative promoter, but the hexanucleotide 5'-ATAAAA-3' located 30 bp upstream of the transcription start site could serve as a presumptive TATA element as observed in the mouse (Fletcher et al., 1992).

Functional analysis of the bovine PGHS-2 promoter. To determine if the isolated 5'-flanking DNA fragment had functional promoter activity, a DNA fragment containing the region -1573/-2 (+1 = transcription start site) was fused into upstream of the firefly luciferase reporter gene in the pGL3.Basic vector (Fig. 4A). The chimeric construct (-1573/-2PGHS.LUC) were transiently transfected in primary cultures of bovine granulosa cells stimulated with forskolin. Results from a time-course study showed that reporter gene activity was very low after 6 h of forskolin stimulation, but gradually increased thereafter and peaked at 36 h (Fig. 4B). Based on these results, a period of 24 or 36 h of forskolin stimulation was selected for subsequent transient transfections.

To identify regions within the -1573/-2 fragment involved in forskolin-regulation of PGHS-2 promoter activity, a series of 5'-deletion mutants were designed (Fig. 4A), subcloned into pGL3.Basic, and transiently transfected in bovine granulosa cells. Results showed that deletion of the promoter region between -1573 and -147 had no effect on forskolin-stimulated reporter gene activity (Fig. 4C). However, the deletion of the region between -147 and -87 resulted in a 56% and 75% decrease in basal and forskolin-stimulated luciferase activities, respectively. Further deletion between -87 and -37 resulted in a complete loss in both basal and forskolin-induced reporter gene

activities, with the -37/-2PGHS.LUC activities being similar to those of the promoter-less construct (Fig. 4C). Thus, the region -147/-37 plays a key role in basal and forskolin-dependent regulation of the bovine PGHS-2 promoter in granulosa cells.

To determine if consensus *cis*-acting elements present within the -147/-37 region were involved in promoter activity, site-directed mutants of the AP2, C/EBP, ATF, and E-box elements were generated and tested in transient transfections (Fig. 5A). Results indicated that mutation of the E-box had marked effect on PGHS-2 promoter activities, with basal and forkolin-stimulated luciferase activities of the mutant decreasing to 8 % and 9%, respectively, of the activities of the -147/-2PGHS.LUC wildtype control (Fig. 5B). In contrast, mutations of other three elements had no comparable effect on promoter activities, but mutation of the C/EBP element resulted in a 29% decrease in forskolin-stimulated reporter gene activity when compared to control (Fig. 5B).

Protein/DNA binding activities within the bovine PGHS-2 promoter region -147/-2. The ability of the -147/-2 PGHS-2 promoter fragment to interact with nuclear proteins was tested by EMSA using extracts prepared from granulosa cells of preovulatory follicles obtained before (0 h post-hCG) or during (18 and 20 h post-hCG) induction of PGHS-2 *in vivo* (Liu and Sirois, 1998). Results showed that all nuclear extracts generated multiple DNA-protein complexes, of which eight were selected and designated as complexes a, b, c, d, e, f, g and h for reference purposes. Some complexes were regulated by hCG stimulation: complexes b, d, e, and f were detected only with the 0 h extract, complexes c and h appeared to be increased after hCG treatment (18 and 20 h), whereas complex a did not vary markedly (Fig. 6A).

To assess the specificity of protein/DNA interactions, and to identify more precisely *cis*-acting elements involved in complex formation, competition

assays were performed using 25 molar excess of unlabelled wildtype -147/-2 fragment, as well as 25-molar excess of -147/-2 fragments containing either a mutated E-box, ATF, C/EBP or AP2 element. Results showed that all complexes generated with the 0 h and 18h extract were competed with excess of cold competitor -147/-2 wildtype fragment, thus demonstrating the specific nature of the interactions (Fig. 6B and C). Interestingly, the use of -147/-2 fragments with point-mutations allowed to localize various complexes to specific *cis*-elements. Four bands (b, e , f, and g; Fig. 6B) were assigned to the E-box at 0 h, but only one of them was still present at 18 h post-hCG (band g; Fig. 6C). An identical DNA/protein interaction pattern was observed with the ATF mutated DNA fragment, which likely relates to the overlapping nature of the E-box and ATF/CRE elements. Complexes a and c were localized to the C/EBP element at 0 and 18 h post-hCG, with the intensity of band c being more pronounced at 18 h. All complexes were competed by the ATF mutated -147/-2 fragment, suggesting that this element was not involved in the formation of any of the observed complexes (Fig. 6B and C). Complexes d and h could not be assigned to any of the four elements.

To confirm binding activities to the E-box and C/EBP element, competitive EMSAs were performed with complementary oligonucleotides (Table 1) containing an E-box or C/EBP element as cold competitor DNA. Results shown in Figure 7A and B confirmed those observed in Figure 6B an C: excess of cold E-box oligonucleotides competed complexes b, e, f, and g at 0 h, and complexe g at 18 h; excess of cold C/EBP oligonucleotides competed complexes a and c at both time-points; and competition with both E-box and C/EBP oligonucleotides abolished all complexes. Interestingly, complex h was competed by the E-box or the C/EBP oligonucleotides, suggesting that both elements are required for the recruitment of this large complex.

USF and C/EBP proteins are involved in the formation of multiple protein/DNA complexes. To determine if the identified complexes contain USF and C/EBP proteins, supershift EMSAs were performed using antibodies against USF-1, the COOH-terminus of USF-2, the amino-terminus of USF-2, C/EBPα, C/EBPß, and C/EBPδ. The DNA fragment -147/-2 was used as labelled probe, and assays were done in the presence of 25-molar excess of cold competitor mutants (E-box or C/EBP mutant in the -147/-2 context) to clearly identify bands interacting with each *cis*-element. Results suggest that complexes interacting at the E-box at 0 h contain USF-1 and USF-2 proteins (Fig. 8A). The complex with the fastest mobility, band b, was markedly shifted by an antibody directed against the COOH terminus USF-2. However, an antibody directed against the NH_2-terminus of USF-2 had no effect on band b, suggesting that this complex contains the previously described NH2-truncated form of the protein in mammalian cells (Sirito et al., 1994). Complexes of higher molecular weight are thought to contains dimers composed of a full-length and a truncated USF protein (bands e and f), or dimers composed of two full-length USF proteins (band g). The only complex localized to the E-box at 18 h post-hCG (band g, Fig. 8B) was shifted by USF antibodies (against the COOH-terminus) in a manner similar to that observed at 0 h. For complexes involving the C/EBP element, band a, which is present at 0 and 18 h, was shown to contain primarily C/EBPß; whereas band c, which is more prominent at 18 h, appeared to contain some C/EBPα (Fig. 8B).

To determine the potential regulation of USF and C/EBP proteins, immunoblot analyses were performed using granulosa cells nuclear extracts isolated before (0 h) and after (18 and 20 h) hCG treatment *in vivo*. Results showed that USF-1 remained relatively constant after hCG treatment, whereas USF-2 and C/EBPß appeared lower at 18 and 20 h, as compared to 0 h (Fig.

9). No C/EBPα and δ was detected by immunoblots (data not shown). Interestingly, a putative truncated form of USF-2 (18,000 Mr) was present at 0 h, but disappeared at 18 and 20 h (Fig. 9), which is in keeping with results from EMSAs.

DISCUSSION

Previous studies have identified a marked difference in the time-course of follicular PGHS-2 induction in species with a short *versus* a long ovulatory process, ranging from 2-4 h post-hCG in rats to 18 h and 30 h post-hCG in cows and mares, respectively (Sirois et al., 1992; Sirois, 1994; Liu et al., 1997; Sirois and Dore, 1997). Interestingly, the gradual delay in timing of PGHS-2 induction in species with progressively longer ovulatory processes was proposed as a putative molecular determinant involved in dictating the species-species length of the ovulatory process (Richards, 1997; Sirois and Dore, 1997). Yet, this remarkable variation in the control of PGHS-2 induction across species is intriguing, considering that it involves the same cell type (granulosa cells) and the same agonist (hCG), and remains unprecedented when compared to other physiological and pathological conditions. This study is the first to focus on the characterization of the molecular mechanisms involved in delayed PGHS-2 gene expression in granulosa cells of a species with a long ovulatory process. The selected *in vitro* model of primary cultures of bovine granulosa cells recapitulates the pattern of induction observed *in vivo* (Tsai et al., 1996; Liu et al., 1997; Sirois and Dore, 1997), as PGHS-2 mRNA and protein became maximal only at 24 h post-hCG. This is in sharp contrast with the more rapid induction reported in rats where PGHS-2 expression has already peaked by 6 h post-hCG (Sirois et al., 1992; Wong and Richards, 1992). Moreover, the time-course transient transfection study of bovine granulosa cells

with a -1573/-2 PGHS-2 promoter/luciferase chimeric construct showed that reporter gene activity was maximal after 24-36 h of forskolin stimulation, which parallels the increase in prostaglandin synthetic activity associated with the induction of the endogenous PGHS-2 gene *in vitro*. In contrast, comparable studies in rats indicated that 6 h of forskolin stimulation was sufficient to induce maximal PGHS-2 promoter activity in rat granulosa cells (Sirois et al., 1992). Thus, the delayed induction of PGHS-2 in bovine granulosa cells *in vitro*, when compared to rat granulosa cells, provides a valuable model to study the regulation of PGHS-2 gene expression in species with a long ovulatory process.

Differences in the primary structure (*cis*-acting elements) of the bovine PGHS-2 promoter, or in the complement of transcription factors present in bovine granulosa cell nuclei, or a combination of both, were considered as potential causes of delayed PGHS-2 induction in this species. The cloning and deletion mutant analyses of the bovine PGHS-2 promoter revealed that the proximal promoter region (-147/-2) was, as in rats (Morris and Richards, 1996), sufficient to confer basal and full forskolin-inducible activities, which suggests that the same region is involved in both species. This region, which spans the first 150 bp upstream of the transcription start site, is relatively conserved in bovine and rats (63% identity in nucleotide sequence), and contains four putative *cis*-elements, including AP2, C/EBP, ATF/CRE and E-box. Deletion and site-directed mutagenesis studies indicated that the E-box was essential, but not sufficient, for the full promoter activity of the bovine PGHS-2 gene in granulosa cells. Mutation of the C/EBP element resulted only in moderate reduction (29%) in forskolin-induced, but not in basal, activity, whereas mutation of ATF/CRE and AP2 elements had no inhibitory effect. It seems likely that additional, yet unidentified, element(s) present between -87 and -147 bp are involved in PGHS-2 promoter activity, because the activity of –147/-

2PGHS.LUC was significantly higher than that of –87/-2PGHS.LUC. However, the central role played by the E-box in the bovine system is in keeping with results observed in rats (Morris and Richards, 1996). Thus, results of promoter activity assays did not provide clues on potential mechanisms responsible for the marked difference in the time-course of PGHS-2 induction in these two species.

One key finding of this study is the presence, and regulation, of multiple complexes of USF-1 and USF-2 proteins binding to the E-box. USF-1 (43 kDa) and USF-2 (44 kDa) are ubiquitous proteins that belong to the Myc family of transcription factors. They are characterized by the highly conserved basic helix-loop-helix and leucine zipper domains responsible for dimerization and DNA binding (Sirito et al., 1994; Ghosh et al., 1997). USF proteins interact with DNA as homodimers and heterodimers, and are known to affect the expression of various genes (Ghosh et al., 1997; Vallet et al., 1997; Sirito et al., 1998). Interestingly, a recent study showed that overexpression of USF-1 and USF-2 homodimers acts as repressor, whereas overexpression of USF-1/USF-2 heterodimers acts as activator of ribosomal RNA gene transcription, suggesting that the relative amount of each form of dimers could play an important role in modulating gene expression (Ghosh et al., 1997). Another proposed mechanism by which the activity of these transcription factors could be regulated is by the presence of a N-terminus truncated form of USF proteins (also called mini-USF) (Sirito et al., 1994). Mini-USF (18 kDa) lacks the transcription activation domain but have the dimerization and DNA binding domains, and would function as a dominant negative mutant (Descombes and Schibler, 1991; Sirito et al., 1994). Such mutants were proved useful experimentally to demonstrate the role of USF in regulating the activity of L-type pyruvate kinase promoter (Iynedjian, 1998), but their natural occurrence has

remained elusive. The present study identifies a first physiological event during which the presence of putative N-truncated USF-2 could serve a repressor role in gene expression. Supershift EMSAs and immunoblot analyses provided complementary evidence for the presence of a putative N-terminus truncated USF in nuclear extracts of granulosa cells isolated prior to hCG (0 h). Any dimers containing a mini-USF would act as a dominant negative mutant (Iynedjian, 1998). Interestingly, hCG treatment was accompanied by the disappearance of N-truncated USF proteins in granulosa cell nuclear extracts (EMSAs and immunoblots), suggesting that removal of repressive mini-USF could serve as an initial step in delayed PGHS-2 induction in species with a long ovulatory process. These results contrast with those observed in rats where, although granulosa cell extracts were shown to contain USF proteins binding to the E-box, there was no evidence of mini-USF in this species (Morris and Richards, 1996). Also, hCG had no effect on the nature of protein complexes binding to the E-box, and on levels of USF proteins in rats (Morris and Richards, 1996). Thus, major differences exist in the nature and regulation of protein/DNA binding activities at the E-box element between species with a rapid *versus* delayed induction of PGHS-2, and the putative repressor role mini-USF in species with delayed PGHS-2 induction is attractive but will require further studies.

Another important difference in protein/DNA binding activities between the bovine and the rat PGHS-2 promoter is at the C/EBP element. In rats, there is no C/EBPß protein in nuclear extracts of granulosa cells obtained prior to hCG treatment (0 h post-hCG), but there is a rapid induction of C/EBPß mRNA and protein after gonadotropin treatment (Sirois et al., 1993). In contrast, high levels of C/EBPß protein are present granulosa cells of bovine preovulatory follicles at 0h post-hCG, and gonadotropin treatment is not

associated with a further increase in protein levels, but rather by a slight decrease in C/EBPß. The functional significance of C/EBPß binding to the CAAT box of the PGHS-2 promoter in granulosa cells remains unclear. In rats, although initial experiments performed in the context of an incomplete proximal PGHS-2 promoter proposed that it was involved in cAMP-responsiveness (Sirois et al., 1993), more recent site-directed mutagenesis experiments done with the full proximal promoter region revealed that binding to the CAAT box was not required for promoter activation in granulosa cells (Morris and Richards, 1996). These results are supported by those observed in C/EBPß deficient mice where the gonadotropin-dependent induction of PGHS-2 in granulosa cells is not compromised (Sterneck et al., 1997). Interestingly, the rapid post-induction down-regulation of PGHS-2 mRNA was not observed in C/EPBß null mice, suggesting that expression of this transcription factor could be involved in the repression of the PGHS-2 gene after its activation (Sterneck et al., 1997). There is evidence for such a role of C/EBPß in vascular endothelial cells where overexpression of the protein was shown to suppress PGHS-2 promoter activity (Inoue and Tanabe, 1997). Thus, the relative high levels of C/EBPß in nuclear extracts of bovine granulosa cells prior to gonadotropin could serve to repress promoter activity in species with a delayed PGHS-2 induction and a long ovulatory process. A transient decrease in C/EBPß levels after gonadotropin treatment could allow activation of gene transcription through the recruitment of additional factors.

Lastly, this study characterizes for the first time the primary structure of the bovine PGHS-2 gene. The intron/exon organization of the bovine PGHS-2 gene is very similar to that of the human (Kosaka et al., 1994), mouse (Fletcher et al., 1992) and equine genes (Boerboom and Sirois, 1998). The overall size of the bovine gene compares with that of other species, and the length of

internal exons 2-9 and of the coding region of exons 1 and 10 are identical amongst bovine, mouse, human and equine PGHS-2 (Fletcher et al., 1992; Kosaka et al., 1994; Boerboom and Sirois, 1998). The transcription start site of the bovine PGHS-2 gene was identified at a guanidine residue, which contrasts with the start site identified in human (cytidine; Kosaka et al., 1994), mouse, rat, and equine PGHS-2 (adenosine; Fletcher et al., 1992; Sirois et al., 1993; Boerboom and Sirois, 1998). This difference in the transcription initiation site contributes, at least in part, to a small variation observed across species in the length of the 5'-UTR, ranging from 122 to 138 bp.

In summary, this study is the first to characterize some of the molecular mechanisms involved in the delayed gonadotropin-dependent activation of PGHS-2 gene expression in species with a long ovulatory process. The report provides the characterization of the bovine PGHS-2 gene, and promoter, as well as a model *in vitro* of primary cultures of granulosa cells in which the regulation of PGHS-2 closely parallels the regulation observed *in vivo*. The marked difference in the time-course of PGHS-2 induction between rats and cattle appears associated with distinct regulation of nuclear proteins/DNA interactions at the level of the promoter. Our results indicate a novel regulation of USF proteins in granulosa cells during ovulation, with N-terminus truncated USF potentially exerting a repressor role on PGHS-2 promoter activity prior to gonadotropin treatment in bovine preovulatory follicles. The distinct pattern of expression of C/EBPß proteins between cattle and rats suggests that this transcription factor could also exert a repressor role in promoter activation before hCG treatment in bovine granulosa cells. Although these new exciting findings provide the basis of novel working hypotheses in need of testing, further effort should also focus on other, yet unidentified, transcription factors involved in the regulation in PGHS-2 gene expression in granulosa cells.

ACKNOWLEDGMENTS

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Fig. 1. **Time- and forskolin-dependent regulation of PGHS-2 mRNA and protein, and PGE_2 synthesis in bovine granulosa cells *in vitro*.** Bovine granulosa cells were cultured for various intervals of time in the absence or presence of forskolin (10 μM). *A*, Semiquantitative RT-PCR analysis of relative PGHS-2 mRNA levels in primary cultures of bovine granulosa cells. RNA samples (5 μg/time-point) were subjected to RT-PCR analysis, as described in Experimental Procedures. Results show changes in PGHS-2 cDNA (1.1 kb) compared to an internal control (GAPDH cDNA; 850 bp). *B*, regulation of PGHS-2 protein by forskolin in bovine granulosa cells *in vitro*. Cell extracts were prepared from cultures of granulosa cells treated with forskolin, and proteins (50 μg/lane) were analyzed by one-dimensional SDS-PAGE and immunoblotting techniques. Arrows on the right indicate migration of intact immunoreactive PGHS-2 protein (Mr = 72,000) and of a breakdown product (Mr = 59,000). The filter was exposed to film at -70 °C for 14 h. *C*, effect of forskolin of cumulative secretion of PGE_2 by granulosa cells. Concentrations of PGE_2 in culture medium were determined by specific radioimmunoassays. Results are presented as means ± SEM of triplicate cultures from three independent experiments. Results presented in panels *A* and *B* are representative of three and two independent experiments, respectively.

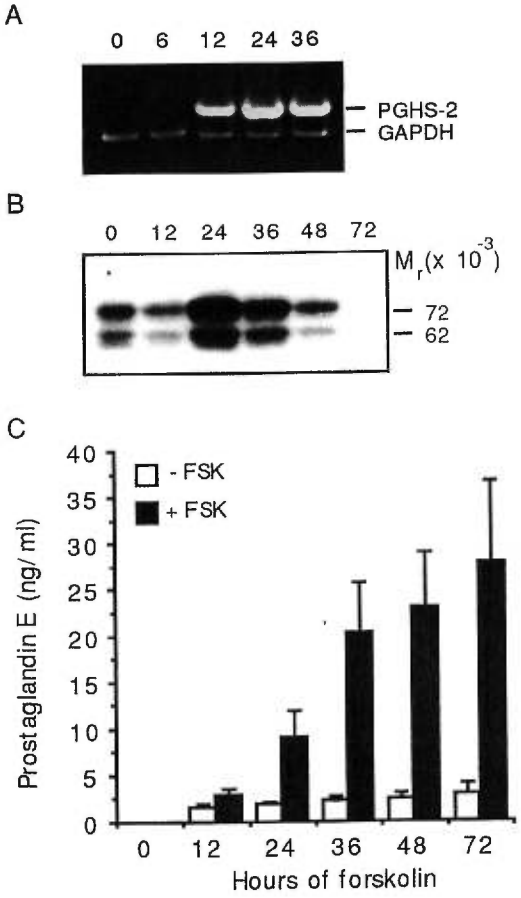
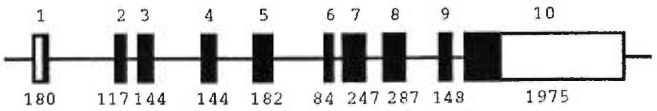

A
0 6 12 24 36
PGHS-2
GAPDH
B
0 12 24 36 48 72
M_r(x 10^{-3})
72
62
C
- FSK
+ FSK
Prostaglandin E (ng/ml)
40
35
30
25
20
15
10
5
0
0 12 24 36 48 72
Hours of forskolin

Fig. 2. **Characterization of the bovine PGHS-2 gene** *A*, schematic representation of the bovine PGHS-2 gene. Exons are shown as *boxes* and introns are represented as *lines*. All elements are drawn to scale. The numbers above the boxes indicate the exon number, whereas those below the boxes show the number of nucleotides in the exon. *B*, exon/intron organization of the bovine PGHS-2 gene. Exonic sequences at each splice junction are shown in capital letters and intronic sequences in lowercase letters. Capital letters above each codon correspond to the encoded amino acid residue. Numbers in parentheses represent the exact size (bp) of the intron as determined by DNA sequencing. The complete nucleotide sequence of the bovine gene (exons and introns) was been submitted to the GenBank (accession number AF31699). *C*, determination of the transcription initiation site of the bovine PGHS-2 gene by primer extension analysis. The labeled anti-sense 24-mer primer was hybridized to RNA samples containing (follicle isolated 24 h post-hCG) and not containing (follicle 0 h post-hCG; negative control) PGHS-2 mRNA, and primer extension was performed as described in *Experimental Procedures*. The extended product was analyzed on 6% polyacrylamide gel and its size determined by comparison with the products of a sequencing reaction using the same primer and a genomic clone spanning this region as the template. A 57-nucleotide extended product was obtained.

A



B

Exon				Intron				Exon
	G	A	A	181	1232	N	P	
1	GGT	GCA	G	gtgag...1(1052)	...tgtag	CA AAT	CCT	2
	T	T	P	1350	1458	E	F	
2	ACC	ACA	C	gtaag...2(109)	...tgcag	CC GAA	TTT	3
	L	T	S	1603	2287	R	S	
3	TTG	ACG	T	gtaag...3(685)	...ttcag	CG AGA	TCA	4
	V	K	G	2432	3076	R	K	
4	GTG	AAA	G	gtgag...4(645)	...tatag	GG AGG	AAA	5
	H	G		3259	3856	V D	L	
5	CAT	GGG		gtgag...5(598)	...tctag	GTG GAC	TTA	6
	Y	Q		3941	4051	M I	N	
6	TAT	CAG		gtttg...6(111)	...tttag	ATG ATT	AAT	7
	L	I	G	4299	4582	E	T	
7	CTG	ATA	G	gtaag...7(284)	...aatag	GA GAA	ACT	8
	G	R		4870	5366	V A	G	
8	GGC	AGG		gtaag...8(497)	...cacag	GTC GCT	GGC	9
	L	T	G	5515	5663	E	K	
9	CTT	ACA	G	gtggg...9(149)	...cacag	GA GAG	AAG	10

C

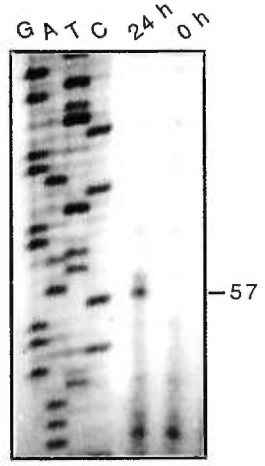


Fig. 3. **Nucleotide sequence of the bovine PGHS-2 promoter from -1650 to +100.** Numbering is relative to the transcription start site (+1) determined by primer extension analysis (Fig. 2). The sequence was submitted to the transcription factor database TFSites (GCG, Wisconsin), and putative *cis*-acting elements located within the first 600 bp upstream of the cap site are underlined. The nucleotide sequence was deposited to GenBank (accession number AF031699).

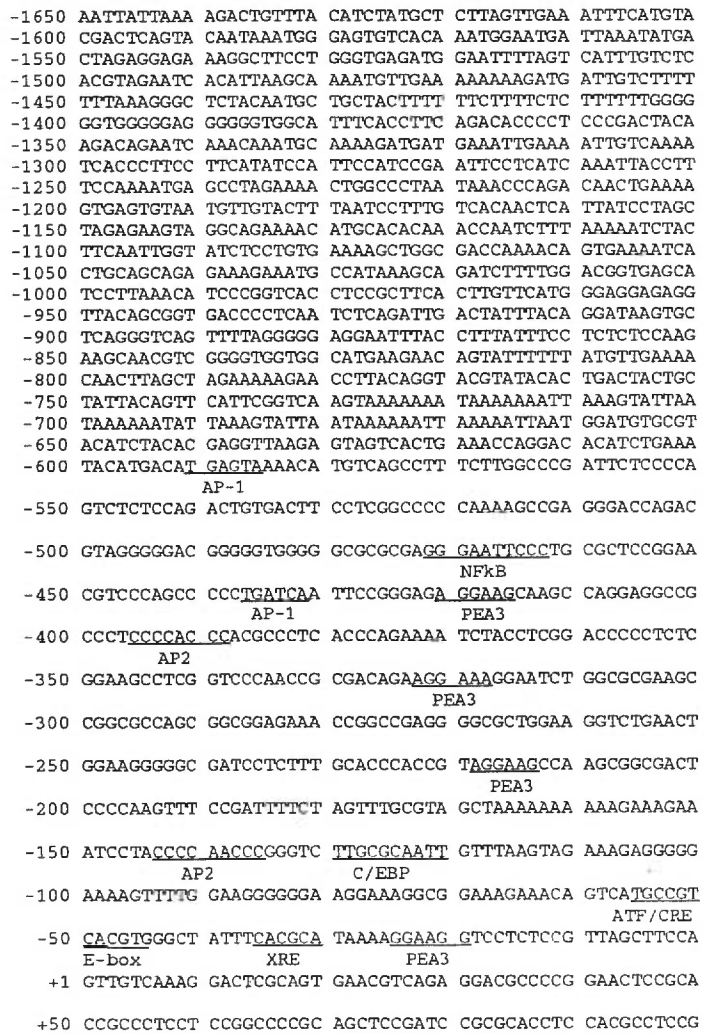

-1650 AATTATTAAA AGACTGTTTA CATCTATGCT CTTAGTTGAA ATTTCATGTA
-1600 CGACTCAGTA CAATAAATGG GAGTGTCACA AATGGAATGA TTAAATATGA
-1550 CTAGAGGAGA AAGGCTTCCT GGGTGAGATG GAATTTTAGT CATTTGTCTC
-1500 ACGTAGAATC ACATTAAGCA AAATGTTGAA AAAAAAGATG ATTGTCTTTT
-1450 TTTAAAGGGC TCTACAATGC TGCTACTTTT TTCTTTTCTC TTTTTTGGGG
-1400 GGTGGGGGAG GGGGGTGGCA TTTCACCTTC AGACACCCCT CCCGACTACA
-1350 AGACAGAATC AAACAAATGC AAAAGATGAT GAAATTGAAA ATTGTCAAAA
-1300 TCACCCTTCC TTCATATCCA TTCCATCCGA ATTCCTCATC AAATTACCTT
-1250 TCCAAAATGA GCCTAGAAAA CTGGCCCTAA TAAACCCAGA CAACTGAAAA
-1200 GTGAGTGTAA TGTTGTACTT TAATCCTTTG TCACAACTCA TTATCCTAGC
-1150 TAGAGAAGTA GGCAGAAAAC ATGCACACAA ACCAATCTTT AAAAATCTAC
-1100 TTCAATTGGT ATCTCCTGTG AAAAGCTGGC GACCAAAACA GTGAAAATCA
-1050 CTGCAGCAGA GAAAGAAATG CCATAAAGCA GATCTTTTGG ACGGTGAGCA
-1000 TCCTTAAACA TCCCGGTCAC CTCCGCTTCA CTTGTTCATG GGAGGAGAGG
 -950 TTACAGCGGT GACCCCTCAA TCTCAGATTG ACTATTTACA GGATAAGTGC
 -900 TCAGGGTCAG TTTTAGGGGG AGGAATTTAC CTTTATTTCC TCTCTCCAAG
 -850 AAGCAACGTC GGGGTGGTGG CATGAAGAAC AGTATTTTTT ATGTTGAAAA
 -800 CAACTTAGCT AGAAAAAGAA CCTTACAGGT ACGTATACAC TGACTACTGC
 -750 TATTACAGTT CATTCGGTCA AGTAAAAAAA TAAAAAAATT AAAGTATTAA
 -700 TAAAAAATAT TAAAGTATTA ATAAAAAATT AAAAATTAAT GGATGTGCGT
 -650 ACATCTACAC GAGGTTAAGA GTAGTCACTG AAACCAGGAC ACATCTGAAA
 -600 TACATGACAT GAGTAAAACA TGTCAGCCTT TCTTGGCCCG ATTCTCCCCA
AP-1
 -550 GTCTCTCCAG ACTGTGACTT CCTCGGCCCC CAAAAGCCGA GGGACCAGAC
 -500 GTAGGGGGAC GGGGGTGGGG GCGCGCGAGG GAATTCCCTG CGCTCCGGAA
NFkB
 -450 CGTCCCAGCC CCCTGATCAA TTCCGGGAGA GGAAGCAAGC CAGGAGGCCG
AP-1 PEA3
 -400 CCCTCCCCAC CCACGCCCTC ACCCAGAAAA TCTACCTCGG ACCCCCTCTC
AP2
 -350 GGAAGCCTCG GTCCCAACCG CGACAGAAGG AAAGGAATCT GGCGCGAAGC
PEA3
 -300 CGGCGCCAGC GGCGGAGAAA CCGGCCGAGG GGCGCTGGAA GGTCTGAACT
 -250 GGAAGGGGGC GATCCTCTTT GCACCCACCG TAGGAAGCCA AGCGGCGACT
PEA3
 -200 CCCCAAGTTT CCGATTTTCT AGTTTGCGTA GCTAAAAAAA AAAGAAAGAA
 -150 ATCCTACCCC AACCCGGGTC TTGCGCAATT GTTTAAGTAG AAAGAGGGGG
AP2 C/EBP
 -100 AAAAGTTTTG GAAGGGGGGA AGGAAAGGCG GAAAGAAACA GTCATGCCGT
ATF/CRE
  -50 CACGTGGGCT ATTTCACGCA TAAAAGGAAG GTCCTCTCCG TTAGCTTCCA
E-box XRE PEA3
   +1 GTTGTCAAAG GACTCGCAGT GAACGTCAGA GGACGCCCCG GAACTCCGCA
  +50 CCGCCCTCCT CCGGCCCCGC AGCTCCGATC CGCGCACCTC CACGCCTCCG

Fig. 4. **Functional analysis of the bovine PGHS-2 promoter in primary granulosa cell cultures**. *A*, bovine PGHS-2 promoter fragments -1573/-2, -491/-2, -324/-2, -147/-2, -87/-2, and -38/-2 were generated as described in Experimental Procedures, and subcloned upstream of the firefly luciferase reporter gene in the pGL3.Basic vector (Basic.LUC). *B*, Time-dependent induction of PGHS-2 promoter activity in bovine granulosa cells. Primary cultures of bovine granulosa cells were transiently transfected with the full-length construct -1573/-2PGHS.LUC, and incubated for 6, 12, 24, 36, 48 and 72 in the presence of forskolin (10 μM), as described under "Experimental Procedures". Cells transfected with the promoterless plasmid pGL3-Basic were stimulated with forskolin for 36 h. Results are presented as luciferase activity normalized per μg of protein (mean ± SEM of triplicate cultures from three independent experiments). *C*, Deletion analysis of the bovine PGHS.2 promoter. Primary cultures of granulosa cells were transfected with equimolar amounts of -1573/-2PGHS.LUC, a series of 5'-deletion mutants, and with the promoterless plasmid pGL3-Basic. All cultures were co-transfected with the SV40 *Renilla* luciferase vector (SV40.pRL) as an internal control to normalize experimental reporter activity. After transfection, cells were incubated for 36 h in the absence or presence of forskolin (10 μM). Results are presented as normalized relative luciferase activity (Firefly/Renilla; means ± SE of triplicate cultures of three experiments).

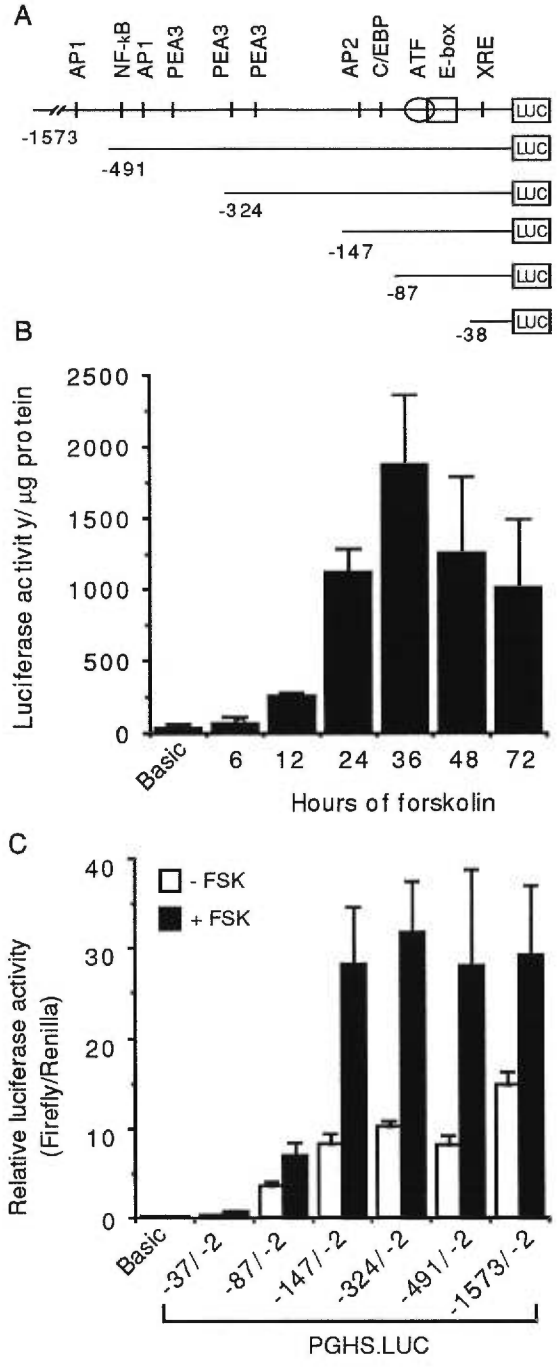
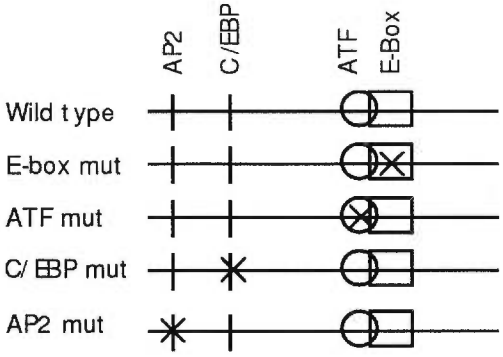

A
AP1
NF-κB
AP1
PEA3
PEA3
PEA3
AP2
C/EBP
ATF
E-box
XRE
LUC
-1573
-491
-324
-147
-87
-38
B
Luciferase activity/μg protein
2500
2000
1500
1000
500
0
Basic
6
12
24
36
48
72
Hours of forskolin
C
Relative luciferase activity
(Firefly/Renilla)
40
30
20
10
0
- FSK
+ FSK
Basic
-37/-2
-87/-2
-147/-2
-324/-2
-491/-2
-1573/-2
PGHS.LUC

Fig. 5. **Effect of site-directed mutagenesis on forskolin-dependent PGHS-2 promoter activity in bovine granulosa cells**. *A*, four site-directed mutants were generated within the context of the -147/-2 PGHS-2 promoter fragment, as described in Experimental Procedures. *B*, primary cultures of bovine granulosa cells were transiently transfected with either the promoterless plasmid pGL3.Basic, the wildtype construct -147/-2PGHS.LUC, or with -147/-2PGHS.LUC constructs containing point mutations with the AP2 (AP2mut), C/EBP (C/EBPmut), ATF/CRE (ATFmut) or E-box (E-boxmut) cis-elements. All cultures were co-transfected with the SV40 *Renilla* luciferase vector (SV40-pRL) as an internal control to normalize experimental reporter activity. After transfection, cells were incubated for 36 h in the absence or presence of forskolin (10 μM). Results are presented as normalized relative luciferase activity (Firefly/Renilla; means ± SE of triplicate cultures of three experiments).

A.


AP2
C/EBP
ATF
E-Box
Wild type
E-box mut
ATF mut
C/EBP mut
AP2 mut

B.

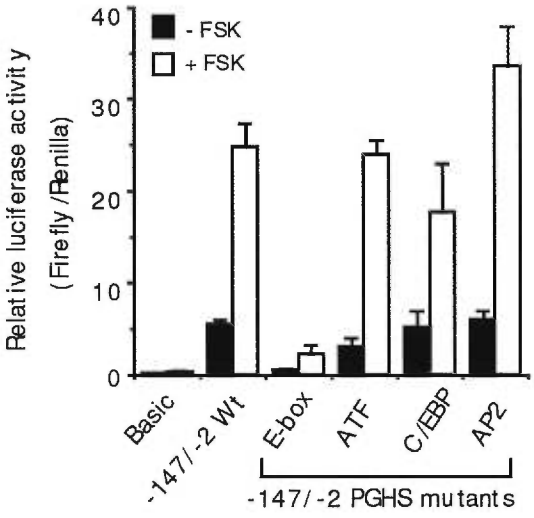

Relative luciferase activity
(Firefly /Renilla)
40
30
20
10
0
- FSK
+ FSK
Basic
-147/-2 Wt
E-box
ATF
C/EBP
AP2
-147/-2 PGHS mutants

Fig. 6. **Gonadotropin-dependent regulation of DNA-binding activities in nuclear extracts of bovine granulosa cells and roles of C/EBP and E-box *cis*-acting PGHS-2 promoter elements.** *A*, extracts of nuclear proteins were prepared from granulosa cells of preovulatory follicles isolated at 0, 18 and 20 h post-hCG, as described in Experimental Procedures. Extracts were incubated with the [^{32}P]-labelled DNA fragment -147/-2, and protein/DNA interactions were tested in electrophoretic mobility shift assays (EMSAs). Binding reactions were resolved by 5% acrylamide/0.5X TBE gel electrophoresis. For reference purposes, eight protein/DNA complexes were designated as bands a, b, c, d, e, f, g and h. *B*, competitive EMSAs were performed in the presence of granulosa cell nuclear extract prepared 0 h post-hCG, [^{32}P]-labelled fragment -147/-2, and 25-molar excess of unlabelled competitor DNA fragments. Competitor DNA included wildtype -147/-2, as well as four site-directed mutants (E-box mutant [E-boxmut], C/EBP mutant [C/EBPmut], ATF/CRE mutant [ATFmut] and AP2 mutant [AP2mut]) generated in the context of the -147/-2 PGHS-2 promoter fragment. *C*, competitive EMSAs were performed under conditions identical to those described in *B*, except that the nuclear extract was prepared from granulosa cells isolated 18 h post-hCG.

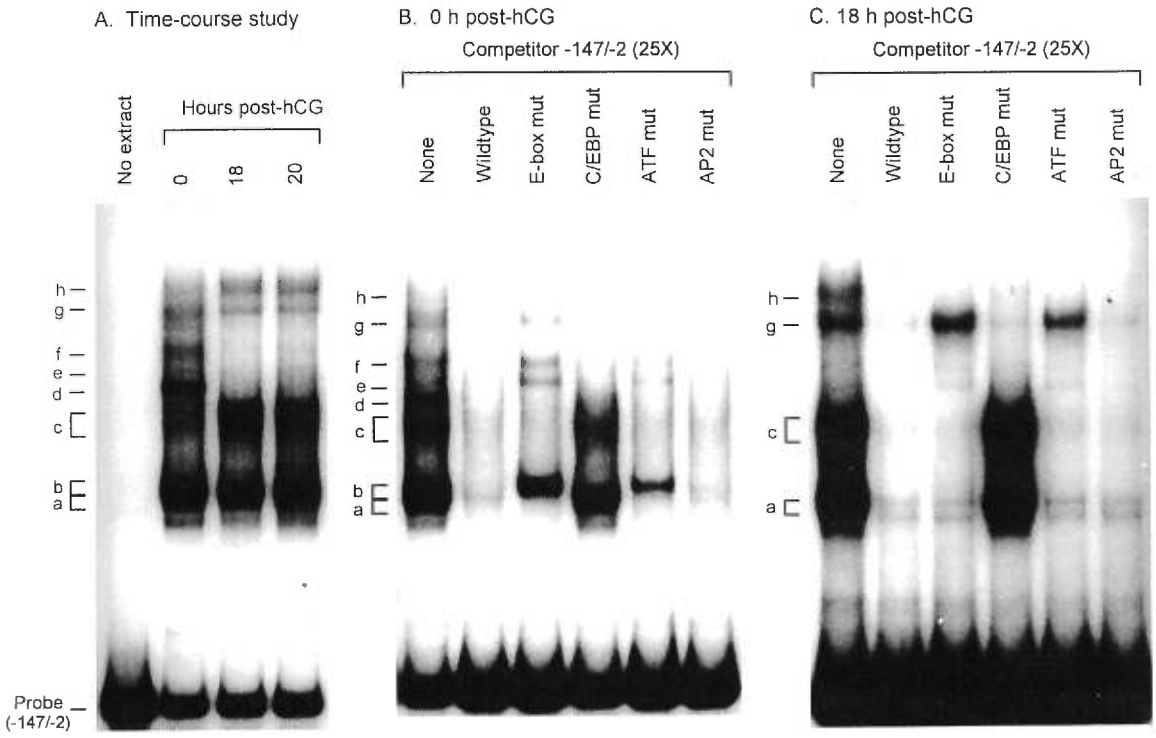

A. Time-course study
No extract
Hours post-hCG
0
18
20
h
g
f
e
d
c
b
a
Probe
(-147/-2)
B. 0 h post-hCG
Competitor -147/-2 (25X)
None
Wildtype
E-box mut
C/EBP mut
ATF mut
AP2 mut
C. 18 h post-hCG
Competitor -147/-2 (25X)
None
Wildtype
E-box mut
C/EBP mut
ATF mut
AP2 mut

Fig. 7. **Competition of DNA-binding activities in bovine granulosa cell nuclear extracts with C/EBP and E-box consensus oligonucleotides.** Competitive EMSAs were performed in the presence of granulosa cell nuclear extract prepared 0 h (*A*) or 18 h (*B*) post-hCG, [^{32}P]-labelled fragment -147/-2, and 50-molar excess of unlabelled competitor DNA fragments. Competitor DNA included wildtype -147/-2, complementary oligonuleotides containing the bovine PGHS-2 C/EBP (C/EBP-b) and E-box (E-box-b) *cis*-elements, or complementary oligonucleotides containing consensus C/EBP (C/EBP-c) and E-box (E-box-c) elements, as described in Table 1. Binding reactions were resolved by 5% acrylamide, 0.5 X TBE gel electrophoresis.

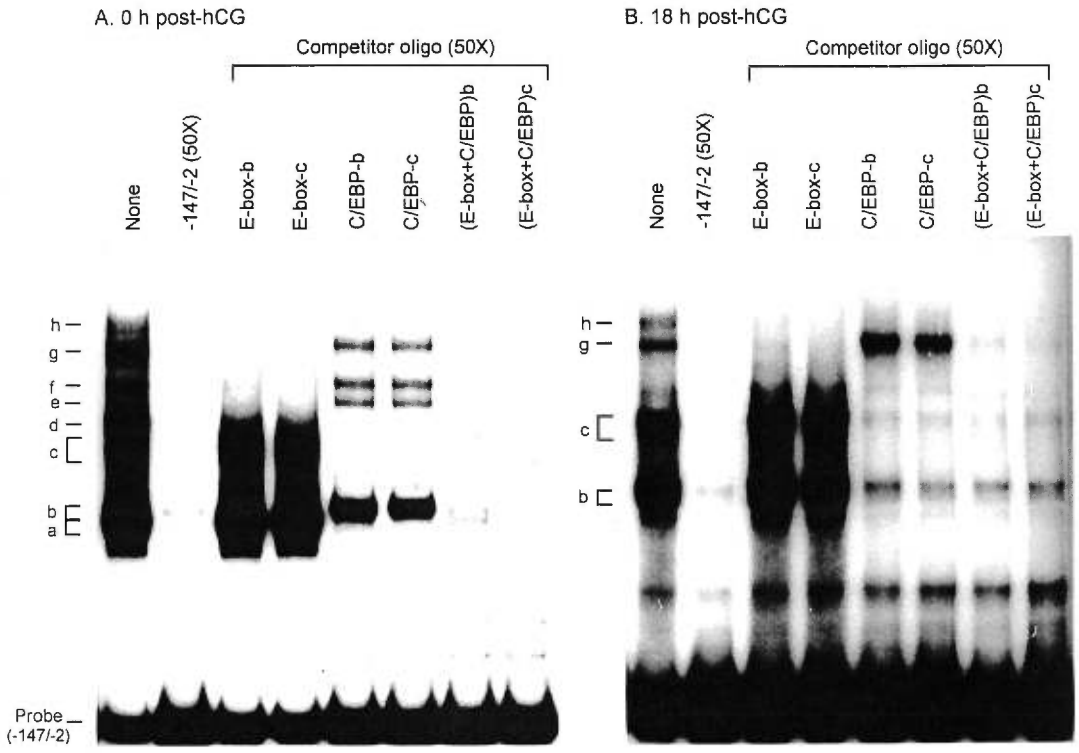

A. 0 h post-hCG
Competitor oligo (50X)
None
-147/-2 (50X)
E-box-b
E-box-c
C/EBP-b
C/EBP-c
(E-box+C/EBP)b
(E-box+C/EBP)c
h
g
f
e
d
c
b
a
Probe
(-147/-2)
B. 18 h post-hCG
Competitor oligo (50X)
None
-147/-2 (50X)
E-box-b
E-box-c
C/EBP-b
C/EBP-c
(E-box+C/EBP)b
(E-box+C/EBP)c
h
g
c
b

Fig. 8. **Effect of antisera against C/EBP and USF proteins on binding activities between granulosa cell nuclear extracts and the bovine PGHS-2 promoter fragment -147/-2.** To determine if USF and C/EBP proteins were involved in nuclear protein/DNA binding, EMSAs were performed in the presence granulosa cell nuclear extract prepared 0 h (*A*) or 18 h (*B*) post-hCG, [^{32}P]-labelled fragment -147/-2, 25-molar excess of unlabelled competitor DNA fragments -147/-2 with a mutated E-box (E-boxmut) or C/EBP (C/EBPmut), and selected polyclonal antibodies. They included antibodies raised against USF-1, the COOH-terminus of USF-2, the N-terminus of USF-2, C/EBPα, C/EBPß and C/EBPδ. Binding reactions were prepared as described under Experimental Procedures, and were resolved by 5% acrylamide, 0.5 X TBE gel electrophoresis.

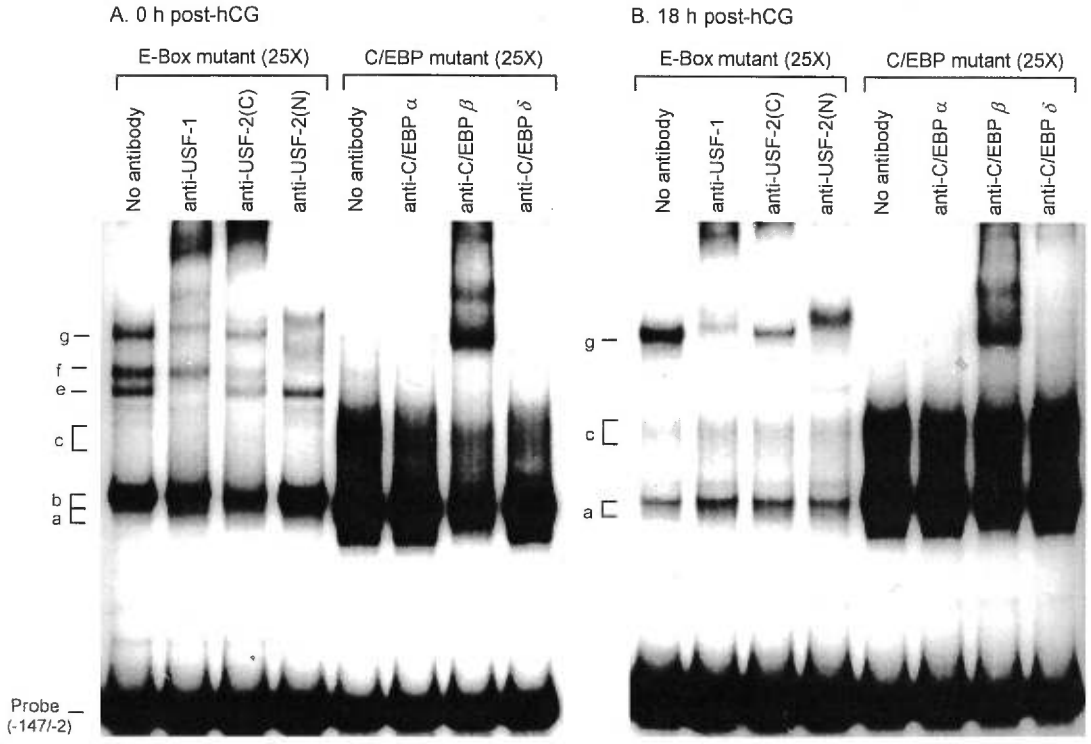

A. 0 h post-hCG
B. 18 h post-hCG
E-Box mutant (25X)
C/EBP mutant (25X)
No antibody
anti-USF-1
anti-USF-2(C)
anti-USF-2(N)
anti-C/EBP α
anti-C/EBP β
anti-C/EBP δ
g
f
e
c
b
a
Probe
(-147/-2)

Fig. 9. **Immunoblot analysis of USF-1, USF-2 and C/EBPß protein content in bovine granulosa cell nuclear extracts prepared 0, 18 and 20 h post-hCG *in vivo*.** Nuclear extracts were prepared from granulosa cells isolated 0, 18 and 20 h post-hCG. Proteins (50 μg/lane) were analyzed by one-dimensional SDS-PAGE and immunoblotting techniques using polyclonal antibodies agains USF-1, USF-2/COOH, the COOH-terminus of USF-2 (anti-USF2(C)), USF-2/NH_2, the N-terminus of USF-2 (anti-USF-2(N)) and C/EBPß, as described under "Experimental Procedures". Markers on the right indicate migration of molecular weight standards. Upper arrow heads on the left indicate migration of respective full-length proteins, whereas lower arrow heads indicate putative N-terminus truncated USF-2 (mini-USF) and C/EBPß.

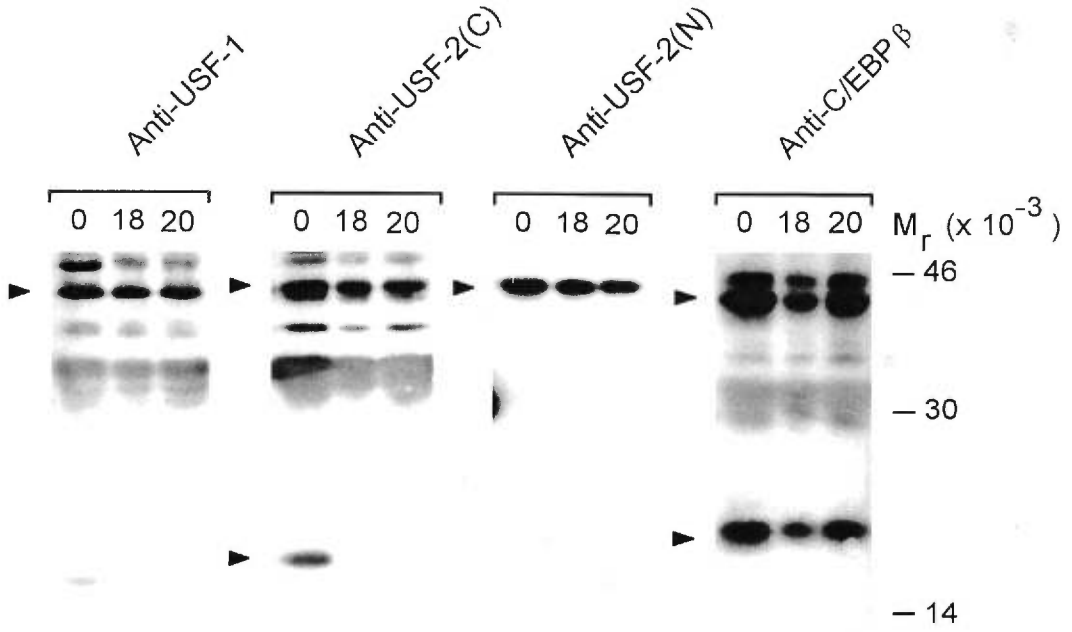

Anti-USF-1
Anti-USF-2(C)
Anti-USF-2(N)
Anti-C/EBP β
0 18 20
0 18 20
0 18 20
0 18 20
M$_r$ (x 10^{-3})
46
30
14

Table I. Sequences of oligonucleotides.

A. Semi-quantitative RT-PCR

1	5'-TGTTCCAGTATGATTCCACCC-3'
2	5'-TCCACCACCCTGTTGCTGTA-3'
3	5'-TGTTCCAGTATGATTCCACCC-3'
4	5'-TCCACCACCCTGTTGCTGTA-3'

B. Deletion mutants of the bovine PGHS-2 promoter

5	5'-AAATGGGAGCTCCACAAATGGAATGATTAAATATGACTA-3'
6	5'-AGAGGTGAGCTCACGGGGGTGGGGGCGCGCGAGGGAATTCCC-3'
7	5'-TCAGACGAGCTCGAAGGAAAGGAATCTGGCGC-3'
8	5'-AAGAAAGAGCTCCTACCCCAACCCGGGTCTTGCGCAATTGTT-3'
9	5'-AAAAGTGAGCTCAGGGGGGGAAGGAAAGGCGGAAAGAAACAGT-3'
10	5'-CGTTTGCTCGAGGGAAGCTAACGGAGAGGACCT-3'
11	5'-CTTTCACGCATAAAAGGAAGGTCCTCTCCGTTAGCTTCCC-3'
12	5'-TCGAGGGAAGCTAACGGAGAGGACCTTCCTTTTATGCGTGAAAGAGCT-3'

C. Site-directed mutants of the bovine PGHS-2 promoter

E-box mutant	forward	5'-GCCGTCACTTTGGCTATTTCACGC-3'
	reverse	5'-ATGACTGTTTCTTTCCGCCTTTCC-3'
C/EBP mutant	forward	5'-GGGTCTTGCTCCCATGTTTAAGTAG-3'
	reverse	5'-GGGTTGGGGTAGGAGCTCGGTACC-3'
ATF mutant	forward	5'-CAGTCATACATTCACGTGGGC-3'
	reverse	5'-TTTCTTTCCGCCTTTCCTTCCCCCC-3'
AP2 mutant	forward	5'-CTACCATGAACCGGGTCTTGCGC-3'
	reverse	5'-GAGCTCGGTACCTATCGATAGAG-3'

D. Competitor oligonucleotides for EMSAs

Bovine E-box	sense	5'-GGGCAGTCATGCCGTCACGTGGGCTATTT-3'
	anti-sense	5'-AAATAGCCCACGTGACGGCATGACTG-3'
Consensus E-box*	sense	5'-CACCCGGTCACGTGGCCTACACC-3'
	anti-sense	5'-GGTGTAGGCCACGTGACCGGGTG-3'
Bovine C/EBP	sense	5'-GGGGTCTTGCGCAATTGTTTAAGTAGAA-3'
	anti-sense	5'-TTCTACTTAAACAATTGCGCAAGAC-3'
Consensus C/EBP*	sense	5'-TGCAGATTGCGCAATCTGCA-3'
	anti-sense	5'-TGCAGATTGCGCAATCTGCA-3'

* Consensus oligonucleotides were obtained from Santa Cruz Biotechnologies.

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7. GENERAL DISCUSSION

My project provides novel information on the expression and regulation of PGHS-2 in granulosa cells of bovine follicles prior to ovulation. Previous reports have shown that PGHS-2 is induced in preovulatory follicles of rats, cows and mares, but only in models of induced ovulation with exogenous hCG (Wong and Richards, 1991; Sirois et al., 1992; Sirois, 1994; Sirois and Dore, 1997). Administration of GnRH was also shown to induce the expression of PGHS-2 in bovine preovulatory follicles (Tsai et al., 1996). However, whether or not PGHS-2 is induced in preovulatory follicles under physiological conditions has not been determined. Results from my first study demonstrate that PGHS-2 mRNA and protein are not present in preovulatory follicles isolated at the onset of estrus, but are markedly induced 24 h after the onset of estrus. The pattern of PGHS-2 expression is very similar to that observed during hCG-induced ovulation, indicating that the induction of PGHS-2 is a physiological event, and is gonadotropin-dependent.

Immunoblot analysis revealed that a 74,000 Mr PGHS-2 protein was selectively induced in granulosa cells after the endogenous LH surge. This is in agreement with previous studies in rats and cows in which the induction of PGHS-2 enzymes by hCG was detected only in granulosa cells of preovulatory follicles (Wong and Richards, 1991; Sirois et al., 1992; Sirois, 1994). Therefore, it is clear that induction of PGHS-2 by gonadotropins is granulosa cell-specific in preovulatory follicles.

Immunohistochemical analyses showed that the PGHS-2 staining was particularly pronounced around the granulosa cell nuclei. Similar results were observed in other cell types, such as fibroblasts and endothelial cells in which PGHS-2 was preferentially located to the nuclear envelop (Morita et al., 1995; Spencer et al., 1998). Differential subcellular localizations of PGHS-1 and -2

are thought to be responsible for distinct roles of these two isoforms in various cellular functions. Prostaglandins derived from PGHS-1 in the endoplasmic reticulum are likely to be secreted and to interact with cell surface receptors in an autocrine or paracrine fashion; whereas prostaglandins produced from PGHS-2 in the nuclear envelop may directly interact with nuclear receptors in an intracrine manner (Kliewer et al., 1995; Yu et al., 1995). A recent study also shows that these two isoforms utilize different phospholipase systems and lipid pools for prostaglandin synthesis (Reddy and Herschman, 1997). However, the physiological functions of nuclear prostaglandin localization in granulosa cells remain unclear.

The delayed induction of PGHS-2 in preovulatory follicles after the endogenous LH surge is consistent with the time-course observed in the model of hCG-induced ovulation in cattle (Sirois, 1994), but sharply contrasts with observations in rats (Sirois et al., 1992). Induction of PGHS-2 is rapid in rats (2-4 h post-hCG) (Sirois et al., 1992), but relatively delayed in cows (18 h post-hCG) and mares (30 h post-hCG) (Sirois, 1994; Sirois and Dore, 1997). This difference in the time-course induction of PGHS-2 suggests a distinct molecular mechanism involved in the activation of PGHS-2 gene during ovulation. However, the time interval from PGHS-2 induction to ovulation is relatively constant in rats, cows and mares (about 10 h), suggesting a common role of PGHS-2 in the regulation of follicular rupture.

The induction of PGHS-2 was associated with increased follicular prostaglandin synthetic activities prior to ovulation. A recent study showed that inhibition of PGHS-2 by the selective inhibitor NS-398 significantly reduced gonadotropin-induced prostaglandin synthesis and ovulation rate in rats (Mikuni et al., 1998). Mice lacking the PGHS-2 gene are infertile because of defective ovulation and fertilization (Lim et al., 1997); whereas the absence of the PGHS-

1 gene in mice does not affect ovarian function (Langenbach et al., 1995), suggesting that prostaglandins produced by PGHS-2 play an important role in follicular rupture.

The precise role of prostaglandins during the ovulatory process remains to be elucidated. Studies in rats suggest their involvement in the activation of interstitial collagenase gene expression necessary for follicular rupture (Reich et al., 1991). Gene targeting studies have demonstrated that PGE receptors (EP1-3), PGF receptor (FP) and PGI receptor (IP) deficient mice have normal fertility (Ushikubi et al., 1998). Therefore, it is unclear which prostaglandins and which receptors are necessary for ovulation. Also, prostaglandins synthesized from PGHS-2 could be involved in the activation of nuclear receptors, such as PPARs (Forman et al., 1995; Kliewer et al., 1995). PPARs have been identified in bovine follicles (Sundvold et al., 1997), whether or not they are regulated by gonadotropins has not been determined. Furthermore, whether PPARs are involved in the regulation of follicular rupture-related genes remains a mystery.

Results from the second study show that the induction of PGHS-2 in bovine follicles during superovulation is time- and follicle size-dependent. The time-course PGHS-2 induction during superovulation was similar to that observed in follicles during a natural estrous cycle (Sirois, 1994; Liu et al., 1997). The expression of PGHS-2 was exclusively observed in follicles that were greater than 8 mm in diameter, suggesting that this size is an important developmental stage for bovine follicles. The study of Xu et al. (1995) showed that LH receptors are only expressed in granulosa cells of follicles greater than 8 mm. Therefore, the gonadotropin-dependent induction of PGHS-2 at this developmental stage is likely related to the coincident expression of LH receptors in granulosa cells.

A large number (24%) of follicles greater than 8 mm are PGHS-2 negative at 24 h post-hCG, which is very similar to the incidence of anovulatory follicles detected by ultrasonography during superovulation in cattle (Laurincik et al., 1993; Pruwantara et al., 1994). One key finding of this study was that these PGHS-2 negative large follicles (> 8 mm) did not luteinize as evidenced by high estradiol and low progesterone levels in their follicular fluid. The presence of PGHS-2 negative large follicles may represent one of the adverse effects of current superovulatory treatments, not only because these follicles remain anovulatory, but also because they increase intraovarian and circulation levels of estradiol.

Interestingly, the expression of PGHS-2 could be used as a maker to differentiate two subpopulations of superovulatory follicles with distinct steroidogenic capacities. PGHS-2 positive follicles were luteinized and had high progesterone and low estradiol, but PGHS-2 negative follicles were not luteinized and had high estradiol and low progesterone levels. This result suggests that the expression of PGHS-2 is closely related to the luteinization process (granulosa cell differentiation). The expression of PGHS-2 has previously been linked to cell differentiation and transformation processes (DeWitt and Smith, 1995; Tsujii and DuBois, 1995).

The second study also indicated that PGHS-2 expression is closely related to the morphology of the cumulus-oocyte-complex (COC). All PGHS-2-positive follicles contained an expanded COC, whereas the majority of small, medium and large PGHS-2-negative follicles isolated at 0 and 24 h post-hCG had a compact unexpanded COC. The only time point at which COC expansion was observed in a considerable population of PGHS-2-negative follicles (40%) was at 18 h post-hCG. It has been shown that oocyte maturation is initiated at about 10-12 h post-LH/hCG (De Loos et al., 1991), suggesting

that COC expansion occurs earlier than PGHS-2 expression. Therefore, it is likely that PGHS-2-negative follicles with an expanded COC at 18 h would become positive shortly thereafter. Although COC expansion was only detected in PGHS-2-positive follicles, the precise role of PGHS-2 in oocyte maturation remains unclear. In cattle, PGHS-2 expression and oocyte maturation could be independent events during ovulation since COC expansion apparently precedes PGHS-2 induction. However, PGHS-2 was detected in ovulated COCs of normal mice, and the lack of PGHS-2 expression in COC could be the cause of fertilization failure in PGHS-2-deficient female mice (Lim et al., 1997).

In the final study, the molecular mechanism involved in the late induction of PGHS-2 in bovine granulosa cells was explored. Studies *in vitro* showed that PGHS-2 protein and transcript were induced by forskolin (cAMP/protein kinase A pathway) in primary cultures of bovine granulosa cells. Induction of PGHS-2 by forskolin *in vitro* was delayed as compared to rats (Sirois et al., 1992), and followed a time-course similar to that observed *in vivo* after LH/hCG (Sirois, 1994; Tsai et al., 1996; Liu et al., 1997). Therefore, this *in vitro* culture system appeared suitable to study the regulation and molecular control of PGHS-2 in granulosa cells.

The bovine PGHS-2 promoter was isolated in our laboratory (Antaya et al., 1997). Comparison of the 5'-flanking region (600 bp) of the mouse, rat, human, equine and bovine PGHS-2 genes revealed numerous regions of homology, including putative binding sites for transcription factors such as NFkB, SP-1, AP-2, PEA3, C/EBP, CREB and E-box binding proteins. Transient transfections of full-length PGHS-2 promoter into primary cultures of granulosa cells revealed a time-dependent induction of promoter activity, with a peak at 24-36 h post-forskolin. This result indicates that the activation of bovine PGHS-

2 promoter is also delayed when compared to the rat promoter (Sirois et al., 1993), suggesting that the late induction of PGHS-2 gene in granulosa cells of bovine preovulatory follicles is transcriptionally controlled through cAMP/protein kinase A pathway.

Deletion analysis of the bovine PGHS-2 promoter identified that the -147/-2 region of the proximal promoter had full-length promoter activity and confered forskolin-induced responsiveness. Four putative *cis*-elements were identified within this region, including AP2, C/EBP, and E-box with an overlapping ATF/CRE. Removal of AP2 and C/EBP elements from this region decreased basal and forskolin-induced promoter activity, whereas further removal of the E-box and ATF/CRE resulted in a complete loss in both basal and forskolin-induced activity of the PGHS-2 promoter in granulosa cells. Site-directed mutation within the C/EBP element (CAAT-box) attenuated forskolin-induced activity although the basal activity of promoter remained unchanged. However, disruption of the E-box lead to 91% loss of promoter activity. These results clearly indicate that the binding of nuclear proteins to E-box is essential but not sufficient for full transcriptional activity of the bovine PGHS-2 promoter in response to forskolin. Disruption of the ATF site had no effect on the forskolin-induced activity, suggesting that the ATF element is not involved in the forskolin-dependent regulation of PGHS-2 transcription. Site-directed mutagenesis within the AP2 site slightly increased both basal and forskolin-induced activities, but the significance of this finding remains to be determined.

Electrophoretic mobility shift assays (EMSA) documented that DNA-protein interactions within the -147/-2 region are regulated by hCG. Multiple DNA-protein complexes were detected in nuclear extracts of granulosa cells isolated prior to hCG treatment (0 h), but several complexes disappeared 18 h post-hCG, whereas one complex increased in density. Further studies showed

the two DNA-protein interaction regions, C/EBP and E-box, were identified by EMSAs within the -147/-2 region. Four DNA-protein complexes were located to the E-box at 0 h, whereas only one DNA-protein complex was identified 18 h post-hCG. Two major DNA-protein complexes were assigned to the C/EBP, with only minor changes upon stimulation of hCG. The pattern of DNA-protein interactions within the proximal PGHS-2 promoter is in sharp contrast with the observations in rats (Sirois et al., 1993; Morris and Richards, 1996). The binding pattern of nuclear proteins to C/EBP changed markedly post-hCG in rats (Sirois et al., 1993), but the binding of transcription factors to E-box remains unchanged (Morris and Richards, 1996), indicating that distinct *trans*-activation mechanisms of PGHS-2 exist between species with a short *versus* a long ovulatory process.

One novel finding of this study was the presence of an N-terminus truncated USF-2 isoform binding to the E-box at 0 h, and the loss of binding at 18 h post-hCG. Results from Western blots further confirmed the presence of N-terminus truncated USF-2 (18 kDa) in the 0 h nuclear extract, and its disappearance at 18 h and 20 h post-hCG. USF-1 (43 kDa) and USF-2 (44 kDa) are ubiquitous proteins, and belong to the Myc family of transcription factors characterized by highly conserved basic helix-loop-helix and leucine zipper domains responsible for dimerization and DNA binding (Sirito et al., 1994; Ghosh et al., 1997). USF proteins interact with DNA as homodimers and heterodimers, and are known to affect the expression of various genes (Ghosh et al., 1997; Vallet et al., 1997; Sirito et al., 1998). The differential dimerization (homo- *versus* heterodimers) of USF proteins has the potential to confer opposite effects on gene expression (Ghosh et al., 1997). Furthermore, the activity of these transcription factors could be regulated by the presence of a N-terminal truncated form of USF proteins (Sirito et al., 1994). These N-truncated

USF or mini-USF (18 kDa, Sirito et al., 1994), which lack the transcription activation domain but have the dimerization and DNA binding domains, would function as a dominant negative mutant (Descombes and Schibler, 1991). Thus, the N-terminus truncated USF-2 isoform expressed in granulosa cell nuclei could potentially serve as a repressor, that would block PGHS-2 transcription. However, in rats, levels of USF proteins did not vary in nuclear extracts of granulosa cells after hCG treatment, and there was no evidence of N-terminus truncated USF binding to the E-box (Morris and Richards, 1996), suggesting an important difference in the *trans*-activation of the PGHS-2 gene between these two different species.

Immunoblot analyses showed a relative high level of C/EBPβ protein in nuclear extracts of granulosa cells isolated at 0 h post-hCG, and the amount of C/EBPβ protein decreased slightly after hCG treatment. In contrast, studies in rats showed that there is no C/EBPβ protein in nuclear extract of granulosa cells obtained prior to hCG, but there is a rapid induction of C/EBPβ mRNA and protein after gonadotropin treatment (Sirois et al., 1993). The functional significance of C/EBPβ binding to the CAAT box of the PGHS-2 promoter in granulosa cells remains unclear. Early studies in rats proposed that C/EBPβ could be involved in cAMP-responsiveness (Sirois et al., 1993). However, recent site-directed mutagenesis experiments done with the full proximal promoter region revealed that binding to the CAAT box was not required for promoter activation in rat granulosa cells (Morris and Richards, 1996). These results are further supported by observations in C/EBPβ deficient mice where the gonadotropin-dependent induction of PGHS-2 in granulosa cells is not compromised (Sterneck et al., 1997). However, the down-regulation of PGHS-2 mRNA was impaired in C/EPBβ null mice, suggesting that expression of this transcription factor could be involved in the repression of the PGHS-2 gene

after its activation (Sterneck et al., 1997). There is evidence for such a role of C/EBPβ in vascular endothelial cells where overexpression of the protein was shown to suppress PGHS-2 promoter activity (Inoue and Tanabe, 1997). Thus, the relative high levels of C/EBPβ in nuclear extracts of bovine granulosa cells prior to gonadotropin could also serve to repress promoter activity in species with a delayed PGHS-2 induction and a long ovulatory process. A transient decrease in C/EBPβ levels after gonadotropin treatment could allow activation of gene transcription.

In summary, this project demonstrated that induction of PGHS-2 in bovine preovulatory follicles is a physiological event triggered by endogenous LH surge. Superovulation was associated with a follicle size-dependent induction of PGHS-2 in bovine follicles. The expression of PGHS-2 was accompanied by other signs of terminal follicular differentiation, including luteinization and cumulus oocyte expansion, suggesting that PGHS-2 expression can be used as a marker for follicular commitment to ovulation. The molecular basis for the late induction of bovine PGHS-2 has been studied. Results have clearly shown that hCG regulates the binding of specific nuclear proteins to the E-box and CAAT-box regions of the bovine PGHS-2 promoter. An N-terminus truncated USF isoform present in granulosa cells bound to PGHS-2 proximal promoter, and is potentially involved in the repression of PGHS-2 gene in bovine granulosa cells.

8. GENERAL CONCLUSIONS

The three major conclusions of my project are:

(1) Induction of PGHS-2 in bovine preovulatory follicles is a physiological event triggered by the endogenous LH surge. The induction of PGHS-2 is time-dependent (16-18 h post-LH surge) and granulosa cell-specific. The expression of PGHS-2 is associated with increased follicular prostaglandin synthetic activities prior to ovulation.

(2) Induction of PGHS-2 is follicle size-dependent (≥ 8 mm) in bovine follicles during superovulation. PGHS-2 expression was closely related to other signs of terminal follicular differentiation, including luteinization and cumulus oocyte expansion. The lack of PGHS-2 expression in ovulatory size-follicles (≥ 8 mm) was associated with the absence of luteinization and COC expansion. Therefore, PGHS-2 could be used as a marker for follicular commitment to ovulation.

(3) Induction of PGHS-2 in bovine granulosa cells *in vitro* is also delayed when compared to rats. A DNA fragment extending from -147 to -2 of bovine PGHS-2 promoter confers full promoter activity in granulosa cells in response to forskolin. An N-terminus truncated USF-2 isoform present only in nuclear extract from 0 h suggests its potentially involvement in the repression of PGHS-2 transcription in bovine follicles.

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