Université de Montréal

CHARACTERIZATION AND REGULATION OF ENZYMES RESPONSIBLE FOR STEROID ACTIVATION, INACTIVATION AND BIOAVAILABILITY DURING THE OVULATORY PROCESS IN THE MARE

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Cette thèse intitulée

CHARACTERIZATION AND REGULATION OF ENZYMES RESPONSIBLE FOR STEROID ACTIVATION, INACTIVATION AND BIOAVAILABILITY DURING THE OVULATORY PROCESS IN THE MARE

présentée par

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RÉSUMÉ

La montée préovulatoire d'hormone lutéinisante (LH) est responsable du déclenchement des changements morphologiques et biochimiques qui vont ultimement être responsables de la relâche de l'ovocyte et de la formation du corps jaune (CL). Lors de cette période, la machinerie stéroïdogène interrompt sa production d'œstrogènes en faveur de la biosynthèse de progestérone, ce qui résulte en une baisse d'œstradiol tard en estrus. Cette baisse en œstrogènes actifs a longtemps été attribuée au manque en potentiel biosynthétique, en particulier à la perte de la cytochrome P450aromatase (CYP19A1), enzyme limitante responsable de l'aromatisation des androgènes. Le traitement de juments avec la gonadotrophine chorionique humaine (hCG) provoque la lutéinisation du follicule préovulatoire et l'ovulation, qui aura alors lieu entre 39 et 48 h après injection. Les échantillons utilisés pour la présente étude ont été isolés à différents temps après le traitement d'hCG, notamment à 0 h, représentant un follicule isolé avant hCG, ainsi qu'à 12, 24, 30, 33, 36 et 39 h post-hCG. Comme référence, un CL au jour 8 du cycle œstral ainsi que différents tissus ont été obtenus en guise de comparaison. L'hypothèse générale de cette thèse est que le changement des concentrations de stéroïdes observé lors du processus ovulatoire est le résultat de la modulation de l'expression de gènes spécifiques, responsables de l'activation, l'inactivation et la bio-disponibilité des stéroïdes.

Le deux premiers papiers examinent la régulation transcriptionnelle de la 17β-hydroxystéroïde déshydrogénase de type 1 (17βHSD1), une protéine responsable de la réduction de l'œstrone en sa version plus active, l'œstradiol, du membre 2B1 de la famille de peptides transporteurs d'anion organiques (OATP2B1), encodé par le gène SLCO2B1 et qui est responsable de l'influx de stéroïdes sulfonés, ainsi que la sulfatase de stéroïdes (STS) qui catalyse l'enlèvement du groupe sulfonate pour en faire des stéroïdes libres. La structure primaire du gène, de l'ADN complémentaire (ADNc) et de la protéine de la 17βHSD1 ont été caractérisées. La structure du gène de la 17βHSD1 était hautement homologue à celle des autres espèces. La structure primaire de l'ADNc et de la protéine de la SLCO2B1 ont aussi été caractérisées. Des analyses RT-

PCR/Southern ont démontré des changements significatifs dans les concentrations de transcrits 17βHSD1, SLCO2B1 et STS dans les follicules préovulatoires lors de l'induction gonadotropine-dépendante de la lutéinisation folliculaire. Des concentrations élevées d'ARN messager de la 17βHSD1, SLCO2B1 et STS étaient présents dans les follicules préovulatoires avant hCG. Une diminution rapide et dramatique des transcrits 17βHSD1 et SLCO2B1 a été détectée dans des préparations de cellules granulosa isolées de follicules obtenus 12 h post-hCG et les concentrations sont demeurées basses, presque indétectables, par la suite. Le transcrit du SLCO2B1 était aussi exprimé et réprimé dans la thèque interne, mais sa diminution était plus graduelle que dans les cellules de la granulosa. La régulation de la STS était quelque peu différente, car son transcrit était hautement exprimé dans les deux types cellulaires et qu'il était significativement réprimé dans les cellules granulosa entre 30 et 39 h post-hCG. Le promoteur de la 17βHSD1 a été séquencé et plusieurs éléments *cis* potentiels ont été identifiés, incluant les séquences liant les facteurs de transcription Sp1, NFκB et GATA. La diminution de liaison des extraits de noyaux de cellules granulosa observée après le traitement de gonadotrophine pourrait expliquer la diminution d'expression du transcrit 17βHSD1 observée dans les follicules préovulatoires lors du processus ovulatoire. La co-localisation du SLCO2B1 et de la STS dans les follicules préovulatoires avant hCG suggère une voie alternative de biosynthèse d'œstrogènes qui impliquerait l'importation et l'utilisation de stéroïdes sulfonés, et que la répression de cette voie ainsi que la voie traditionnelle utilisant le cholestérol comme point de départ, constituerait une base moléculaire pour la diminution de biosynthèse d'œstrogènes observée lors de cette période.

Les troisième et quatrième papiers examinent la régulation de gènes impliqués dans l'inactivation et l'exportation d'œstrogènes lors du processus ovulatoire et catalysés par la 17βHSD4, une enzyme responsable de l'oxydation de l'œstradiol en œstrone, ainsi que la sulfotransférase d'œstrogènes (EST), qui catalyse la sulfoconjugaison des œstrogènes, et la protéine « multidrug resistance-1 » (MRP1), encodée par le gène ABCC1 et qui est impliquée dans l'exportation des stéroïdes sulfonés. Les structures

primaires des ADNc et des protéines de la 17βHSD4, EST et ABCC1 ont été caractérisées. Les analyses par RT-PCR/Southern, d'immunobuvardage et immunohistochimiques, ont démontré des changements significatifs dans les concentrations de 17βHSD4, EST et ABCC1 lors du processus ovulatoire. De basses concentrations présentes avant traitement ont été augmentées après hCG et sont demeurées élevées par la suite dans les cellules granulosa. Ces résultats démontrent que la diminution d'œstradiol observée avant l'ovulation n'est pas seulement due à la diminution en potentiel biosynthétique, mais aussi à l'augmentation de l'expression de protéines responsables de l'inactivation et de l'exportation d'œstrogènes. De plus, ces résultats démontrent qu'il est peut-être nécessaire d'éliminer les œstrogènes actifs lors de ce processus.

Le dernier papier visait à caractériser une autre 17β-hydroxystéroide déshydrogénase, 17βHSD5, faisant partie de la famille des aldo-kéto réductases et qui s'est avérée posséder une activité 20α-HSD, soit la AKR1C23. Les structures primaires de l'ADNc et de la protéine de la AKR1C23 ont été déterminées et des analyses RT-PCR/Southern, d'immunobuvardage et d'immunohistochimie ont été utilisées pour examiner la régulation de la AKR1C23 lors du processus ovulatoire. Les concentrations de AKR1C23 étaient basses dans les follicules préovulatoires avant hCG, puis une augmentation significative a été détectée dans les follicules obtenus entre 12 et 39 h post-hCG. Considérant son activité 20αHSD, qui corrèle avec les concentrations de 20α-dihydroprogestérone dans la liqueur folliculaire, ces résultats suggèrent un rôle pour une enzyme qui métabolise la progestérone dans les cellules granulosa du follicule préovulatoire lors du processus ovulatoire.

Globalement, ces résultats démontrent que lors du processus de lutéinisation/ovulation folliculaire chez la jument, la modulation des gènes impliqués dans l'activation, l'inactivation et la bio-disponibilité des stéroïdes sexuels favorise un environnement cellulaire sans œstrogènes actifs, ainsi que la présence d'une enzyme

ayant la capacité de métaboliser la progestérone et ce, surtout dans les cellules de la granulosa.

Mots-clés: Cheval, ovaire, follicule préovulatoire, hCG, stéroïdes, 17β-hydroxystéroïde déshydrogénase, sulfoconjugaison, transport de stéroïdes sulfonés, sulfatase de stéroïdes, aldo-kéto réductase.

SUMMARY

The preovulatory rise in luteinizing hormone (LH) is responsible for triggering the morphological and biochemical changes that ultimately lead to the release of the oocyte and the formation of the corpus luteum (CL). During this period, the steroidogenic machinery interrupts production of estrogens in favor of biosynthesis of progesterone, resulting in a late-estrus drop in plasma 17β-estradiol. This decrease in active estrogens has long been believed to be due to a lack of estrogen biosynthetic potential, in particular due to the loss of cytochrome P450aromatase (CYP19A1), the rate-limiting enzyme that aromatizes androgens. Treatment of mares with human chorionic gonadotropin (hCG) causes preovulatory follicles to undergo luteinization, with ovulation usually occurring 39 to 48 h later. Samples used in this study were obtained at different times relative to hCG treatment, namely at 0 h, which represents a follicle isolated prior to hCG treatment, and at 12, 24, 30, 33, 36, and 39 h after hCG. For reference, a CL sample at day 8 of the cycle along with various other equine tissues were used for comparison. The overall hypothesis of this thesis is that the change in steroid concentrations observed during the ovulatory process is a result of the programmed modulation of the expression of specific genes responsible for the activation, inactivation and bioavailability of steroids.

The first and second papers examine the transcriptional regulation of 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1), a protein responsible for the reduction of estrone to the more biologically potent 17β-estradiol; member 2B1 of the organic anion transporting polypeptide family (OATP2B1), encoded by the SLCO2B1 gene and responsible for the importation of sulfoconjugated steroids; as well as steroid sulfatase (STS), an enzyme responsible for the hydrolysis of sulfoconjugated steroids. The primary structures of the equine 17βHSD1 gene, cDNA and protein were characterized. The 17βHSD1 gene structure was highly homologous to that of other species. The primary structures of the equine SLCO2B1 cDNA and protein were also characterized. RT-PCR/Southern blot analyses demonstrated significant changes in

steady-state levels of 17βHSD1, SLCO2B1 and STS transcripts in preovulatory follicles during the gonadotropin-dependent induction of follicular luteinization/ovulation. Elevated concentrations of 17βHSD1, SLCO2B1 and STS mRNA were present in equine preovulatory follicles prior to hCG treatment. A rapid and dramatic decrease in 17βHSD1 and SLCO2B1 transcripts was detected in isolated preparations of granulosa cells obtained 12 h post-hCG and remained low, almost undetectable, thereafter. Levels of SLCO2B1 mRNA were also expressed and downregulated in the theca interna, in a pattern more gradual than what was observed in the granulosa cell layer. The regulation of STS was slightly different in that levels were high in both cell types, but STS mRNA was downregulated significantly 30-39 h post-hCG in granulosa cells. The equine 17βHSD1 promoter was sequenced and several putative *cis*-acting elements were identified, including sequences that bind the transcription factors Sp1, NFκB and GATA. The decrease in granulosa cell nuclear extract binding observed after gonadotropin treatment may account for the downregulation in 17βHSD1 transcript observed in preovulatory follicles during the ovulatory process. The co-localization of SLCO2B1 and STS in preovulatory follicles prior to hCG treatment suggest an alternate pathway of estrogen biosynthesis and that the gonadotropin-dependent downregulation of both pathways during the ovulatory process provides an additional molecular basis for the decreased 17β-estradiol biosynthesis observed during that period.

The third and fourth papers examine regulation of genes involved in estrogen inactivation and export during the ovulatory process as catalyzed by 17βHSD4, which is responsible for the oxidation of 17β-estradiol to estrone; as well as estrogen sulfotransferase (EST), which catalyzes the sulfoconjugation of estrogens; and multidrug resistance protein-1 (MRP1), encoded by the ABCC1 gene and involved in the efflux of sulfoconjugated steroids. The primary structures of the 17βHSD4, EST and ABCC1 cDNA and proteins were characterized. RT-PCR/Southern blot analyses in combination with immunoblot and immunohistochemistry demonstrated a significant change in steady-state levels of 17βHSD4, EST and ABCC1 during the ovulatory process. Low concentrations present prior to hCG treatment were upregulated after hCG

treatment and remained high thereafter, with the upregulation occurring mainly in the granulosa cell layer. These results indicate that the decrease in 17β-estradiol observed prior to ovulation is not only due to the decrease in biosynthetic potential, but also to upregulation of proteins involved in estrogen inactivation and export. Moreover, active estrogens may need to be eliminated during this process.

The last paper was aimed at characterizing the regulation of another 17βHSD, 17βHSD5, an aldo-keto reductase which was later demonstrated to possess progesterone-metabolizing activity, AKR1C23. The primary structures of the AKR1C23 cDNA and protein were determined and RT-PCR/Southern blot analyses as well as immunoblot and immunohistochemistry were used to examine the regulation of AKR1C23 during the ovulatory process. While low levels of AKR1C23 were present in preovulatory follicles prior to hCG treatment, a significant increase in AKR1C23 was observed in granulosa cell samples isolated from preovulatory follicles obtained 12-39 h post-hCG. Considering its 20αHSD activity, which correlates with follicular fluid concentrations of 20α-DHP, these results suggest the need for a progesterone-metabolizing enzyme in granulosa cells of preovulatory follicles during the ovulatory process.

Overall, these results demonstrate that, during the process of follicular luteinization/ovulation in the mare, the modulation of genes involved in steroid activation, inactivation and bioavailability favors a cellular environment without active estrogens and the presence of a progesterone-metabolizing protein occurring mainly in the granulosa cell layer.

Keywords: Horse, ovary, preovulatory follicle, hCG, steroids, 17β-hydroxysteroid dehydrogenase, sulfoconjugation, sulfoconjugated steroid transport, steroid sulfatase, aldo-keto reductase.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

17βHSD	17β-hydroxysteroid dehydrogenase
20α-DHP	20α-dihydroprogesterone
20αHSD	20α-hydroxysteroid dehydrogenase
3βHSD/KSI	3β-hydroxysteroid dehydrogenase/ketosteroid isomerase
3'-UTR	3'-untranslated region
5'-UTR	5'-untranslated region
A4	androstenedione
ABC	ATP-binding cassette
AKR	aldo-keto reductase
APS	adenosine-5'-phosphosulfate
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
bp	base pair
cAMP	cyclic adenosine monophosphate
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CL	corpus luteum
CHO	Chinese hamster ovary
CRE	cAMP response element
CREB	CRE binding protein
CREM	CRE modulator
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
E1	estrone
E2	17β-estradiol
eCG	equine chorionic gonadotropin
EMSA	electromobility shift assay
ER	estrogen receptor
EST	estrogen sulfotransferase
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GRE	glucocorticoid response element
GSH	glutathione
hCG	human chorionic gonadotropin
HEK293	human embryonal kidney 293
IL	interleukin
kb	kilobase
kDa	kilodalton
LDL	low-density lipoprotein
LH	luteinizing hormone
MDR	multidrug resistance
mRNA	messenger RNA
MRP	MDR-associated protein
NAD	nicotinamide adenine dinucleotide

NADP	nicotinamide adenine dinucleotide phosphate
NBD	nucleotide-binding domain
NFκB	nuclear factor-κB
nt	nucleotide
OATP	organic anion transporting polypeptide
P4	progesterone
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PKA	protein kinase A
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
recFSH	recombinant FSH
RNA	ribonucleic acid
RT	reverse transcriptase
SCP2	sterol carrier protein 2
SDR	short-chain dehydrogenase/ reductase
SF-1	steroidogenic factor-1
SLCO	solute carrier/OATP
SREBP	sterol regulatory element binding protein
StAR	steroid acute regulator
STS	steroid sulfatase
T	testosterone
TNFα	tumour necrosis factor α

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INTRODUCTION

1. Overview of the equine estrous cycle

The estrous cycle, in the mare, is characterized by a series of tightly regulated events, including a follicular phase, also termed estrus, that culminates in the release of the oocyte from the follicle, as well as a luteal phase or diestrus, in which a temporary luteal gland affects further follicular recruitment, until it eventually regresses (figure 1).

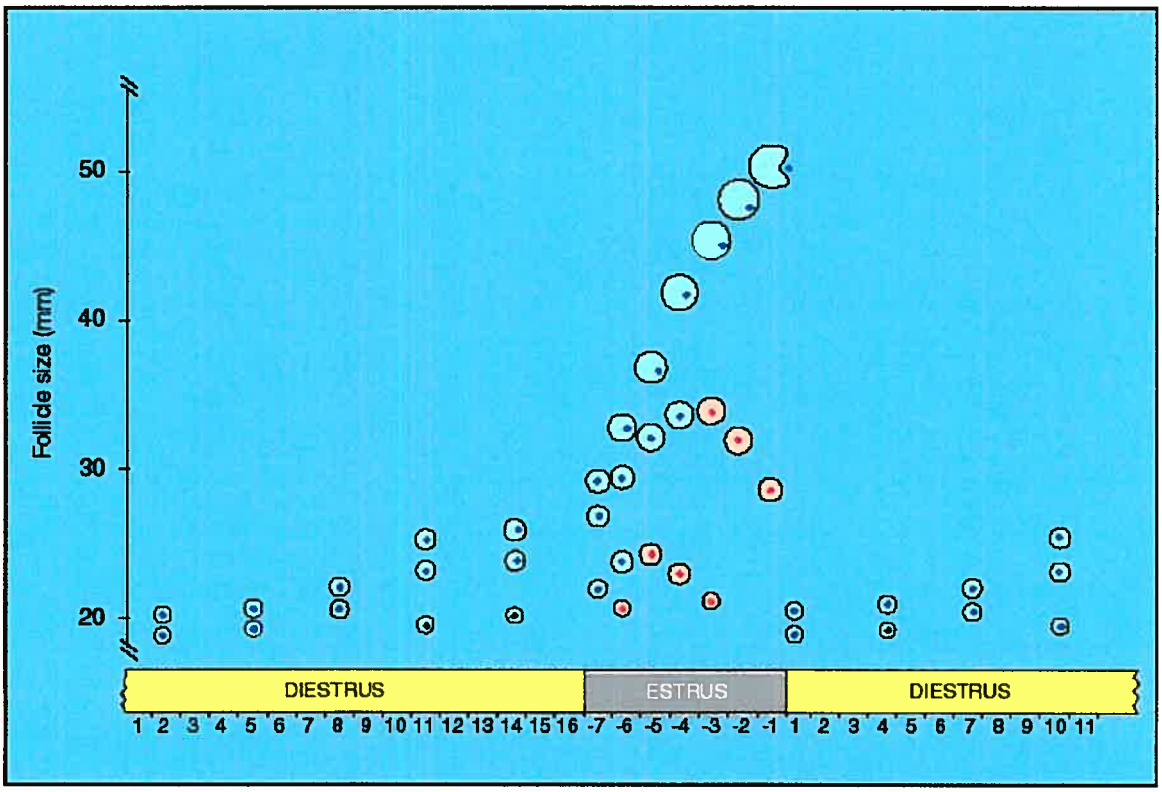

FIGURE 1: Estrous cycle in the mare depicting a major follicular wave (adapted from reference 1).

The mare provides an interesting model for the study of gonadotropin-dependent luteinization/ovulation for many reasons. Being a seasonal polyestrous animal, the mare goes through an ovulatory season which is accompanied by several estrous cycles, typically occurring during spring and summer, followed by an anovulatory season (1). Daylight is a key factor in the initiation of the ovulatory season, as melatonin has been shown to affect gonadotropin releasing hormone (GnRH) secretion (2, 3). Also, this

animal develops relatively large preovulatory follicles, reaching 40 to 45 mm in diameter. Therefore, follicular development can be precisely monitored using ultrasound imagery (4).

The estrous cycle (figure 1), defined as the duration between two ovulatory events, lasts on average 22 days, which includes a relatively long follicular phase (estrus of about 6.5 days) and a luteal phase (diestrus) averaging about 15 days (1). These events are largely dictated by gonadotropin secretion from the anterior pituitary, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropins are heterodimeric peptide hormones, composed of a common α-subunit and a hormone-specific β-subunit that are non-covalently bound (5). Typical anterior pituitary gonadotropins, such as FSH and LH, are found in all mammals and their secretion is mediated by the pulsatile release of GnRH by the hypothalamus (6, 7). The growth of ovarian follicles and the differentiation of ovarian granulosa cells are complex processes that depend on the sequential stimulation by FSH and LH. Primates and equids are particular in that they will also produce their own placental gonadotropins, referred to as human chorionic gonadotropin (hCG) and equine chorionic gonadotropin (eCG), respectively. While hCG is necessary for the early pregnancy to continue, the biological function of eCG remains a matter of speculation.

1.1 Morphological and hormonal changes during the equine estrous cycle

Day 0 of the estrous cycle is defined as the day of ovulation, which has been shown to occur less than 48 h before the end of estrus (3). The rupture of the ovarian follicle and the expulsion of the oocyte result in morphological and biochemical changes leading to the formation of a transient endocrine organ called the corpus luteum (CL). In most species, both the granulosa cells and the theca interna contribute to this organ, however, this is not the case in the mare (8). The theca interna of the equine preovulatory follicle has been shown to degenerate prior to ovulation, resulting in a CL that is uniquely composed of granulosa cells (9). By day 3 of the estrous cycle, the CL is completely formed and progesterone production is at its maximum. LH appears to be responsible for maintaining the CL, as well as stimulating the biosynthesis of

progesterone (3). If a conceptus is present, it will release a yet undefined factor that will maintain the CL beyond its normal cyclical lifespan of 15-16 days and suppress the upregulation of oxytocin receptors in the endometrium, until day 38-40 where the CL is thought to be maintained by eCG secretion from the chorion resulting in the sustained progesterone biosynthesis required for the maintenance of gestation (10). However, in the event that gestation does not occur, the pituitary-released oxytocin will cause the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus, resulting in CL regression. This occurs approximately 14 days after ovulation.

During the estrous cycle, a pool of follicles of five different classes is present (reviewed by 11). These are classified on their dependency and sensitivity to gonadotropins, and the transition from one class to the next is tightly regulated via growth factors, peptide hormones, and steroid hormones. The first class of follicles, the primordial follicles, consist of one primary oocyte surrounded by a layer of flattened cells. These follicles remain dormant until development is initiated and the granulosa cells begin to proliferate. The second class of follicles, the primary follicles, are now surrounded by a single layer of granulosa cells and enveloped by a basal lamina that separates the granulosa cells from the surrounding stromal/thecal elements. The mechanisms involved in the reinitiation of growth of primordial follicles are not well understood, yet it is believed that follicles leave the primordial pool in an ordered fashion (11). The third class of follicles comprises preantral or secondary follicles. These have on average two layers of granulosa cells, expressing FSH-receptors, as well as a layer of cubic cells surrounding the granulosa cells called the theca interna. Preantral follicles are exposed to the blood supply, with a network of vessels forming just outside the basal lamina, hence, they are exposed to various factors in circulation, including gonadotropins (1, 11).

Around the time of ovulation, levels of FSH start to rise (figure 2), resulting in the recruitment of secondary follicles and the emergence of the first follicular wave (12). In mares, two types of follicular waves have been described, minor waves where the largest follicle does not attain the diameter of the dominant follicle and major waves

characterized by the presence of both dominant and subordinate follicles (13). The first wave of follicles can be characterized as minor and is typically not sustained, resulting in atresia (3). The ovulatory follicle is part of the fourth class of follicles and most often emerges from the second wave of follicular growth, which is usually initiated at mid-diestrus (figure 1) (3). After the preantral follicle develops six or seven granulosa cell layers, as well as a more pronounced thecal layer with accompanying LH-receptors, the formation of small fluid-filled cavities begins. These cavities eventually aggregate and result in the formation of a single fluid-filled space, termed the antrum. At this stage, the newly formed antral or tertiary follicles have a more complex morphology. They are composed of multiple granulosa and theca interna layers, as well as a new type of fibroblastic cell, called the theca externa. The granulosa cells surrounding the oocyte are now referred to as the *cumulus oophorus*, or cumulus cells.

Several days after the second peak in FSH and emergence of the second follicular wave, the two largest follicles enlarge to mean diameters of 22 and 19 mm (12). This marks the beginning of continued growth of the largest follicle to become dominant and reduced or terminated growth of the remaining follicles to become subordinate follicles (12). Even though the difference in diameter between the two largest follicles remains constant, the surface area of the largest follicle increases relative to the next-largest follicle. During this time, secretory products of the largest follicle suppress circulating FSH concentrations thereby starving the smaller follicles of gonadotropin support, which causes their regression; these atretic follicles comprise the fifth class of follicles (12). The dominant follicle-produced factors are primarily steroids and proteins of the TGFβ family.

In most mammals, the onset of follicular luteinization is triggered by a sharp rise and fall in LH, characterized as an LH surge. This is not the case in mares (figure 2). As it continues to develop, the dominant follicle also responds to the rising LH concentrations by producing sufficient 17β-estradiol to exert positive feedback stimulation on LH secretion. This results in a gradual increase in LH, beginning 6 or 7 days before ovulation and peaking approximately one day after ovulation (figure 2) (3, 14).

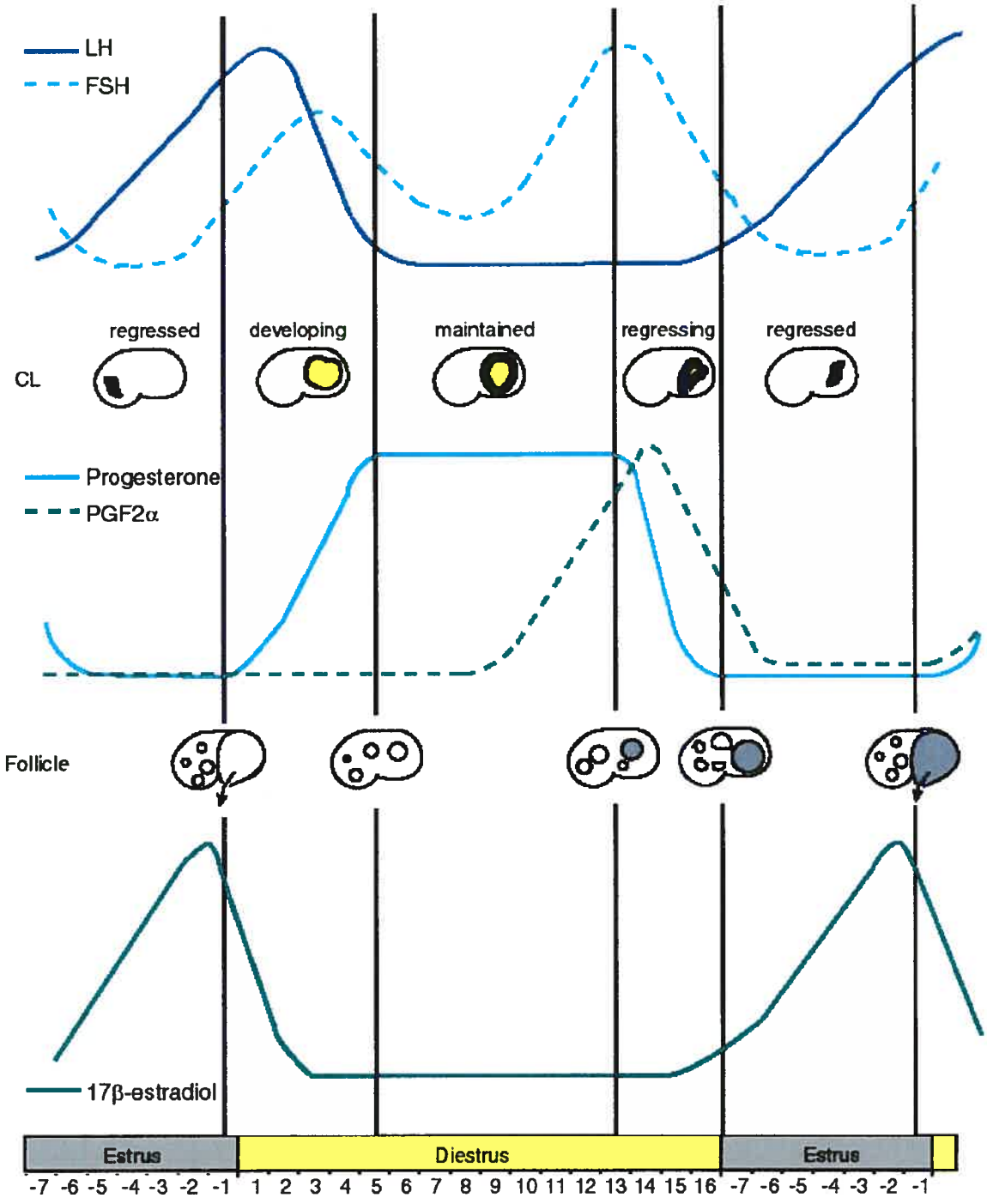

FIGURE 2: Hormonal changes during the equine estrous cycle (adapted from reference 1).

This suggests that in horses, the ovulatory process is initiated when a threshold concentration of gonadotropins is reached. During the peri-ovulatory period, the preovulatory follicle responds to LH by markedly increasing its antrum size and blood flow (9). Granulosa cells begin secreting progesterone and decrease their secretion of estrogen, which accounts for the late-estrus drop in plasma estrogen concentration and the small rise in plasma progesterone just before ovulation. A cascade of events which include prostaglandin synthesis (15), progesterone action on the follicle (16), and consequent expression of proteolytic enzymes such as ADAMTS (16) expression results in the break-down of the follicular-ovarian membranes and expulsion of the oocyte.

1.2 Cellular aspects of luteinization

Luteinization is characterized as being a differentiation process by which the diverse cells of the preovulatory follicle acquire the functional and morphological characteristics of luteal cells. This process is initiated by LH in the mare, and begins approximately 2 days prior to ovulation (17). It has been shown that ovulation in mares can be induced during the follicular phase by the administration of hCG when the preovulatory follicle reaches approximately 35 mm, and the duration of the ovulation, defined as the interval from hCG injection to follicular rupture, has been demonstrated to be approximately 36-48 h (18). Before hCG, the granulosa cell layer is composed of 4-9 compact layers of small elongated cells with some evidence of mitosis, whereas, the theca interna layer is characterized by many polyhedral cells that have a plump appearance and high level of vascularization indicative of steroidogenesis (9). The administration of hCG results in a dramatic increase in intracellular spacing of the granulosa cell layer, more than doubling its thickness after 39 h, with the maximum expansion of the granulosa cell layer being reached after 24 h. Mitotic figures can no longer be observed and there is a significant increase in mucoid substances, the best known of which is hyaluronan, is detected between the granulosa cells (9). The theca interna has been shown to undergo significant thinning, with fewer cells present and the presence of occasional pyknotic nuclei. A dramatic increase in edema, hemorrhage and hyperemia, as well as in the number of blood vessels is also observed, reaching a maxima at 36 and 39 h post-hCG (9). These

data, particularly the incipient degeneration of the theca, support the hypothesis that the granulosa cells are the sole contributor to the CL.

The differentiation process from granulosa to granulosa-lutein cells involves many changes at the cellular level. Granulosa-lutein cells become hypertrophied, becoming the largest steroidogenic cell type in the body (17). Gap junctions that allowed rapid communication between granulosa cells during follicular development are lost (17), but are reestablished later on in the process (19). The smooth endoplasmic reticulum and golgi apparatus become more prominent, and the size and complexity of mitochondria increase (17). The cytoskeleton is also affected by the process of luteinization and is believed to play an important role in steroidogenesis (8). Changes include the acquisition of smooth muscle actin by the granulosa cells, as well as desmin, cytokeratin and vimentin (8). Although it is not the case in all species (17, 20, 21), the absence of mitotic figures observed during hCG-induced luteinization in the mare (9) may reveal the occurrence of granulosa cell exit from the cell cycle and complete cessation of division resulting in their terminal differentiation after the LH surge, as seen in the rat (22).

1.3 Luteinization-associated modulation of gene expression

The mechanisms involved in signal transduction following LH are complex and their study is still in relative infancy. However, some generalizations can be made about what is known thus far. The protein kinase A (PKA) pathway has been linked to the luteinization process. The binding of LH to its receptor, a seven-transmembrane domain G-protein-coupled receptor, leads to its activation and the subsequent production of cyclic adenosine monophosphate (cAMP) from adenylate cyclase and adenosine triphosphate (ATP) (7). The intracellular messenger, cAMP, then binds to the regulatory subunits of the inactive PKA holoenzyme and activates PKA's catalytic subunits by causing their dissociation from the holoenzyme complex. They are then free to catalyze the phosphorylation of serine and threonine residues of various proteins, including cAMP response element modulator (CREM), nuclear factor-κB (NFκB), cAMP response element binding protein (CREB), as well as many other nuclear receptors (7).

The activation of CREB the via its phosphorylation by PKA results in the activation of many genes, such as steroid acute regulator (StAR) (23), CYP11A1 (8), as well as the Niemmann-Pick C1 protein involved in the low density lipoprotein (LDL) pathway (24). Numerous other transcription factors, including steroidogenic factor-1 (SF-1), liver receptor homolog-1, sterol regulatory element binding proteins (SREBPs), and the GATAs, have been shown to be involved in the luteinization process (8). The complexity of the regulation of expression of this multiplicity of factors is beyond the scope of the present review.

As previously mentioned, the follicle wall undergoes dramatic changes during luteinization, as seen in its vascularization, as well as in its structural integrity. Many vasoactive and inflammatory agents have been shown to be involved in the ovulatory process (25). Vascular endothelial growth factor has been linked to the increased vascular permeability and shown to stimulate angiogenesis in response to hypoxia in the inner follicular compartments (26), whereas P-selectin is known to play a critical role in the initial steps of leukocyte recruitment from the bloodstream during inflammation and has been shown to be upregulated during luteinization (27). These effects may be secondary to prostaglandin synthesis, which has also been shown to play a key role in the ovulatory process (15). The structural remodeling of the follicle during luteinization has been investigated over the past decade. Many proteolytic activities have been identified and shown to be regulated. They include the plasminogen activators, which have been shown to activate collagenases (26), the metalloproteinases (16, 26), as well as the metalloproteinase inhibitors (26). The rodent model, in which luteal cells undergo terminal differentiation during the process of luteinization, have been shown to lose cyclin D2 expression after LH, with a resulting increase in cell cycle inhibitors (22). The progesterone receptor (PR) has also been shown to be upregulated, supporting the hypothesis that progesterone action is required for luteinization to occur (28). Indeed, the PR knockout mouse is incapable of ovulation (29).

In section 2.2, the molecular biology of ovarian steroid biosynthesis will be addressed, it is nonetheless noteworthy to mention certain key aspects of the regulation

of steroidogenic enzymes during the process of luteinization. The most notable functional change observed during luteinization is the shift in ovarian steroid biosynthesis, from 17β-estradiol to allow the large-scale synthesis of progesterone. In a simplified view of the regulation of steroidogenic enzymes, and this holds true for many species including the mare, the LH surge results in the upregulation of enzymes involved in progesterone biosynthesis, such as StAR and CYP11A1 (30-41), whereas those downstream of progesterone, the expression of CYP17A1 and CYP19A1 are turned off (36, 42-47).

2. Steroid hormones

Steroids are a subclass of a large family of chemical compounds known as terpenoids (48). They are all derivatives of a perhydrocyclopentanophenanthrene ring system, with a skeleton formed of four interconnected rings of 17 carbon atoms (Figure 3). A few polar hydroxyl groups may be attached to this ring structure, but they are not numerous enough to render a steroid water-soluble. Thus, as with oxygen, carbon dioxide and fatty acids, steroid hormones diffuse rapidly through the lipid portions of membranes.

Steroid compounds exhibiting hormonal activity have been classified into six families, namely progestins, glucocorticoids, mineralocorticoids, androgens, estrogens and vitamin D, having one of four parent molecules (figure 3, reviewed in 48). Progestins all consist of 21 carbons, having two keto groups on carbons 3 and 20. Glucocorticoids and mineralocorticoids are similar to progestins, however, they have an additional hydroxyl group on carbon 21, as well as a side-chain composed of two carbons at position 17 and the unsaturation of the bond between carbons 4 and 5. Androgens are composed of 19 carbons with a keto group present at position 3 and a hydroxyl group present at position 17. Estrogens are different as their A-ring has been aromatized. They also have two hydroxyl groups present at carbons 3 and 17 (48).

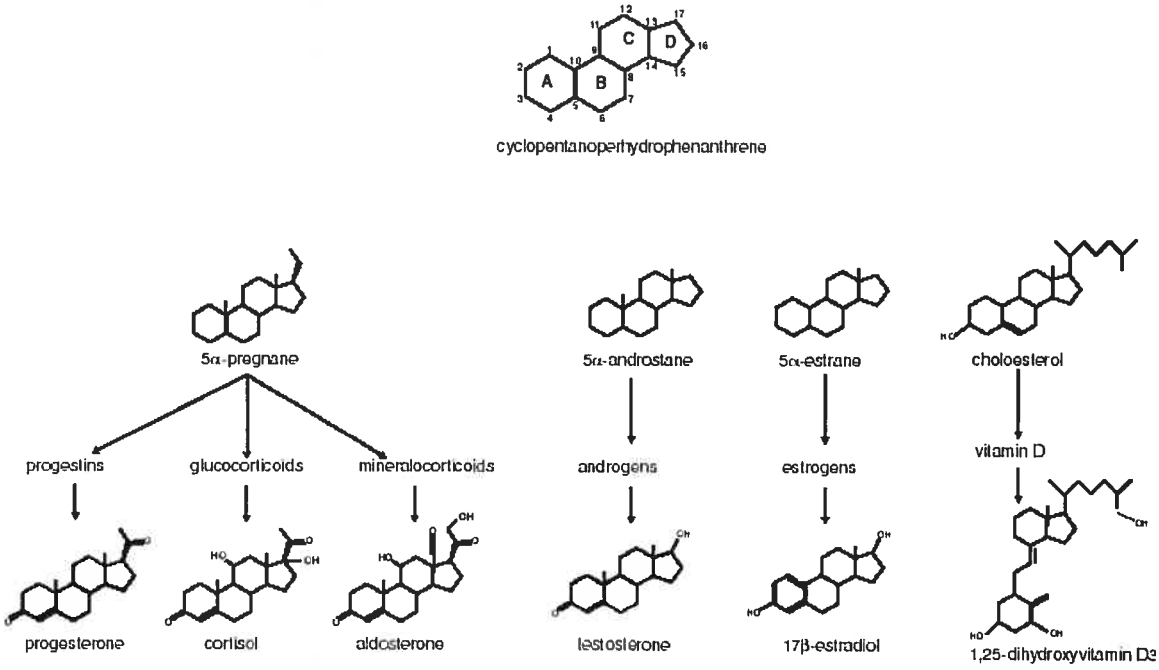

FIGURE 3: Structure of the steroid hormone backbone and parent molecules (adapted from reference 48).

Specific compounds are named according to their substituent's suffixes and their position on the parent molecule. Also, the Greek letters α and β are added depending on whether the substituent is above or below the plane of the ring, respectively. For example, the addition of a hydroxyl group at position 17 and below the plane of an androstane parent molecule, as well as a keto group at position 3 and the unsaturation of the A ring at position 4, will result in a compound systematically named 17β-hydroxyandrost-4-en-3-one or testosterone (48).

2.1 Role of steroid hormones in physiological processes

The majority of steroids are produced by specialized cells in specialized tissues, such as the adrenal and gonads, and their actions can be local as well as have an effect in distant tissues (reviewed in 49). Peripheral tissues have also been shown to produce or inactivate steroids, however, the action of these steroids is usually restricted to the site of expression. The adrenal cortex has been shown to express significant amounts of

many different steroids, including aldosterone, cortisol, corticosterone, dehydroepiandrosterone (DHEA), and androstenedione. The zona glomerulosa is responsible for the secretion of the mineralocorticoid, aldosterone, and its effects include the regulation of electrolyte metabolism, namely sodium, potassium, and hydrogen ions, and the way they are handled by the kidneys. The zona fasciculata and zona reticularis of the cortex are responsible for the production of other adrenal steroids. Cortisol is a glucocorticoid and has important effects on the metabolism of glucose and other organic nutrients, it has also been shown to facilitate the body's response to stress, regulate the immune system, and exert mineralocorticoid effects at high concentrations. DHEA and androstenedione are androgens that are much less potent than testosterone, but are involved in several physiological functions in females (49).

The gonads strongly express the enzymes involved in sex steroid biosynthesis (reviewed in 49). The testes are capable of producing large amounts of androgens, such as testosterone, which play an important role in the differentiation and growth of the reproductive tract, external genitalia, as well as certain regions of the brain. After puberty, they direct the development and maintenance of secondary sex characteristics, as well as sexual behavior (49). The ovaries are the main site of estrogen and progestin biosynthesis. During development, estrogens stimulate the growth of female external genitalia, as well as mammary gland development and the formation of mammary ducts and fat deposition. They are also responsible for stimulating bone growth and for the ultimate cessation of bone growth (49). After sexual maturation, and as noted above, during the follicular phase of the cycle, 17β-estradiol is secreted by the granulosa cells of the preovulatory follicle. As described earlier, these steroids have both positive and negative feedback effects on gonadotropin secretion. Estrogens also modulate the growth of smooth muscle and proliferation of the epithelial linings of the reproductive tract. They increase contractions and ciliary activity in the uterine tubes, myometrial contractions and responsiveness to oxytocin in the uterus, as well as stimulate the secretion of abundant and clear cervical mucus and prepare of the uterine endometrium for progesterone actions by increasing the number of progesterone receptors (49).

Progesterone, on the other hand, is mainly produced by the CL and placenta, although its biosynthesis begins in the preovulatory follicle prior to ovulation. Physiologically, progesterone is necessary for gestation, as it decreases contractions of the uterine tubes and myometrium. In humans, it also stimulates secretions from the endometrial glands, induces thick and sticky mucus from the cervix, and decreases proliferation of vaginal epithelial cells. At the level of the hypothalamus, high concentrations of progesterone inhibit GnRH secretion, which results in a negative-feedback inhibition of FSH and LH secretion thereby preventing LH surges during the luteal phase and gestation (49).

2.1.1 Estrogens and cell cycle progression

The cell cycle is composed of four major phases (figure 4) (reviewed in 49, 50). The interphase is considered to be the interval between the end of one division and the appearance of the structural and gene expression changes that indicate the beginning of the next division. In a dividing cell population, interphase is the longest phase of the cycle. The S phase (synthesis) occurs after the first interval, G_1, and is characterized by extensive DNA replication. Following DNA synthesis, there is a second interval (G_2) before cytokinesis begins. The final phase, the M phase, includes the mitotic and cytokinetic events that result in two daughter cells. There are numerous control points in the cell cycle and at each of these, certain events must occur in order for the cell to proceed to the next phase. One of particular significance is the transition between G_1 and S while another is that between G_2 and M. The phosphorylation of cyclin-dependent kinases and consequent phosphorylation of specific substrates have been shown to dictate the progress through the cell cycle. When cells undergo terminal differentiation, they exit the cell cycle and enter a phase known as G_0.

Estrogens have been shown to increase DNA synthesis in the rodent mammary gland and uterus by recruiting non-cycling cells to the cell cycle, whereas cells that were already cycling had a reduced G_1 phase length (51). Breast cancer cells have been extensively used to study the regulation of the cell cycle by sex steroids. They were

shown to be most responsive to estrogens, through the estrogen receptors, early during the G_1 phase, right after mitosis (51-53).

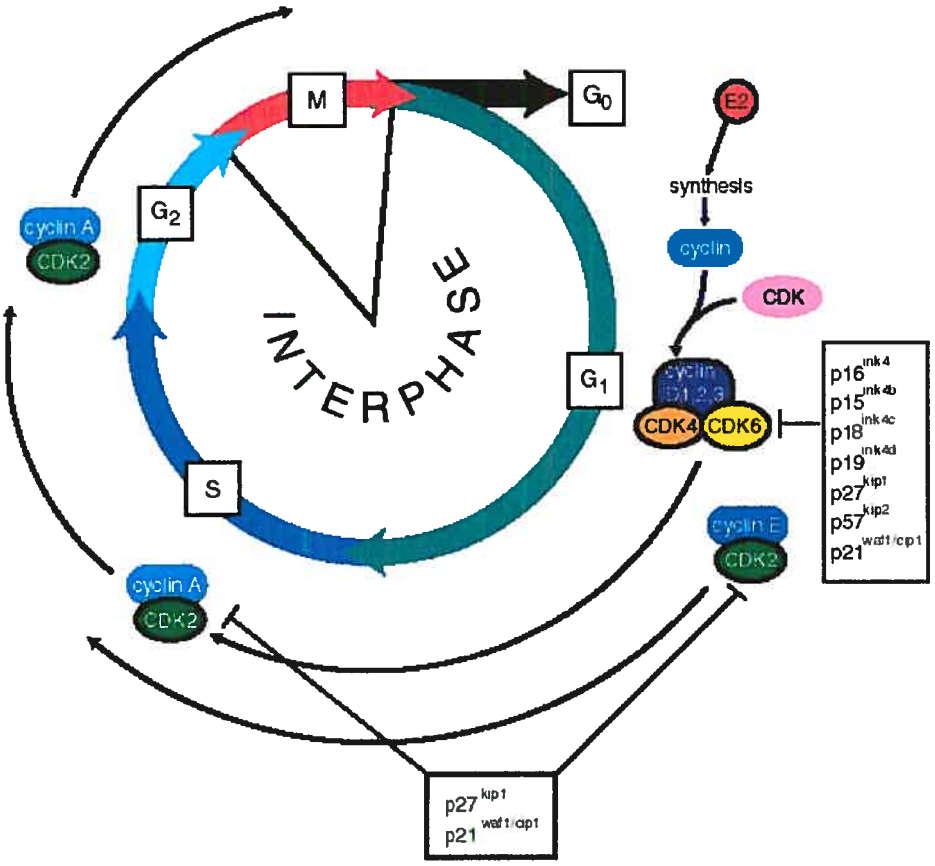

FIGURE 4: Simplified cell cycle (adapted from reference 50).

The regulation of the immediate-early gene c-myc is one of the earliest effects detectable after estrogen treatment and this holds true in many cell systems, including rat uteri (54, 55), normal breast epithelial cells and breast cancer cells (56, 57). Mitogens, such as estrogens, have been shown to be responsible for mediating the association of cyclin D1 with cyclin-dependent kinases (CDKs) and inducing their kinase activity. This results in the hyperphosphorylation, with the help of the cyclin E-CDK2 complex, of tumour suppressor proteins, such as retinoblastoma (58). This hyperphosphorylation has been shown to cause the release of the E2F transcription factors, responsible for the transcription of many proteins involved in cell cycle

progression (59). Recent work demonstrates the positive effects of estrogens at the G_2/M phase of the cell cycle in ovarian granulosa and breast cancer cells (60).

2.2 Molecular biology of ovarian steroid hormone biosynthesis

Steroidogenic tissues, including the adrenals, placenta, brain and gonads, all rely on cholesterol as a precursor for the biosynthesis of a range of steroid hormones (48). Although *de novo* cholesterol synthesis is possible in steroidogenic cells, the principal sources of cholesterol appear to be the plasma lipoproteins (61). The initiation of steroid biosynthesis has been shown to begin in the mitochondria. It is believed that cholesterol destined for steroid conversion is transported to these organelles in vesicles that travel along the cytoskeletal fibers, and that proteins like sterol carrier protein 2 (SCP2) may then promote the transfer of cholesterol from the vesicles to the mitochondria (62-64).

The biosynthesis of ovarian steroid hormones requires the involvement of several enzymes sequestered in specific subcellular compartments. These can be classified into two major classes of proteins, the cytochrome P450 heme-containing proteins and the hydroxysteroid dehydrogenases (65). The accepted nomenclature for the cytochrome P450 enzymes is CYP, followed by an Arabic number representing the P450 family and a letter indicating the subfamily (66).

Early on in follicular development, the principal ovarian steroidogenic cells, the theca interna and granulosa cells, contribute to the production of active estrogens, as first described in Armstrong and Dorrington's two-cell theory (67). Although the two-cell theory applies to the mare, the role of each cell type is somewhat different. It can be hypothesized, however, that the characteristics of the essential steroidogenic enzymes, such as activity and subcellular localization, are conserved. The first and rate-limiting step in the production of all steroid hormones is the transfer of cholesterol from the outer to the inner mitochondrial membrane via the actions of StAR, and its subsequent conversion to pregnenolone by CYP11A1 (figure 5) (65).

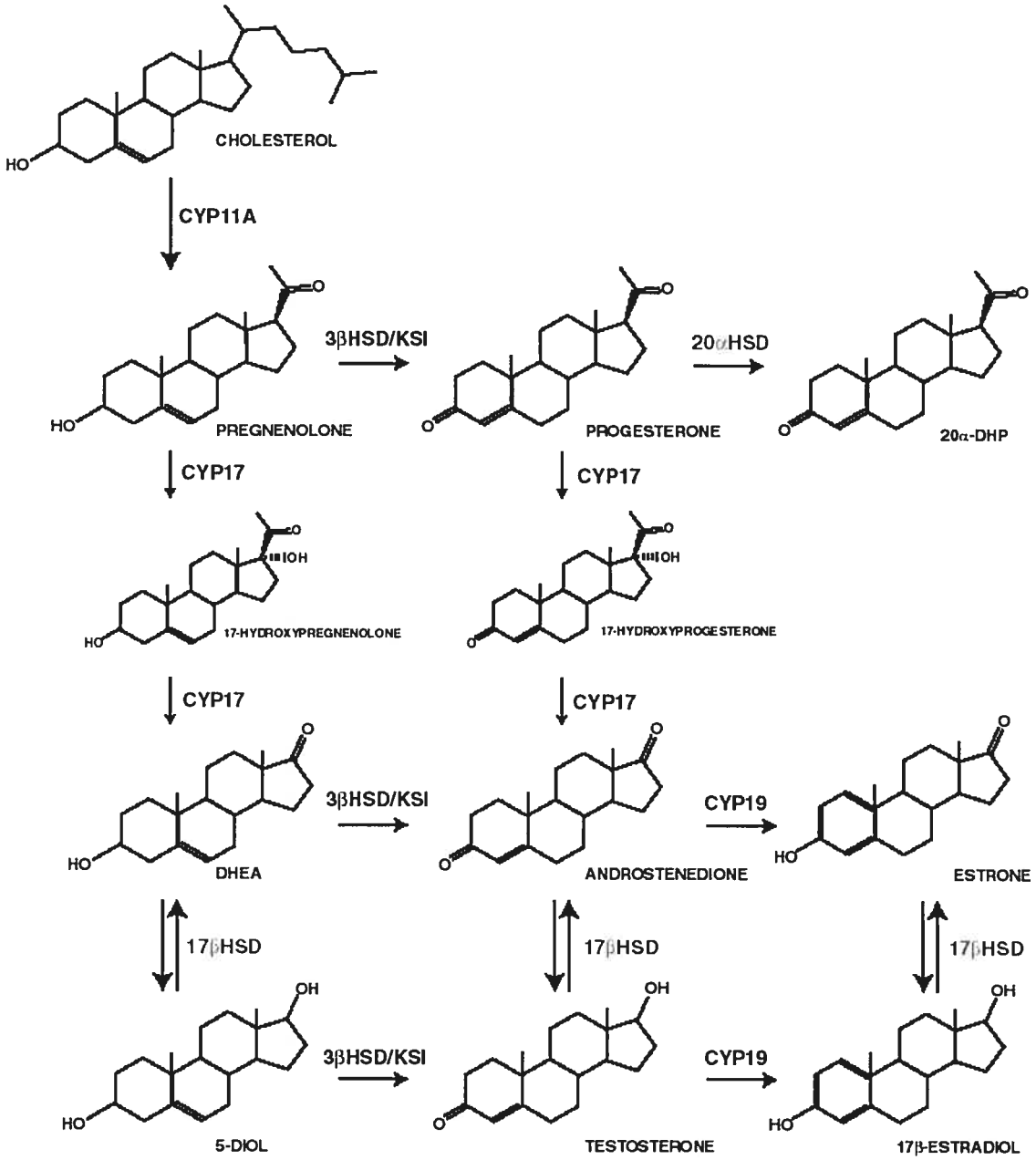

FIGURE 5: Simplified steroidogenesis (adapted from reference 68).

Although it is well established that StAR is involved in the import of cholesterol, some evidence supports the use of mitochondrial contact sites in this process (69). In the mare, preovulatory levels of StAR and CYP11A1 are highest in the theca interna, whereas luteinization results in a switch in cell-type expression to granulosa cells (30, 41) as the theca cells degenerate. The product of CYP11A1, pregnenolone, can then either be acted upon by the enzyme 3β-hydroxysteroid dehydrogenase/ketosteroid

isomerase (3βHSD/KSI) catalyzing its conversion to progesterone or be converted to DHEA via the actions of the CYP17A1 protein (65). The equine preovulatory follicle expresses these enzymes in the granulosa cells and theca interna, respectively. Therefore, pregnenolone from the theca may either traverse the basement membrane of the follicle and further modified by 3βHSD/KSI in granulosa cells (30) or may remain in the thecal layer and undergo catalysis by CYP17A1 (43). DHEA then diffuses to the granulosa cell layer where enzymes like 3βHSD, CYP19A1 and the 17β-hydroxysteroid dehydrogenases (17βHSD) catalyze its conversion to active estrogens (65).

2.2.1 Steroidogenesis from cholesterol to estrone

Many enzymes in the steroidogenic pathway are important for the production of estrogens. However, since the focus of this work is aimed at characterizing the final steps in estrogen biosynthesis as well as its inactivation, only a brief overview of the molecular characterization of the steroidogenic enzymes involved in the multi-step process of cholesterol conversion to estrone will be described.

2.2.1.1 Steroidogenic acute regulator (StAR)

StAR is responsible for the translocation of cholesterol from the outer to the inner mitochondrial membrane (70). It has been cloned and characterized in many species, including the mare (41), mouse (71), rat (72), human (73), cow (74), sheep (31), pig (75), and hamster (76). Two isoforms of the equine cDNA were identified and found to measure 1599- and 2918 base pairs (bp) in length (41). Both transcripts had open reading frames of 855 nucleotides, but were variable in their 5'- and 3'-untranslated regions (UTRs) (41). Once translated, the equine protein was composed of 285 amino acids, 86-90% identical to the StAR proteins from the species mentioned above (41).

StAR is believed to be active in its 37-kDa precursor form. However, this cytosolic phospho-protein exhibits a relatively short half-life. Once translocated across the mitochondrial membrane, along with cholesterol, it is truncated to a 30-kDa form that is more stable than its full-length counterpart, yet its role remains unclear (77-79). The role

of StAR in steroid biosynthesis has been extensively demonstrated *in vivo* (78), as well as in cell cultures (73, 80).

Many studies have addressed the transcriptional regulation of StAR in the ovary. In the mare, the effects of gonadotropins on StAR transcript expression were examined in follicles isolated at different times after an ovulatory dose of hCG and visualized by Northern blot analysis (41). In that study, no significant effect of gonadotropin treatment was discernable in intact follicle wall samples. Nonetheless, when individual cell compartments were examined, a significant increase in StAR transcript was observed 30-39 h post-hCG in granulosa cells, while the high levels present in theca interna prior to hCG significantly decreased 36 h after treatment. In vitro studies demonstrated that gonadotropins and activators of the PKA pathway upregulated StAR expression in granulosa cells from rat (33, 72, 81), porcine (82, 83), bovine luteal cells (40), and human (32) and PGF2α repressed StAR expression (40). Other *in vivo* studies demonstrated that StAR is regulated in a gonadotropin-dependent and stage-specific manner in developing follicles (32, 33, 38, 84). High levels of StAR mRNA have been detected in the corpus luteum of many species, including the mare (41) and previous studies have also shown a decrease in StAR expression in the corpus luteum during luteal regression (31, 32, 38, 40, 74, 75, 84-86).

2.2.1.2 Cytochrome P450 side-chain cleavage (CYP11A1)

The first rate-limiting and hormonally regulated enzymatic conversion in the biosynthesis of steroids from cholesterol is dictated by cytochrome P450 side-chain cleavage (CYP11A1) in tandem with its associated electron-transport chain (61, 87). CYP11A1 has been shown to be localized to the matrix side of the inner mitochondrial membrane and to catalyze the conversion of cholesterol to pregnenolone, a common precursor to all steroid hormones (61, 87). Its cDNA has been cloned and characterized in a number of different species, including the mare (30). The equine transcript measures 1837 nucleotides (nt), with an open reading frame of 1560 nt and encoding a 520-amino acid protein that is highly homologous to other mammalian orthologues.

The transcriptional regulation of the CYP11A1 gene has been shown to be under gonadotropin control. In the mare, studies using Northern blot analyses have demonstrated that the theca interna is the main site of CYP11A1 transcript expression prior to hCG treatment, with a downregulation occurring 30-39 h after hCG. In granulosa cells, an increase in CYP11A1 mRNA is observed 39 h post-hCG (30). A high level of CYP11A1 expression was also noticeable in equine corpora lutea (30).

2.2.1.3 Cytochrome P450 17α-hydroxylase/C17-20 lyase (CYP17A1)

CYP17A1, previously known as cytochrome P450 17α-hydroxylase/C17-20 lyase, is responsible for the conversion of pregnenolone to DHEA and has also been shown to convert progesterone to androstenedione, in a two-step reaction. It has been cloned in various species, including the mare (43). When expression of the equine CYP17A1 transcript was examined, a single 2.4-kb mRNA band was detectable by Northern blot. The regulation of CYP17A1 mRNA in preovulatory follicles after hCG treatment revealed that high levels were present prior to gonadotropin treatment and that these significantly decreased 36-39 h post-hCG. The signal was restricted to theca interna, and was undetectable in both granulosa cells and corpora lutea, indicating that the thecal layer is responsible for CYP17A1 activity.

2.2.1.4 3β-Hydroxysteroid dehydrogenase/ ketosteroid isomerase (3βHSD/KSI)

The dually functional enzyme 3βHSD/KSI converts Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids, first through catalyzing the dehydrogenation of hydroxysteroids, and by subsequent isomerization of the Δ^5-ketosteroid product to yield the α,β-unsaturated ketones. This enzyme has been shown to catalyze the conversion of pregnenolone to progesterone, as well as DHEA to androstenedione. It is therefore clear that 3βHSD/KSI is essential for the biosynthesis of all steroid hormones, including glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogens (88-91). It has been localized to the endoplasmic reticulum as well as the mitochondrial membrane and is expressed in many tissues, including the gonads, adrenal cortex, placenta, and in certain peripheral tissues that are not traditionally recognized as steroidogenic (88-93). In other species, such as the human and rat, many isoforms of the enzyme have been isolated

(94-96). In the horse, only a single 1612-bp 3βHSD/KSI cDNA has been cloned comprising an open reading frame of 1119 nt, that translates to a 373 amino acid protein (30). In the rat, the adrenal 3βHSD/KSI transcript was shown to be upregulated by ACTH and downregulated by corticosterone (97), whereas the ovarian 3βHSD/KSI appeared to be upregulated by hCG and downregulated by prolactin (98). The expression of equine 3βHSD/KSI mRNA, however, does not appear to be under LH control (30). It was demonstrated by Northern blot that 3βHSD was highly expressed in the granulosa cells of the equine preovulatory follicle, as well as in corpora lutea, and that no significant difference in 3β-HSD/KSI mRNA expression was detectable after hCG treatment. No signal was detected in the theca interna.

2.2.1.5 Cytochrome P450 aromatase (CYP19A1)

CYP19A1 catalyzes one of the final steps in estrogen biosynthesis, via the conversion of androstenedione and testosterone to estrone and 17β-estradiol, respectively. The CYP19A1 gene is interesting in that it has at least nine different untranslated first exons (99). They are referred to as exons I.1, I.2, I.3, I.4, I.5, I.6, PII, 2a, and 1f, and are alternatively spliced into a common 5'-splice acceptor site located 38 bp upstream of the translation start site in exon 2. The CYP19A1 gene has been cloned in a number of species, including the human (99) and pig (100), and cDNAs including promoters 1f and PII have been cloned and characterized in the mare (43). The equine 2682-bp promoter 1f transcript has been shown to encode a 503 amino acid-protein that is highly conserved compared to other species. The predicted equine protein has many conserved features, including a membrane-spanning region, an I helix, a heme-binding region and a putative cAMP-dependent protein kinase phosphorylation site.

The transcriptional regulation of CYP19A1 has also been characterized in various species. In the mare, a biphasic pattern of expression was detected in granulosa cells of preovulatory follicles by Northern blot analysis following hCG treatment (43). When individual promoters were examined, it was demonstrated that activation of PII was responsible for CYP19A1 expression prior to the ovulatory stimulus and in corpora

lutea, whereas the increase in transcript observed 30-39 h post-gonadotropin treatment was attributable to promoter 1f activation. No expression was detected in theca interna. NFκB-specific inhibitors were shown to suppress basal promoter II activity in cultured human granulosa cells, indicating a potential role for NFκB in ovarian aromatase expression (101). Also, activation of peroxisome proliferator-activated receptor (PPAR)-γ and retinoid X receptor, resulted in decreased NFκB/promoter II interactions, and resulted in a downregulation of aromatase expression (101).

The activity of different promoters has been used to explain the tissue-specific distribution of the CYP19A1 transcripts. It also accounts for differences in the time and site of expression, as demonstrated by the differential expression in early versus mid-pregnancy porcine placentas, as well as foetal versus adult human liver (100, 102). CYP19A1 expression has been detected in the gonads (103), brain (104), adipose tissue (105, 106), and skin (107). The targeted disruption of the CYP19A1 gene in mice results in arrested folliculogenesis, no corpora lutea, elevated levels of LH, FSH and testosterone, and infertility (108).

2.2.2 17β-Hydroxysteroid dehydrogenases

The biological potency of androgens and estrogens is modulated by the family of 17β-HSDs. They are responsible for the oxidation or reduction of steroids at the C17 position; the keto-forms being inactive and the hydroxy-forms being active and able to activate their cognate receptors (109). Twelve 17βHSDs have been identified thus far, differing in their substrate specificities, cofactor preference, subcellular localization, and tissue distribution (figure 6). All 17βHSDs are coded by different genes, with distinct amino acid sequences, therefore, they should not be referred to as isozymes. Although 17βHSDs have been shown to be capable of both oxidation and reduction reactions *in vitro*, they are unidirectional enzymes *in vivo* (110, 111). It is well recognized that NAD^+ and NADPH are the most abundant forms of intracellular nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate cofactors, respectively, thereby dictating the direction of 17βHSD activity. Indeed, certain 17βHSDs prefer a

phosphorylated cofactor (NADP(H)), while enzymes responsible for the oxidative reactions prefer NAD(H) (110, 111).

Enzyme type	Gene name	Other names	Human		Mouse	
			Function	Chromosome	Function	Chromosome
1	HSD17B1 HSD17BP1		E1→E2	17q21 ϕ17q21	E1→E2 A4→T	11
2	HSD17B2		E2→E1 T→A4 20α-DHP→P4	16q24.1-q24.2	E2→E1 T→A4	8
3	HSD17B3		A4→T	9q22	A4→T	13
4	HSD17B4	MF2	β-oxidation of fatty acids, E2→E1	5q21	β-oxidation of fatty acids, E2→E1	18
5	HSD17B5 AKR1C3	DD3, HAKRe	A4→T P4→20α-DHP	10p14-p15	A4→T	13
6	HSD17B6		N/A	N/A	androgen inactivation	N/A
7	HSD17B7 HSD17B7P2	PRAP	E1→E2 cholesterol synthesis	1q23, ϕ10p11, ϕ1q24	E1→E2 cholesterol synthesis	1
8	HSD17B8	HKE6, FABGL	E2→E1 androgen inactivation	6p21.3	E2→E1 androgen inactivation	17
9	HSD17B9		N/A	N/A	E2→E1 retinol oxidation	10
10	HSD17B10	HADH2, SCHAD, ERAB	estrogen and androgen inactivation, β-oxidation of fatty acids	Xp11.2	estrogen and androgen inactivation, β-oxidation of fatty acids	X
11	HSD17B11	Pan1b, retSDR2	estrogen and androgen inactivation	4q22.1	N/A	5
12	HSD17B12	KAR, KIK1	3-ketoacyl-CoA reductase fatty acid synthesis	11p11.2	3-ketoacyl-CoA reductase fatty acid synthesis	2

FIGURE 6: The different 17βHSDs. E1: estrone; E2: 17β-estradiol; A4: androstenedione; T: testosterone; P4: progesterone; 20α-DHP: 20α-dihydroprogesterone; N/A: not available (adapted from reference 109).

The 17βHSDs are readily grouped into two families based on protein architecture: the short-chain dehydrogenase/reductase (SDR) family (112) and the aldo-keto reductase (AKR) family (113). Members of the SDR family are often multimeric and share several amino acid sequence motifs, such as the Rossman fold motif (TGxxxGxG) involved in cofactor binding, a NAG domain involved in structural stabilization and located between the cofactor binding site and the active site, an active centre (YxxSK), and a site that dictates the direction of the reaction (PGxxxT) that is located C-terminal to the active site (112). Even though these domains are highly conserved among SDR

members, sequence similarity outside the conserved regions may be as low as 20% (112).

Members of the AKR superfamily are monomeric proteins of approximately 320 amino acids, that lack a Rossman fold motif yet still bind nicotinamide cofactors (113-118). Over one hundred AKR proteins have been classified into fourteen subfamilies (AKR1-AKR14) with substrates ranging from aliphatic and aromatic aldehydes, isoflavinoids, monosaccharides, steroids, prostaglandins and polycyclic aromatic hydrocarbons. The AKR1 family is the largest and contains the aldehyde reductases, the aldose reductases, the hydroxysteroid dehydrogenases and the 5β-reductases. Isoforms of the AKR1C family have been demonstrated to share at least 84% amino acid sequence identity, and to exhibit 3α-, 17β- and 20α-hydroxysteroid dehydrogenase activities (119). In the past, the multifunctional nature of AKRs has led to a fair bit of confusion regarding their nomenclature, as has been demonstrated for the AKR1C3 gene, which has also been called human liver 3αHSD type II, 17βHSD type V, dihydrodiol dehydrogenase (DD) type X, and prostaglandin F synthase (120). However, a website is now available (www.med.upenn.edu.akr/) that will designate a specific AKR name to proteins according to their amino acid sequence and functionality, which is expected to clarify the identity of individual proteins.

2.2.2.1 17β-Hydroxysteroid dehydrogenase type 1 (17βHSD1)

Type 1 17βHSD is a member of the SDR family and was the first 17βHSD to be characterized. It was first isolated from the human placenta (121, 122) and has since become the most well characterized 17βHSD. Type 1 has also been called placental 17βHSD, estradiol 17βHSD, and 17β, 20α-HSD. When adult mouse tissues were examined for 17βHSD1 mRNA expression, the transcript was detected in the granulosa cells of growing follicles, in the epithelial cells of the prostate, in the germ cells of the seminiferous tubules, as well as in the sebaceous glands of the dorsal skin and the intermediate lobe melanotrophs of the pituitary gland (123). In human and non-human primates, the transcript could only be detected in the placenta, ovary and mammary

gland (124-126), perhaps due to a limited number of tissues studied. Elevated levels of 17βHSD1 have also been detected in uterine leiomyomas from women as compared with the surrounding myometrium (127). Elevated 17β-estradiol levels due to overactive 17βHSD1 or an improper balance of oxidative versus reductive 17βHSDs have been shown to support the development of breast cancer in humans (128-130).

2.2.2.1.1 Cloning and characterization of 17βHSD1

The genomic structure of the 17βHSD1 gene is well conserved in all mammals where it has been studied. It is relatively short, containing six exons and five introns, and spans approximately 3 kb. The human 17βHSD gene (HSD17B1) is located on chromosome 17q11-q21 and is found in two tandem copies, only the 3'-duplicate being the functional gene (131-133). It has been cloned in the gibbon, orangutan, and chimpanzee, and in these primates the 17βHSD1 gene is also found in tandem duplication (131). However, this is not the case for the mouse, tree shrew, and macaque (figure 7) (131, 134).

While exons 1 and 2 are highly conserved, the sizes of introns 3 and 4 vary slightly between species, and the 3'-end of exon 6 has been shown to exhibit considerable variability from one species to the next (131). The 17βHSD1 transcript has also been described in various species, including the human (121), rat (135), and mouse (134). Two major mRNA species have been identified in the human placenta, having lengths of 1.3 and 2.2 kb. The major 1.3-kb transcript is also the form that is hormonally regulated. The open reading frame ranges from 894 to 1032 bp, depending on the species, and encodes proteins of 34-37 kDa. The variability observed in the last exon of the 17βHSD1 gene is responsible for the varying lengths in 17βHSD1 protein.

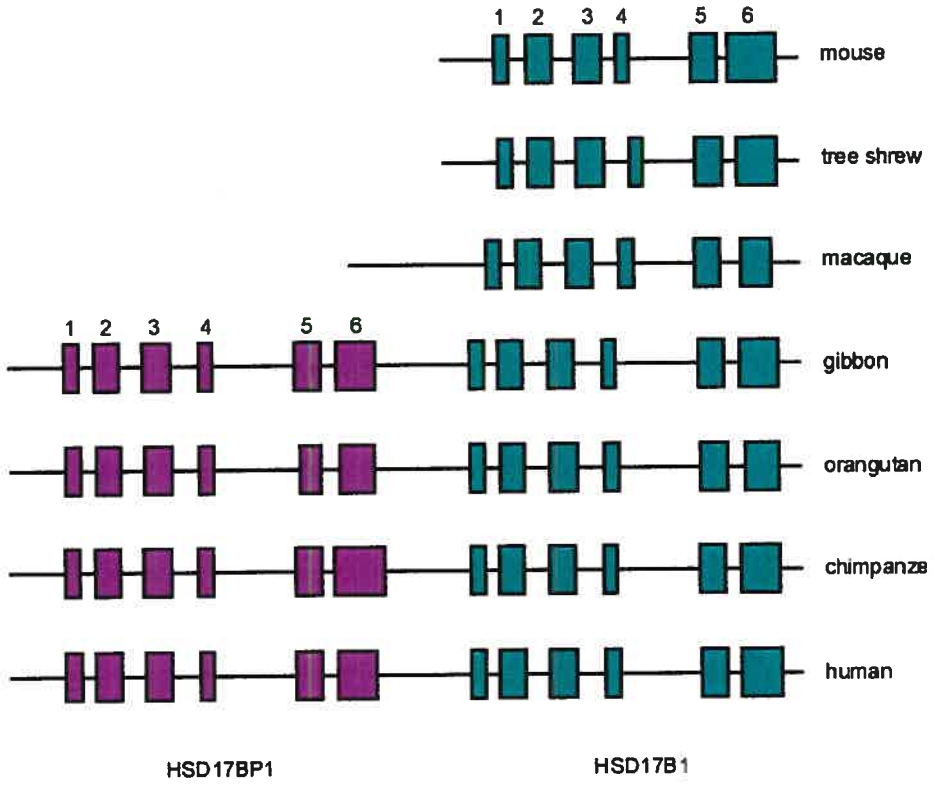

FIGURE 7. Comparison of the genomic structure of HSD17B1 genes. HSD17BP1 is the pseudogene of HSD17B1 (adapted from reference 131).

2.2.2.1.2 Biochemistry and enzymology of 17βHSD1

The 17βHSD1 protein has been crystallized and its three dimensional structure has been solved (136). It is a soluble protein that has been shown to functions as a homodimer and to utilize NADPH as a cofactor (137). In humans and non-human primates, estrone has been shown to be its main substrate, with a Km ranging from 0.4 μM to 8.6 μM, depending on the study (111, 121). Therefore, in these species, the sole purpose for 17βHSD1 appears to be the biosynthesis of 17β-estradiol. In rodents, its substrate specificity is broader and has been demonstrated to include androgens (134, 138).

2.2.2.1.3 Transcriptional regulation of 17βHSD1

Few studies examining the transcriptional regulation of 17βHSD1 have been published. However, the use of immature hypophysectomized rats allowed the study of the gonadotropin-dependent regulation of 17βHSD1 in ovaries by *in situ* hybridization and immunohistochemistry (135). These rats were treated for two days with recombinant FSH (recFSH), resulting in the induction of 17βHSD1 expression in granulosa cells of antral follicles. When rats were first pre-treated with diethylstilbestrol for five days, and then treated with recFSH, a decrease in ovarian 17βHSD1 expression was observed. Activin-A, and not inhibin, has also been shown to modulate 17βHSD1 transcript expression in rat cultured granulosa cells (139). The transcriptional regulation of the 17βHSD1 gene in choriocarcinoma cells has also been studied and was shown to be under the control of growth factors such as basic fibroblast growth factor, epidermal growth factor, and transforming growth factor α (140), as well as retinoic acids (141, 142), and cAMP (143-145). More recently, promoter activity assays have revealed that Sp1 may be important in 17βHSD1 transcription, while AP-2 interferes with Sp1 binding and GATA-3, through binding to the silencer region, may downregulate 17βHSD1 transcription (146).

2.2.2.2 17β-Hydroxysteroid dehydrogenase type 2 (17βHSD2)

Type 2 17βHSD was the second 17βHSD to be cloned and is also part of the SDR superfamily (147). The human and mouse 17βHSD2 genes have been cloned and localized on chromosomes 16q24.1-q24.2 and 8, respectively. The 17βHSD2 mRNA sequences have been cloned from the human placenta (147), mouse (148), rat (149) and partially in the marmoset. The cDNA for human 17βHSD2 codes for a 387 amino acid protein, that contains a N-terminal signal anchor motif and a C-terminal endoplasmic reticulum motif. This enzyme was shown to oxidate androgens and estrogens, utilizing NAD+ as a cofactor, at a two-fold higher rate than the reverse reduction reaction (147, 150, 151), as well as catalyze the conversion of 20α-dihydroprogesterone (20α-DHP) to progesterone (147). It has been detected in the human placenta, liver, gastro-intestinal tract, kidney, uterus, breast, and prostate, consistent with its role in steroid hormone

inactivation (152). Aberrant regulation of 17βHSD2 expression has been associated with endometriosis and inflamed gastric tissues, as well as with colon, prostate and breast cancer (130, 153-155).

2.2.2.3 17β-Hydroxysteroid dehydrogenase type 3 (17βHSD3)

Type 3 17βHSD has also been extensively characterized. It is mainly expressed in Leydig cells of the testes and has been shown to catalyze the reduction of androstenedione to testosterone, using NADPH as a cofactor (156, 157). The human gene (HSD17B3) has been cloned and shown to possess 11 exons, spanning 60 kb and located on chromosome 9q21 (156), which translates to a 310 amino acid protein, with a molecular weight of 34 kDa that is subcellularly localized to microsomes. Specific mutations in the 17βHSD3 gene have been linked to a condition called male pseudohermaphroditism, and characterized by the absence of male internal reproductive structures (156). Although 17βHSD3 expression has mainly been characterized in the testes, it has also been detected in human adipose tissue (158), primary osteoblast-like cells (159), and the brain temporal lobe (160).

2.2.2.4 17β-Hydroxysteroid dehydrogenase type 4 (17βHSD4)

The most unique 17βHSD is definitely type 4. It is the only 17βHSD to be located in peroxisomes and is unique in containing many functional domains (161-163). It has been shown to catalyze the oxidation of 17β-estradiol, as well as be involved in the β-oxidation of fatty acids, act as a hydratase towards 2-enoyl-acyl-CoA and facilitate the transfer of cholesterol between membranes. Although type 4 17βHSD is found in many tissues, it seems to be localized to specific cell types. For example, in the brain of rodents, 17βHSD4 is confined to Purkinje cells and the anterior pituitary (164). In the eye of chicks, its expression is limited to the retinal pigment epithelium (165), whereas high levels of 17βHSD4 are present in the lung bronchial epithelium (166). In porcine reproductive tissues, immunohistological data demonstrates that the type 4 protein is localized to the granulosa cells of preovulatory follicles, to the Leydig cells of the testes, as well as the luminal and glandular epithelium of the uterus (167, 168). Mutations or a

deficiency in 17βHSD4 have been associated with D-specific multifunctional protein deficiency (169), Zellweger syndrome (169-171) and Stiff-man syndrome (172).

2.2.2.4.1 Cloning and characterization of 17βHSD4

The cloning of the 17βHSD4 gene and cDNA was achieved using degenerate PCR-primers designed according to the partial amino acid sequence of a 32-kDa protein isolated from porcine uteri (173). An initial 405-bp fragment was amplified from porcine endometrial cDNA, with an open reading frame that translated to a peptide that was identical to the amino acid sequence obtained by Edman degradation of the 32-kDa protein. This cDNA was used as a probe to identify a 3-kb cDNA from a porcine kidney cDNA library. The sequence was also confirmed in porcine uterus samples and shown to encode a 737-amino acid protein of approximately 80 kDa (174). This protein had also been identified and cloned during studies on peroxisomal β-oxidation of bile acid intermediates in rats and humans, and named D-specific hydroxyacyl-coenzyme A dehydrogenase/hydratase (170, 175-181). Since then, the 17βHSD4 cDNA has been cloned in the human (161), mouse (164), chicken (165), guinea pig (182), zebrafish, and macaque. Its genomic structure was identified in humans, and shown to be composed of 24 exons, spanning more than 100 kb (figure 8) and localized on chromosome 5q2 (170, 183, 184).

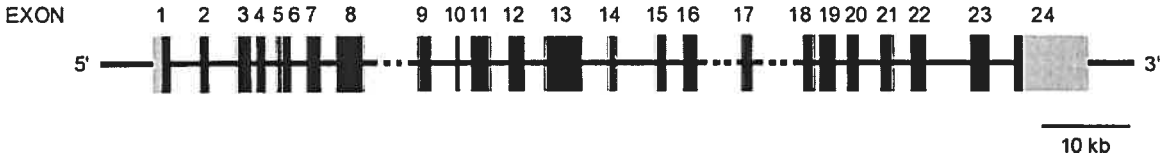

FIGURE 8. Human 17βHSD4 gene structure (adapted from reference 167).

The three domain structure of 17βHSD4 is unique among all 17βHSDs. The first domain, as encoded by the first twelve exons, has high homology with other 17βHSDs of the SDR family (118, 185, 186). The center domain reveals high homology with several fatty acid hydratases (187), while the C-terminal domain has 40% identity with the SCP2-domain of the SCPX protein (188, 189). However, the exon/intron structures

and the sizes of the first two genes are far from been analogous. This is not the case for the third domain, which appears to be part of the 17βHSD4 protein as a result of a gene fusion.

2.2.2.4.2 Biochemistry and enzymology of 17βHSD4

The 80-kDa protein now known as 17βHSD4 has three functional domains, all involved in either steroid oxidation or β-oxidation of fatty acids and has been shown to possess a three amino acid peroxisomal targeting signal (Ala-Lys-Ile) at its C-terminus (figure 9) (163). Certain peroxisomal proteins have been shown to be cleaved from larger precursors in the course of translocation (190), after the sequence Ala-[Ala/Val]-Pro (191). This cleavage sequence is present in the porcine 17βHSD4 enzyme (186), but not in the mouse (164) and rat (175, 179). Interestingly, three populations of the 17βHSD4 protein, composed of a full-length 80-kDa protein, as well as truncated 32- and 45-kDa proteins, have been detected in all three species. The extent of posttranscriptional processing is dependent on the tissues in which the protein was expressed (192).

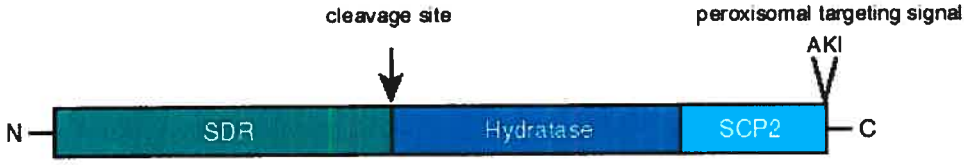

FIGURE 9: Structure of the 17βHSD4 protein (adapted from reference 167).

The activities of the three domains were independently expressed for investigation (189). The SDR domain (amino acids 1-323 in the porcine protein), catalyzed the oxidation of both 17β-estradiol and acetoacetyl-CoA. The conversion of 17β-estradiol to estrone by the full length protein equaling kinetic rapidity to that observed for either the purified or the recombinant 32-kDa proteins, with Kms of 0.4, 0.2 and 0.3 μM, respectively (189). Although the velocity of 17β-estradiol oxidation is several fold lower than that of fatty acyl-CoA, the affinity for 17β-estradiol and conversion rate to estrone is similar to that of other 17βHSDs involved in the oxidation of estrogens (147). The

hydratase domain (amino acids 324-596) was also shown to have Km values against 2-enoyl-acyl-CoA similar to other enzymes involved in the β-oxidation of fatty acids (193, 194), and the C-terminal SCP2-like domain (amino acids 597-737) was shown to facilitate the transfer of 7-dehydrocholesterol and phosphatidylcholine between membranes *in vitro* (189). Five Asn-X-Ser/Thr glycosylation sites have been shown to be present in the 80-kDa protein, however, no studies have yet shown that the 17βHSD4 protein is glycosylated (195).

2.2.2.4.3 Transcriptional regulation of 17βHSD4

The transcriptional regulation of 17βHSD4 appears to be under the control of many regulators, including modulators of steroid and fatty acid metabolism. The abundance of 17βHSD4 transcript and protein were both increased by progesterone in the porcine uterus (196), whereas certain peroxisome proliferator ligands, such as gemfibrozil and di-*n*-butyl phthalate, caused an increase in 17βHSD4 mRNA and protein expression, while the agonist WY-14643 regulated 17βHSD4 protein expression pretranslationally in rat liver (197). As the ligands in question activate the PPARα subtype, results indicate that 17βHSD4 can also be induced via this nuclear receptor.

2.2.2.5 17β-Hydroxysteroid dehydrogenase type 5 (17βHSD5)

The enzyme designated 17βHSD type 5 is unique, as it is the only 17βHSD to belong to the AKR family. The human 17βHSD5 gene (AKR1C3; HSD17B5) was cloned and localized to chromosome 10p15-p14, whereas the mouse gene (AKR1C6) was localized to chromosome 13 (198). The gene structure for both species is conserved among all known aldo-keto reductases, i.e. the gene has nine exons and eight introns. The 972-bp open reading frame of the human 17βHSD5 transcript encodes a cytosolic, 323 amino acid protein that is highly labile and whose 17βHSD activity is lost following homogenization (199). It has been shown to convert androstenedione to testosterone in intact cells, as well as possess high 20αHSD activity towards progesterone. The mouse protein, however, is more stable and retains 17βHSD activity in cell homogenates as well as once purified. It does not catalyze the conversion of progesterone to 20α-DHP to

the same extent as the human protein (199). Both proteins are similar in utilization of NADPH as a cofactor.

Tissue distribution analyses have shown that the human 17βHSD5 protein is located in the theca interna and luteal cells of the human ovary, in the epithelial cells of the endometrium, in the mammary gland, and prostate, as well as in the Leydig cells of the testis (200, 201). In the mouse, the 1.7-kb 17βHSD5 transcript was detected in the liver, kidney, testis, and stomach (202). In the past decade, many attempts have been made to clarify the AKR nomenclature and more work needs to be done to catalog the numerous varied enzymatic activities published so far. Therefore, knowing that human 17βHSD5 also has 20αHSD activity, it is important to realize that other studies examining proteins with 20αHSD activity, may also be describing the 17βHSD5 protein. For this reason, an additional section (2.2.3) has been added to account for the AKRs with 20αHSD activity.

2.2.2.6 17β-Hydroxysteroid dehydrogenase type 6

The sixth type of 17βHSD was identified in a rat prostate expression cDNA library, whose clones metabolized 5α-androstane-3α,17β-diol to androsterone (203). This enzyme is also part of the SDR family, with the unusual property of 65% homology with retinol dehydrogenase 1 and the capacity to recognize free retinol as a substrate (203, 204). A 2-kb mRNA band was detected in rat liver and prostate when Northern blot analyses were performed (203). The human 17βHSD6 gene has not been identified, although isolation of the human cDNA has been frequently and unsuccessfully attempted using a rat cDNA probe. Interestingly, these trials yielded many members of the human retinal dehydrogenase family, none of which had any 17βHSD activity (205).

2.2.2.7 17β-Hydroxysteroid dehydrogenase type 7

Type 7 17βHSD was initially described as prolactin receptor-associated protein and is now known as the main 17β-estradiol producing-enzyme of the corpus luteum (206). The cDNA for 17βHSD7 has been cloned in numerous species, including the rat (206),

mouse (207), human (208), marmoset (125), and only partially in the rabbit (209). The human 17βHSD7 gene (HSD17B7) is localized on chromosome 10p11.2 and consists of nine exons and eight introns, and spans 22 kb. Its cDNA codes for a membrane-bound 37 kDa protein that preferentially utilizes NADPH as a cofactor for the reduction of 17β-estradiol (208), but has also been shown to inactivate dihydrotestosterone (DHT) (205). Interestingly, 17βHSD7 has also been shown to participate in postsqualene cholesterol biosynthesis (210) and further investigation is required to determine its importance to the pathway of *de novo* cholesterol synthesis. RT-PCR has localized the human 17βHSD7 transcript at high levels in the placenta, as well as in the ovary, breast, testes and prostate (208), and the marmoset 17βHSD7 transcript, detected by Northern blot, was also highly expressed in the liver and upregulated during the luteal phase and early gestation in the corpus luteum (125). In the rodent model and rabbit, highest expression was observed in the corpus luteum during the second half of gestation or between days 10 and 14 in a pseudopregnant animal, respectively (206, 207, 209).

2.2.2.8 17β-Hydroxysteroid dehydrogenase type 8

Type 8 17βHSD is a protein that had been fairly well characterized in the mouse, as Ke6, and known for its role in the development of polycystic kidney disease (211, 212). Since then, its gene and cDNA has been cloned in humans (213) and mice (211). The human 17βHSD8 gene (HSD17B8; HKe6) is located on chromosome 6p21.3, in the leukocyte antigen region (214), and codes for a 968-nt transcript. The 777-nt open reading frame translates to a 259-amino acid protein that is highly homologous to the mouse protein (213). It has been shown to catalyze the conversion of 17β-estradiol to estrone, using NAD+ as a cofactor in mice (212), and its mRNA has been localized to the ovary, testis, uterus, mammary gland, prostate, pituitary, lung, kidney and liver (212, 215). Its wide tissue distribution is likely due to its estrogen-inactivating potential and may contribute to eliminating estrogenic effects in non-target tissues.

2.2.2.9 17β-Hydroxysteroid dehydrogenase type 9

As with 17βHSD6, type 9 has not yet been cloned in humans and although rat type 6 and mouse type 9 17βHSDs share close similarity to each other, they are not interspecies homologues. The mouse 17βHSD9 gene was identified and localized to chromosome 10. Few studies have been done to characterize 17βHSD9, however, it was found to be part of the SDR family and possess estrogen inactivating activity, as well as be involved in retinol oxidation (216).

2.2.2.10 17β-Hydroxysteroid dehydrogenase type 10

Type 10 17βHSD was first identified from the human brain as a single-domain multifunctional enzyme known as short chain L-3-hydroxyacyl coenzyme A dehydrogenase (217). Its deficiency has been linked to isoleucine degradation deficiency and Alzheimer's disease (218). Its gene was mapped to chromosome Xp11.2. The type 10 protein was shown to act as a multimer of four identical units and utilize NAD^+ for the oxidation of L-3-hydroxyacyl-CoA, while also catalyzing the conversion of 17β-estradiol to estrone (217). It is unique in that it is the only 17βHSD to be localized to the mitochondria, due to an N-terminal mitochondrial targeting sequence. Its cDNA has also been cloned in the mouse and rat, both translating to 261-amino acid proteins, of 27 kDa, that are highly conserved when compared to each other as well as with the human protein (219). Additional studies have localized the human enzyme to the liver and gonads, and have demonstrated low levels of expression in skeletal muscle (220).

2.2.2.11 17β-Hydroxysteroid dehydrogenase type 11

Type 11 17βHSD was first identified when a human database was screened for the conserved domains of SDRs, and it has also been called Pan1b and retSDR2 (221, 222). Transfected Chinese hamster ovary (CHO)-K1 cells have been shown to rapidly metabolize androstane-3α,17β-diol,5α-androstane,7β-diol (221), as well as 17β-estradiol, but not glucocorticoids (223). Northern blot analysis showed high levels of a 1.9-kb 17βHSD11 transcript in human tissues, including the pancreas, kidney, liver, lung, adrenal, ovary, and heart (222). Since then, it has been cloned in the mouse and

Northern blot analyses have shown 17βHSD11 to be highly expressed in the mouse kidney and lung, followed by the heart, spleen, liver and brain, whereas very low levels were observed in the thymus, testis and ovary (224).

The human 17βHSD11 gene (HSD17B11) has been cloned and characterized, and mapped to chromosome 4q22.1 (221). In contrast to some other 17βHSDs, information on its transcriptional regulation is available. Its promoter has been characterized, and a putative steroidogenic factor-1 (SF-1) half-site was identified in the 5' regulatory region. The intracellular second messenger, cAMP, has been shown to downregulate 17βHSD11 mRNA expression in mouse Y1 cells, which was accentuated by the addition of retinoic acid (222), whereas PPARα was shown to induce 17βHSD11 expression in the mouse intestine (225).

2.2.2.12 17β-Hydroxysteroid dehydrogenase type 12

A twelfth 17βHSD (17βHSD12) was identified by its sequence similarity with type 3 17βHSD (226). The human 17βHSD12 gene (HSD17B12) has been cloned and characterized, and shown to share extensive homology with its type 3 counterpart (226). It has been mapped to chromosome 11p11.2 and is composed of eleven exons, spanning 240 kb. The cDNA for 17βHSD12 has been cloned in many species, including the cow (227), human (228, 229), macaque, and mouse (228). The open reading frame of the 17βHSD12 transcript is 939 nt in length and codes for a 312 amino acid microsomal protein. This protein had previously been studied for its ketoacyl-coenzyme A reductase activity and its involvement in fatty acid metabolism (229), but it has since then been shown to catalyze the reduction of estrone to 17β-estradiol in transfected human embryonal kidney 293 (HEK293) cells (226). Using real-time PCR, the human 17βHSD12 transcript was shown to be highly expressed in the mammary gland and ovary, with a lower yet visible expression in the uterus, vagina, cervix and placenta (226).

2.2.3 20α-Hydroxysteroid dehydrogenase (20αHSD)

As previously mentioned, 20αHSD is part of the large and poorly characterized AKR superfamily that catalyzes the conversion of progesterone to its less active form 20α-dihydroprogesterone (20α-DHP). Although it is difficult to be certain that a 20αHSD that was previously reported as such, has not also been referred to and published as 17βHSD type 5 or even prostaglandin F synthase. This review will address what is known of published 20αHSDs. The characterization of 20αHSDs has remained largely at the level of the corpus luteum, where its expression is likely involved in regulating the availability of progesterone for activation of progesterone receptors. Tissue distribution analyses for the rat 1.2-kb transcript described its expression in the corpus luteum, with it being practically undetectable in uterus, kidney, lung, and heart (98). An enzyme with 20αHSD activity was also present in human ovarian epithelial tumours (230), and 20αHSD mRNA and activity were detected in the liver, brain, mammary gland, ovary, and placenta (231-235). High levels of 20αHSD have also been found in the human adrenal by *in situ* hybridization (236). When mouse tissues were examined for the occurrence of 20αHSD mRNA, it was detectable at high levels in luteal cells, Leydig cells, in the zona reticularis of the female adrenal cortex but not the male, as well as in the skin, liver, and kidney (237). This study further revealed a differential expression of the 20αHSD transcript in male versus female extra-gonadal tissues, suggesting that this progesterone-inactivating enzyme may be required at higher levels in females to protect tissues from higher circulating progesterone concentrations.

2.2.3.1 Cloning and characterization of 20αHSD

The first protein to exhibit 20αHSD activity was purified from the rat ovary more than four decades ago (238-240), but cloning of the rabbit and rat cDNAs was not achieved until the early nineties (241, 242). These displayed a high homology with other mammalian members of the AKR superfamily, such as 3αHSD. The bovine testicular 20αHSD has also been cloned and was found to be identical to aldose reductase, but surprisingly, it does not utilize progesterone as a substrate (243). More recently, cloning of the human (AKR1C1) and goat 20αHSDs was accomplished using a cDNA library

from human skin fibroblasts (244) and total mRNA extracts from goat corpora lutea and placenta (245), respectively. Both the human 1.3-kb cDNA and the goat 1.1-kb cDNA encoded 323-amino acid proteins. The rat and mouse 20αHSD genes have also been cloned and characterized (AKR1C8) (246, 247). The rat gene is located on chromosome 17, whereas the mouse gene is located on chromosome 13 and spans approximately 18 kb. Both genes have nine exons, which is conserved among most AKRs.

2.2.3.2 Biochemistry and enzymology of 20αHSD

Human 20αHSD is a cytosolic 37-kDa protein that has been shown to catalyze the reduction of progesterone in transfected HEK 293 cells using NADPH as a cofactor, with a Km value of 0.6 μM (244), and like other members of the AKR superfamily, contains the common (α/β)8-barrel three-dimensional fold (248). It showed only negligible reductive activity towards testosterone, DHT, and 17β-estradiol, as well as very low oxidative activity towards estrone, androstenedione and 20α-DHP (244). As opposed to 20αHSDs from other species having dual enzymatic activities, the rat protein only catalyzes the inactivation of progesterone (239).

2.2.3.3 Transcriptional regulation of 20αHSD

As previously mentioned, the role of ovarian 20αHSD has been studied primarily during luteolysis and at the end of gestation. Given its role in inactivating progesterone, it is not surprising that 20αHSD is completely silenced throughout gestation, while dramatically upregulated at the end of pregnancy in the rodent model (249, 250). The transcriptional regulatory mechanisms have been studied. A 2.5-kb fragment of the rat promoter was isolated and shown to contain one CRE, two Nur77 response elements, two putative AP1 sites and one progesterone response element half-site (247). The transcription start site and a TATA box were also identified. Cultured rat granulosa cells were used to establish both GnRH and FSH as positive regulators of 20αHSD activity (251), whereas forskolin treatment of luteal cells transfected with the rat 20αHSD promoter resulted in a downregulation of luciferase activity (247). In pregnant rats, prolactin and progesterone were shown to downregulate 20αHSD mRNA expression

(98, 252), whereas prostaglandin $F_{2\alpha}$ increased 20αHSD activity via the transcription factor Nur77, thereby reducing circulating concentrations of progesterone (253, 254). At the end of gestation, the decline in progesterone is important for the initiation of labor (249), indeed parturition does not occur in mice bearing an inactivating mutation of 20αHSD.

3. Sulfoconjugation

The biotransformation of molecules through sulfoconjugation (figure 10) represents a basic metabolic conversion of primary importance. The addition of a highly charged sulfonate group (SO^{3-}) results in dramatic changes in a substance's physicochemical properties, including conformational changes in both low- and high-molecular weight molecules, increased water solubility, and alteration of the pKa to approach 1.5, providing the potential for ionization at any pH found in living organisms (255).

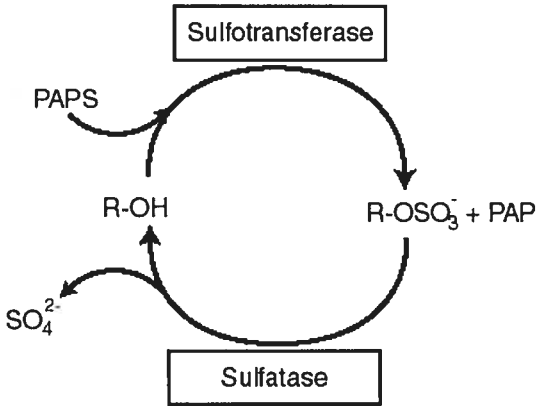

FIGURE 10: Sulfoconjugation (adapted from reference 256).

3.1 Role of sulfoconjugation in physiological processes

Sulfoconjugation has been described in many systems (256). The sulfoconjugation of proteins involved in cell surface and connective tissue structures, proteoglycans and glycosaminoglycans, has been linked to the maintenance of tissue hydration, cation composition, and elasticity (257). Sulfoconjugation has also been shown to affect the functionality of secreted and membrane proteins (258, 259), the biological activity of

glycoprotein hormones (260, 261), as well as the interactions between glycolipids and cation transport systems, extracellular matrix proteins, and blood coagulation systems (262). It also plays an important role in the biotransformation of xenobiotics and endogenous substrates, such as steroids.

3.2 Properties of sulfoconjugated steroids

The sulfoconjugation of steroids occurs in a wide variety of tissues including male and female reproductive tissues, liver, kidney, brain and adrenal cortex (263). The addition of a highly charged sulfonate group also affects the biophysical properties of steroid hormones (figure 11). Since they are now negatively charged, they can no longer diffuse freely across the lipid bilayer and will no longer bind their cognate receptors (264).

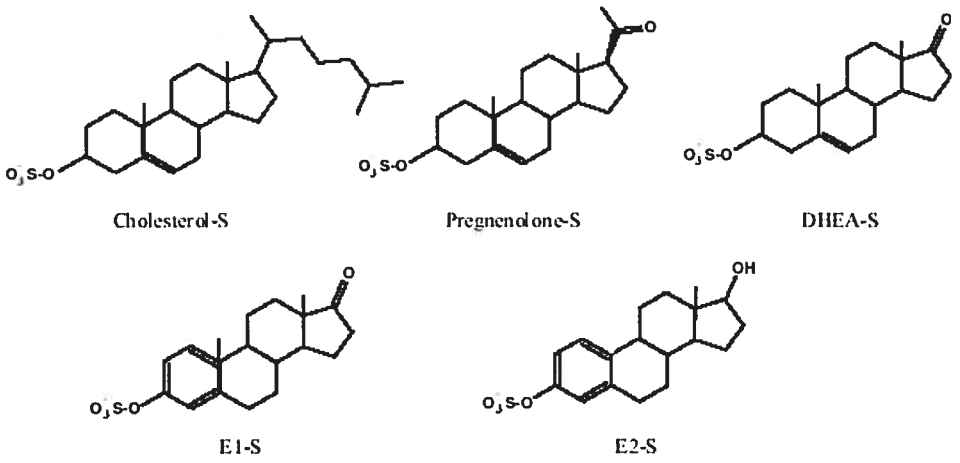

FIGURE 11: Steroid sulfoconjugates (adapted from reference 265).

Although most sulfoconjugated steroids are considered inactive, they are transported in the blood complexed with serum proteins and have the potential to be hydrolyzed by sulfatases, giving rise to free and active steroids. DHEA-S has been shown to be quantitatively the most abundant circulating steroid hormone in humans (266, 267), while E1-S is the major circulating form of plasma estrogen, both steroids having relatively longer half-lives than their unconjugated counterparts (268-271). At a systemic level, sulfonated estrogens can circulate in the blood and reach estrogen target

tissues, where they can be converted to receptor-active free estrogens by the enzyme steroid sulfatase (272). It has also been reported that the main steroid present in the urine of pregnant mares and in the ejaculate of stallions is E1-S, which can reach concentrations of 67 ng/mL during the breeding season (268).

3.3 Sulfotransferases

Sulfotransferases are classified depending on their location, either cytosolic or are membrane-bound. The cytosolic or soluble sulfotransferases have been shown to catalyze the sulfoconjugation of a wide variety of endogenous compounds, including hormones and neurotransmitters, as well as drugs and xenobiotics. The membrane-bound sulfotransferases are involved at the level of posttranslational modification of macromolecules. Enzymes that carry out the transfer of a sulfonate group must interact with two substrates, i.e. the sulfonate donor and acceptor molecules. Sulfotransferases employ 3'-phosphoadenosine-5'-phosphosulphate (PAPS), as a sulfonate donor to the hydroxyl site of the acceptor molecule (255). PAPS synthesis requires a ready supply of sulfate, either available from the diet or from the catabolism of proteins and sugar sulfates (255). PAPS synthase is a fusion protein containing an ATP sulfurylase moiety at its carboxy-terminus, that induces the conversion of ATP and SO_4^{2-} to adenosine-5'-phosphosulfate (APS) (273). It further contains an APS kinase moiety at its amino-terminus that converts APS to PAPS (273).

Membrane-associated sulfotransferases have been localized to the trans-Golgi complex where they act on carbohydrates, peptides, and proteins. The extensively studied sulfoconjugation of carbohydrates takes place in the Golgi network, on sugar residues of lipids and proteins passing through the secretory pathway (274). This biotransformation results in the change of a common structural motif into a unique recognition site for specific receptors and for lectin (260), and may also regulate the circulatory half-life of glycoprotein hormones (261). Tyrosine sulfoconjugation is also important for the appropriate cellular processing and biological activity of many proteins (275).

More than 40 cytosolic sulfotransferases have been identified in mammals thus far. In previous nomenclature systems, the naming of these sulfotransferases was complicated by tremendous overlap in their substrate specificities. Through rigorous biochemical and genetic characterization, a new system known as the SULT nomenclature has been developed. All known cytosolic sulfotransferases are now classified in five different SULT families (SULT for humans; Sult for rodents), each sharing less than 40% similarity with each other (276). The SULT1 family catalyzes the sulfoconjugation of phenolic drugs and catecholamines (SULT1A), thyroid hormones (SULT1B) and xenobiotics (SULT1C), as well as estrogenic steroids (SULT1E). The SULT2 family members sulfonate neutral steroids (SULT2A) and sterols (SULT2B). The SULT3 family has been characterized as catalyzing the production of sulfamates, whereas members of the SULT4 and SULT5 families have not yet been characterized (276). Although all SULT isozymes are products of different genes, they appear to be clustered on the same chromosomes, suggesting that their emergence has probably resulted from gene duplication (256). The sulfotransferases involved in the sulfoconjugation of steroids are typically found as homodimers in solution (277), and are characterized as high-affinity and low capacity enzymes, thereby resulting in much slower activity than other molecular modifiers such as phosphotransferases (255). Crystal structures of a few sulfotransferases have been solved, including mouse estrogen sulfotransferase (Sult1e1) (278), human dopamine/catecholamine sulfotransferase (SULT1A3) (279), human hydroxysteroid sulfotransferase (SULT2A3) (280), and human estrogen sulfotransferase (SULT1E1) (281).

3.3.1 Hydroxysteroid sulfotransferases

Hydroxysteroid sulfotransferases were first isolated from rat (282) and human (283) livers. These 33-35 kDa proteins were shown to catalyze the sulfoconjugation of many steroids, however, their affinity for DHEA was much higher, hence the name DHEA sulfotransferase (SULT2A1). The first SULT2A1 cDNAs were cloned from the liver of rat (284, 285), human (286-288), and mouse (289), as well as from the human fetal adrenal (290) and guinea pig adrenal gland (291). The purified human SULT2A1 protein exhibits a Km of 0.993 μM towards DHEA and 2.0 μM towards 17β-estradiol,

and only negligibly sulfonates cholesterol (256, 292). SULT2A1 has been shown to be highly expressed in the adrenal gland (293), steroidogenic organs (adrenal and ovary), androgen-dependent tissue (prostate), tissues of the gastro-intestinal tract (stomach, small intestine, and colon), and the liver, whereas its expression was undetectable in the skin (294).

In the past decade, a new hydroxysteroid sulfotransferase has been cloned, that encodes two isozymes, SULT2B1a and SULT2B1b (256). These subtypes are products of the alternative use of exon 1 and only differ at their amino-terminus (256). Even though the SULT2B1s are considered hydroxysteroid sulfotransferases, they are structurally different from their SULT2A1 counterpart, sharing only 37% amino acid identity. Their substrate specificities are also slightly narrower than SULT2A1, with pregnenolone being the major substrate for SULT2B1a (292), and cholesterol being the major substrate for SULT2B1b (256). SULT2B1b is also more widely expressed than SULT2B1a (294). The human genes for both SULT2A1 and SULT2B1 have been identified, with the latter mapped to chromosome 19q13.4, 500 kb telomeric to the SULT2A1 gene (295). Rat SULT2A1 expression has been shown to be repressed by androgens through the hepatocyte nuclear factor-1, and requiring the presence of the C/EBP and OCT-1 elements (296, 297), whereas bile acids and the estrogen-related receptor α have been shown to induce SULT2A1 expression (267, 298). More recently, SF-1 and GATA-6 were shown to be positive regulators of SULT2A1 promoter activity in HEK293 cells (299).

3.3.2 Estrogen sulfotransferase (EST)

The sulfoconjugation of estrogens and as a result, their inactivation, is catalyzed by the cytosolic enzyme estrogen sulfotransferase (EST; SULT1E1). It has been purified from several tissues of a number of species and has been shown to catalyze the sulfoconjugation at a specific site on estrogenic compounds, namely their 3-hydroxyl or phenolic groups. SULT1E1 has been identified in both male and female reproductive tissues, including the Leydig cells of the mouse and human testis (300, 301) and the granulosa cells of the rat preovulatory follicle (302), as well as the kidneys, liver, brain,

and adrenal cortex (272). Marked differences in tissue expression have been observed depending on the species studied. SULT1E1 activity was detected at high levels in the guinea pig adrenals and at low levels in the testis, whereas in the mouse, the levels of SULT1E1 activity were reversed (303). Its expression has also been shown to vary depending on physiological status. For example, estrogen sulfotransferase activity in the human and porcine endometrium was cyclical (304, 305), whereas an increase was observed in the uterus and placenta of mice during pregnancy (306), and in the chorion at midgestation in the guinea pig (307). SULT1E1 knockout mice have demonstrated an important role for estrogen sulfoconjugation in reproductive processes, as these mice displayed reduced fertility, with increased estrogen sensitivity resulting in abnormalities in Leydig cell and seminiferous tubule development (308).

3.3.2.1 Cloning and characterization of EST

The genes for the guinea pig (309) and human (310) SULT1E1s have been cloned and characterized. Both genes reveal a high similarity in genomic structure, consisting of eight exons and seven introns, with introns 3 to 7 being identical, and both genes spanning approximately 20 kb. The cDNA for estrogen sulfotransferase has also been cloned in many species, including the cow (311), rat (296), guinea pig (312), human (313), and mouse (314). The mRNAs for the mouse, rat and bovine SUL1E1 have open reading frames of 888 nt, with variable 5'- and 3'-UTRs. The human open reading frame is slightly shorter, at 885 nt, and encodes a 35-kDa, 294-amino acid protein.

3.3.2.2 Biochemistry and enzymology of EST

As previously mentioned, SULT1E1 has been shown to specifically catalyze the sulfoconjugation of estrogenic compounds on their 3-hydroxyl groups. It will not, however, sulfonate either the 16α- or 17β-hydroxyl groups of phenolic steroids (315-317). The SULT1E1 enzymes, isolated from various species, have molecular weights ranging from 30 kDa, for the mouse protein, to 36 kDa for both the bovine and human proteins (272, 306, 318, 319), and multiple charge isoforms have been observed for the guinea pig and bovine proteins (320, 321). The porcine enzyme has been shown to display a Km of 24 nM towards estrone (318), whereas the guinea pig enzyme has a

slightly lower affinity in uterine and chorionic extracts with a Km of 100 nM (322). However, when overexpressed in CHO-K1 cells, the guinea pig SULT1E1 protein has a Km of 60, 70 and 40 nM towards estrone, 17β-estradiol and estriol, respectively (317). The guinea pig SULT1E1 has also been shown to bind pregnenolone, however, this steroid is not sulfoconjugated by SULT1E1 (323). The human estrogen sulfotransferase has a Km for estrogens in the nanomolar range, but has also been shown to sulfonate DHEA. This discrepancy between the human and mouse proteins has been attributed to a tyrosine present in the binding pocket of the mouse enzyme that prevents DHEA from binding (324). The GxxGxxK motif, that has been shown to be responsible for PAPS binding, is present in all steroid and phenol sulfotransferases, including SULT1E1, and is located in a highly conserved region at the carboxy-terminus. The importance of this site has been confirmed by mutational analysis in which modification of three residues abrogates sulfotransferase activity (325).

3.3.2.3 Transcriptional regulation of EST

The cloning of the human and guinea pig genes has allowed analysis of the SULT1E1 promoter (326). A conventional TATA box variant has been identified 26 nt upstream of the cap site of the guinea pig promoter. Two copies of an estrogen-response element half-site (AGGTCA), a glucocorticoid response element (GRE) half-site (TGTTCT), and a sequence motif analogous to the AP-1 binding sites (TGACTCA) have also been shown to be present (326). Estradiol, progesterone, cortisol and testosterone all induce promoter activity in the guinea pig. In addition to the GRE half-site, the human promoter contains a thyroid response element (TGAAC) half-site. Mouse Sult1e1 expression has been shown to be induced by DHT (327), as well as LH, and the treatment of Leydig cells with this gonadotropin was sufficient to maintain Sult1e1 expression (300). LH has also been shown to upregulate Sult1e1 mRNA expression in rat granulosa cells (302).

3.4 Sulfatases

It was generally believed that sulfoconjugation served principally to eliminate newly charged sulfonated molecules in the urine in mammals. The more recent discovery that a

widely spread enzyme, that functions in hydrolysis of the sulfonate moiety, has led researchers to examine the role of sulfoconjugation in a new light. Sulfatases, which cleave sulfate esters in biological systems, play key roles in regulating the biological activity of a wide range of molecules, including small cytosolic steroids.

3.4.1 Steroid sulfatase (STS)

Steroid sulfatase (STS), previously known as aryl sulfatase C, is responsible for the removal or hydrolysis of the sulfonate group from aryl and alkyl steroid sulfates. This enzyme plays an important role in regulating the formation of biologically active steroids. STS activity has been detected in microsomes of the rat liver (328), brain (329, 330) and testis (331), in human leukocytes (332), osteoblast cell lines (333, 334), and in rat and human ovarian granulosa cells (335, 336). STS protein was detected by immunocytochemistry in cultured human skin fibroblasts (337), ovarian clear cell adenocarcinomas (338), the glandular epithelium of the endometrium (339), as well as the secretory cells of the fallopian tubes (340). A relatively common genetic disorder, X-linked ichthyosis, has been linked to the non-functional STS protein resulting from point and/or deletion mutations in the STS gene (341, 342).

3.4.1.1 Cloning and characterization of STS

The gene for human STS has been cloned and mapped to the distal short arm of the X-chromosome (343). These studies have further shown that it is a pseudoautosomal gene and escapes X-inactivation. A STS pseudogene is also present, but it is located on the Y-chromosome. Because of the deletion of several exons as well as part of its promoter sequence, not to mention the numerous additional stop codons and several large insertions, this pseudogene is transcriptionally inactive.

The active gene consists of 10 exons, spanning 146 kb, with introns ranging from 102 bp to 35 kb (343). The cDNA for STS has been cloned in the human (344), mouse (345), macaque, and rat (346) and even though there is only one gene for STS, certain reports suggest that more than one isoform of the protein may exist in rodents and humans.

3.4.1.2 Biochemistry and enzymology of STS

STS is a membrane-bound protein, composed of 583 amino acids, with a signal peptide from amino acid 21 to 23, as well as four potential glycosylation sites of which at least two are used (347). Depending on the extent of glycosylation, the STS protein has a molecular weight of approximately 65 kDa (347). Its activity was first characterized in rat liver microsomes (328), whereas the purified protein has been shown to hydrolyse aryl sulfates, such as E1-S, as well as alkyl sulfates, including DHEA-S and pregnenolone-S (348-350). STS has been localized by immunocytochemistry to the rough endoplasmic reticulum, the Golgi cisternal, the trans-Golgi reticulum, and at lower levels in the plasma membranes and components of the endocytic pathway (337). Electron microscopy revealed its presence on the nuclear envelope (351). Its crystal structure has also been elucidated demonstrating that STS is an integral membrane protein with at least two segments spanning the lipid bilayer (figure 12), and that it occurs as monomers and dimers (352).

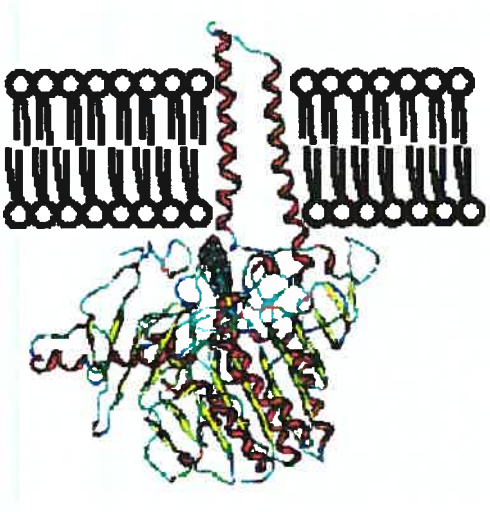

FIGURE 12: Crystal structure of human STS (adapted from reference 265)

3.4.1.3 Transcriptional regulation of STS

Few reports have addressed the transcriptional regulation of the STS gene (353). The STS promoter has been isolated and many putative transcriptional elements have been

identified. The promoter is unusual, as it lacks a TATA box, it is not GC-rich, and lacks binding sites for Sp1. Four upstream regulatory elements have been identified and shown to act as enhancers (353). The inflammatory cytokine interleukin (IL)-1β decreases STS activity as well as mRNA expression in human endometrial stromal cells and vascular smooth muscle cells derived from human aortas (354, 355). While cytokines, tumour necrosis factor (TNF)α and IL-6, have been shown to upregulate STS enzyme activity in MCF-7 breast cancer cells, this regulation was as a result of posttranslational modifications of the STS protein (356). A marked difference in STS protein expression was also noted during the menstrual cycle in women, with highest expression being detected in tissues obtained during the early luteal phase of the cycle (340).

3.5 Transporters of sulfoconjugated steroids

High levels of steroid sulfoconjugates are present in the blood. As previously noted, they lose the ability to diffuse freely across the lipid bilayer when they become negatively charged in the range of pHs present in mammalian tissues. The internalization of circulating steroid sulfoconjugates by cells therefore requires the presence of transporter, while newly sulfoconjugated steroids are expected to require an efflux transporter to exit the cell. Indeed, recent findings have demonstrated that sulfonated steroids, such as E1-S, traverse the cell membrane by transporter-dependent mechanisms (357).

3.5.1 Influx transporters and organic anion transporting polypeptides

The organic anion transporting polypeptides (OATP for humans; Oatp for rodents) belong to the growing family of organic anion/prostaglandin influx transporters that mediate the transport of numerous endogenous and exogenous amphipathic compounds across membranes in a sodium-independent manner (358). The nomenclature for the genes encoding OATPs has been approved by the HUGO Gene Nomenclature Committee, and classifies them within the solute carrier/OATP (SLCO for humans; Slco for rodents) family (359).

Oatp1 (Oatp1a1) was the first member of the OATP superfamily to be identified, and it was isolated as part of a sodium-independent bromosulfophthalein and taurocholate uptake system of the rat liver (360). Since then, at least 14 rat and 11 human SLCO genes have been identified (359). All cloned SLCO family members encode OATP proteins containing between 643 amino acids, as seen for the prostaglandin transporters (OATP2A1), and 722 amino acids for Oatp4a1, with amino acid identities ranging between 24% and 82% (361-363). Based on hydropathy plots, all OATPs have 12 putative transmembrane domains (360), including a large extracellular loop between transmembrane domains 9 and 10 that contains many conserved cysteine residues that resemble zinc finger domains of DNA-binding proteins (364). Loops 2 and 5 also contain putative N-glycosylation sites, whereas the OATP superfamily signature domain is located at the border between extracellular loop 3 and transmembrane domain 6 (figure 13).

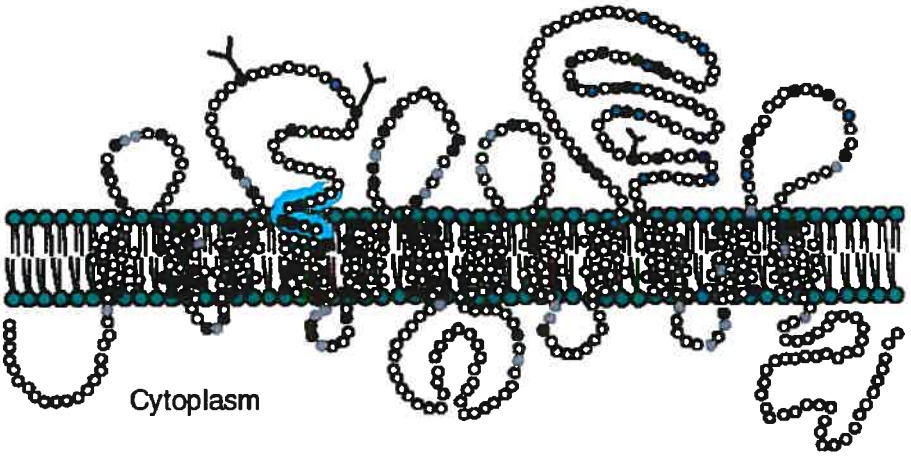

FIGURE 13: Typical transmembrane domain structure of OATPs. Black dots represent conserved amino acids, whereas conserved charged amino acids are in gray. Conserved cysteines are in dark blue, potential glycosylation sites are marked with a Y, while the light blue region represents the location of the OATP superfamily signature motif (adapted from reference 365).

OATPs have been shown to transport a number of different substrates, with specificities sometimes overlapping, and sometimes distinct (366). For example, Oatp1a1 mediates the transmembrane transport of bile salts (367-369), steroid hormones

and their conjugates (368-372), thyroid hormones (373), as well as organic cations (370, 374), whereas Oatp1a5 transports all of the above substrates with the addition of prostaglandin E2 (365). The tissue distribution of various OATPs is widespread, although some OATPs have restricted expression patterns (Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2, OATP1A2, OATP1B1, OATP1B3, and OATP1C1) as opposed to other OATPs (OATP2B1, OATP3A1 and OATP4A1) which appear to be ubiquitous (365). Although few studies examining the transcriptional regulation of the SLCO genes have been published, it has been demonstrated that Slco1a4 gene expression is induced by such disparate stimuli as phenobarbital and pregnenolone-16-α-carbonitrile in rats (375).

3.5.1.1 Cloning and characterization of SLCO2B1

All SLCOs have a highly similar gene structure, with almost identical exon lengths (376). The human SLCO2B1 gene has been cloned and characterized, and it has been mapped to chromosome 11 (365). The cDNA for SLCO2B1 has also been cloned in many species, including the human (361), rat (377), and mouse. The tissue distribution for human SLCO2B1 revealed that it was most highly expressed in the liver, followed by the placenta, lung and spleen, and low in the heart, brain, kidney, pancreas, testis, ovary and small intestine (378, 379). SLCO2B1 has been shown to be the major SLCO expressed in the human mammary gland, being localized in the myoepithelium surrounding the ductal epithelial cells (380).

3.5.1.2 Biochemistry and enzymology of OATP2B1

Although SLCO2B1 expression is widespread, it is fairly selective in the substrates that it transports. OATPs appear to have a transport mechanism that is dependent on anion exchange (365), meaning that a molecule is exported for every molecule imported. As previously mentioned, OATP2B1 is relatively selective in the substrates it transports. It has been shown to mediate influx of E1-S, DHEA-S, benzylpenicillin and bromosulfophthalein, but not to transport bile salts, glucoronidated estrogens, leukotrienes or prostaglandins (365, 379). The OATP2B1 protein has been shown to mediate the uptake of E1-S in HEK293 cells in a pH-dependent manner. At a pH of 5.0,

the cells imported E1-S at a V_{max} of 2135.8 pmol/mg and a K_m of 13.1 μM, whereas when the pH was increased to 7.4, these values decreased to 300 pmol/mg and 8.09 μM, respectively (381, 382). While it can be seen that at an acidic pH the rate of import is higher, this notwithstanding, the affinity for the substrate is slightly higher at a neutral pH. OATPs have also been shown to confer polarity to the substrates they transport. This supposition is based on the localization of OATP2B1 to the apical membrane of small intestinal epithelial cells (381), the basal syncytiotrophoblast membrane (383), as well as the basolateral membranes of the human liver (379).

3.5.1.3 Transcriptional regulation of SLCO2B1

There are no known reports on the transcriptional regulation of SLCO2B1, there is evidence for regulation during differentiation and for regulation by ligands. SLCO2B1 mRNA levels were upregulated upon differentiation of trophoblasts to syncytia (383), and pregnenolone sulfate was shown to decrease E1-S uptake (383), whereas prostaglandins A1 and A2 were shown to increase the uptake of E1-S and DHEA-S in transfected CHO-K1 cells, without affecting the Km (380).

3.5.2 Efflux transporters and members of the ATP-binding cassette family

The ATP-binding cassette (ABC for humans; Abc for rodents) family has been growing since the identification of its first member, P-glycoprotein, by recognition of its overexpression in tumour cells which had acquired the multidrug resistance (MDR) phenotype (384). Although it was first believed to be the only protein responsible for the MDR phenotype, these hopes were quickly shattered when P-glycoprotein-specific inhibitors were unsuccessful in allowing chemotherapeutic agents to stop tumour proliferation in this model (385). These studies did lead to the discovery and elucidation of new roles of several additional members of the ABC family, called the MDR-associated proteins (MRP for humans; Mrp for rodents), that have been shown to be involved in the MDR phenotype (reviewed in 386, 387, 388). For example, P-glycoprotein, also referred to as MDR1, now known to be associated with the drug biodistribution in normal cells (389, 390) as well as conferring resistance to a broad range of apoptotic stimuli (391).

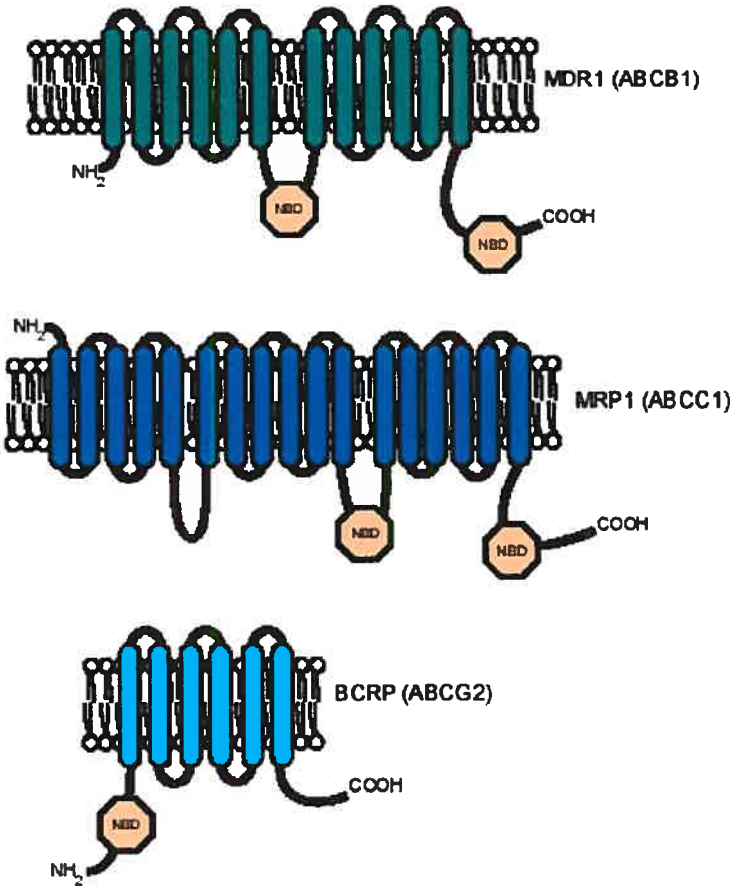

FIGURE 14: Predicted transmembrane structure of three classes of ABC transporters (adapted from reference 392).

The family of ABC genes represent the largest subgroup of the transmembrane proteins identified thus far that mediate the efflux of a wide array of substrates through the hydrolysis of ATP (393). They are named according to the sequence and organization of their nucleotide-binding domains (NBD), as well as domain structure similarities and the number of transmembrane domains (figure 14) (386). The first identified ABC transporter, MDR1, is a product of the ABCB1 gene and has twelve transmembrane domains. Other members of the ABC family, such as MRP4 (ABCC4), MRP5 (ABCC5) and BSEP (ABCB11) share a transmembrane structure similar to MDR1. A second type of ABC transporter is much larger, comprising seventeen transmembrane domains. It includes MRP1 (ABCC1), MRP2 (ABCC2), MRP3

(ABCC3) and MRP6 (ABCC6). A half-transporter has also been reported, breast cancer resistance protein (BCRP) coded by the ABCG2 gene, has six transmembrane domains and only one NBD.

Not only are these efflux transporters responsible for the transport of drugs and xenobiotics, they have also been shown to transport hundreds of unrelated substrates, including hormones and lipids. Even though a few of these ABC transporters have the potential to be important in reproductive biology, only MRP1 (ABCC1) will be further addressed.

3.5.2.1 Cloning and characterization of ABCC1

ABCC1 was first identified in 1992 when its cDNA was found to be overexpressed in an MDR lung cancer cell line (394). It was only when the sequence of this 1531-amino acid human protein was analyzed that it was classified as part of the ABC superfamily. Subsequent to its cloning, MRP1 was shown to be identical to another 190 kDa protein (p190) that had received attention because of its overexpression in a mutidrug-resistant leukemia cell line (395). The human ABCC1 gene has been mapped to chromosome 16p13.1 (396). The cDNA for ABCC1 has been cloned and characterized in many species, including the human (394), mouse (397), rat (398), bovine (399), dog (400), and macaque (401). Marked differences in expression of mouse Abcc1 mRNA were observed depending on the tissue and gender of the mice. Its highest expression, as detected by branched DNA assays, was present in the testis, ovary, placenta, kidney and liver (402). In the latter tissues expression was substantially greater in females when compared to males (402). ABCC1 expression has also been identified in the blood vessel endothelia and in the apical synthiotrophoblast of human term placenta (403). When HeLa cells, as well as other cell types, were transfected with MRP1 cDNA, a MDR phenotype was achieved, suggesting that MRP1 may be involved in resistant cancer cells (404-408). In support of this notion is the unfavorable clinical outcome associated with increased MRP1 and MRP3 expression in advanced ovarian serous papillary adenocarcinoma (409).

3.5.2.2 Biochemistry and enzymology of MRP1

MRP1 is a seventeen transmembrane domain protein sharing structural similarities with other MRPs, such as MRP2, MRP3, and MRP6. Although detectable levels of MRP1 were shown to be present in intracellular membrane compartments of some cell types, cell fractionation studies have demonstrated that the protein was predominantly localized to the plasma membrane (410-413). The binding and subsequent hydrolysis of ATP by the NBDs (figure 13) is believed to be the driving force for substrate transport (414). These NBDs share two sequence motifs, called Walker A and Walker B, as well as an active transport family signature motif located between both Walker motifs (414). Although MRP1 is predicted to have a molecular weight of 171 kDa from its cDNA, it is usually detected at around 190 kDa due to posttranslational N-linked glycosylation on Asn^{19}, Asn^{23} and Asn^{1006} (412).

MRP1 has been shown to transport a variety of substrates similar to those transported by the OATPs. Most of these being glutathione (GSH)-, glucorinide-, or sulfate-conjugated molecules, including E1-S, where transport appears to be stimulated by GSH (357). MRP1 has also been shown to be the principal transporter of leukotriene LCT_4, which mediates inflammatory responses (415).

3.5.2.3 Transcriptional regulation of ABCC1

The transcriptional regulation of ABCs has best been characterized for ABCB1, although a few reports do address the regulation of ABCC1. All of the human ABCs examined to date lack an appropriately positioned TATA box, while most of their rodent counterparts are TATA-dependent (392). The ABCC1 promoter has been described and shown to contain the GC elements essential for promoter activity, further, the transcription factor, Sp1, interacts with these elements (416). The ABCC1 promoter has also been shown to contain a putative AP-1 site that has been shown to interact with a complex containing c-jun and junD (417). Finally, the transcription of the human ABCC1 gene has been shown to be repressed by active p53 (418), whereas a loss in p53 expression has been associated with increased MRP1 in colorectal cancer (419).

HYPOTHESES AND OBJECTIVES

The process of follicular luteinization/ovulation in the mare has been shown to be triggered by a critical concentration of circulating LH. This process is accompanied by changes in steroid biosynthetic pathways, from estrogens to progesterone, resulting in a late-estrus decline in 17β-estradiol. The overall hypothesis of this thesis is that the change in steroid concentrations observed during the ovulatory process is a result of the programmed modulation of the expression of specific genes responsible for the activation, inactivation and bioavailability of steroids.

The general objective was to determine whether certain enzymes known to possess steroid activating and inactivating potential, as well as the proteins responsible for steroid bioavailability, were regulated by gonadotropins during the ovulatory process. The specific objectives were:

1. To clone and characterize the expression of 17βHSD1 during the gonadotropin induction of ovulation in the mare.
2. To clone and characterize the expression of OATP2B1 and STS during the gonadotropin induction of ovulation in the mare.
3. To clone and characterize the expression of 17βHSD4 during the gonadotropin induction of ovulation in the mare.
4. To clone and characterize the expression of EST and MRP1 during the gonadotropin induction of ovulation in the mare.
5. To clone and characterize the expression of an aldo-keto reductase (AKR1C23) with potential 17βHSD5 and 20αHSD activities during the gonadotropin induction of ovulation in the mare.
6. To integrate these data into a model that describes the regulation of steroid activation, inactivation and bioavailability during the ovulatory process in the mare.

SUMMARY OF ARTICLE ONE

Title: Molecular cloning of equine 17β-hydroxysteroid dehydrogenase type 1 and its downregulation during follicular luteinization in vivo. **Kristy A. Brown**, Khampoune Sayasith, Nadine Bouchard, Jacques G. Lussier and Jean Sirois. *Submitted to Journal of Molecular Endocrinology (Feb. 2006).*

Thesis author's contribution to this work: As the primary author, I was responsible for all aspects of this article. Secondary and tertiary authorship credits reflect technical guidance for electromobility shift assays as well as genomic library screening. Fourth and fifth authorships reflect co-directorship and directorship, respectively.

Summary:

- The equine 17βHSD1 cDNA was characterized by a combination of RT-PCR, 5'-RACE and genomic cloning.
- Sequence analysis revealed an open reading frame that encoded a 308-amino acid protein, 72-75% homologous to mammalian orthologues. Variability exists in the carboxy-terminus of all mammalian 17βHSD1 proteins, and the equine protein was shown to be 20 amino acids shorter than human 17βHSD1.
- The complete equine 17βHSD1 gene was isolated from an equine genomic library and sequenced. The equine genomic structure is highly similar to all other mammalian 17βHSD1 genes identified thus far, as it has 6 exons and 5 introns. Variability is present in the length of the last exon relative to other species.
- The regulation of the 17βHSD1 mRNA was examined in equine preovulatory follicles by semi-quantitative RT-PCR in samples obtained at different times after hCG. Results from Southern blot analyses revealed high levels of expression prior to hCG, with a rapid and dramatic decrease observed at 12 h post-hCG and remaining low, almost undetectable, in all subsequent samples. 17βHSD1 mRNA expression and regulation occurred selectively in the granulosa cell layer.

- Of all of the tissues examined, only the preovulatory follicle and a placental sample were shown to express the 17βHSD1 transcript.
- The hCG-dependent regulation of nuclear extract binding to the equine 17βHSD1 promoter was studied by electromobility shift assay (EMSA) using nuclear extracts from granulosa cells isolated prior to hCG treatment as well as at 30 h post-hCG. Results demonstrate a decrease in protein binding to promoter binding sites at 30 h post-hCG protein extracts versus those obtained prior to hCG. Competition assays identified NFκB, GATA and Sp1 as putative *cis*-acting elements in the activation of the equine 17βHSD1 promoter.

Work's contribution to the advancement of science:

- This work characterizes for the first time the primary structure of the equine 17βHSD1 gene, cDNA and protein.
- It demonstrates a rapid downregulation of the 17βHSD1 transcript in granulosa cells of equine preovulatory follicles visible 12 h after hCG.
- It proposes that a decrease in promoter binding by potential *cis*-acting elements such as NFκB, GATA and Sp1 is involved in the downregulation of transcript expression after hCG.
- Overall, it supports the hypothesis that the downregulation of 17βHSD1 provides an additional biochemical basis, beyond CYP19A1 downregulation, for the decrease in 17β-estradiol observed during the ovulatory process.

Molecular Cloning of Equine 17β-Hydroxysteroid Dehydrogenase Type 1 and Its Downregulation during Follicular Luteinization In Vivo

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Abbreviated title: Regulation of 17βHSD1 in preovulatory follicles

Key Words: 17βHSD1, granulosa cells, preovulatory follicles, luteinization, ovary, horse

The 17βHSD1 genomic and cDNA nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers DQ418450 and DQ418451, respectively.

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ABSTRACT

The type 1 form of 17β-Hydroxysteroid dehydrogenase (17βHSD1) was the first isoform to be identified and is capable of converting estrone to 17β-estradiol. This study was aimed at characterizing the molecular structure of the equine 17βHSD1 gene and cDNA, as well as its molecular regulation during hCG-induced follicular luteinization/ovulation in vivo. The equine 17βHSD1 gene was cloned from an equine genomic library and shown to have a conserved genomic structure composed of six exons. Its cDNA sequence was also identified and coded for a 308-amino acid protein, 72.1 to 74.5% homologous to other mammalian orthologs. RT-PCR/Southern blot analyses were performed to study the regulation of the 17βHSD1 transcript in equine preovulatory follicles isolated between 0 and 39 h after hCG treatment. Results demonstrated the presence of high 17βHSD1 mRNA expression prior to hCG treatment with a marked decrease observed 12 h after hCG ($P < 0.05$). Analyses on isolated preparations of granulosa and theca interna cells identified the granulosa cell layer as the site of 17βHSD1 transcript expression and downregulation ($P < 0.05$). A 1412-bp fragment of the equine 17βHSD1 proximal promoter was sequenced and shown to contain many putative transcription factor binding sites. Electomobility shift assays (EMSA) using a fragment of the proximal promoter (-222/-22) and nuclear extracts prepared from granulosa cells isolated prior to hCG (0 h post-hCG) revealed the presence of a major complex and results from competition assays suggest that NFκB, GATA and Sp1 cis-elements are involved. Interestingly, a notable decrease in DNA binding was observed when granulosa cell nuclear extracts isolated 30 h post-hCG were used, which paralleled the decrease in 17βHSD1 transcript after hCG treatment. Thus, this study is the first to report the gonadotropin-dependent downregulation of 17βHSD1 transcript expression in a monoovulatory species, the presence and regulation of protein/DNA interactions in the proximal region of the 17βHSD1 promoter during gonadotropin treatment, and the characterization of the primary structure of the equine 17βHSD1 cDNA and gene.

INTRODUCTION

The biosynthesis of ovarian steroid hormones requires a complex enzymatic cascade that ultimately involves the enzyme 17β-hydroxysteroid dehydrogenase (17βHSD) in the production of both androgen and estrogen secretory products. Twelve types of 17βHSDs have thus far been described, and numbered in the order in which their DNA sequences were determined. They are all members of the short-chain dehydrogenase/reductase superfamily, except for type 5, which belongs to the aldo-keto reductase superfamily (Adamski & Jakob, 2001, Baker, 2001, Luu-The, 2001, Luu-The *et al.*, 2005). These 17βHSDs catalyze the interconversion between less active 17-ketosteroids (*i.e.* low receptor affinity) and more active 17β-hydroxysteroids (*i.e.* high receptor affinity) such as estrone and 17β-estradiol, respectively (Peltoketo *et al.*, 1999, Penning, 1997). The occurrence of different substrate specificities, cofactor preference, subcellular localizations and tissue distributions allows 17βHSDs to dictate the biological potency of androgens and estrogens in mammals.

Type 1 17βHSD (17βHSD1) was the first 17βHSD to be characterized. Its cDNA was first cloned from human placenta and was shown to encode a cytosolic protein of 327 amino acids (Luu The *et al.*, 1989, Peltoketo *et al.*, 1988). It has been shown to preferentially catalyze the reduction of estrone to 17β-estradiol using NADP(H) as a cofactor in humans (Dumont *et al.*, 1992, Lin *et al.*, 1992), whereas its substrate specificity in rodents is broader as it includes androstenedione (Mustonen *et al.*, 1997, Nokelainen *et al.*, 1996). Tissue distribution analyses revealed 17βHSD1 transcript expression in the ovary, placenta, breast, endometrium, prostate, skin and adipose tissue (Dumont *et al.*, 1992, Peltoketo *et al.*, 1988).

In mammals, the preovulatory surge in LH is responsible for the process of follicular luteinization, which is accompanied by dramatic changes in follicular steroidogenesis, including the decreased biosynthesis of 17β-estradiol (Fortune, 1994, Murphy, 2000, Zeleznik, 1994). The marked decrease in expression of cytochrome P450 enzymes, like P450 aromatase (CYP19A1) and P450 17α-hydroxylase/C17-20 lyase

(CYP17A1), has been used to explain this loss in 17β-estradiol biosynthetic capacity (Fortune, 1994, Liu *et al.*, 1999, Richards, 1994). To date, no attempts have been made to elucidate the regulation of enzymes required for the activation of these estrogens, such as 17βHSD1, during the luteinization process in mono-ovulatory species. The present study uses the equine preovulatory follicle as a model to investigate the regulation of 17βHSD1 during gonadotropin-induced ovulation/luteinization. The specific objectives were to clone the equine 17βHSD1 gene and cDNA, and determine the regulation of its mRNA in preovulatory follicles following hCG treatment, as well as begin characterizing interactions at the level of the 17βHSD1 promoter.

MATERIALS AND METHODS

Materials

The QuickHyb hybridization solution and the equine genomic library were obtained from Stratagene Cloning Systems (LaJolla, CA); [α-^{32}P]dCTP was purchased from PerkinElmer Canada Inc. (Woodbridge, Ontario, Canada); the Prime-a-Gene labeling system and pGEM-T easy Vector System I were obtained from Promega Corp. (Madison, WI); the Expand High Fidelity DNA Polymerase was purchased from Roche Diagnostics (Laval, Québec, Canada); the plasmid pcDNA3.1, SuperScript II reverse transcriptase, TRIzol total RNA isolation reagent, 1-kb DNA ladder, 5'-rapid amplification of cDNA ends (RACE) system (Version 2.0), and synthetic oligonucleotides were obtained from Invitrogen Life Technologies (Burlington, Ontario, Canada); the Qiagen OneStep RT-PCR System was purchased from Qiagen Inc. (Mississauga, Ontario, Canada); Biotrans nylon membranes (0.2 μm) were obtained from ICN Pharmaceuticals, Inc. (Montréal, Québec, Canada); Bio-Rad Protein Assay and all electrophoretic reagents were obtained from Bio-Rad Laboratories (Richmond, CA); human chorionic gonadotropin (hCG) was purchased from The Butler Co. (Columbus, OH); poly (dI/dC) was obtained from Amersham Pharmacia Biotech (Baie d'Urfé, PQ, Canada). Polyclonal antibodies against p65 NFκB and GATA-4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Characterization of the equine 17βHSD1 complementary DNA (cDNA) and gene

The equine 17βHSD1 cDNA was characterized by a combination of RT-PCR, 5′-rapid amplification of cDNA ends (5′-RACE) and genomic cloning. A cDNA fragment was first isolated by RT-PCR using sense primer 1 and anti-sense primer 2 designed by sequence alignments of 17βHSD1 homologues from other species, 100 ng of RNA obtained from a preovulatory follicle isolated prior to hCG administration (0 h; see below), and the Qiagen OneStep RT-PCR System as directed by the manufacturer (Fig. 1Aa). As a second approach, the 5′-end of equine 17βHSD1 was characterized using the 5′-RACE system version 2.0 (Invitrogen Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using anti-sense primer 3 (Fig. 1Ab) and 3 μg of RNA from a preovulatory follicle isolated prior to hCG administration. The first 5′-RACE/PCR reaction was accomplished with sense abridged anchor primer 4 (Invitrogen Life Technologies) and anti-sense primer 5, whereas the second 5′-RACE/PCR reaction employed the sense abridged universal amplification primer 6 (Invitrogen Life Technologies) and anti-sense primer 7 (Fig. 1Ab). PCR reactions consisted of 35 cycles of 94 C for 30 s, 56 C for 1 min, and 72 C for 1 min. RT-PCR and 5′-RACE cDNA products were subcloned into the pGEM-T Easy plasmid vector (Promega), and sequenced by the Service de Séquençage de l'Université Laval (Québec, Canada).

Despite numerous attempts to clone the remaining 3' end of the open reading frame, they remained unsuccessful. As a second approach, an equine genomic library (Stratagene) was screened with the equine RT-PCR 17βHSD1 cDNA fragment, as previously described (Liu *et al.*, 1999). The probe was labeled with [α-^{32}P]dCTP using the Prime-a-Gene labeling system (Promega) to a final specific activity greater than 1 x 10^8 cpm/μg DNA, and hybridization was performed at 68 C with QuickHyb hybridization solution (Stratagene). Positive clones were plaque purified through secondary and tertiary screenings, and DNA sequencing was performed commercially as described above. The equine 17βHSD1 genomic sequence was used to design an oligonucleotide 3' to the coding region. This primer, as well another oligonucletide

designed from the 5' end of the cDNA (primers 8 and 9; Fig. 1b), allowed RT-PCR amplification of the complete open reading frame, thereby confirming the contiguous cDNA sequence presented herein (Fig. 1c). The complete equine genomic sequence was obtained after a number of sequencing reactions using equine 17βHSD1-specific primers. A comparison of the cDNA and resulting genomic DNA sequences was used to determine the gene structure of equine 17βHSD1 as well as the exon-intron junctions (Fig. 2).

Equine tissues

Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares, 3-10 years old and weighing approximately 375-450 kg, as previously described (Sirois & Dore, 1997). Briefly, when preovulatory follicles reached 35 mm in diameter during estrus, the ovulatory process was initiated by injection of hCG (2500 IU, iv). Ovariectomies were then performed via colpotomy using an ovariotome at 0, 12, 24, 30, 33, 36 or 39 h post-hCG (n=4-6 mares/time point). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna, as previously described (Sirois *et al.*, 1991). Ovariectomies were also performed on day 8 of the estrous cycle (day 0 = day of ovulation) to obtain corpora lutea (n = 3 mares). Testicular tissues were obtained from the Large Animal Hospital of the Faculté de médecine vétérinaire (Université de Montréal) following a routine castration, whereas other non-ovarian tissues were collected at a local slaughterhouse. All animal procedures were approved by the institutional animal use and care committee.

RNA extraction and semi-quantitative RT-PCR/ Southern analysis

Total RNA was isolated from tissues with TRIzol reagent (Invitrogen Canada Inc.), according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada). The OneStep RT-PCR System (Qiagen) was used for semi-quantitative analysis of 17βHSD1 and rpL7a mRNA levels (control gene) in equine tissues. Reactions were performed as directed by the

manufacturer, using sense (5′-ACCTTGCAGTTGG ACGTGAGAGA-3′) and anti-sense (5′-TCGCGGTACATCTGCTCGCAGT-3′) primers specific for equine 17βHSD1. Sense (5′-ACAGGACATCCAGCCCAAACG-3′) and anti-sense (5′-GCTCCTTTGTCTTCCGAGTTG-3′) primers specific for equine rpL7a were designed from a published sequence deposited in GenBank (accession no. AF508309). These reactions resulted in the production of 17βHSD1 and rpL7a DNA fragments of 503 and 516 bp, respectively. Each reaction was performed using 100 ng of total RNA, and cycling conditions were one cycle of 50 C for 30 min and 95 C for 15 min, followed by a variable number of cycles of 94 C for 1 min, 60 C for 1 min and 72 C for 1 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification, and were 26 and 18 cycles for 17βHSD1 and rpL7a, respectively. Following PCR amplification, samples were subjected to electrophoresis on 2% TAE-agarose gels, transferred to nylon membranes, and hybridized with corresponding radiolabeled 17βHSD1 and rpL7A cDNA fragments using QuikHyb hybridization solution (Stratagene). Membranes were exposed to a phosphor screen, and signals were quantified by means of a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA).

Granulosa Cell Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)

Equine granulosa cells were obtained from preovulatory follicles isolated at 0 and 30 h post-hCG and nuclear extracts were prepared as described (Liu *et al.*, 1999, Sirois *et al.*, 1993). Protein concentration in each extract was determined by the method of Bradford (Bradford, 1976). EMSAs were performed as described (Liu *et al.*, 1999, Sirois *et al.*, 1993), with minor modifications. Briefly, extracts of nuclear proteins (0.5 μg/reaction) were incubated with 40,000 cpm of end-labeled –222/-22 17βHSD1 promoter fragment and 1 μg of poly(dI/dC) (Amersham Biosciences) in a final volume of 20 μl of binding buffer containing 15 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$,5mM dithiothreitol, and 12% (v/v) glycerol. Cold oligonucleotide pairs, both wild type and mutated, were used in 50 times molar excess in order to determine the identity of bound sequence. When antibodies (Santa Cruz Biotechnology) were used in supershift EMSAs, the nuclear extract was first incubated for 1 hour on ice

with the antiserum prior to the addition of other reagents. Binding complexes were resolved by 5% acrylamide, 0.5 x TBE gel electrophoresis.

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG administration on levels of 17βHSD1 mRNA in samples of follicle wall, corpora lutea, theca interna, and granulosa cells. 17βHSD1 transcript levels were normalized with the control gene rpL7a before analysis. When ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple comparisons of individual means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC).

RESULTS

Characterization of the equine 17βHSD1 cDNA, gene, and protein

To clone the equine 17βHSD1 transcript, RT-PCR was performed on ovarian RNA using oligonucleotide primers designed by sequence alignment of 17βHSD1 homologs in other species. The resulting cDNA fragment (Fig. 1A) was sequenced and found to be highly homologous to 17βHSD1 transcripts identified thus far. The 5'-RACE reactions yielded a cDNA product corresponding to the remaining 5'-end coding regions, as well as the 5'-untranslated region (Fig. 1Ab). An equine genomic library was screened with a cDNA probe obtained by RT-PCR. The genomic sequence of equine 17βHSD1 was determined by performing several sequencing reactions (GenBank accession number DQ418450) and used to derive the 3' end of the equine 17βHSD1 cDNA, as well as the genomic structure. It was determined to have six exons (Fig. 2a), identical to what is observed for the human and mouse genes. The exon-intron junctions were also shown to be conserved (Fig 2b). After sequencing of the 17βHSD1 gene, equine-specific primer were designed and used to amplify a RT-PCR product that extended the entire length of the cDNA open reading frame, thereby confirming that all RT-PCR products were derived from the same transcript (Fig. 1Ac). The deduced 982-bp primary transcript encoded a 924-bp open reading frame (Fig. 1A, GenBank accession number DQ418451), which predicted a protein of 308 amino acids.

The predicted protein is highly conserved when compared with marmoset (AAG01115), rat (AAH86365), and mouse (CAA61770) 17βHSD1 proteins. Equine 17βHSD1 has 73.3% identity at the amino acid level and a 81.2% identity at the nucleic acid level relative to human 17βHSD1 (NP_000404, Fig. 3). Homology was lost when the carboxy-terminus of the proteins was examined and the proteins also exhibited variability in sizes; the equine 17βHSD1 is 20 amino acids shorter than human 17βHSD1, which is 16 amino acids shorter than both rodent proteins presented (Fig. 3). The marmoset protein may be incomplete in its amino-terminus.

Tissue distribution of equine 17βHSD1 mRNA

RT-PCR /Southern blot analyses were used to evaluate the expression of equine 17βHSD1 mRNA in various tissues. High levels of 17βHSD1 transcript were detected in a preovulatory follicle isolated prior to hCG and in a placenta sample; whereas the message was very low or absent in all other tissues examined (Fig. 4A). Abundance of the control gene rpL7a remained constant in all tissues studied (Fig. 4B).

Regulation of 17βHSD1 mRNA in preovulatory follicles

The regulation of equine 17βHSD1 mRNA in preovulatory follicles isolated during estrus between 0-36 h after hCG treatment and in corpora lutea on day 8 of the estrous cycle was examined by RT-PCR/Southern blot. The results clearly demonstrated a dramatic decrease in 17βHSD1 transcript expression in equine follicles during the hCG-induced ovulatory/luteinization process. Elevated levels of 17βHSD1 mRNA were observed prior to hCG (0 h) with a pronounced downregulation observed 12 h post-hCG. Levels remained low, almost undetectable, in samples isolated between 24 and 36 h post-hCG, as well as in day 8 corpus luteum (Fig. 5A). No variation was observed in levels of rpL7a transcript in follicle wall and corpora lutea preparations (Fig. 5B). When results from multiple follicles and corpora lutea were expressed as ratios of 17βHSD1 to rpL7a, a significant decrease in 17βHSD1 transcript was observed in follicles between 12 and 36 h after hCG treatment and in corpora lutea ($P < 0.05$) (Fig. 5C).

In order to determine to contributions of the different steroidogenic cell types that make up the follicle wall, granulosa and theca interna cells isolated from follicles obtained between 0 and 39 h after hCG treatment (Fig. 6). The results revealed that the granulosa cell layer was the sole contributor of 17βHSD1 transcript expression. In this cell type, a significant decrease in 17βHSD1 mRNA was observed 12-39 h after hCG ($P < 0.05$; Fig.6A). 17βHSD1 mRNA expression was low to absent in all theca interna samples examined (Fig. 6B).

Binding Activity of Nuclear Extract Proteins to the 17βHSD1 Proximal Promoter

Genomic cloning led to the characterization of a 1.4-kb fragment of 5'-flanking DNA region (Fig. 7). Use of transcription start site prediction software (www.fruitfly.org/seq_tools/promoter.html) identified a putative transcription start site 31 bp upstream of the ATG start codon (Fig. 7). Putative *cis*-acting elements located within 300 bp upstream of the trancription start site were identified with the TRANSFAC database (http://motif.genome.jp/) and included a C/EBP, an AP-2, two GATA, a NFκB, two Sp1, and a CRE element. To determine whether hCG affected the binding of putative transcriptional regulators to the –222/-22 fragment (+1 representing the transcriptional start site) of the 17βHSD1 promoter, nuclear extracts from granulosa cells isolated from preovulatory follicles obtained at 0 and 30 h post hCG were used in EMSA. Results demonstrated that a major protein/DNA complex was formed with nuclear extracts at 0 h post-hCG (Fig. 8A, lane 2). The use of 30 h post-hCG nuclear extracts did not affect the migration of the band, but resulted in a net decrease in protein binding (Fig. 8A, lane 3). To characterize the specificity of protein/DNA interactions, EMSAs were performed using molar excess of unlabeled oligonucleotides containing various putative transcription factor binding sites present within the –222/-22 fragment (Fig. 8A, lanes 4-7). Results showed that competitors containing Sp1-binding sites reduced protein/promoter complex formation (Fig. 8A, lane 5), whereas no effect was observed when competitors containing the first GATA or C/EBP and AP-2 elements were used (Fig. 8A, lanes 4 and 6). Interestingly, when an oligonucleotide containing both the NFκB and the second GATA binding sites, the signal was completely abolished (Fig. 8A, lane 7).

To further confirm the specificity of the binding to the putative binding sites, individual transcription factor-specific regions were mutated and again, used as competitors. The previously observed NFκB/GATA competition was greatly reduced when either sites were mutated, indicating a putative collaborative binding instance (Fig. 8B, lanes 4 and 5). To investigate the potential presence of the NFκB protein in the binding complex, supershift EMSAs were performed using antibodies specific to the p50 and p65 subunits of NFκB, as well as an antibody specific for GATA-4. The intensity of the major band decreased when the anti-p65 antibody was used (Fig. 8B, lanes 7 versus 2, band [a]). No change in intensity was discernable when the anti-50 or anti-GATA4 antibodies were used (Fig. 8B, lanes 6 and 8 versus lane 2, band [a])

DISCUSSION

This is the first study to identify hCG as a negative regulator of 17βHSD1 mRNA expression during follicular luteinization in granulosa cells of a monoovulatory species. Follicular luteinization/ovulation has previously been associated with dramatic changes in steroidogenic enzyme expression. Enzymes responsible for androgen and estrogen biosynthesis have been shown to be down-regulated, whereas the expression of those responsible for enhanced progesterone synthesis are up-regulated (Fortune, 1994, Richards, 1994, Ronen-Fuhrmann *et al.*, 1998, Sandhoff *et al.*, 1998). This study thereby provides an additional molecular basis for the decrease in 17β-estradiol production. Previous investigations of 17βHSD1 in the ovary have included its detection by Northern blot in the rat (Ghersevich *et al.*, 1994), RT-PCR in humans (Nelson *et al.*, 2001), *in situ* hybridization and Southern blotting in mice (Pelletier *et al.*, 2004, Sha *et al.*, 1997), as well as in human corpora lutea by immunohistochemistry (Vaskivuo *et al.*, 2002). It has been detected in granulosa cells of developing follicles in immature and mature rats (Akinola *et al.*, 1997), and its regulation during the ovulatory process has been examined in rodents, however, it was limited by the use of immature hypophysectomized rats (Ghersevich *et al.*, 1994).

This study characterizes the hCG-dependent downregulation of 17βHSD1 transcript expression in a series of preovulatory follicles from a monoovulatory species. Previous reports using immature hypophysectomized rats have demonstrated that recombinant FSH had a stimulatory effect on 17βHSD1 transcript and protein expression, and that further treatment with hCG resulted in a downregulation of 17βHSD1 mRNA, visible after one day of treatment (Ghersevich *et al.*, 1994). The present study shows that this downregulation is already visible 12 h after hCG treatment in the equine preovulatory follicle. Therefore, the results presented herein are consistent with the previous report and further establishes the rapidity of 17βHSD1 transcript downregulation.

The molecular control of the 17βHSD1 gene in granulosa cells has remained largely uncharacterized. It has been shown, however, that AP-2 can interfere with Sp1 binding, and that GATA-3 can prevent transcription of constructs containing the17βHSD1 proximal promoter in choriocarcinoma cells (Piao *et al.*, 1997). Further, retinoic acids and activin-A have been demonstrated to induce 17βHSD1 mRNA in human JEG-3 cells and cultured rat granulosa cells, respectively (Ghersevich *et al.*, 2000, Piao *et al.*, 1997, Zhu *et al.*, 2002). The present study demonstrates for the first time the gonadotropin-dependent decrease in nuclear extract binding to the 17βHSD1 proximal promoter and identifies NFκB as a putative *cis*-acting element in 17βHSD1 transcriptional regulation. Interestingly, NFκB has also been demonstrated to activate transcription of the CYP19A1 gene (Fan *et al.*, 2005). CYP19A1 encodes the cytochrome P450 enzyme aromatase, whose role in estrogen biosynthesis has largely been characterized (Simpson *et al.*, 2005). In that study, it was shown that activation of NFκB resulted in an up-regulation of CYP19A1's promoter II activity and that this may be due to the direct interaction of the p65 subunit of NFκB with the CYP19A1 promoter as identified by chromatin immunoprecipitation (Fan *et al.*, 2005). Also, peroxisome proliferator-activated receptor-γ and retinoid X receptor were speculated of down-regulating aromatase expression, when stimulated simultaneously, by disrupting the p65/promoter interaction (Fan *et al.*, 2005, Mu *et al.*, 2001). Considering that the

regulation of CYP19A1, who's transcript levels are high in equine preovulatory follicles at 0 h and drop dramatically after hCG (Boerboom *et al.*, 1999), is identical to the regulation observed for 17βHSD1, further studies will be needed to unravel whether similar or distinct transcriptional mechanisms are involved.

The 17βHSD1 cDNA has previously been cloned in various species, including human (Peltoketo *et al.*, 1988), rat (Ghersevich *et al.*, 1994), and mouse (Nokelainen *et al.*, 1996). This study presents the equine cDNA and gene structure. Its genomic structure is consistent with that of other species, as they all have been demonstrated to have six exons and five introns. A pseudogene present upstream of the 17βHSD1 gene has been identified in humans, orangutan, chimpanzees, and gibbons, however, it is not conserved in all species (Keller *et al.*, 2005). It will be interesting to determine if this is also the case in horses. Also, the sizes of introns 3 and 4 vary slightly between species and the 3'-end of exon 6 has been shown to exhibit considerable variability from one species to the next (Keller *et al.*, 2005), as is the case in the mare. This variability is discernable when examining the amino acid sequence of the carboxy-terminus and the length of the 17βHSD1 proteins.

This study also investigates the expression of 17βHSD1 mRNA in equine tissues and establishes high levels of 17βHSD1 transcript in preovulatory follicles prior to hCG. While high levels of equine 17βHSD1 mRNA were also observed in a placenta sample, which is consistent with the high 17βHSD1 mRNA and protein observed in placentae of humans and non-human primates (Castagnetta *et al.*, 1997, Lin *et al.*, 1992, Schwabe *et al.*, 2001), this is not the case for rodents, however (Akinola *et al.*, 1997). It has also been detected by *in situ* hybridization in mice in granulosa cells of growing follicles, the intermediate lobe melanotrophs of the pituitary, in epithelial cells of the prostate and in germ cells of the testis (Pelletier *et al.*, 2004). It is further found the epithelium of normal and cancerous breast tissue of women by *in situ* hybridization (Miettinen *et al.*, 1999, Oduwole *et al.*, 2004, Soderqvist *et al.*, 1998).

In summary, this study is the first to characterize the primary structure of the equine 17βHSD1 cDNA and gene, to demonstrate the regulation of this gene during follicular luteinization in a monoovulatory species, to identify the preovulatory gonadotropin signal as a negative regulator of equine 17βHSD1 mRNA expression, and to propose NFκB and Sp1 as putative *cis*-acting elements in 17βHSD1 promoter activation. Considering the estrogen activating activity of 17βHSD1, its gonadotropin-dependent downregulation provides an additional molecular basis for the decrease in 17β-estradiol biosynthetic capacity observed during the process of ovulation/luteinization.

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LEGENDS FOR FIGURES

FIG.1. Cloning strategy for equine 17βHSD1. A, The open reading frame (ORF) of the equine 17βHSD1 cDNA is depicted as an open box whereas the 5′- and 3′-untranslated regions (UTR) are shown as lines; the size in base pairs of each element is given in parenthesis. Equine 17βHSD1 was characterized by a combination of RT-PCR (a), 5′-RACE (b) and a second round of RT-PCR with an antisense primer obtained through genomic cloning (c), as described in *Materials and Methods*; arrows and numbers show the relative position, orientation and identity of oligonucleotides used in each cloning procedure. B, List of oligonucleotides used for equine 17βHSD1 cloning. The abridged anchor primer (4) and abridged universal amplification primer (6) are components of the 5′-RACE system (Invitrogen Life Technologies). C, Sequence of 17βHSD1 cDNA. 5'- and 3'-UTRs are shown in *lowercase* letters, whereas the ORF is presented in *uppercase* letters with the start (ATG) and stop (TGA) codons in *bold*. The complete nucleotide sequence of the equine 17βHSD1 cDNA was submitted to GenBank (accession number DQ418451).

FIG. 2. Equine 17βHSD1 gene structure. A, Schematic representation of the equine 17βHSD1 gene structure. Exons are shown as boxes and introns are presented as lines. All elements are drawn to scale. Roman numerals indicate the exon number, whereas numbers in parentheses show the number of nucleotides in the exon. B, Exon/intron boundaries of the equine 17βHSD1 gene. Exonic sequences at each splice junction are presented in *uppercase* letters whereas intronic sequences are shown in *lowercase* letters. Numbers in parentheses represent the exact size of the intron. The complete nucleotide sequence of the equine 17βHSD1 gene was submitted to GenBank (accession number DQ418450).

FIG. 3. Equine 17βHSD1 predicted amino acid sequence. A, The amino acid sequence of equine (equ) 17βHSD1 is aligned with the human (hum), macaque (maq), rat and mouse (mou) homologues. Identical residues are marked with a printed period, hyphens indicate gaps in protein sequences created to optimize alignment, and numbers on the

right refer to the last amino acid on that line. The percentage presented at the end of each sequence represents that sequence's homology to the equine protein.

FIG. 4. Expression of 17βHSD transcript in equine tissues. RNA extracts were prepared from various equine tissues, and samples (100 ng) were analyzed for 17βHSD1 and rpL7a (control gene) by semi-quantitative RT-PCR/Southern blotting, as described in *Materials and Methods*. A, expression of 17βHSD1 mRNA in equine tissues. B, expression of rpL7a mRNA in equine tissues. The number of PCR cycles for each gene was within the linear range of amplification, and they represented 26 and 18 cycles for 17βHSD1 and rpL7a, respectively. The follicle wall extract was prepared from a preovulatory follicle obtained prior to (i.e. 0 h) hCG treatment. Numbers on the right indicate the size of the PCR fragment.

FIG. 5. Downregulation of 17βHSD1 mRNA by hCG in equine preovulatory follicles. RNA extracts were prepared from the wall of preovulatory follicles isolated between 0 and 36 h after hCG, and from corpora lutea (CL) isolated on day 8 of the estrous cycle. RNA samples (100 ng) were analyzed for 17βHSD1 and rpL7a by semiquantitative RT-PCR/Southern blotting, as described in *Materials and Methods*. A, Regulation of 17βHSD1 mRNA in equine follicles (one representative follicle per time point). B, Constitutive expression of rpL7a mRNA in the same follicles. Numbers on the right depict the size of the PCR fragment. C, Relative changes in 17βHSD1 mRNA in equine follicles after hCG treatment. The 17βHSD1 signal was normalized with the control gene rpL7a, and results are presented as a ratio of 17βHSD1 to rpL7a (n = 5-6 distinct follicles [i.e. animals] per time point, and n = 3 corpora lutea). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P < 0.05$).

FIG. 6. Cell type-dependent expression and regulation of 17βHSD1 mRNA in equine preovulatory follicles. RNA extracts were prepared from granulosa cells (A) and theca interna (B) isolated from equine preovulatory follicles between 0-39 h post-hCG, and samples (100 ng) were analyzed for 17βHSD1 and rpL7a by semi-quantitative RT-

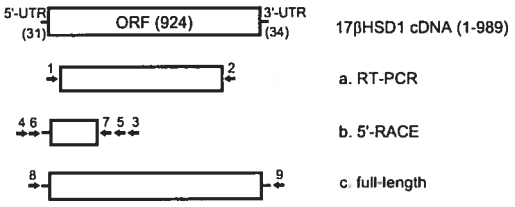
PCR/Southern blotting, as described in *Materials and Methods*. *Autoradiograms* show representative results of 17βHSD1 and rpL7a mRNA levels (one sample per time point). The 17βHSD1 signal was normalized with rpL7a, and results are presented as a ratio of 17βHSD1 to rpL7a (mean ± SEM; n = 4 samples [i.e. mares] per time point). *Bars* marked with an asterisk are significantly different from 0 h post-hCG ($P < 0.05$).

FIG. 7. Isolation and characterization of the equine 17βHSD1 promoter. Numbering is relative to the putative transcription start site (+1) and the ATG translation start codon is in *bold*. Putative *cis*-acting elements located within the 300 bp upstream of the start site are presented above their respective sequences. The probe used in binding assays is *double-underlined*. The nucleotide sequence of the 1.4 kb promoter fragment is part of the genomic sequence deposited to GenBank (accession number DQ418450).

FIG. 8. Gonadotropin-dependent regulation of DNA binding activities in equine granulosa cell nuclear extacts. A, nuclear protein extracts were prepared from granulosa cells of follicles isolated before (0 h) and after (30 h) hCG treatment (lanes 2 and 3), as described in *Materials and* Methods. Extracts were incubated with ^{32}P-labeled 17βHSD1 promoter fragment -222/-22, and protein/DNA interactions were studied by EMSAs. For reference purposes, the major protein/DNA complex is designated as *band a*. Competitive EMSAs were performed in the presence of various unlabeled competitor DNA (lanes 4-7). B, competitive EMSAs and supershift assays were performed in the presence of nuclear extracts prepared from granulosa cells from follicles isolated prior to hCG-treatment, ^{32}P-labeled oligonucleotide NFκB/GATA and various unlabeled competitor wild-type and mutated DNA, as well as with NFκB and GATA-specific antibodies. C, sequences of competitor wild-type and mutated (Δ) oligonucleotides.

FIGURE 1

A



B

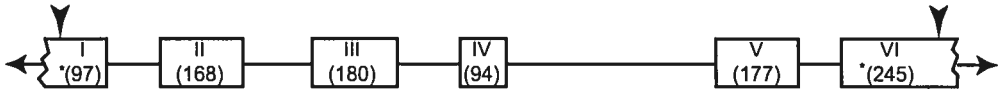
1. 5'-CAGATCCATCCCAGAGCTTCAAAGT-3'
2. 5'-GCTCGGTGGTGAAGTAGCGCAG-3'
3. 5'-ATCAAGCCTCCCATGCT-3'
4. 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'
5. 5'-CCCGCATTACACACCAGCACGT-3'
6. 5'-GGCCACGCGTCGACTAGTAC-3'
7. 5'-GCGTCTCTCACGTCCAACTGCA-3'
8. 5'-ACTCCCAGCCTCTCCTGGACCGC-3'
9. 5'-TGGGCGGCAGGACGAGCGCCAA-3'

C

```
1    gcactcccag cctctcctgg accgccgcct cATGGACCGC ACCATCGTGC TCATCACCGG CTGCTCTTCC GGCATCGGCC TGCACCTGGC
91   CCTGCGTCTG GCATCTGACC CATCCCGGAG TTTCAAAGTG TATGCCACGC TGCGGGACCT GACAGCGCAG GGCCCACTGT GGGAGATGGC
181  CCGGGCCCGA GGGTGCCCTC CCGGCTCCCT GGAGACCTTG CAGTTGGACG TGAGAGACGC AGATTCCGTG GCCGCTGCCC GGGCACGTGT
271  GACCGAGGGC CGCGTGGACG TGCTGGTGTG TAATGCGGGC CGGGGCCTGC TCGGGCCGCT CGAGCTGCAC CCGGCGGGCG CCGTGGCCTC
361  CGTGCTGGAC GCGAACGTGG CCGGGACGGT GCGGACGATT CAGGCCTTCC TGCCGGACAT GAAGCGCCGC CGCTCGGGAC GCATATTGGT
451  GACCGGGAGC ATGGGAGGCT TGATGGGGCT GCCCTTCAAT GCCATTTACT GCGCCAGCAA GTTCGCGATC GAAGGTTTAT GTGAGAGTCT
541  GGCGGTTCTG CTGCAGCCCT TTGGGGTCCA CGTGAGCCTC ATTGAGTGCG GCCCGGTGCA CACCGCCTTC CAGGAAAAGC TGGAGGGCGG
631  CCCGGGCGGG GCGCTGGACG GCGCGGACGC CGACACCCGC GACCTCTTCT CCCGCTACCT GCGCCACTGC GAGCAGATGT ACCGCGAGGT
721  GGCACAGGAC CCGGAGGCGG TGACGGAGGT CTTCCTCACC GCGCTGCGCG CCCCGCGGCC GGCCCTGCGC TACTTCAGCA CCGAGCGCTT
811  CCTGCCCCTG GTCCAGCTGC GCCTCTCCGA CCCCAGCGGC CGCAGCTACG TCGCCGCCAT GCACAGCGCG GTGTTCGCCG ACGAGCCCGC
901  CCAGGACCCC GACGGCGCCC GGGCCGAGGC CAGAGACGGA GAAGCCGGGG AGCCCTGAgc ttggcgctcg tcctgccgcc ca
```

FIGURE 2

A.



B.

Exon		Intron		Exon
			G[001]CACTC...	1
1	...TCAAAG[128]gtacgt...	1 (108 bp)	...ctccagT[129]GTATG...	2
2	...TGCTGG[296]gtgagt...	2 (143 bp)	...ctgtagT[297]GTGTA...	3
3	...TGATGG[476]gtgagt...	3 (131 bp)	...tcgaagG[478]GCTGC...	4
4	...GGTCCA[570]gtgagt...	4 (443 bp)	...ccgcagC[571]GTGAG...	5
5	...GACGGA[747]agtggg...	5 (89 bp)	...gccgcaG[748]GTCTT...	6
6	...AATAAT			

FIGURE 3

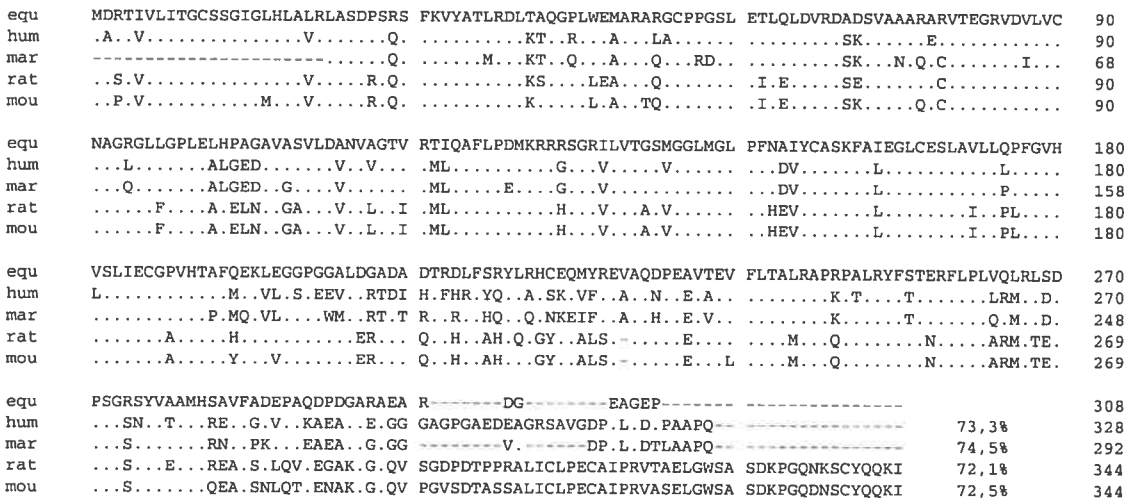

equ MDRTIVLITGCSSGIGLHLALRLASDPSRS FKVYATLRDLTAQGPLWEMARARGCPPGSL ETLQLDVRDADSVAAARARVTEGRVDVLVC 90
hum .A..V.....V.....Q. .....KT..R...A...LA..... .....SK.....E..... 90
mar ----------.....Q. .....M...KT..Q...A...Q...RD.. .....SK...N.Q.C.....I... 68
rat ..S.V.....V.....R.Q. .....KS....LEA...Q..... .I.E.....SE.....C..... 90
mou ..P.V.....M...V.....R.Q. .....K.....L.A..TQ..... .I.E.....SK.....Q.C..... 90
equ NAGRGLLGPLELHPAGAVASVLDANVAGTV RTIQAFLPDMKRRRSGRILVTGSMGGLMGL PFNAIYCASKFAIEGLCESLAVLLQPFGVH 180
hum ...L.....ALGED.....V..V... .ML.....G...V.....V..... ...DV.....L.....L..... 180
mar ...Q.....ALGED..G....V..... .ML.....E....G...V..... ...DV.....L.....P..... 158
rat .....F....A.ELN..GA...V..L..I .ML.....H...V...A.V..... ..HEV.....L.....I..PL.... 180
mou .....F....A.ELN..GA...V..L..I .ML.....H...V...A.V..... ..HEV.....L.....I..PL.... 180
equ VSLIECGPVHTAFQEKLEGGPGGALDGADA DTRDLFSRYLRHCEQMYREVAQDPEAVTEV FLTALRAPRPALRYFSTERFLPLVQLRLSD 270
hum L.....M..VL.S.EEV..RTDI H.FHR.YQ..A.SK.VF..A..N..E.A.. .....K.T....T.....LRM..D. 270
mar .....P.MQ.VL....WM..RT.T R..R..HQ..Q.NKEIF..A..H..E.V.. .....K.....T.....Q.M..D. 248
rat .....A.....H.....ER... Q..H..AH.Q.GY..ALS.-.....E.... ....M...Q.....N.....ARM.TE. 269
mou .....A.....Y...V.....ER... Q..H..AH...GY..ALS.-.....E...L ....M...Q.....N.....ARM.TE. 269
equ PSGRSYVAAMHSAVFADEPAQDPDGARAEA R-------DG--------EAGEP------- ---------- 308
hum ...SN..T...RE..G.V..KAEA..E.GG GAGPGAEDEAGRSAVGDP.L.D.PAAPQ-- ---------- 73,3% 328
mar ...S.....RN..PK...EAEA..G.GG --------V.------DP.L.DTLAAPQ-- ---------- 74,5% 292
rat ...S...E...REA.S.LQV.EGAK.G.QV SGDPDTPPRALICLPECAIPRVTAELGWSA SDKPGQNKSCYQQKI 72,1% 344
mou ...S.....QEA.SNLQT.ENAK.G.QV PGVSDTASSALICLPECAIPRVASELGWSA SDKPGQDNSCYQQKI 72,5% 344

FIGURE 4

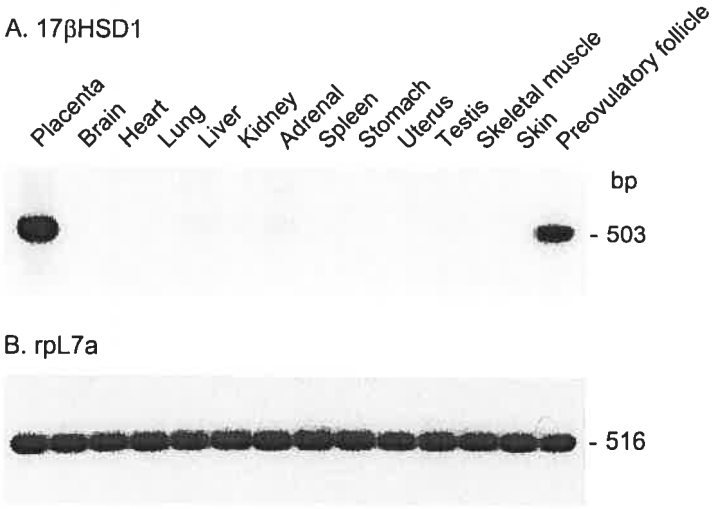

A. 17βHSD1
Placenta
Brain
Heart
Lung
Liver
Kidney
Adrenal
Spleen
Stomach
Uterus
Testis
Skeletal muscle
Skin
Preovulatory follicle
bp
- 503
B. rpL7a
- 516

FIGURE 5

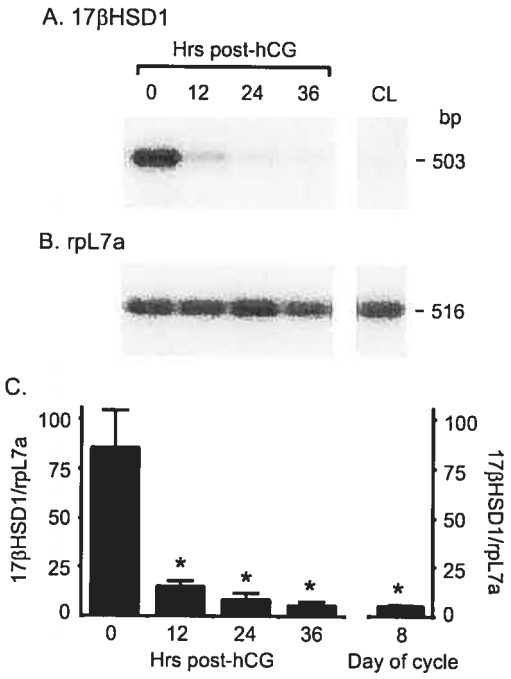

A. 17βHSD1
Hrs post-hCG
0
12
24
36
CL
bp
503
B. rpL7a
516
C.
17βHSD1/rpL7a
100
75
50
25
0
*
0
12
24
36
8
Hrs post-hCG
Day of cycle

FIGURE 6

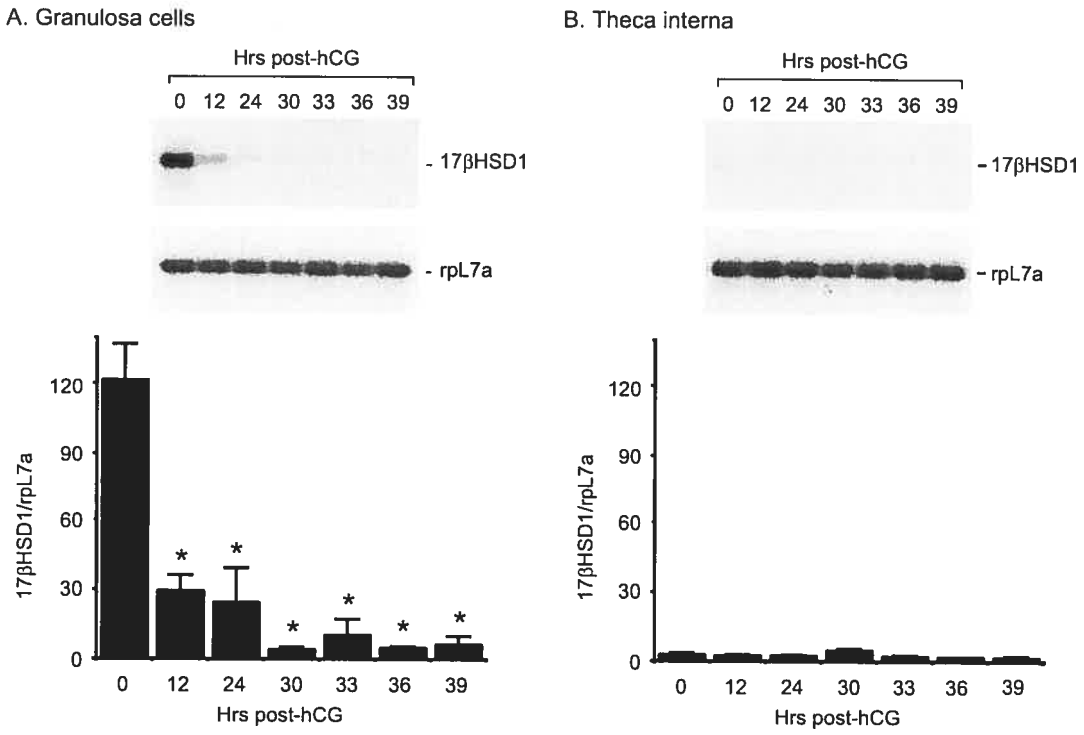

A. Granulosa cells
Hrs post-hCG
0 12 24 30 33 36 39
- 17βHSD1
- rpL7a
17βHSD1/rpL7a
120
90
60
30
0
0 12 24 30 33 36 39
Hrs post-hCG
B. Theca interna
Hrs post-hCG
0 12 24 30 33 36 39
- 17βHSD1
- rpL7a
17βHSD1/rpL7a
120
90
60
30
0
0 12 24 30 33 36 39
Hrs post-hCG

FIGURE 7

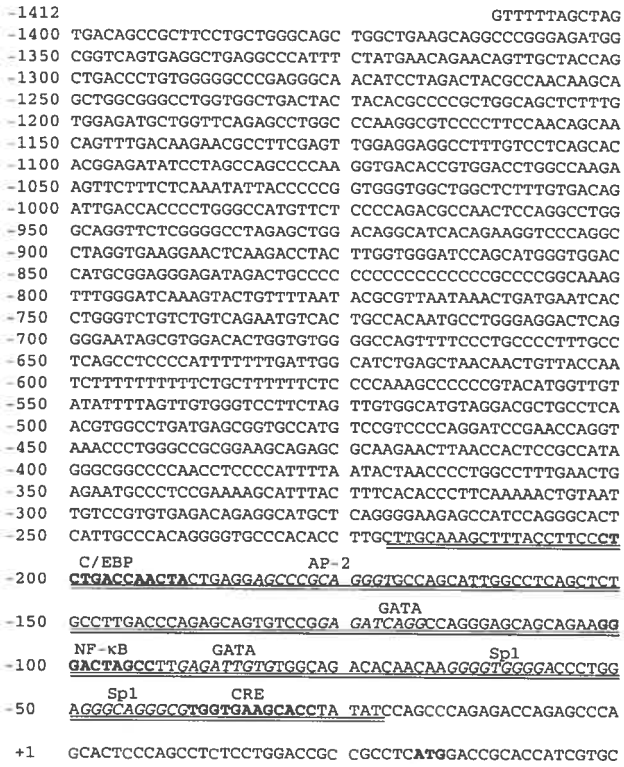
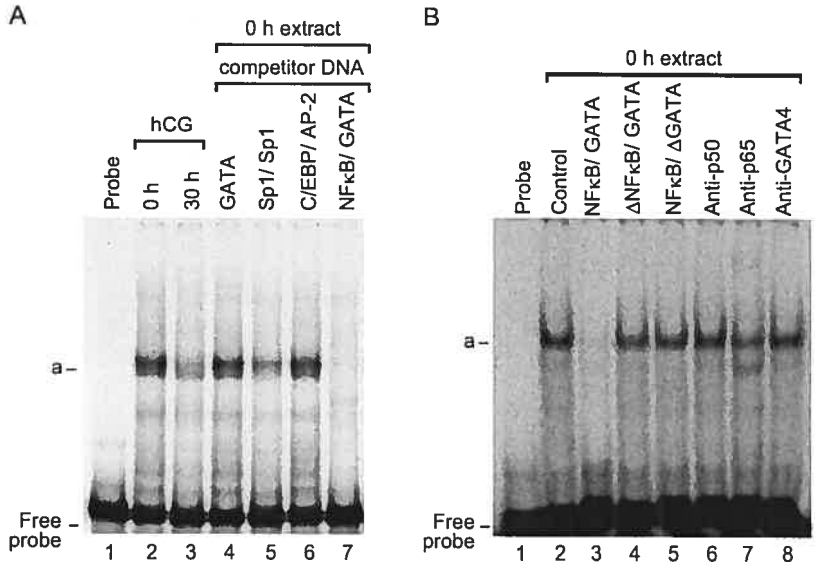

-1412 GTTTTTAGCTAG
-1400 TGACAGCCGCTTCCTGCTGGGCAGC TGGCTGAAGCAGGCCCGGGAGATGG
-1350 CGGTCAGTGAGGCTGAGGCCCATTT CTATGAACAGAACAGTTGCTACCAG
-1300 CTGACCCTGTGGGGGCCCGAGGGCA ACATCCTAGACTACGCCAACAAGCA
-1250 GCTGGCGGGCCTGGTGGCTGACTAC TACACGCCCCGCTGGCAGCTCTTTG
-1200 TGGAGATGCTGGTTCAGAGCCTGGC CCAAGGCGTCCCCTTCCAACAGCAA
-1150 CAGTTTGACAAGAACGCCTTCGAGT TGGAGGAGGCCTTTGTCCTCAGCAC
-1100 ACGGAGATATCCTAGCCAGCCCCAA GGTGACACCGTGGACCTGGCCAAGA
-1050 AGTTCTTTCTCAAATATTACCCCCG GTGGGTGGCTGGCTCTTTGTGACAG
-1000 ATTGACCACCCCTGGGCCATGTTCT CCCCAGACGCCAACTCCAGGCCTGG
-950 GCAGGTTCTCGGGGCCTAGAGCTGG ACAGGCATCACAGAAGGTCCCAGGC
-900 CTAGGTGAAGGAACTCAAGACCTAC TTGGTGGGATCCAGCATGGGTGGAC
-850 CATGCGGAGGGAGATAGACTGCCCC CCCCCCCCCCCCCGCCCCGGCAAAG
-800 TTTGGGATCAAAGTACTGTTTTAAT ACGCGTTAATAAACTGATGAATCAC
-750 CTGGGTCTGTCTGTCAGAATGTCAC TGCCACAATGCCTGGGAGGACTCAG
-700 GGGAATAGCGTGGACACTGGTGTGG GGCCAGTTTTCCCTGCCCCTTTGCC
-650 TCAGCCTCCCCATTTTTTTGATTGG CATCTGAGCTAACAACTGTTACCAA
-600 TCTTTTTTTTTTCTGCTTTTTTCTC CCCAAAGCCCCCCGTACATGGTTGT
-550 ATATTTTAGTTGTGGGTCCTTCTAG TTGTGGCATGTAGGACGCTGCCTCA
-500 ACGTGGCCTGATGAGCGGTGCCATG TCCGTCCCCAGGATCCGAACCAGGT
-450 AAACCCTGGGCCGCGGAAGCAGAGC GCAAGAACTTAACCACTCCGCCATA
-400 GGGCGGCCCCAACCTCCCCATTTTA ATACTAACCCCTGGCCTTTGAACTG
-350 AGAATGCCCTCCGAAAAGCATTTAC TTTCACACCCTTCAAAAACTGTAAT
-300 TGTCCGTGTGAGACAGAGGCATGCT CAGGGGAAGAGCCATCCAGGGCACT
-250 CATTGCCCACAGGGGTGCCCACACC TTGCTTGCAAAGCTTTACCTTCCCT
C/EBP
AP-2
-200 CTGACCAACTACTGAGGAGCCCGCA GGGTGCCAGCATTGGCCTCAGCTCT
GATA
-150 GCCTTGACCCAGAGCAGTGTCCGGA GATCAGGCCAGGGAGCAGCAGAAGG
NF-κB
GATA
Sp1
-100 GACTAGCCTTGAGATTGTGTGGCAG ACACAACAAGGGGTGGGGACCCTGG
Sp1
CRE
-50 AGGGCAGGGCGTGGTGAAGCACCTA TATCCAGCCCAGAGACCAGAGCCCA
+1 GCACTCCCAGCCTCTCCTGGACCGC CGCCTCATGGACCGCACCATCGTGC

FIGURE 8



C

C/EBP/AP-2	5'-CCTTCC**CTCTGACCAACTA**CTGAGG*AGCCCGCAGGGT*GC-3'
Sp1/Sp1	5'-CAA*GGGGTGGGGA*CCCTGGA*GGGCAGGGCGT*GGTG-3'
GATA	5'-GTGTCCG*GAGATCAGG*CCAGGG-3'
NFκB/GATA	5'-GCAGAA**GGGACTAGCC**TT*GAGATTGTGT*GGCAG-3'
ΔNFκB/wtGATA	5'-GCAGAA**GAAGTGCAAC**TT*GAGATTGTGT*GGCAG-3'
wtNFκB/ΔGATA	5'-GCAGAA**GGGACTAGCC**TT*AATGCCAGGT*GGCAG-3'

SUMMARY OF ARTICLE TWO

Title: Down-regulation of messenger ribonucleic acid encoding an importer of sulfoconjugated steroids during human chorionic gonadotropin-induced follicular luteinization in vivo. **Kristy A. Brown**, Nadine Bouchard, Jacques G. Lussier and Jean Sirois. *Submitted to Journal of Steroid Biochemistry & Molecular Biology (April 2006).*

Thesis author's contribution to this work: As the primary author, I was responsible for all aspects of this article. Secondary authorship credits reflect technical contributions to STS cDNA cloning. Tertiary and quaternary authorships reflect co-directorship and directorship, respectively.

Summary:

- The equine SLCO2B1 cDNA was characterized by a combination of RT-PCR, 5'- and 3'-RACE reactions on RNA samples from preovulatory follicles isolated prior to hCG treatment. A 686-bp fragment of the equine STS cDNA was obtained by performing a 5'-RACE reaction using antisense oligonucleotides obtained from a previously cloned equine STS fragment.
- Sequence analysis of the SLCO2B1 revealed an open reading frame that encoded a 709-amino acid OATP2B1 protein, 86% homologous to the bovine orthologue. All putative OATP domains were conserved in the equine protein, including the OATP superfamily signature motif and the transmembrane domains.
- The regulation of the equine SLCO2B1 and STS transcripts was examined in equine preovulatory follicles by semi-quantitative RT-PCR in samples obtained at different times after hCG. Results from Southern blot analyses revealed high levels of SLCO2B1 and STS mRNA expression prior to hCG. A rapid and dramatic decrease in SLCO2B1 levels was observed at 12 h post-hCG in isolated granulosa cell preparations where it remained low, almost undetectable, in all subsequent samples. This decrease in SLCO2B1 mRNA was more gradual in the theca interna, becoming

significant only at 30 h post-hCG. A significant decrease in STS transcript expression was observed in granulosa cells from 24 to 39 h post-hCG.

Work's contribution to the advancement of science:

- This work characterizes for the first time the primary structure of the equine SLCO2B1 cDNA and protein.
- It demonstrates a rapid downregulation of the SLCO2B1 transcript in granulosa cells of equine preovulatory follicles visible 12 h after hCG, as well as the gradual decrease in SLCO2B1 mRNA in theca interna.
- It demonstrates the significant downregulation of STS transcript expression from 24 to 39 h post-hCG, selectively occurring in granulosa cells.
- Overall, it supports the hypothesis that an alternate pathway of ovarian estrogen biosynthesis is present during which the importation of sulfoconjugated steroids by OATP2B1 and their hydrolysis to free steroids by STS is involved. Its downregulation during the ovulatory process may provide a further additional biochemical mechanism for the decrease in 17β-estradiol observed during this period.

Down-Regulation of Messenger Ribonucleic Acid encoding an Importer of Sulfoconjugated Steroids during Human Chorionic Gonadotropin-Induced Follicular Luteinization In Vivo

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ABSTRACT

Members of the organic anion transporting polypeptide (SLCO/OATP) superfamily are capable of importing anionic compounds across the lipid bilayer in a sodium-independent manner. Member 2B1 has been shown to transport few substrates, two of which are dihydroepiandrosterone-3-sulfate (DHEA-S) and estrone-3-sulfate. Steroid sulfatase (STS) catalyses the hydrolysis of these steroids into their unconjugated counterparts. The objective of this study was to investigate the regulation of SLCO2B1 and STS mRNAs during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The equine SLCO2B1 cDNA was cloned and shown to encode a 709-amino acid protein (OATP2B1) that is highly conserved when compared to mammalian orthologs. RT-PCR/Southern blot analyses were performed to study the regulation of SLCO2B1 and STS transcripts in equine preovulatory follicles isolated between 0 and 39 h after hCG treatment. Results showed high levels of SLCO2B1 mRNA expression before hCG, with a marked decrease observed in follicles obtained 24-39 h post-hCG ($P < 0.05$). Analyses of isolated granulosa and theca interna cells identified high mRNA expression in both cell types prior to hCG treatment, with granulosa cells showing a more rapid SLCO2B1 mRNA down-regulation. No significant change in STS mRNA was observed in intact follicle walls. However, when both cell types were isolated, a significant decrease in STS mRNA was observed in granulosa cells 24-39 h post-hCG. Collectively, these results demonstrate that the hCG-dependent induction of follicular luteinization is accompanied by the down-regulation of SLCO2B1 and STS transcripts. Considering that OATP2B1 can import sulfoconjugated DHEA and estrogens, and that STS can remove the sulfonate moiety from these steroids, their down-regulation in luteinizing preovulatory follicles may provide an additional biochemical basis for the decrease in ovarian 17β-estradiol biosynthesis after the LH surge.

INTRODUCTION

Organic anion transporting polypeptides (OATPs) are part of a growing superfamily of sodium-independent transporters termed the solute carrier family (SLCO/OATP) (Hagenbuch & Meier, 2003). Based on hydropathy plots, the SLCO genes encode 12-transmembrane domain proteins, called OATPs and have been shown to collectively import a wide variety of compounds including bile salts, steroid conjugates, thyroid hormones, and xenobiotics, just to name a few (Hagenbuch & Meier, 2003). In addition to endogenous and exogenous amphipathic compounds, OATPs can mediate the transport of numerous inhibitors and antagonists (Cvetkovic *et al.*, 1999, Eckhardt *et al.*, 1996, Gao *et al.*, 2000, Hsiang *et al.*, 1999, Ishizuka *et al.*, 1998, Pang *et al.*, 1998, Reichel *et al.*, 1999). The SLCO2B1 gene encodes the 709-amino acid protein OATP2B1, previously named OATP-B (Hagenbuch & Meier, 2004). This transporter has been shown to import a limited number of substrates including dihydroepiandrosterone-3-sulfate (DHEA-S) and estrone-3-sulfate (E1-S), but unlike most other OATPs it will not transport bile salts, prostaglandins or glucoronidated steroids. Previous reports characterizing SLCO/OATP2B1 have primarily focused on its role as a pH-sensitive transporter in the small intestine, and its role in the clearance of organic anions by the human liver and the uptake of sulfoconjugated steroids by the placenta and mammary gland (Kullak-Ublick *et al.*, 2001, Nozawa *et al.*, 2004, Pizzagalli *et al.*, 2003, St-Pierre *et al.*, 2002, Ugele *et al.*, 2003).

The sulfoconjugation of steroids occurs in a wide variety of tissues including male and female reproductive tissues, liver, kidney, brain and adrenal cortex (Strott, 1996). DHEA sulfotransferase (SULT2A1) is highly expressed in the adrenal gland and catalyzes the sulfonation of DHEA to DHEA-S (Rainey *et al.*, 2002). DHEA-S has been shown to be quantitatively the most abundant circulating steroid hormone in humans (Nieschlag *et al.*, 1973, Seely *et al.*, 2005), while E1-S is the major circulating form of plasma estrogen, both steroids having relatively longer half-lives than their unconjugated counterparts (Noel C. T. *et al.*, 1981, Pasqualini *et al.*, 1986, Pasqualini *et al.*, 1989). Sulfoconjugation of steroids changes them from being hydrophobic to hydrophilic molecules, thereby preventing them from diffusing freely across the lipid bilayer and

necessitating a transport system like OATPs. In addition, estrogen sulfoconjugates can no longer bind to the estrogen receptor, rendering them biologically inactive. Steroid sulfatase, also called arylsulfatase C, is the enzyme responsible for the removal of the sulfonate moiety from sulfoconjugated steroids, such as DHEA-S and E1-S, and is part of a superfamily comprising 12 different mammalian sulfatases (Bond *et al.*, 1997, Ferrante *et al.*, 2002). Human STS has been successfully purified and crystallized. It is a membrane-bound protein of the endoplasmic reticulum, consisting of approximately 583 amino acids with a signal peptide of 21-23 amino acids, and exhibiting N-linked glycosylation at two of the possible four sites (Hernandez-Guzman *et al.*, 2001, Stein *et al.*, 1989). This enzyme has been shown to be expressed in human placenta, skin fibroblasts, breast tissues, and human fallopian tubes (Burns, 1983, Dibbelt & Kuss, 1986, Noel H. *et al.*, 1983, Purohit *et al.*, 1998, Stein *et al.*, 1989, Suzuki *et al.*, 1992, Vaccaro *et al.*, 1987, van der Loos *et al.*, 1984, Yanaihara *et al.*, 2001).

In mammals, follicular development is stimulated by follicle stimulating hormone and is accompanied by an increase in 17β-estradiol biosynthesis. The LH surge is responsible for follicular luteinization, a process that is accompanied by dramatic changes in follicular steroidogenesis, including the decreased production of 17β-estradiol (Fortune, 1994, Murphy, 2000, Zeleznik & Benyo, 1994). This loss in 17β-estradiol biosynthetic capacity has been explained by a marked decrease in the expression of key steroidogenic enzymes involved in the follicular production of active estrogens (Fortune, 1994, Richards, 1994). However, there has been no attempt to determine whether proteins responsible for sulfoconjugated steroid import and hydrolysis, such as SLCO/OATP2B1 and STS, respectively, are also regulated during the luteinization process. In the present study the equine preovulatory follicle is used as model to investigate the regulation of SLCO2B1 and STS mRNAs during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The specific objectives were to clone equine SLCO2B1 and STS, and determine the expression of their mRNAs in preovulatory follicles after hCG treatment.

MATERIALS AND METHODS

Materials

The Prime-a-Gene labeling system and pGEM-T Easy Vector System I were purchased from Promega Corp. (Madison, WI). The [α-^{32}P]dCTP was purchased from PerkinElmer Canada, Inc. (Woodbridge, ON, Canada), and the QuickHyb hybridization solution was obtained from Stratagene Cloning Systems (La Jolla, CA). The TRIzol total RNA isolation reagent, SuperScript II reverse transcriptase, 1-kilobase DNA ladder, synthetic oligonucleotides, and 5'-rapid amplification of cDNA ends (RACE) system (Version 2.0) were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). The Qiagen OneStep Reverse Transcription-Polymerase Chain Reaction (RT-PCR) System was obtained from Qiagen, Inc. (Mississauga, ON, Canada). The Expand High Fidelity DNA Polymerase was purchased from Roche Diagnostics (Laval, PQ, Canada). Biotrans nylon membranes (pore size, 0.2 mm) were obtained from ICN Pharmaceuticals, Inc. (Montréal, PQ, Canada), and all electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA). The hCG was obtained from The Buttler Co. (Columbus, OH).

Cloning of the equine SLCO2B1 and STS cDNA

The full length equine SLCO2B1 transcript was characterized using a multistep cloning strategy (Fig. 1). A first 1187-base pair (bp) reverse transcription-PCR product (RT-PCR1; Fig. 1Aa) was obtained from pooled equine ovarian RNA samples isolated from four preovulatory follicles 0 h post-hCG. Ovarian tissues were isolated and RNA was extracted as previously described (Kerban *et al.*, 1999). RT-PCR was performed using the OneStep RT-PCR system (Qiagen) as directed by the manufacturer, using 500 ng of RNA and oligonucleotide primers designed by sequence alignments of known SLCO2B1 species homologues (Fig. 1B; primers 1 and 2). Following agarose gel electrophoresis, the RT-PCR product was excised and ligated into the PGEM-T Easy plasmid vector (Promega), and proper recombinant plasmids were identified from transformed bacterial colonies using standard techniques (Sambrook *et al.*, 1989). Sequencing of the insert was performed by the Service de Séquençage de l'Université Laval (Québec, PQ, Canada) using vector-based T7 and SP6 oligonucleotide primers.

The same techniques were used to clone a second fragment of 1104 bp (RT-PCR2; Fig. 1Ab). Sequences obtained from the initial RT-PCR products served to design specific oligonucleotides for 5'- and 3'-Rapid Amplification of cDNA Ends (RACE) procedures. 5'-RACE was performed using the 5'-RACE System, Version 2.0 kit (Invitrogen) as directed by the manufacturer, using 5 μg of pooled ovarian tissue RNA (as described above) and SLCO2B1-specific primers for reverse transcription (primer 5; Fig. 1) and PCR (primers 7 and 9), along with forward primers supplied with the kit (primers 6 and 8; Fig. 1). The longest product obtained (Fig. 1Ac) was isolated and sequenced as described above. The 3'-RACE was performed as previously described (Boerboom *et al.*, 2000), except 5 μg of pooled ovarian tissue RNA (as described above) was used as a template for the initial RT reaction. Briefly, an RT reaction was performed using a poly-dT oligonucleotide with anchor sequences at its 5' end (Fig. 1B; primer 10). This was followed by nested PCR reactions using oligonucleotide primers that bound to the anchor sequence in conjunction with SLCO2B1-specific forward primers (Fig. 1B; primers 11-14). The product of the second PCR reaction (Fig. 1Ad) was isolated and sequenced as described above.

The partial STS sequence was obtained by 5'-RACE using the 5'-RACE system, Version 2.0 kit (Invitrogen), as described above. Primers were designed from a short 244 bp equine STS sequence available in Genbank (Accession #AF133204). They included a first primer (5' – GTAAAGGCGGGTGATGG - 3') used for the RT reaction, as well as two additional primers (5' – CCCCGTGACAGAAACACACGTG – 3' and 5' – GTGTGTGGAA AAACATCCATTGGTG – 3') used during the first and second PCR reactions, respectively.

Equine tissues and RNA extraction

Testicular tissues were obtained from the Large Animal Hospital of the Faculté de médecine vétérinaire (Université de Montréal) following a routine castration, whereas other non-ovarian tissues were collected at a local slaughterhouse. Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from

Standardbred and Thoroughbred mares as previously described (Sirois & Dore, 1997). Briefly, when preovulatory follicles reached 35 mm in diameter during estrus, the ovulatory process was induced by injection of hCG (2500 IU, iv) and ovariectomies were performed via colpotomy using an ovariotome at 0, 12, 24, 30, 33, 36, or 39 h post hCG (n = 4-6 mares / time point) (Sirois & Dore, 1997). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna. Ovariectomies were also performed on day 8 of the estrous cycle (day 0, day of ovulation) to obtain corpora lutea (n = 3 mares). All animal procedures were approved by the institutional animal use and care committee. Total RNA was isolated from tissues with TRIzol reagent (Invitrogen Canada Inc.), according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada).

Semiquantitative RT-PCR and Southern analysis

The OneStep RT-PCR System (Qiagen) was used for semi-quantitative analysis of SLCO2B1, STS and rpL7a mRNA levels in equine tissues. Reactions were performed according to the manufacturer's directions, using sense (5' – TGCCAGACAA GGACATCAAGGC - 3') and antisense (5' - CACAGGGAAGCCTCGGAATCC - 3') primers specific for equine SLCO2B1, sense (5' - TTACCTTCACGTGCACACGGCC - 3') and antisense (5' - GTGGGTGCCAGCGCACAGCAT - 3') primers specific for equine STS, and sense (5' - ACAGGACATCCAGCCCAAACG - 3') and antisense (5' - GCTCCTTTGTCTTCCGAGTTG - 3') primers specific for the equine control gene ribosomal protein L7a (rpL7a). These reactions resulted in the production of SLCO2B1, STS and rpL7a DNA fragments of 442, 518 and 516 bp, respectively. Each reaction was performed using 100 ng total RNA, and cycling conditions were one cycle of 50 C for 30 min and 95 C for 15 min, followed by a variable number of cycles of 94 C for 1 min, 59 C for 1 min, and 72 C for 1 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification and was 20 cycles for SLCO2B1, 24 cycles for STS and 15 cycles for rpL7a. Following PCR amplification, samples were electrophoresed on 2% TAE-agarose gels, transferred to nylon membranes, and hybridized with corresponding radiolabeled SLCO2B1, STS and rpL7a

cDNA fragments using QuickHyb hybridization solution (Stratagene). Membranes were exposed to a phosphor screen, and signals were quantified on a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA).

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG administration on levels of SLCO2B1 and STS mRNA in samples of follicle wall, corpora lutea, theca interna, and granulosa cells. SLCO2B1 and STS mRNA levels were normalized with the control gene rpL7a before analysis. When ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for the comparisons of individual means with control (0 h post hCG). Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC).

RESULTS

Characterization of equine SLCO2B1 and STS

To clone the equine SLCO2B1 transcript, RT-PCR was performed on ovarian RNA using oligonucleotide primers designed by sequence alignment of known SLCO2B1 species homologs. The resulting cDNA fragment (Fig. 1Aa) was sequenced and found to be highly homologous to SLCO2B1 transcripts identified thus far. A combination of 5'- and 3'-RACE reactions yielded cDNA products corresponding to all remaining coding regions, as well as the 5'- and 3'-UTRs (Fig. 1, Ab and Ac). The deduced SLCO2B1 3434-bp primary transcript encoded a 2127-bp open reading frame (Fig. 2; GenBank accession number DQ213041), which predicted a protein of 709 amino acid residues (OATP2B1). This 709-amino acid protein is highly conserved when compared with human (NP_009187) and bovine (NP_777268) homologs. The equine OATP2B1 has 86.2% identity at the amino acid level (Fig. 3) and a 88.1% identity at the nucleic acid level when compared to bovine OATP2B1. All putative transmembrane domains and conserved amino acids from the OATP-signature motif appear to be present in the equine enzyme (Fig. 3).

A 686-bp fragment of the equine STS cDNA was isolated using the 5'-RACE technique (Genbank accession number DQ367333). This sequence translated to a 228-amino acid peptide, being 82% identical to amino acids 258-583 of the human protein (NP_000342).

Tissue distribution of equine SLCO2B1 and STS mRNAs

To study to tissue distribution of equine SLCO2B1 and STS, various equine tissues were obtained and the expression of SLCO2B1 and STS was examined by RT-PCR/Southern blot. Results showed that the SLCO2B1 and STS transcripts were expressed in many of the tissues studied (Fig. 4A and 4B). Levels of SLCO2B1 mRNA were high in liver and kidney; moderate in placenta, brain, spleen and a preovulatory follicle isolated prior to hCG; low in heart, testes, skin and skeletal muscle; and very low or absent in lung, adrenal, stomach and uterus. Levels of STS transcript were high in liver and spleen; moderate in placenta, brain, lung, adrenal and a preovulatory follicle isolated prior to hCG; low in kidney, uterus, testes and skin; and very low or absent in heart, stomach and skeletal muscle. However, levels of the control gene rpL7a remained relatively constant in all tissues studied (Fig. 3C).

Regulation of SLCO2B1 and STS transcripts in preovulatory follicles and corpora lutea

The regulation of SLCO2B1 and STS mRNAs in preovulatory follicles was studied by RT-PCR/Southern blot, using follicles isolated during estrus at 0, 12, 24, and 36 h after the administration of an ovulatory dose of hCG. Total RNA was extracted from the follicle wall (theca interna with attached granulosa cells), as well as from three corpora lutea obtained on day 8 of the estrous cycle. Levels of equine SLCO2B1 mRNA were high in equine preovulatory follicles prior to treatment with hCG (0 h), but were clearly repressed from 24 h to 36 h post hCG, and remained low in the corpus luteum at day 8 of the cycle (Fig. 5A). When results from multiple follicles and corpora lutea were expressed as ratios of SLCO2B1 to rpL7a, a significant decrease in SLCO2B1 transcript was detected in follicles from 24 to 36 h post hCG (Fig. 5C). Levels of STS transcript, however, remained high in all the samples studied (Fig. 5D), with no significant change observed when the results from multiple follicles and corpora

lutea were expressed as a ratio of STS to L7a ($P < 0.05$; Fig. 5F). No change in rpL7a transcript was detected after gonadotropin treatment (Fig. 5B and 5E).

In order to determine which cell type in the equine follicle wall was responsible for the expression of the SLCO2B1 and STS transcripts, granulosa and theca interna cells were isolated from follicles obtained between 0 and 39 h post hCG (Fig. 6). Results indicated that both cell types were responsible for SLCO2B1 expression, with the repression of transcript expression occurring earlier in granulosa cells than theca interna. In granulosa cells, the decrease in SLCO2B1 mRNA was significant at 12 h post hCG and it remained low ($P < 0.05$; Fig. 6Aa), whereas results for theca interna demonstrated a significant decrease in SLCO2B1 transcript only from 30 h post hCG followed by a further decline at 36 and 39 h post-hCG ($P < 0.05$; Fig. 6Ab). Although STS mRNA expression remained constant in preparations of intact follicle wall, some changes were observed when isolated granulosa cells and theca interna were studied. In granulosa cells, levels in STS mRNA significantly decreased 24-39 h post hCG ($P < 0.05$; Fig. 6Ba), whereas a modest increase in STS transcript levels was observed in theca interna at 39 h post hCG (Fig. 6Bb).

DISCUSSION

This study demonstrates for the first time that hCG-induced follicular luteinization is accompanied by a decrease in mRNA expression of both SLCO2B1 and STS in granulosa cells, as well as a down-regulation of SLCO2B1 in the theca interna layer. Follicular luteinization/ovulation has previously been associated with various changes in steroidogenic enzyme expression. The expression of enzymes involved in androgen and estrogen biosynthesis, cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) and cytochrome P450 aromatase (CYP19A1), are downregulated; whereas the expression of those responsible for enhanced progesterone synthesis, steroidogenic acute regulatory protein (StAR) and cytochrome P450 side-chain cleavage enzymes (CYP11A1), are induced (Fortune, 1994, Richards, 1994, Ronen-Fuhrmann *et al.*, 1998, Sandhoff *et al.*, 1998). Such changes have also been observed in the mare during hCG-induced luteinization/ovulation, with *CYP17A1* being expressed in theca interna cells,

and 3β-hydroxysteroid dehydrogenase and *CYP19A1* being expressed exclusively in the equine granulosa cell layer (Boerboom & Sirois, 2001, Boerboom *et al.*, 1999, Kerban *et al.*, 1999). There have been no previous reports, however, on the regulation of proteins/enzymes responsible for the import and hydrolysis of sulfoconjugated steroids. Previous investigations of the expression of SLCO/OATP2B1 in the ovary have primarily been limited to its detection by Northern blot (Kullak-Ublick *et al.*, 2001). Whereas, the presence of STS in the ovary has mainly been detected by examining its activity in rat ovaries, cultured primary granulosa cells and granulosa cell lines (Clemens *et al.*, 2000) as well as by immunohistochemical analyses of ovarian clear cell adenocarcinomas (Okuda *et al.*, 2001).

The regulation of SLCO2B1 and STS in the ovary has not been addressed and there is little information available from any tissue on the mechanisms involved in their regulation. This study identifies hCG as a potential transcriptional modulator of both SLCO2B1 and STS expression in granulosa and theca interna cells of the preovulatory follicle. High levels of SLCO2B1 transcript were observed in granulosa cells with a rapid downregulation of SLCO2B1 mRNA occurring after hCG, this being reminiscent of the *CYP19A1* regulation during follicular luteinization/ovulation (Boerboom *et al.*, 1999). A marked and significant decrease in SLCO2B1 expression in theca interna was also observed at 36 and 39 h post-hCG, this being very similar to the down-regulation of *CYP17A1* observed in this cell layer at this time (Boerboom *et al.*, 1999). Studies in other tissues have shown that there is an 8-fold increase in SLCO2B1 transcript expression during differentiation of human placental trophoblast to syncytia (St-Pierre *et al.*, 2002). In the mouse liver, Slco2b1 mRNA was shown to be down-regulated by ligands of peroxisome proliferator-activated receptor α and nuclear factor erythroid 2-related factor 2 activators (Cheng *et al.*, 2005).

The present study is also the first to demonstrate the down-regulation of STS mRNA expression in granulosa cells. The promoter region of the human STS gene has been identified and localized to the X chromosome (Yen *et al.*, 1988). It is unusual, however, in that it lacks a TATA box, is not rich in GC, and lacks binding sites for Sp1

and other known transcription factors. The inflammatory cytokine interleukin-1β (IL-1β) has been shown to down-regulate STS transcript expression in human endometrial stromal cells, as well as in vascular smooth muscle cells derived from human aortas (Matsuoka *et al.*, 2002, Nakamura *et al.*, 2003). Interestingly, IL-1β has been shown to be upregulated after LH treatment in cultured human granulosa cells (Chen *et al.*, 2000) and in granulosa cells obtained 6 h after gonadotropin treatment in the mare (Martoriati & Gerard, 2003). It will be interesting to determine whether IL-1β is an intermediate in the gonadotropin-dependent down-regulation of STS in follicular cells. The regulation of the STS enzyme has also been demonstrated through posttranslational modifications (Newman *et al.*, 2000) and more studies will need to be done in order to determine if this is also a contributing factor in the regulation of STS during follicular luteinization.

Previous investigations using *Xenopus laevis* oocytes and basal syncytiotrophoblast membrane vesicles to study the uptake of various substrates by OATP2B1 have demonstrated that DHEA-S and E1-S were potential substrates for the transporter, and that pregnenolone sulfate partially inhibited OATP2B1-mediated transport of E1-S in the oocyte expression system (Kullak-Ublick *et al.*, 2001, St-Pierre *et al.*, 2002). STS has also been shown to hydrolyse sulfoconjugated DHEA, E1, pregnenolone and cholesterol (Dibbelt & Kuss, 1983, Egyed & Oakey, 1985). It has been subcellularly localized to the endoplasmic reticulum, and characterized as being mushroom-shaped, with highly hydrophobic helices capable of traversing lipid bilayers and an active site near the membrane surface, facing the lumen (Hernandez-Guzman *et al.*, 2001). Interestingly, 3β-hydroxysteroid dehydrogenase and type II cytochrome P450 enzymes, like CYP17A1 and CYP19A1, are also located in the endoplasmic reticulum (ER) (Fournet-Dulguerov *et al.*, 1987, Thomas *et al.*, 1989). Therefore, SLCO/OATP2B1 and STS may be responsible for supplying pregnenolone and DHEA to steroidogenic enzymes within the ER and may contribute to the biosynthesis of 17β-estradiol observed prior to the onset follicular luteinization. Results from the present study suggest that the decrease in estrogens observed during LH-induced follicular luteinization/ovulation may not only be a result of decreased de novo steroid synthesis, but also due to a decrease in estrogen precursor import and hydrolysis.

This study also investigated the co-localized expression of the SLCO2B1 and STS genes in the ovary and established abundant levels of SLCO2B1 and STS in preovulatory follicles prior to hCG. High levels of SLCO2B1 transcript were also observed in the liver, brain and spleen. This finding is consistent with results in humans where Northern blot analyses identified liver and spleen as tissues where SLCO2B1 was highly expressed (Kullak-Ublick *et al.*, 2001). Expression of STS mRNA was high in spleen, kidney and brain. It has been demonstrated, however, that the organ and tissue distribution of STS varies considerably from species to species (Reed *et al.*, 2005).

In summary, this study is the first to characterize the primary structure of equine SLCO/OATP2B1, to demonstrate the regulation of the SLCO2B1 and STS genes during follicular luteinization, and to identify ovulatory levels of gonadotropins as a negative regulator of SLCO2B1 and STS expression. The extent of these enzyme's contributions to the elevated 17β-estradiol levels prior to the LH rise has remained uncharacterized, but their elevated expression clearly suggests a potential role. Moreover, considering their respective activities, it is not unreasonable to propose that their down-regulation in luteinizing preovulatory follicles may also contribute to the decrease in ovarian 17β-estradiol observed after the LH rise. Further studies will be needed to unravel the role and regulators of SLCO2B1 and STS gene expression in ovarian function.

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FIGURE LEGENDS

Fig. 1. Cloning of equine SLCO2B1. A, cloned cDNA fragments. Each fragment is schematically represented, with its identity indicated on the right and its position in the deduced transcript sequence indicated in parentheses. Lines indicated untranslated regions (UTRs); open boxes designate the open reading frame (ORF). Lengths of the deduced transcript and its structural elements are indicated in base pairs (bp). Arrows indicate the position and orientation of the oligonucleotides employed in the cloning processes, with numbers indicating their identity. B, Oligonucleotides used in the various cloning procedures.

Fig. 2. Deduced nucleotide sequences for equine SLCO2B1. The open reading frame is presented in *uppercase* letters, whereas the untranslated regions are shown in *lowercase* letters. The translation initiation codon (ATG) and termination codon (TGA) are denoted in *bold underlined*. Numbers on the *right* refer to the last nucleotide on that line. This sequence has been deposited in Genbank with accession number DQ213041.

Fig. 3. Deduced primary structure of the equine OATP2B1 protein and comparison with known OATP2B1 homologs. Identical residues are indicated by a printed period, numbers on the *right* refer to the last amino acid residue on that line. The predicted amino acid sequence of the equine OATP2B1 protein is aligned with human (hum; GenBank accession number NP_009187) and cow (NP_777268) homologs (A). *Bold underlined* residues are part of the OATP-superfamily signature; *boxed* sequences, identified with roman numerals, are putative transmembrane domains (Hagenbuch & Meier, 2003).

Fig. 4. Expression of SLCO2B1 and STS mRNA in equine tissues. Total RNA was extracted from various equine tissues, and samples (100 ng) were analyzed for SLCO2B1, STS and rpL7a (control gene) content by a semi-quantitative RT-PCR/Southern blotting techniques, as described in *Materials and Methods*. A, Expression of SLCO2B1 mRNA in equine tissues. B, Expression of STS mRNA in equine tissues. C, Expression of rpL7a mRNA in equine tissues. The number of PCR

cycles for each gene was within the linear range of amplification, and they represented 20, 24 and 15 cycles for SLCO2B1, STS and rpL7a, respectively. Numbers on the *right* indicate the size of the PCR fragment.

Fig. 5. Regulation of SLCO2B1 and STS transcript by hCG in equine preovulatory follicles. Preparations of follicle wall were obtained from preovulatory follicles isolated between 0-36 h after hCG and of corpora lutea (CL) isolated on day 8 of the estrous cycle. Samples (100 ng) of total RNA were analyzed for SLCO2B1, STS and rpL7a content by a semi-quantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. A, Regulation of SLCO2B1 mRNA in equine follicles (one representative follicle per time point). B, Constitutive expression of rpL7a mRNA in the same follicles. C, Relative changes in intensity of SLCO2B1 signal was normalized with the control gene rpL7a (mean ± SEM; five or six distinct follicles, i.e. animals per time point and three different corpora lutea). D, Regulation of STS mRNA in equine follicles. E, Constitutive expression of rpL7a mRNA in the same follicles. Numbers on the *right* indicate the size of the PCR fragment. F, Relative changes in intensity of STS signal was normalized with the control gene rpL7a (mean ± SEM; five or six distinct follicles, i.e. animals per time point and three different corpora lutea). *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$).

Fig. 6. Regulation of SLCO2B1 (A) and STS (B) mRNA in equine granulosa and theca interna cells. Preparations of granulosa cells (a) and theca interna (b) were isolated from equine preovulatory follicles between 0-39 h after hCG treatment, and samples (100 ng) of total RNA were analyzed for SLCO2B1, STS and rpL7a content by a semi-quantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. The SLCO2B1 and STS signals were normalized with the control gene rpL7a, and results are presented as a ratio of either SLCO2B1 to rpL7a (A) or STS to rpL7a (B) (mean ± SEM; n = 4 samples; *i.e.* mares per time point). *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$). *Insets* show representative results of SLCO2B1, STS and rpL7a mRNA levels from one sample per time point.

FIGURE 1

A

B

1. 5'-ATGGGACCCAGGATAGGGCCAG-3'
2. 5'-TTGGGCAGGAAGGTGGCCATGC-3'
3. 5'-GCCATCCCCTACTTCTTCTTCC-3'
4. 5'-GAAGAAGAACTGGAGGCCGATG-3'
5. 5'-ACACTTGGCCGCGAGTCCTGA-3'
6. 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'
7. 5'-GCTTTGCCTCCAGGTGTGTCT-3'
8. 5'-GGCCACGCGTCGACTAGTAC-3'
9. 5'-CATGGCCTTGATGTCCTTGTCT-3'
10. 5'-GTACCGGATCCTCTAGAGAGCTCGTCGACCT
    CGAGGAATTCAAGCTTTTTTTTTTTTTTTTTTT-3'
11. 5'-CTGCCATTGACACCACCTGTGT -3'
12. 5'-GTACCGGATCCTCTAGAGAGCTC-3'
13. 5'-CTATGACAACGACCTGCTCCGA-3'
14. 5'-GTCGACCTCGAGGAATTCAAGCTT-3'

FIGURE 2

```
cgattcacag agagggaagg aacaagtgac tgagggagac aggcgcttgg agagtggggt caagttgaac aggacttcct aacccaaacc  90
tgggagaggt ggctgctatc cagcgtggcc caagaggcac cagcctagcg cgtggagtcc ccaggttcgc acagccctgc gctgtggctc  180
gcggctgctg tctccaggag cgcccgataa ggtctcactc catttctcct gctcagctcc tgtcttgaaa ctgcaatagg ggctggaact  270
cactgcacgc agtggtcATG GGACCCAGGA TAGGGCCGGT GAGTGAGGGG CCCCAGATGC CAGACAAGGA CATCAAGGCC ATGATGGGCA  360
CAGAAGACAC ACCTGGAGGC AAAGCCAGCC CAGACCCTCA GGACTCGCGG CCAAGTGTGT TCCACAGCAT CAAGGTCTTC GTCCTGTGCC  450
ACAGCCTGCT GCAGCTGGCG CAGCTCATGA TCTCCGGCTA CCTCAAGGGC TCCATCTCCA CAGTGGAGAA ACGCTTCGGC CTCTCCAGTC  540
AGACCTCTGG GCTGCTGGCC GCCTTCAACG AGGTGGGGAA CACAGCCCTG ATTGTGTTTG TGAGCTATTT TGGCAGCCGG GTGCATCGGC  630
CCCGACTGAT CGGCTGTGGG GCTATCCTTG TAGCCCTGGC GGGCCTGCTC ATGGCTCTCC CACACTTCAT TTCGGAGCCA TACCGCTTCG  720
ACAACACCAG TCCTGCGGAC ATGCCGCAGG ATTCCGAGGC TTCCCTGTGC CTGCCCTCAA CCTCTGCCCG GGCCCCAGCC TCCTCCAACA  810
GCAGCTGCTC AAGCCACACG CAGGCCCAGC ATCTGGTCGC GGTGGGGATC ATGTTCACGG CACAGACCGT GCTTGGGGTG GGCGGGGTGC  900
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TGTTGGGACC AGGCATGGCC TACGGGCTGG GCGGCCTCAT GCTACGCCTT TATGTGGACA TTAACCGAAT GCCAGAAGGA GGTATCAACC  1080
TGACATCGAA GGACCCCCGA TGGGTGGGTG CCTGGTGGCT GGGCTTCCTC ATCTCGGCTG GAGTCGTGGC CCTGGCTGCC ATCCCCTACT  1170
TCTTCTTCCC CAGAGAGATG CCCAAGGAGA AACACGAGCT TCGCTTCCGG CGAAGGGTCT TGGCAGTTTC AGCCTCACCT GTCAGCAAAG  1260
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CCATGGTGGC GGGCATGGCC ACCTTCCTGC CCAAGTTCTT GGAGCGCCAG TTTTCCGTCA CAGCATCCTT TGCCAACCTG CTCATAGGCT  1530
GTGTCACCAT CCCCTTGGCC ATCATGGGCA TCGTGATGGG GGGCTTCCTG GTCAAGCGCC TCCGCCTGGG CCCCATGCGC TGCAGCATCC  1620
TTTGCCTACT GGGGATGCTG TTCTGCCTCC TTCTCAGCCT GCCCCTCTTC TTCATGGGCT GCTCCACCCA CCACATCGCA GGCATCATCC  1710
ACCAGCCTGG CGCGCAGCCT GGGCTGGAGC GGTTTCCAGG CTGCATGGAG CCCTGCTCCT GCCCATCGGA CGACTTTAAC CCTGTCTGCG  1800
AGCCCAGCAG CCGTGTGGAG TACCTCACGC CCTGCCACGC GGGCTGCACA AGCCGGATGG TCCAGGAAGC TCTGGACAAA AGTCAGGTTT  1890
TCTACACCAA CTGCAGCTGC GTGGCAGGGG GCAGCCCTGT GCTGGCGGGC TCCTGCGACT CAGCCTGCAG CCACCTGGTG CTGCCCTTCA  1980
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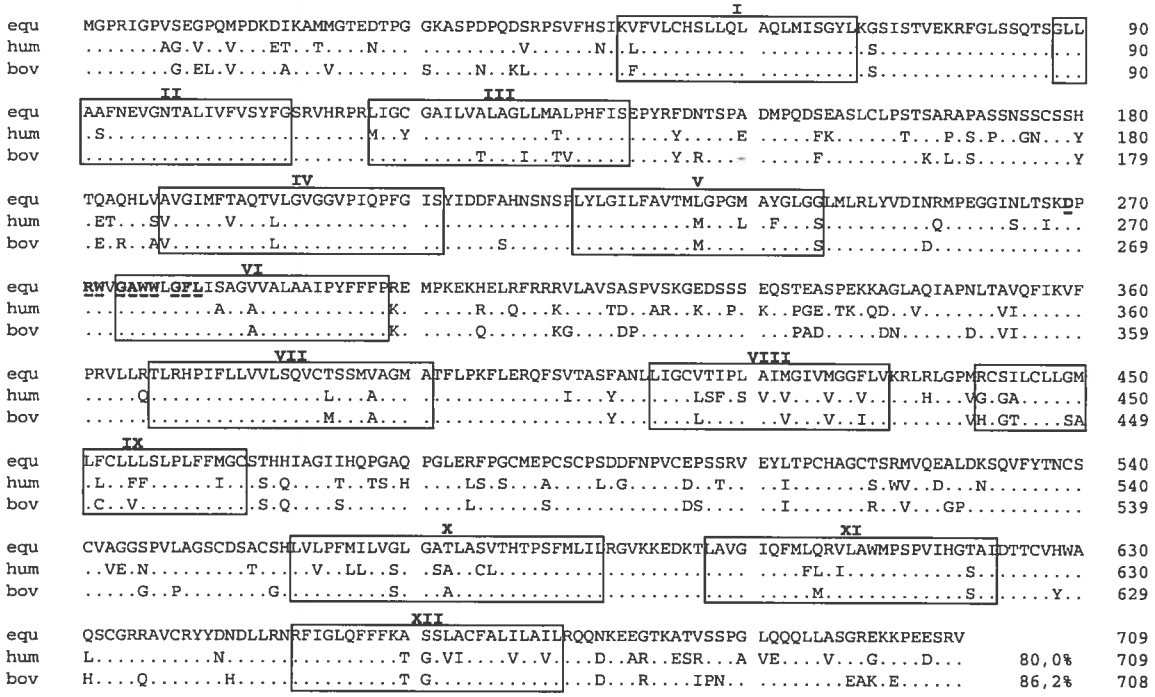
FIGURE 3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II
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V
VI
VII
VIII
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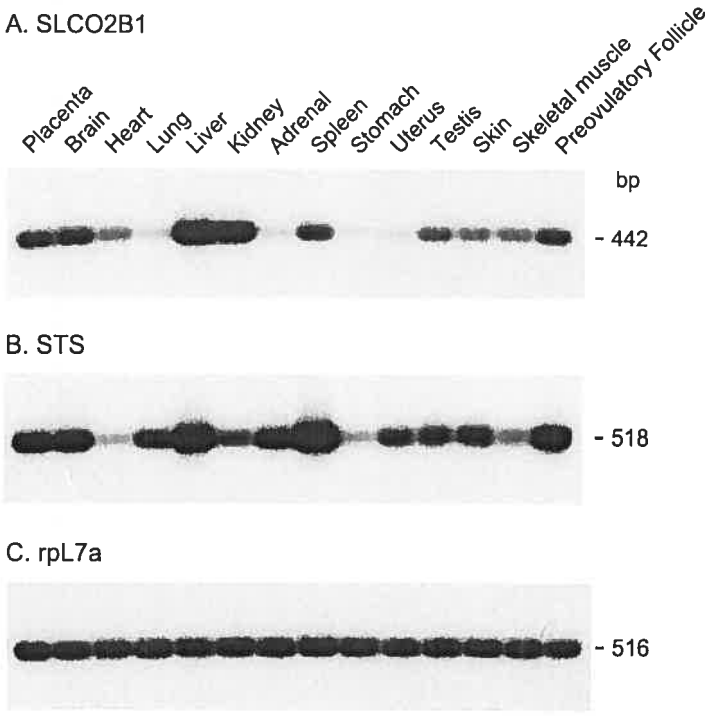
FIGURE 4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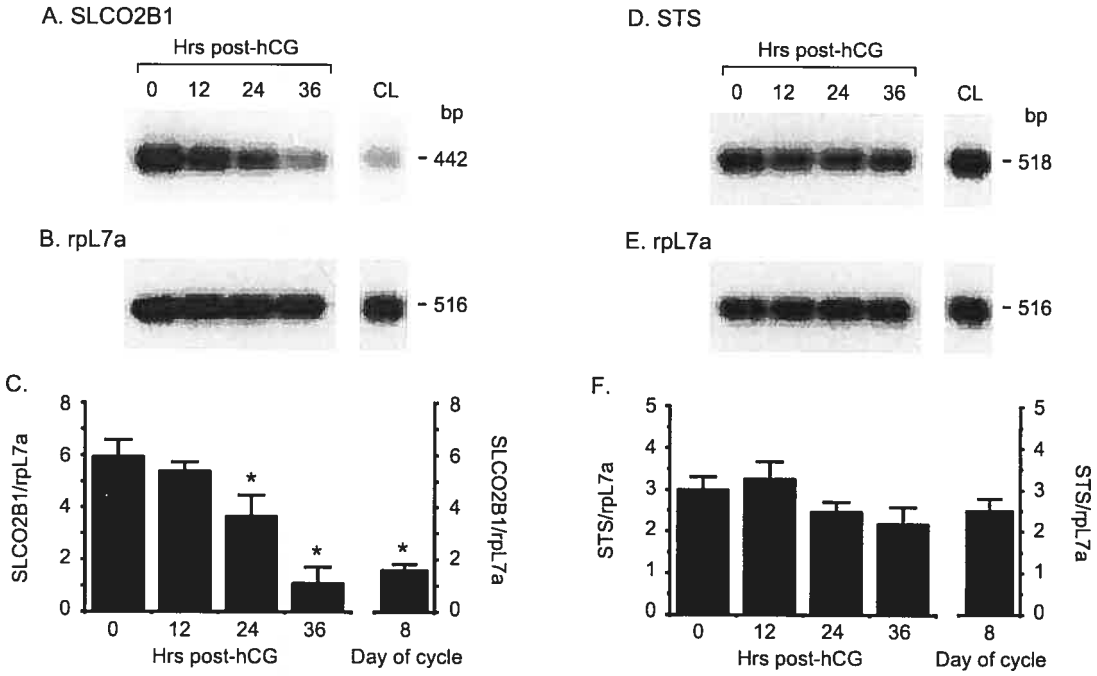
Placenta
Brain
Heart
Lung
Liver
Kidney
Adrenal
Spleen
Stomach
Uterus
Testis
Skin
Skeletal muscle
Preovulatory Follicle
bp
- 442
B. STS
- 518
C. rpL7a
- 516

FIGURE 5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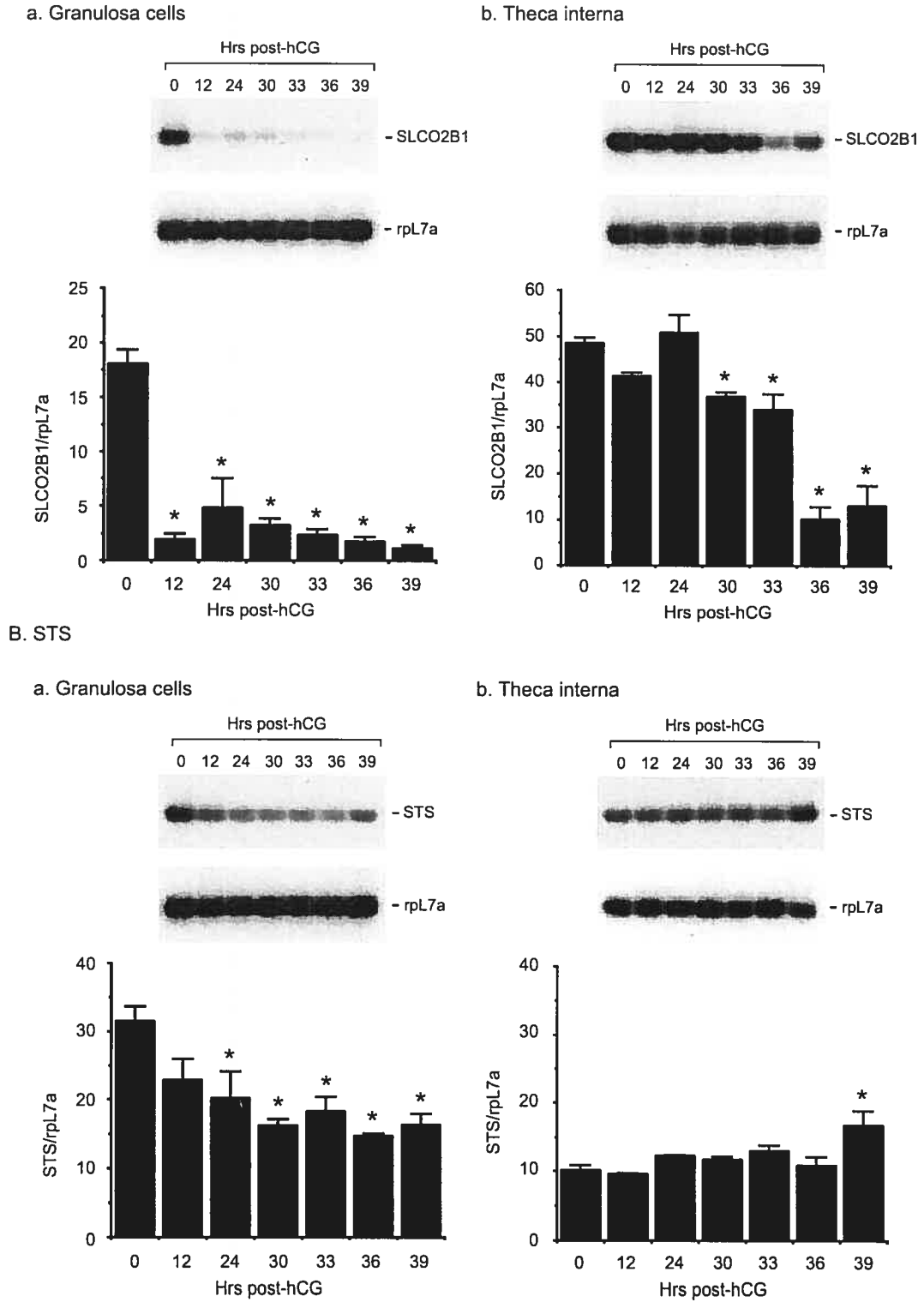
bp
- 442
B. rpL7a
- 516
C.
SLCO2B1/rpL7a
Hrs post-hCG
Day of cycle
D. STS
- 518
E. rpL7a
- 516
F.
STS/rpL7a
Hrs post-hCG
Day of cycle

FIGURE 6


A. SLCO2B1
a. Granulosa cells
b. Theca interna
Hrs post-hCG
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- SLCO2B1
- rpL7a
SLCO2B1/rpL7a
Hrs post-hCG
B. STS
a. Granulosa cells
b. Theca interna
- STS
STS/rpL7a

SUMMARY OF ARTICLE THREE

Title: Human chorionic gonadotropin-dependent regulation of 17β-hydroxysteroid dehydrogenase type 4 in preovulatory follicles and its potential role in follicular luteinization. **Kristy A. Brown**, Derek Boerboom, Nadine Bouchard, Monique Doré, Jacques G. Lussier and Jean Sirois. 2004 *Endocrinology* 145(4):1906-15.

Thesis author's contribution to this work: As the primary author, I was responsible for all aspects of this article. Secondary authorship credits reflect the cloning of the equine 17βHSD4 cDNA, whereas tertiary authorship credits reflect technical support. Quaternary authorship credits reflect immunohistochemical support, whereas fifth and sixth authors represent co-directorship and directorship credits, respectively.

Summary:

- The equine 17βHSD4 cDNA was characterized by a combination of RT-PCR and cDNA library screening of pooled equine ovarian RNA.
- Sequence analysis of the 17βHSD4 transcript revealed an open reading frame that encoded a 735-amino acid protein, which was highly conserved relative to its mammalian orthologues. The three putative functional domains were conserved in the equine protein, as were the potential glycosylation sites.
- The regulation of the equine 17βHSD4 transcript was examined in equine preovulatory follicles by semi-quantitative RT-PCR in samples obtained at different times after hCG. Results from Southern blot analyses revealed basal levels of 17βHSD4 mRNA expression prior to hCG, with a significant increase in 17βHSD4 expression from 24 to 36 h in intact follicle wall preparations. This increase was mostly due to the granulosa cell layer contribution to 17βHSD4 mRNA expression.
- The regulation of the equine 17βHSD4 protein in preovulatory follicles revealed an increase between 24 and 39 h post-hCG of the full length form of the protein, reflecting observations of the mRNA abundance. Immunohistochemical data

confirmed the cellular localization of the protein as well as its qualitative induction in granulosa cells.

Work's contribution to the advancement of science:

- This work characterizes, for the first time, the primary structure of the equine 17βHSD4 cDNA and protein.
- It demonstrates the induction of the 17βHSD4 transcript and protein in preovulatory follicles occurs between 24 and 39 h after hCG and that regulation occurs predominantly in granulosa cells. In addition, this study describes the full-length protein as being the major form of regulated 17βHSD4 during the ovulatory process.
- Overall, it supports the hypothesis that estrogen-inactivating enzymes are induced during the ovulatory process. This may provide evidence that active estrogens need to be eliminated during this process.

Human Chorionic Gonadotropin-Dependent Regulation of 17β-Hydroxysteroid Dehydrogenase Type 4 in Preovulatory Follicles and Its Potential Role in Follicular Luteinization*

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ABSTRACT

The 17β-hydroxysteroid dehydrogenase type 4 (17β-HSD4) has a unique multidomain structure, with one domain involved in estradiol-17β inactivation. The objective of the study was to investigate the regulation of 17β-HSD4 during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The equine 17β-HSD4 cDNA was cloned, and shown to encode a 735-amino acid protein that is highly conserved (81-87% identity) when compared to other mammalian orthologs. RT-PCR/Southern blot analyses were performed to study the regulation of 17β-HSD4 transcripts in equine preovulatory follicles isolated between 0 and 39 h after hCG. Results showed the presence of basal 17β-HSD4 mRNA expression prior to hCG treatment, but an increase was observed in follicles obtained after 24 h post-hCG ($P < 0.05$). Analyses of isolated preparations of granulosa and theca interna cells identified basal mRNA expression in both layers, but granulosa cells appeared as the predominant site of follicular 17β-HSD4 mRNA induction. A specific polyclonal antibody was raised against a fragment of the equine protein and used to study the regulation of the 17β-HSD4 protein. Immunoblots showed an increase of full-length 17β-HSD4 protein in follicles after 24 h post-hCG ($P < 0.05$), in keeping with mRNA results. Immunohistochemical data confirmed the induction of the enzyme in follicular cells after hCG. Collectively, these results demonstrate that the gonadotropin-dependent induction of follicular luteinization is accompanied by an increase in 17β-HSD4 expression. Considering the estrogen-inactivating function of 17β-HSD4, its regulated expression in luteinizing preovulatory follicles appears as a potential complementary mechanism to reduce circulating levels of estradiol-17β after the LH surge.

INTRODUCTION

The 17β-hydroxysteroid dehydrogenases (17β-HSD) are key regulators of the biological potency of androgens and estrogens in mammals, as they catalyze the interconversion of less active 17-ketosteroids such as androstenedione and estrone (i.e. low receptor affinity) into more active 17β-hydroxysteroids like testosterone and 17β-estradiol (i.e. high receptor affinity), and vice versa (1-2). The bi-directional nature of these 17β-HSD reductive (activation) or oxidative (inactivation) activities, which require NADP(H) and NAD(H) as cofactors, is thought to serve as a molecular switch to regulate hormone action in target tissues. Thus far, at least 11 members of the 17β-HSD family have been identified and numbered according to the time of their cloning. They all belong to the short-chain dehydrogenase/reductase (SDR) superfamily, except for 17β-HSD type 5 which belongs to the aldo-keto reductase (AKR) superfamily (3-5). Marked differences in substrate specificity, tissue distribution, subcellular localization and reductive *versus* oxidative catalytic preferences are observed among 17β-HSDs. The 17β-HSD type 1 (17β-HSD1), the first one to be characterized, predominantly favors the reductive reaction and functions as an estradiol-17β biosynthetic enzyme in the human placenta and developing ovarian follicle (6-8). In contrast, 17β-HSD type 2 (17β-HSD2) is primarily an oxidative enzyme involved in androgen and estrogen inactivation in numerous tissues (9, 10), whereas the 17β-HSD type 3 is a reductive enzyme catalyzing testosterone production in the testis (11). Other 17β-HSDs also exhibit preferential reductive (types 5, 7) or oxidative (types 4, 6, 8, 9-11) activities, and their specific patterns of expression in classical steroidogenic and peripheral tissues have been associated with various physiological and pathological processes (2-5, 12).

The 17β-HSD type 4 isoform (17β-HSD4) is recognized as an unusual 17β-HSD because of its multifunctional domain structure and its unique localization to peroxisomes (13-15). It is a 80 kDa protein with three distinct functional domains: a N-terminal dehydrogenase domain sharing homology with other members of the SDR superfamily, a central hydratase domain similar to that of peroxisomal enzymes involved in β-oxydation of fatty acids, and a C-terminal sterol carrier protein 2-like domain that potentially facilitates lipid and sterol transfer between membranes (16-19). The N-

terminal dehydrogenase domain is responsible not only for the oxidation of estradiol-17β, but also for the dehydrogenation of fatty acyl-CoA. The full-length protein can be cleaved post-translationally at its N-terminus, resulting in a 32 kDa fragment containing the dehydrogenase domain, but the extent of the processing was shown to vary greatly among tissues. The enzyme is expressed ubiquitously but little is known about the physiological regulators of 17β-HSD4 expression (20-23). Mutated forms of 17β-HSD4 have been associated with a fatal peroxisomal genetic disorder in humans (24, 25), and the intact protein has recently been identified as a possible antigen in a neurological autoimmune disease (26).

In mammals, the preovulatory period is accompanied by dramatic changes in ovarian follicular steroidogenesis as the LH surge triggers follicular luteinization, a process during which the predominant steroid produced switches from estradiol-17β to progesterone (27-29). The loss in estradiol-17β biosynthetic capacity after the LH surge has been explained by a marked decrease in expression of key steroidogenic enzymes involved in the follicular production of active estrogens (28, 30). However, there has been no attempt to determine whether an enzyme with estradiol-17β inactivating capacity such as 17β-HSD4 is also regulated during the luteinization process. In the present study, the equine preovulatory follicle is used as model to investigate the regulation of 17β-HSD4 during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The specific objectives were to clone equine 17β-HSD4, and determine the expression of its mRNA and protein in preovulatory follicles after hCG treatment.

MATERIALS AND METHODS

Materials

The Access RT-PCR kit, Prime-a-Gene labeling system, and pGEM-T easy Vector System I were obtained from Promega Corp. (Madison, WI); the QuickHyb hybridization solution and ExAssist/SOLR system were purchased from Stratagene Cloning Systems (LaJolla, CA); [α-^{32}P]dCTP was obtained from Mandel Scientific-New England Nuclear Life Science Products (Mississauga, Ontario, Canada); TRIzol total RNA isolation reagent, 1-kb DNA ladder, and synthetic oligonucleotides were purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada); Biotrans

nylon membranes (0.2 μm) were obtained from ICN Pharmaceuticals, Inc. (Montréal, Québec, Canada); Hybond-P PVDF membranes, Rainbow molecular weight markers, ECL plus, horseradish peroxidase linked donkey anti-rabbit secondary antibody, pGEX-2T vector, BL-21 protease-deficient *E. Coli* strain, and glutathione Sepharose beads were purchased from Amersham Pharmacia Biotech (Baie D'Urfé, Québec, Canada); the Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, CA); Bio-Rad Protein Assay and all electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA); hCG was obtained from The Buttler Co. (Columbus, OH); diaminobenzidine tetrahydrochloride was purchased from Sigma Chemical Co. (St-Louis, MO).

Cloning of the equine 17β-HSD4 complementary DNA (cDNA)

The isolation of the equine 17β-HSD4 cDNA was performed by a combination of RT-PCR and cDNA library screening. A cDNA fragment was first isolated using 500 ng of pooled equine ovarian RNA, oligonucleotide primers (sense primer 5′-AAAGCAGTGGCCAACTATGA TTCAG-3′ and anti-sense primer 5′-TTGCTGGCATTGTCAAAGTCACAGA-3′) designed by sequence alignments of known 17β-HSD4 species homologues, and the Access RT-PCR kit (Promega) as directed by the manufacturer. Ovarian RNA samples were prepared from preovulatory follicles isolated before or after hCG treatment, and a corpus luteum obtained on day 8 of the cycle [day 0 = day of ovulation]), as previously described (31). The cDNA fragment was subcloned into the pGEM-T Easy plasmid vector (Promega), and sequenced by the Service de Séquençage de l'Université Laval (Québec, Canada). As a second approach, an equine cDNA library prepared with mRNA from a preovulatory follicle obtained 36 h post-hCG was screened with the equine RT-PCR 17β-HSD4 cDNA fragment, as described (32). The probe was labeled with [α-^{32}P]dCTP using the Prime-a-Gene labeling system (Promega) to a final specific activity greater than 1 x 10^8 cpm/μg DNA, and hybridization was performed at 68 C with QuickHyb hybridization solution (Stratagene). Positive clones were plaque purified through secondary and tertiary screenings, pBluescript phagemids containing the cloned DNA inserts were

excised *in vivo* with the Ex-Assist/SOLR system (Stratagene), and DNA sequencing was performed commercially as described above.

Equine tissues and RNA extraction

Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares, 3-10 years old and weighing approximately 375-450 kg, as previously described (32). Briefly, when preovulatory follicles reached 35 mm in diameter during estrus, the ovulatory process was induced by injection of hCG (2500 IU, iv) and ovariectomies were performed via colpotomy using an ovariotome at 0, 12, 24, 30, 33, 36 or 39 h post-hCG (n=4-6 mares/time point). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna, as previously described (33). Ovariectomies were also performed on day 8 of the estrous cycle (day 0 = day of ovulation) to obtain corpora lutea (n = 3 mares). Testicular tissues were obtained from the Large Animal Hospital of the Faculté de médecine vétérinaire (Université de Montréal) following a routine castration, whereas other non-ovarian tissues were collected at a local slaughterhouse. All animal procedures were approved by the institutional animal use and care committee. Total RNA was isolated from tissues with TRIzol reagent (Invitrogen Canada Inc.), according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada).

Semiquantitative RT-PCR and Southern analysis

The Access RT-PCR System (Promega) was used for semi-quantitative analysis of 17β-HSD4 and rpL7a mRNA levels (control gene) in equine tissues. Reactions were performed as directed by the manufacturer, using sense (5′-TAAGCCACTTCCCAGAACGGG-3′) and anti-sense (5′-AGCTC CCCACCCTTAGAGGG-3′) primers specific for equine 17β-HSD4. Sense (5′-ACAGGACATCCAGCCCAAACG-3′) and anti-sense (5′-GCTCCTTTGTCTTCCGAGTTG-3′) primers specific for equine rpL7a were designed from a published sequence deposited in GenBank (accession no. AF508309). These

reactions resulted in the production of 17β-HSD4 and rpL7a DNA fragments of 624 and 516 bp, respectively. Each reaction was performed using 100 ng of total RNA, and cycling conditions were one cycle of 48 C for 45 min and 94 C for 2 min, followed by a variable number of cycles of 94 C for 30 sec, 58 C for 1 min and 68 C for 2 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification, and were 15 and 18 cycles for 17β-HSD4 and rpL7a, respectively. Following PCR amplification, samples were electrophoresed on 2% TAE-agarose gels, transferred to nylon membranes, and hybridized with corresponding radiolabeled 17β-HSD4 and rpL7A cDNA fragments using QuikHyb hybridization solution (Stratagene). Membranes were exposed to a phosphor screen, and signals were quantified on a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA).

Production of an anti-equine 17β-HSD4 antibody

A pair of sense (5′-GATGGATCCAGAGTTCATTTGCGGGGCTCC-3′) and anti-sense (5′-CAGAATTCCTGATGTGGCCGTTGATGCCGC-3′) primers that incorporated a *BamHI* and an *EcoRI* restriction site, respectively, was designed from the equine 17β-HSD4 open reading frame to generate a fragment spanning the region from Arg^{121} to Ser^{322}. The fragment was amplified by PCR using the Expand High Fidelity polymerase (Roche Molecular Biochemicals), and following the manufacturer's protocol. The fragment was isolated after electrophoresis, digested with *Bam HI* and an *EcoRI*, subcloned into pGEX-2T in frame with the GST coding region (Amersham Pharmacia Biotech), and sequenced to confirm its identity. Protease deficient E. Coli BL-21 (Amersham Pharmacia Biotech) were transformed with the 17β-HSD4/pGEX-2T construct, expression of recombinant 17β-HSD4/GST fusion protein was induced with IPTG, and bacterial protein extracts were obtained after sonication and centrifugation, as described (34, 35). The 17β-HSD4/GST fusion protein was purified by affinity on glutathione Sepharose beads (Amersham Pharmacia Biotech), digested with thrombin to release the 17β-HSD4 fragment, resolved by one-dimensional SDS-PAGE, transferred

on nitrocellulose, and stained with Ponceau S Red (33, 34). The 17β-HSD4 band (M_r = 21,800) was cut and used to immunize rabbits.

Cell extracts and immunoblot analysis

Cell extracts were prepared as previously described (35). Briefly, tissues were homogenized and sonicated on ice in TED buffer (20 mM Tris, pH 8.0, 50 mM EDTA, 0.1 mM DEDTC) containing 1.0% Tween. The sonicates were centrifuged at 16,000 x g for 15 min at 4 C. The recovered supernatant (whole cell extract) was stored at –80 C until electrophoretic analyses were performed. Protein concentration was determined by the method of Bradford (36) (Bio-Rad Protein Assay). Samples (60 μg of proteins) were resolved by one-dimensional SDS-PAGE, and electrophoretically transferred to PVDF membranes (35). Membranes were incubated with the polyclonal anti-equine 17β-HSD4 antibody (1:1,000), and immunoreactive proteins were visualized on Kodak film X-OMAT AR after incubation with the horseradish peroxidase linked donkey anti-rabbit secondary antibody (1:10,000 dilution) and the enhanced chemiluminescence system (ECL plus), and following the manufacturer's protocol (Amersham Pharmacia Biotech). Autoradiograph images were digitized using a ScanMaker 4 flatbed scanner (Microtek Lab, Inc., Redondo Beach, CA), and signal intensities were quantified using the ImageQuant software version 1.1 (Molecular Dynamics).

Immunohistochemical localization of 17β-HSD4

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, Ca), as previously described (37). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 μm-thick sections were prepared and deparaffined through graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with diluted normal goat serum for 20 min at room temperature. The anti-17β-HSD4 antibody was diluted in PBS (1:1,000 dilution) and applied, and sections were incubated overnight at 4 C. Control sections were incubated with PBS. After rinsing in PBS for 10 min, a biotinylated goat anti-rabbit antibody (1:222 dilution; Vector Laboratories, Burlingame, Ca) was applied, and

sections were incubated for 45 min at room temperature. Sections were washed in PBS for 10 min, and incubated with the avidin DH-biotinylated horseradish peroxidase H reagents for 45 min at room temperature. After washing with PBS for 10 min, the reaction was revealed using diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Sections were counterstained with Gill's hematoxylin stain and mounted.

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG on levels of 17β-HSD4 mRNA in samples of follicle wall, corpora lutea, theca interna and granulosa cells. 17β-HSD4 mRNA levels were normalized with the control gene rpL7a prior to analysis. One-way ANOVA was also used to test the effect of time after hCG on levels of 17β-HSD4 protein in follicle wall preparations. When ANOVAs indicated significant differences ($P < 0.05$), the Dunnett's test was used for multiple comparisons of individual means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC).

RESULTS

Characterization of the equine 17β-HSD4 cDNA

A 651-bp equine 17β-HSD4 cDNA fragment was initially isolated by RT-PCR with primers designed by sequence alignments of known 17β-HSD4 species homologues (Fig. 1). When this fragment was used as a probe to screen an equine follicular cDNA library, five positive clones were obtained from a screen of approximately 125,000 phage plaques, of which two (clones 4 and 5) were selected for purification and DNA sequencing. Results revealed that the equine 17β-HSD4 cDNA was composed of a 5′-untranslated region of 103 bp, an open reading frame of 2208 bp (including the stop codon), and a 3′-untranslated region of 341 bp containing two overlapping 5′-AATAAA-3′polyadenylation signals (Fig. 1).

The coding region of equine 17β-HSD4 encodes a 735-amino acid protein, which is identical in length to that of mouse (38), rat (22) and guinea pig 17β-HSD4 (39), but one and two amino acids shorter than the human (13) and porcine protein (40),

respectively (Fig. 2). The equine 17β-HSD4 sequence was shown to be highly conserved when compared to other species homologues, with a greater than 81% identity observed at the amino acid level, and greater than 82% identity at the nucleic acid level. The N-terminal dehydrogenase, central hydratase, and C-terminal SCP 2-like domains that characterize the multifunctional domain structure of 17β-HSD4 are conserved in the equine protein (Fig. 2). Moreover, the localization of equine 17β-HSD4 to peroxisomes is also predicted based on the Ala-Lys-Leu tripeptide peroxisomal targeting signal present at the C-terminus (Fig. 2) (15, 41). The equine protein was shown to contain several consensus glycosylation sites (Fig. 2), as observed in other species (13, 38, 40), but their biological significance remains unclear since other reports indicate that 17β-HSD4 is not glycosylated (42).

Tissue distribution of equine 17β-HSD4 mRNA

The expression of equine 17β-HSD4 mRNA in various equine tissues was examined by RT-PCR/Southern blot. Results revealed a ubiquitous pattern of expression of the 17β-HSD4 transcript, but levels varied across tissues (Fig. 3). High levels of 17β-HSD4 mRNA were observed in testis and in a preovulatory follicle isolated 36 h after hCG, moderate levels were detected in brain, heart, liver, kidney and skeletal muscle, and low to very low levels were present in other tissues studied (Fig. 3).

Regulation of 17β-HSD4 mRNA in preovulatory follicles

RT-PCR/Southern blot was used to study the regulation of the 17β-HSD4 transcript in preovulatory follicles isolated during estrus between 0 and 36 h after hCG treatment, and in corpora lutea obtained on day 8 of the estrous cycle. Results clearly showed that 17β-HSD4 mRNA was regulated in equine follicles during the hCG-induced ovulatory/luteinization process. Levels of equine 17β-HSD4 mRNA were low but detectable in equine follicles prior to hCG treatment (0 h), and increased at 24 and 36 h post-hCG (Fig. 4A). This increase appeared transient as levels of 17β-HSD4 in a day-8 corpus luteum returned to those present before hCG treatment (Fig. 4A). When results from multiple follicles and corpora lutea were expressed as ratios of 17β-HSD4

to rpL7a, a significant increase in 17β-HSD4 transcript was detected in follicles at 24 and 36 h post hCG ($P < 0.05$), but not in follicles at 12 h post hCG and in corpora lutea (Fig. 4C). No significant change was observed in levels of rpL7a transcript in follicle wall and corpora lutea preparations (Fig. 4B).

To determine the relative contribution of each cellular compartment in the expression of follicular 17β-HSD4 transcript, isolated preparations of granulosa and theca interna cells were obtained from follicles isolated between 0 and 39 h post hCG (Fig. 5). Results revealed that both cell types were involved in 17β-HSD4 mRNA expression within the follicle, with the granulosa cell layer apparently providing the predominant site of 17β-HSD4 induction. In granulosa cells, a significant increase in 17β-HSD4 mRNA was observed at 30, 36 and 39 h post hCG ($P < 0.05$; Fig. 5A). In contrast, the increase in 17β-HSD4 transcript in theca interna was more modest, became significant only at 24 h post-hCG ($P < 0.05$), and levels tended to decrease at 36 and 39 h after hCG treatment.

Regulation of 17β-HSD4 protein in preovulatory follicles

To determine whether the increase in 17β-HSD4 mRNA in equine preovulatory follicles after hCG treatment was associated with changes in protein levels, a specific anti-equine 17β-HSD4 antibody was produced and used in immunoblots to study the regulation of the 17β-HSD4 protein in follicles isolated between 0 and 39 h post hCG. Results showed that the antibody recognized three major immunoreactive signals; a 80,000 M_r band believed to correspond to the full-length protein, and 45,000 and 32,000 M_r bands thought to represent proteolytic fragments (Fig. 6A). Interestingly, Western blot analyses revealed that the 80,000 M_r signal increased significantly between 24 and 39 h post hCG, as compared to 0 h ($P < 0.05$; Fig. 6). In contrast, the 45,000 M_r band remained constant between 0 and 30, and decreased at 36 and 39 h post-hCG, whereas no significant difference was observed across time for the 32,000 M_r band (Fig. 6).

To determine the cellular localization of 17β-HSD4 protein expression in preovulatory follicles, immunohistochemistry was performed on sections of equine follicles isolated 0 and 39 h h after hCG treatment (n = 4 follicles per time point).

Results showed that follicles isolated prior to hCG treatment (0 h) had a very compact granulosa cell layer, and light but detectable 17β-HSD4 staining was observed in the granulosa and theca layers (Fig. 7A). The administration of hCG caused a marked expansion of the granulosa cell layer, and severe oedema and vascular changes in theca layers, as previously characterized (43). A marked increase in 17β-HSD4 immunoreactivity was observed in granulosa and theca cells of follicles isolated 39 h post-hCG (Fig. 7B-D). Also, the elevated levels of 17β-HSD4 mRNA detected by RT-PCR in the testis (Fig. 3) appeared associated with intense 17β-HSD4 immunostaining in Leydig cells of equine testicular sections (Fig. 7E).

DISCUSSION

This study demonstrates for the first time that the process of luteinization/ovulation induced by hCG in preovulatory follicles is accompanied by an increase in 17β-HSD4, a multifunctional protein involved in steroid and fatty acid metabolism (16-19). The process of follicular luteinization has previously been associated with dramatic changes in expression of other steroidogenic enzymes and proteins. Most notably, they include a marked decrease or loss in P45017α and P450AROM which contribute in reducing estrogen biosynthesis, and an increase in steroidogenic acute regulatory protein (StAR) and cytochrome P450 cholesterol side-chain cleavage (P450SCC) which promote enhanced progesterone synthesis (28, 30, 44, 45). Although differences in cellular localization and time-course regulation of these enzymes have been observed among species, they are clearly recognized as part of the common biochemical basis for the switch in predominant estradiol-17β to progesterone production occurring after the LH surge, including in the equine preovulatory follicle (31, 46, 47). Results from the present study suggest that the induction of an estradiol-17β inactivating enzyme like 17β-HSD4 may represent a novel and complementary mechanism contributing to the reduction in levels of active estrogens during follicular luteinization.

Previous investigations on the expression of 17β-HSD4 in the ovary have primarily been limited to tissue distribution analyses using commercial human RNA

membranes (13, 48), or Northern blots and immnohistochemistry performed on various porcine tissues collected randomly at the slaughterhouse (42). Overall, these studies revealed that 17β-HSD4 was expressed at low to moderate levels in ovarian tissues, which agrees with levels observed in the wall of equine preovulatory follicles collected prior to hCG treatment. However, this is the first study to investigate the expression of the enzyme in a developmental series of preovulatory follicles collected during gonadotropin treatment, and to establish the presence of high levels of 17β-HSD4 36 h post-hCG. High levels of 17β-HSD4 were also observed in the equine testis, with expression located primarily in Leydig cells as observed in the pig (42). The ubiquitous pattern of expression of the enzyme in equine tissues, as well as differences in the predominant sites of 17β-HSD4 expression among tissues, are also in keeping with observations in other species (13, 21, 42, 48, 49). However, the presence of moderate levels of 17β-HSD4 in equine liver, heart, kidney and muscle compares with reports in human (13, 48).

The control of 17β-HSD4 expression *in vivo* remains largely uncharacterized. Progesterone has been identified as a positive regulator of the enzyme in the pig uterus (21, 50), but it is not clear if this effect is conserved in other species (51). Several environmental toxicants acting as peroxisome proliferators have also been shown to affect steroid metabolism and upregulate the expression of 17β-HSD4, a peroxisomal enzyme (23, 52). The net effect of these chemicals in female rats was to reduce serum levels of estradiol-17β, which was originally proposed to involve the increased expression of estrogen-inactivating 17β-HSD4 in the liver (23). Interestingly, peroxisome proliferators have also recently been shown to suppress P450AROM and increase 17β-HSD4 in cultured rat granulosa cells, providing evidence for a concomitant effect in the ovary (51, 53). The present study identifies high/ovulatory levels of gonadotropins as a physiological regulator of 17β-HSD4 in the preovulatory follicle. Basal expression and hCG-dependent regulation of follicular 17β-HSD4 mRNA was observed in both granulosa and theca interna cells, but the up-regulation of the transcript clearly appeared more pronounced in the granulosa cell compartment. The development and use of an equine anti-17β-HSD4 antibody revealed the presence of three

immunoreactive bands thought to correspond to full-length (80,000 M_r band) and proteolytic C-terminal (45,000 M_r band = hydratase + SCP2-like domain) and N-terminal (32,000 M_r band = hydrogenase domain) fragments, based on reports in other species (18, 54). The hCG-dependent increase in full-length 17β-HSD4 protein (80,000 M_r band) detected by immunoblots were in keeping with results identified for the transcript. However, the precise biochemical basis for the apparent reduction in the 45,000 M_r band and unchanged low levels of the 32,000 M_r band remains unclear, and will require further investigations. Likewise, the relatively robust increase in 17β-HSD4 immmunoreactivity observed in theca cells at 39h post-hCG was somewhat surprising given the relatively low levels of transcripts at that time, and additional studies will be needed to resolve this issue.

The multifunctional nature of the 17β-HSD4 protein led investigators to question the predominant role of the enzyme under physiological conditions. Because the rate of fatty acyl-coA oxidation is several fold higher than that for estradiol-17β, some proposed that the enzyme may be primarily involved in fatty acid metabolism, and that estrogen inactivation may represent only a secondary activity *in vivo* (4, 18, 19). However, the enzymatic parameters (V_{max}, K_m) of 17β-HSD4 for estradiol-17β oxidation is very similar to that of 17β-HSD2, another 17β-HSD isoform expressed in numerous tissues, essentially involved in inactivating circulating androgens and estrogens, and generally thought to protect peripheral tissues from excessive steroid hormone actions (9, 10, 18). Based on previous investigations and on results from the present study, it is tempting to propose that the predominant physiological role of 17β-HSD4 may be tissue- and stage-dependent. For example, its expression in liver may serve primarily functions linked to lipid metabolism, whereas the gonadotropin-dependent induction of 17β-HSD4 in preovulatory follicles may relate predominantly to estrogen metabolism. There is a tremendous amount of estradiol-17β in the follicular fluid of equine preovulatory follicles, with estimates ranging from 60 to 135 μg (2-3 μg of estradiol-17β/ml; total volume of fluid = 30-45 ml [55-57]). The concentration of the hormone in equine follicles (7.3-11.0 μM) is therefore several fold higher than the reported K_m for this substrate in other species (0.2 μM; 13, 18). Thus, the dramatic

reduction in levels of estradiol-17β during luteinization may result not only from a decrease in expression of biosynthetic enzymes, but also from the induction of an intra-ovarian 17β-HSD4-dependent estrogen inactivating system. However, one should not exclude that the other domains of the multi-functional protein are also involved in ovarian functions. The localization of 17β-HSD4 in peroxisomes, which are known to play a major role in cholesterol biosynthesis (58, 59), and the presence of a SCP 2-like transfer domain in the protein remain intriguing and point to a potential implication in cholesterol transport.

In summary, this study is the first to characterize the primary structure of equine 17β-HSD4, to demonstrate the regulation of the enzyme during follicular luteinization, and to identify ovulatory levels of gonadotropins as a positive regulator of 17β-HSD4 expression. The precise physiological significance and molecular control of 17β-HSD4 induction in preovulatory follicles will remain to be elucidated. Interestingly, the luteinization/ovulatory process is also accompanied by an induction in follicular progesterone synthesis, progesterone receptors, and peroxisome proliferator-activated receptors (PPARs) (31, 59-62). Given the putative role of progesterone and PPARs in 17β-HSD4 expression (21, 23, 50, 52, 53), it will be important to determine whether they are intermediates in the gonadotropin-dependent induction of the enzyme in follicular cells. With its relatively large size (40-45 mm in diameter) and long ovulatory process (39-42 h post hCG), the equine preovulatory follicle will provide an interesting model to investigate some of these issues.

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LEGENDS FOR FIGURES

Fig. 1. Nucleotide sequence of equine 17β-HSD4. The equine 17β-HSD4 cDNA was cloned by a combination of RT-PCR and library screening, as described in *Materials and Methods*, and shown to consist of a 5'-untranslated region of 103 bp (lowercase letters), an open reading frame of 2208 bp (uppercase letters), and a 3'-untranslated region of 341 bp (lowercase letters). The internal 17β-HSD4 cDNA fragment obtained by RT-PCR is shown in bold types, the translation initiation (ATG) and stop (TGA) codons are underlined, two overlapping consensus polyadenylation signals are double-underlined, and numbers on the right refer to the last nucleotide on that line. The nucleotide sequence was submitted to GenBank (accession number AY499667).

Fig. 2. Predicted amino acid sequence of equine 17β-HSD4 and comparisons with other mammalian homologues. The amino acid sequence of equine (equ) 17β-HSD4 is aligned with the human (hum), mouse (mou), rat, guinea pig (gpg) and pig homologues. Identical residues are marked with a printed period, hyphens indicate gaps in protein sequences created to optimize alignment, and numbers on the right refer to the last amino acid on that line. Box regions include a N-terminal short chain dehydrogenase/reductase (SDR), a central hydratase, and a C-terminal sterol carrier protein 2 (SCP2)-like domain. Five consensus N-linked glycosylation sites are indicated in bold types (the asparagine residue is marked with an asterisk), and the consensus AKL peroxisomal targeting signal located at the extremity of the C-terminus is underlined.

Fig. 3. Expression of 17β-HSD4 mRNA in equine tissues. Total RNA was extracted from various equine tissues, and samples (100 ng) were analyzed for 17β-HSD4 and rpL7a (control gene) content by a semiquantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. *A*, expression of 17β-HSD4 mRNA in equine tissues. *B*, expression of rpL7a mRNA in equine tissues. The number of PCR cycles for each gene was within the linear range of amplification, and they represented 15 and 18 cycles for 17β-HSD4 and rpL7a, respectively. Numbers on the right indicate the size of the PCR fragment.

Fig. 4. Regulation of 17β-HSD4 transcript by hCG in equine preovulatory follicles. Preparations of follicle wall were obtained from preovulatory follicles isolated between 0 and 36 h after hCG, and of corpora lutea (CL) isolated on day 8 of the estrous cycle. Samples (100 ng) of total RNA were analyzed for 17β-HSD4 and rpL7a content by a semiquantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. *A*, regulation of 17β-HSD4 mRNA in equine follicles (one representative follicle per time point). *B*, constitutive expression of rpL7a mRNA in the same follicles. Numbers on the right indicate the size of the PCR fragment. *C*, relative changes in 17β-HSD4 transcripts in equine follicles after hCG treatment. The intensity of 17β-HSD4 signal was normalized with the control gene rpL7a (n = 5-6 distinct follicles [i.e. animals] per time point, and n = 3 different corpora lutea). Bars marked with an asterisk are significantly different from 0 h post-hCG ($P < 0.05$).

Fig. 5. Regulation of 17β-HSD4 mRNA in equine granulosa and theca interna cells. Preparations of granulosa cells (A) and theca interna (B) were isolated from equine preovulatory follicles between 0-39 h post-hCG, and samples (100 ng) of total RNA were analyzed for 17β-HSD4 and rpL7a content by a semi-quantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. The 17β-HSD4 signal was normalized with the control gene rpL7a, and results are presented as a ratio of 17β-HSD4 to rpL7a (mean ± SEM; n = 4 samples [i.e. mares] per time point). *Bars* marked with an asterisk are significantly different from 0 h post-hCG ($P < 0.05$). *Inserts* show representative results of 17β-HSD4 and rpL7a mRNA levels from one sample per time point.

Fig. 6. Regulation of 17β-HSD4 protein by hCG in equine preovulatory follicles. Protein extracts were prepared from preovulatory follicles isolated 0, 12, 24, 30, 36 and 39 h after hCG treatment, and analyzed by one-dimensional SDS-PAGE and immunoblotting using a specific polyclonal antibody raised against a fragment of the equine 17β-HSD4 protein, as described in *Materials and Methods.* A, Results from

protein extracts (60 μg/lane) of one representative follicle per time point are shown. Markers on the right indicate the migration of molecular weight (M_r) standards. B, Extracts from follicles isolated between 0 and 39 h post-hCG (n = 4 distinct follicles [i.e. mares] per time point) were analyzed by immunoblotting, and the relative intensity of 80,000 and 45,000 M_r bands were quantified by densitometry. Results are presented as mean ± SEM, and bars marked with an asterisk are significantly different from 0 h post-hCG ($P < 0.05$).

Fig. 7. Immunohistochemical localization of 17β-HSD4 in equine preovulatory follicles. Immunohistochemistry was performed on formalin-fixed sections of preovulatory follicles isolated 0 and 39 h after hCG treatment and of equine testicular tissues, as described in *Materials and Methods.* Results show relatively weak but detectable 17β-HSD4 staining in granulosa and theca interna cells of a preovulatory follicle obtained 0 h post-hCG (A), but a marked increase in signal intensity in both cell types of distinct follicles isolated 39 h post-hCG (B, C and D). E, Intense 17β-HSD4 immunoreactivity is observed in Leydig cells of equine testicular sections. F, Control staining from the follicular tissue presented in D was negative when the primary antibody was replaced with PBS. Magnification, ×200 (A-C, and E) and ×400 (D and F).

FIGURE 1

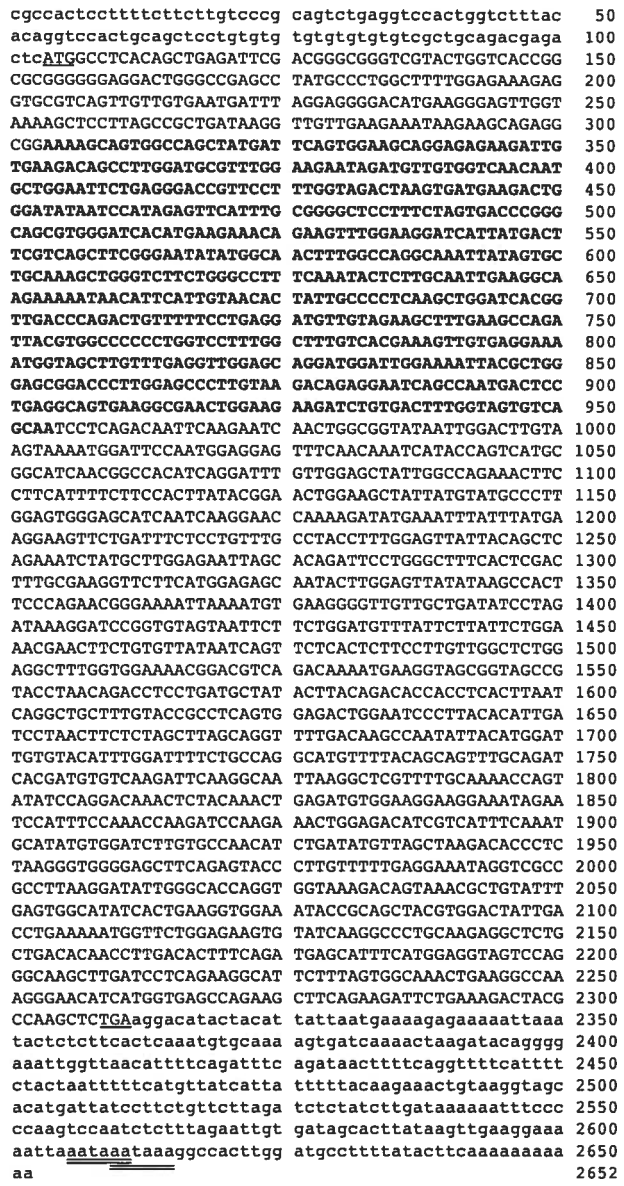
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FIGURE 2

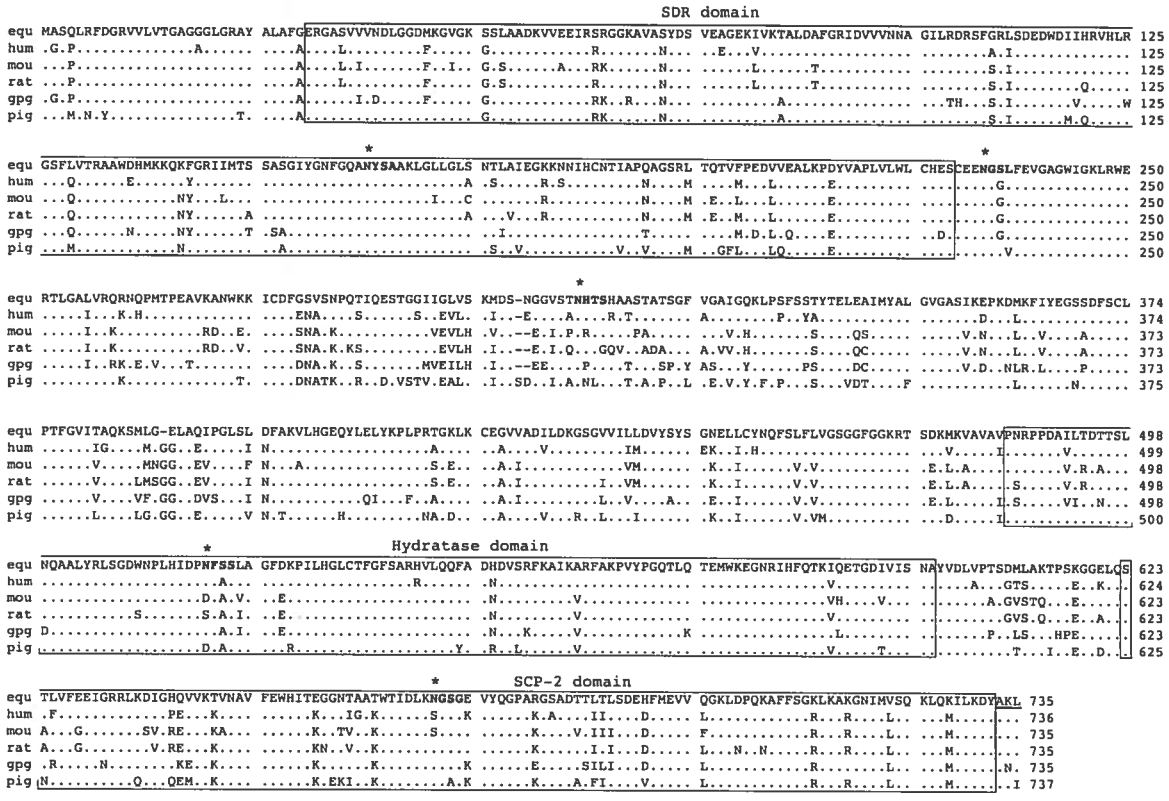
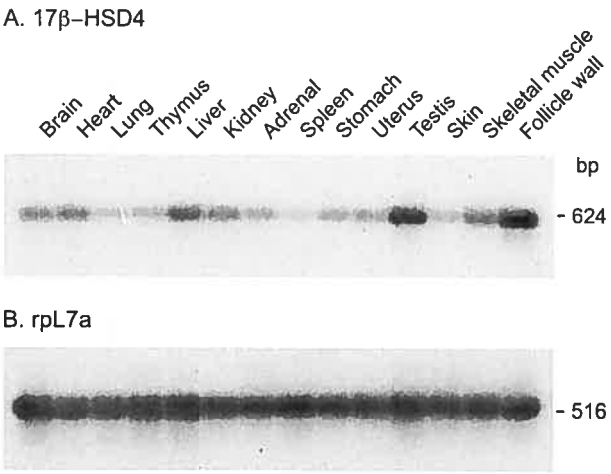


FIGURE 3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Brain
Heart
Lung
Thymus
Liver
Kidney
Adrenal
Spleen
Stomach
Uterus
Testis
Skin
Skeletal muscle
Follicle wall
bp
- 624
B. rpL7a
- 516

FIGURE 4

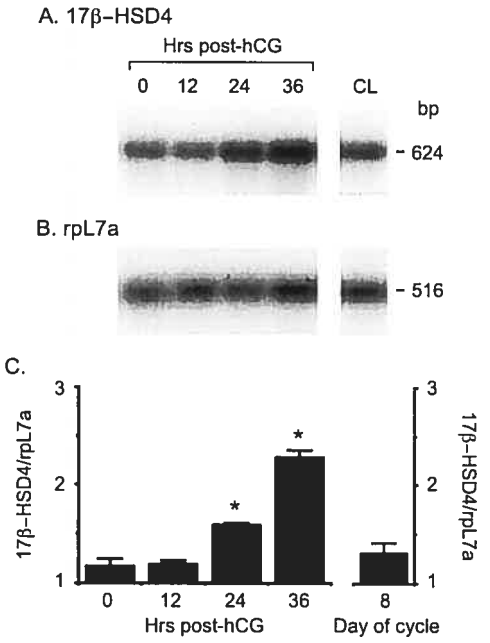

A. 17β-HSD4
Hrs post-hCG
0 12 24 36
CL
bp
624
B. rpL7a
516
C.
17β-HSD4/rpL7a
3
2
1
*
*
0 12 24 36
Hrs post-hCG
8
Day of cycle

FIGURE 5

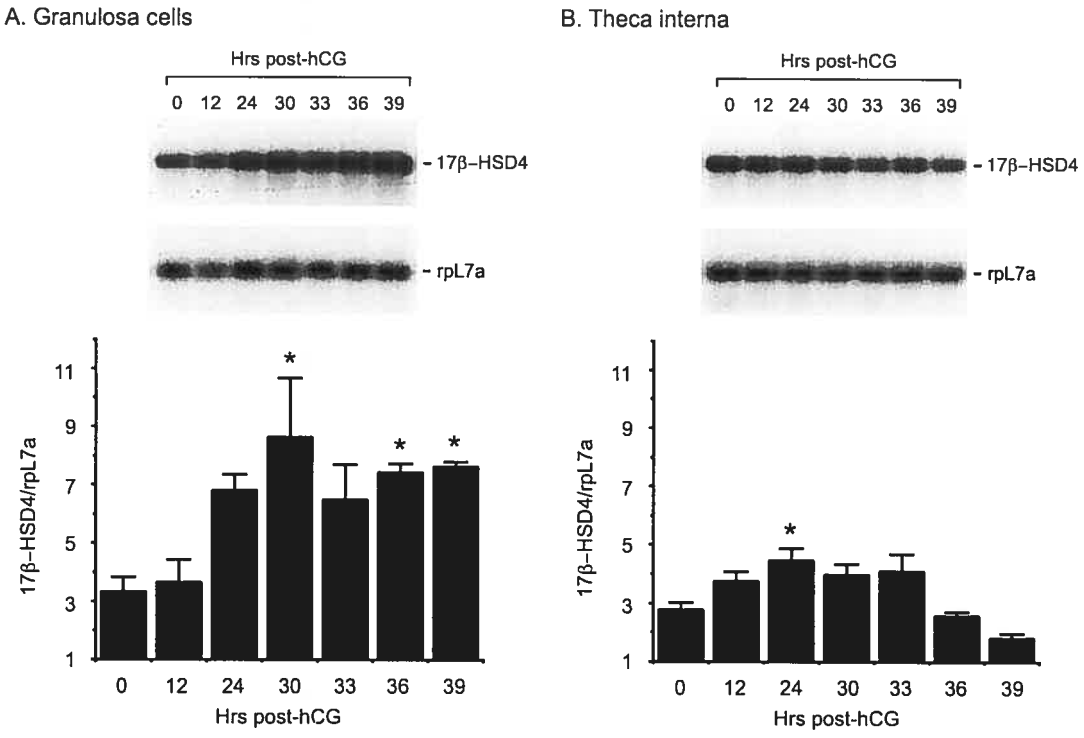

A. Granulosa cells
B. Theca interna
Hrs post-hCG
0 12 24 30 33 36 39
17β-HSD4
rpL7a
17β-HSD4/rpL7a
1
3
5
7
9
11
*
Hrs post-hCG

FIGURE 6

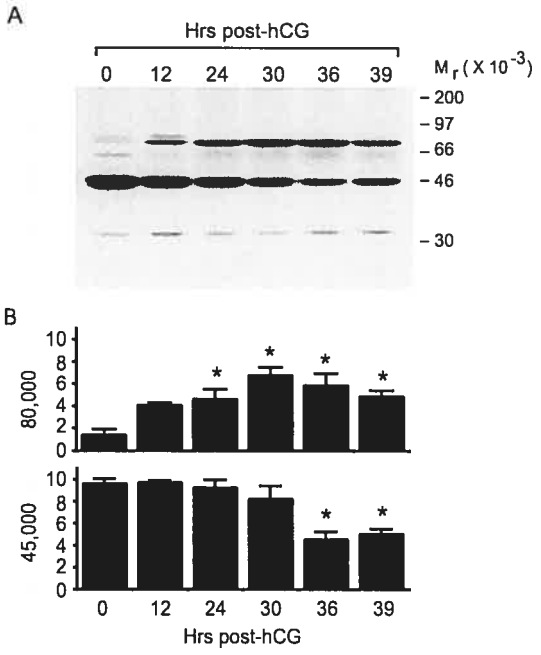
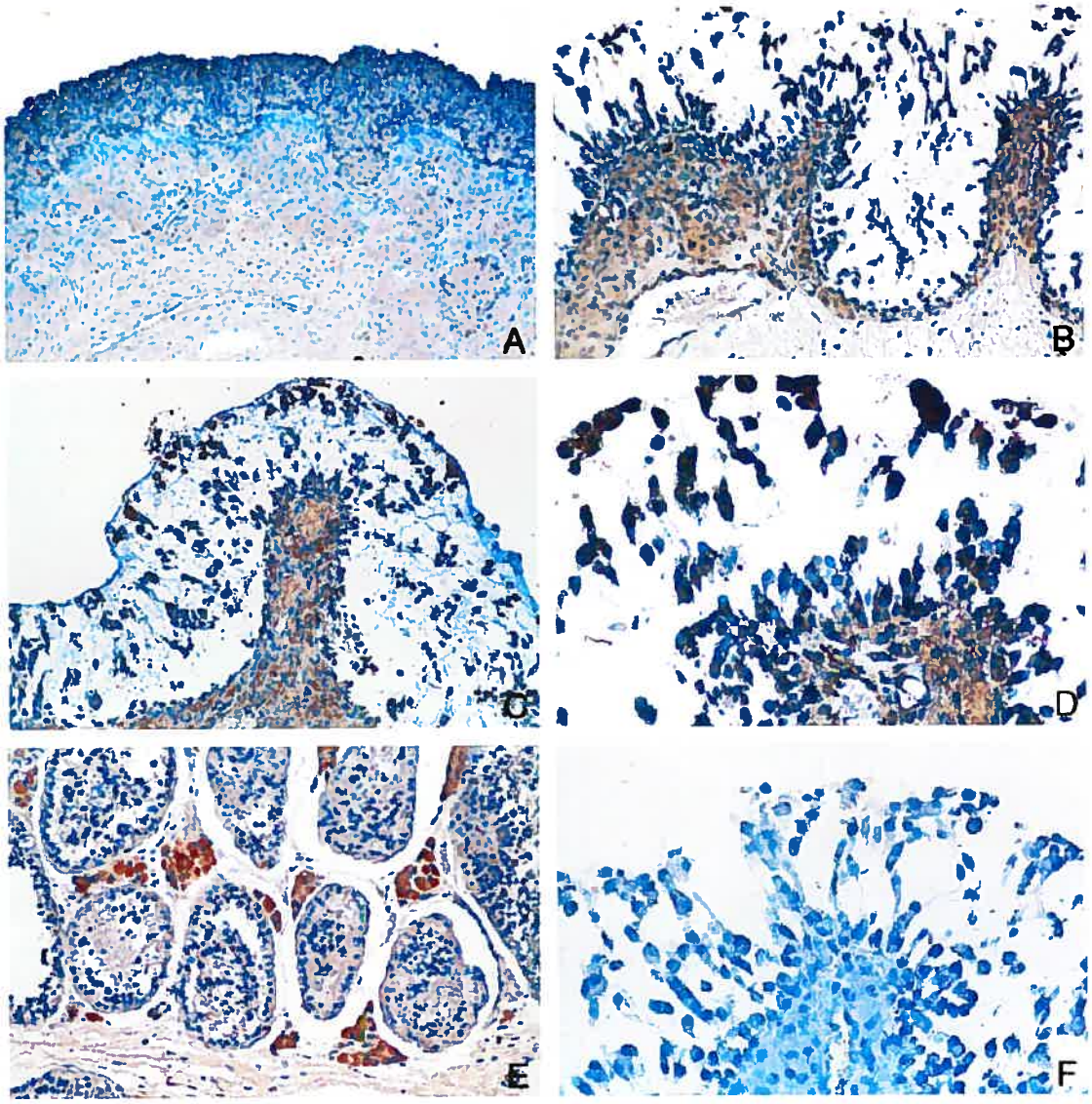

A
Hrs post-hCG
0
12
24
30
36
39
M_r (X 10^{-3})
200
97
66
46
30
B
80,000
45,000
10
8
6
4
2
0
*
0
12
24
30
36
39
Hrs post-hCG

FIGURE 7



SUMMARY OF ARTICLE FOUR

Title: Human chorionic gonadotropin-dependent upregulation of genes responsible for estrogen sulfoconjugation and export in granulosa cells of luteinizing preovulatory follicles. **Kristy A. Brown**, Monique Doré, Jacques G. Lussier and Jean Sirois. *Submitted to Endocrinology (April 2006).*

Thesis author's contribution to this work: As the primary author, I was responsible for all aspects of this article. Secondary authorship credits reflect support for immunohistochemical analyses. Tertiary and quaternary authorship credits reflect co-directorship and directorship, respectively.

Summary:

- The equine EST and ABCC1 cDNAs were characterized by a combination of RT-PCR and 3'-RACE reactions on pooled RNA from equine preovulatory follicles.
- Sequence analysis of the EST transcript revealed an open reading frame that encoded a 296-amino acid protein, which was more than 80% identical to the human orthologue. The P-loop motif, which is highly conserved in all sulfotransferases, was also present in the equine EST protein, as well as other amino acids known to be involved in the sulfoconjugation of estrogens.
- Sequence analysis of the ABCC1 transcript revealed an open reading frame that encoded a 1531-amino acid protein, called MRP1, which was more than 90% identical to the bovine orthologue. The Walker motifs, involved in nucleotide binding, as well as the signature amino acids of the active transport family and the putative glycosylation sites, were highly conserved in the equine MRP1 protein.
- The regulation of the equine EST and ABCC1 transcripts was examined in equine preovulatory follicles by semi-quantitative RT-PCR in samples obtained at intervals after hCG. Results from Southern blot analyses revealed very low basal levels of EST mRNA expression prior to hCG, with a dramatic increase observable from 30 to 39 h post-hCG occurring selectively in the granulosa cell layer. Levels of ABCC1

transcript were detectable in preovulatory follicles prior to hCG, but increased significantly in granulosa cells between 12 and 39 h post-hCG.

- The localization of the equine EST and MRP1 proteins in preovulatory follicles as examined by immunohistochemistry, revealed that EST expression to be near undetectable in follicles prior to hCG treatment with higher levels being seen in both cell types 39 h after hCG. Some localization for MRP1 was observed in granulosa cells at 0 h post-hCG with a dramatic increase detectable at 39 h post-hCG.

Work's contribution to the advancement of science:

- This work characterizes for the first time the primary structure of the equine EST and ABCC1 cDNAs and proteins.
- It demonstrates the gonadotropin-dependent induction of the EST and ABCC1 transcripts between 30 and 39 h, and between 12 and 39 h post-hCG, respectively, as well as shows the co-localized expression of the equine EST and MRP1 proteins in granulosa cells of preovulatory follicles after hCG.
- Overall, it supports the hypothesis that enzymes involved in estrogen sulfoconjugation and export are induced during the ovulatory process. This may provide additional evidence that active estrogens need to be eliminated during this process.

Human Chorionic Gonadotropin-Dependent Upregulation of Genes Responsible for Estrogen Sulfoconjugation and Export in Granulosa Cells of Luteinizing Preovulatory Follicles

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Short Title: EST and ABCC1 induction in preovulatory follicles

Key words: EST, ABCC1, MRP1, granulosa cells, preovulatory follicles, luteinization, ovary, horse

K.A.B., M.D., J.G.L., and J.S. have nothing to declare.

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ABSTRACT

Estrogen sulfotransferase (EST) is responsible for the sulfoconjugation of estrogens, thereby changing their physical properties and preventing their action via the estrogen receptors. These sulfoconjugated steroids no longer diffuse freely across the lipid bilayer, instead, they are exported by members of the ATP-binding cassette family, like ABCC1. The objective of this study was to investigate the regulation of EST and ABCC1 during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The transcripts for EST and ABCC1 were cloned by reverse transcription-polymerase chain reaction (RT-PCR) and the regulation of their mRNAs was studied in preovulatory follicles obtained during estrus at 0, 12, 24, 30, 33, 36 and 39 h after hCG. Results obtained from RT-PCR/Southern blot analyses showed significant changes in steady-state levels of both EST and ABCC1 mRNA after hCG treatment ($P<0.05$). In granulosa cells, a significant increase in EST transcript was observed 30-39 h post-hCG. Similarly, ABCC1 transcript levels were induced in granulosa cells 12-39 h post-hCG. In contrast, no significant changes in either EST or ABCC1 were detected in theca interna samples after hCG. The increase in EST and ABCC1 transcripts observed in granulosa cells was reflected in preparations of intact follicle walls, suggesting that the granulosa cell layer contributes the majority of EST and ABCC1 expression in preovulatory follicles. The present study demonstrates that follicular luteinization is not only accompanied by a decrease in 17β-estradiol biosynthesis, but also with an increase in expression of genes responsible for estrogen inactivation and elimination from granulosa cells, such as EST and ABCC1, respectively.

INTRODUCTION

The biotransformation of molecules through sulfoconjugation results in striking changes in their physicochemical properties. The addition of a highly charged sulfonate group (SO_3^-), which remains fully ionized at any pH found in biological systems, causes hydrophobic compounds to be converted to hydrophilic ones, thereby resulting in increased water solubility as well as conformational changes in both low- and high-molecular-weight molecules (1). Sulfotransferases have been classified into two different groups depending on whether they are membrane-bound or soluble. More than 44 soluble or cytosolic sulfotransferases have been identified in mammals, with substrates ranging from endogenous compounds like hormones and neurotransmitters to drugs and xenobiotics (2). The current nomenclature classifies the cytosolic sulfotransferases into five SULT families, sharing less than 40% similarity with each other. The SULT1 family members act on phenolic drugs and cathecolamines (SULT1A), thyroid hormones (SULT1B), xenobiotics (SULT1C), and estrogenic steroids (SULT1E) (3).

Estrogen sulfotransferase (EST, encoded by SULT1E1) is a 30- to 36-kDa protein that catalyses the sulfoconjugation of estrogens at the 3-hydroxyl position. It utilizes 3'-phospho-adenosine-5'phosphosulfate (PAPS) as its sulfonate source, which is produced in a two step ATP-dependent reaction by the enzyme PAPS synthetase (4, 5). Once sulfonated, 17β-estradiol (E2-S) no longer binds to its cognate receptor (6) and its ability to diffuse freely across the lipid bilayer is greatly compromised. The first member of the ATP-binding cassette superfamily, P-glycoprotein, was identified in tumor cells exhibiting resistance to multiple chemotherapeutic agents (7). The ABC superfamily has since then been largely characterized, representing the largest family of transmembrane proteins, and demonstrated as being responsible for the ATP-dependent transport of many substrates, including hormones, lipids, drugs, and other toxins. Most ABC transporters are unidirectional, moving compounds from the cytoplasm to the outside of the cell (8). Member C1 of the ATP-binding cassette superfamily (ABCC1) translates into a 17-transmembrane-domain 190-kDa glycoprotein, called multidrug resistance protein 1 (MRP1), whose role as the primary transporter of many organic anions has

been largely characterized (9). It was first identified in the small cell lung carcinoma cell line NCI-H69 and has been shown to transport many glutathione-conjugated drugs, as well as leukotrienes and more recently, has been demonstrated to export estrone-3-sulfonate (E1-S) and E2-S (10-13).

In mammals, follicular luteinization/ovulation is triggered by a surge in luteinizing hormone (LH) and is characterized by numerous physical and biochemical changes, including the decreased production of 17β-estradiol (14-16). This loss in 17β-estradiol biosynthetic capacity has been explained by a marked decrease in the expression of key steroidogenic enzymes involved in the follicular production of active estrogens (15, 17). However, little is known about the regulation of enzymes/proteins responsible for the inactivation and elimination of estrogens, as mediated by EST and ABCC1, respectively, during this period. In the present study, the equine preovulatory follicle is used as model to investigate the regulation of EST and ABCC1 during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The specific objectives were to clone equine EST and ABCC1, determine the expression of their mRNAs, as well as EST protein in preovulatory follicles after hCG treatment, and correlate their expression with the presence of sulfoconjugated steroids in the follicular fluid.

MATERIALS AND METHODS

Materials

The Prime-a-Gene labeling system, pGEM-T Easy Vector System I was purchased from Promega Corp. (Madison, WI). The [a-32P]dCTP was purchased from PerkinElmer Canada, Inc. (Woodbridge, ON, Canada), and the QuickHyb hybridization solution was obtained from Stratagene Cloning Systems (La Jolla, CA). The TRIzol total RNA isolation reagent, SuperScript II reverse transcriptase, 1-kilobase DNA ladder, and synthetic oligonucleotides were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). The Qiagen OneStep Reverse Transcription-Polymerase Chain Reaction (RT-PCR) System was obtained from Qiagen, Inc. (Mississauga, ON, Canada). The Expand High Fidelity DNA Polymerase was purchased from Roche Diagnostics (Laval, PQ, Canada). Biotrans nylon membranes (pore size, 0.2

mm) were obtained from ICN Pharmaceuticals, Inc. (Montréal, PQ, Canada), and all electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA). The hCG was obtained from The Buttler Co. (Columbus, OH). The equine-specific antibodies were produced by New England Peptide, Inc. (Gardner, MA). The Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA). The diaminobenzidine tetrahydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO).

Cloning of the equine EST and ABCC1 cDNAs

The equine EST transcript was isolated in fragments using a multistep cloning strategy (Fig. 1A). A 515-base pair (bp) reverse transcription-PCR product (RT-PCR) (Fig. 1Aa) was initially cloned from pooled equine ovarian RNA samples isolated from a preovulatory follicle, and an ovulatory follicle (36 h post-hCG treatment). Ovarian tissues were isolated and RNA was extracted as previously described (18). RT-PCR was performed using the OneStep RT-PCR System (Qiagen) as directed by the manufacturer, using 500 ng of RNA and oligonucleotide primers designed by sequence alignments of known EST species homologues (primers 1 and 2; Fig. 1B). Following agarose gel electrophoresis, the RT-PCR product was excised and ligated into the PGEM-T Easy plasmid vector (Promega), and proper recombinant plasmids were identified from transformed bacterial colonies using standard techniques (19). Sequencing of the insert was performed by the Service de Séquençage de l'Université Laval (Québec, PQ, Canada) using vector-based T7 and Sp6 oligonucleotide primers. Sequences obtained from the initial RT-PCR product served to design specific oligonucleotides for a second RT-PCR (primer 4; Fig. 1B) and 3'-Rapid Amplification of cDNA Ends (RACE) reactions (primers 6 and 8; Fig. 1B). The second RT-PCR also required the design of a sense oligonucleotide from sequence alignments of species homologues (primer 3; Fig. 1B), and yielded a 290-bp fragment, that partially overlapped the initial RT-PCR product and included the translation initiation codon (Fig. 1Ab). The 3'-RACE was performed as previously described (20), except 5 μg of pooled ovarian tissue RNA (as described above) was used as a template for the initial RT reaction. Briefly, an RT reaction was performed using a poly-dT oligonucleotide with anchor sequences at its 5'

end (primer 5; Fig. 1Ac). This was followed by nested PCR reactions using oligonucleotide primers that bound to the anchor sequence in conjunction with EST-specific forward primers. The product of the second PCR reaction (Fig. 1Ac) was isolated and sequenced as described above. A clone encompassing the entire coding region was isolated by RT-PCR (primers 10 and 11; Fig 1Ad) and found to correspond with the deduced primary EST transcript reported herein (Fig. 1A).

Due to the large size of the equine ABCC1 transcript, it was cloned using a multistep cloning strategy (Fig. 1C). A 981-base pair (bp) reverse transcription-PCR product (RT-PCR) (Fig. 1Ca) was initially cloned from pooled equine ovarian RNA samples isolated from a preovulatory follicle, and an ovulatory follicle (36 h post-hCG treatment). RT-PCR was performed using the OneStep RT-PCR System (Qiagen) as directed by the manufacturer, using 500 ng of RNA and oligonucleotide primers designed by sequence alignments of known ABCC1 species homologues (Fig. 1C). Subcloning and sequencing were done as described above. Sequences obtained from the initial RT-PCR product served to design a specific oligonucleotide for a second RT-PCR reaction (primer 4; Fig. 1Cb). The second RT-PCR also required the design of a sense oligonucleotide from sequence alignments of species homologues (primer 3; Fig. 1D) and yielded a 440-bp fragment, that partially overlapped the initial RT-PCR product and included the translation initiation codon (Fig. 1Cb). A third RT-PCR product was obtained, again, using sense and antisense primers designed by sequence alignments of known ABCC1 homologues (primers 5 and 6; Fig. 1Cc). The 1267-bp fragment was sequenced and used for the design of a second equine-specific primer (primer 7; Fig. 1D). The fourth and fifth RT-PCR products were obtained by using equine-specific sense primers (primers 7 and 9; Fig. 1D) and antisense primers designed from sequence alignment of known homologues (primers 8 and 10). These yielded 1359- (Fig. 1Cd) and 1029-bp (Fig. 1Ce) fragments, respectively. Finally, a sixth RT-PCR reaction was done using equine-specific sense and antisense primers (primers 11 and 12, Fig. 1D) and yielding a 1228-bp fragment that was then sequenced (Fig. 1Cf).

Equine tissues and RNA extraction

Testicular tissues were obtained from the Large Animal Hospital of the Faculté de médecine vétérinaire (Université de Montréal) following a routine castration, whereas other nonovarian tissues were collected at a local slaughterhouse. Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares as previously described (21). Briefly, when preovulatry follicles reached 35 mm in diameter during estrus, the ovulatory process was induced by injection of hCG (2500 IU, iv) and ovariectomies were performed via colpotomy using a chain écraseur at 0, 12, 24, 30, 33, 36, or 39 h post hCG (n = 4-6 mares / time point; ovulation occurring 36-48 h post hCG) (21, 22). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna. Ovariectomies were also performed on d 8 of the estrous cycle (d 0, day of ovulation) to obtain corpora lutea (n = 3 mares). All animal procedures were approved by the institutional animal use and care committee. Total RNA was isolated from tissues with TRIzol reagent (Invitrogen Canada Inc.), according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada).

Semiquantitative RT-PCR and Southern analysis

The OneStep RT-PCR System (Qiagen) was used for semi-quantitative analysis of EST, ABCC1 and rpL7a mRNA levels in equine tissues. Reactions were performed according to the manufacturer's directions, using sense (5' – CTGAAACAGATGGCAT CTCC - 3') and antisense (5' - GGTTTCCTTCCCAGAAATTGC - 3') primers specific for equine EST, sense (5' - AGCAGGACGAGCAGGACAACG - 3') and antisense (5' - GAGTCCACCGTGTCCAGCTCC - 3') primers specific for equine ABCC1, and sense (5' - ACAGGACATCCAGCCCAAACG - 3') and antisense (5' – GCTCCTTTGTCTTC CGAGTTG - 3') primers specific for equine rpL7a. These reactions resulted in the production of EST, ABCC1 and rpL7a DNA fragments of 359, 631 and 516 bp, respectively. Each reaction was performed using 100 ng total RNA, and cycling conditions were one cycle of 50 C for 30 min and 95 C for 15 min, followed by a variable number of cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 2 min. The

number of cycles used was optimized for each gene to fall within the linear range of PCR amplification and was 26 cycles for EST, 20 cycles for ABCC1 and 18 cycles for rpL7a. Following PCR amplification, samples were electrophoresed on 2% TAE-agarose gels, transferred to nylon membranes, and hybridized with corresponding radiolabeled EST, ABCC1 and rpL7a cDNA fragments using QuickHyb hybridization solution (Stratagene). Membranes were exposed to a phosphor screen, and signals were quantified on a Storm imaging system using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA).

Protein extracts, anti-equine EST and MRP1 antibodies and immunoblot analysis

Liver and preovulatory follicle extracts were prepared as previously described (23). Briefly, tissue was homogenized and sonicated on ice in TED buffer [20 mm Tris (pH 8.0), 50 mm EDTA, and 0.1 mm diethyldithiocarbamic acid) containing 1.0% Tween. The sonicate was centrifuged at 16,000 x g for 15 min at 4 C. The recovered supernatant (whole cell extract) was stored at -80 C until electrophoretic analyses were performed. Protein concentration was determined by the method of Bradford (24) (Bio-Rad protein assay). Samples (50 μg proteins) were resolved by one-dimensional SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (23). The equine-specific anti-EST polyclonal antibody was generated (New England Peptide, Inc.) using a peptide fragment encompassing amino acids Tyr^{281}-Ile^{296}, whereas the equine-specific anti-MRP1 antibody was generated (New England Peptide, Inc.) using a peptide fragment encompassing amino acids Glu^{1262}-Arg^{1277}. Membranes were either incubated with the polyclonal anti-equine EST antibody (1:1,000) or anti-MRP1 antibody (1:1,000), and immunoreactive proteins were visualized on Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) after incubation with the horseradish peroxidase-linked donkey antirabbit secondary antibody (1:10,000 dilution) and the enhanced chemiluminescence system (ECL Plus), following the manufacturer's protocol (Amersham Pharmacia Biotech).

Immunohistochemical localization of EST

Immunohistochemical staining was performed using the Vectastain ABC kit

(Vector Laboratories, Inc.), as previously described (21). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 μm-thick sections were prepared and deparaffined through a graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with diluted normal goat serum for 20 min at room temperature. The anti-EST antibody (1:100 dilution) and the anti-MRP1 (1:5,000 dilution) were diluted in PBS and applied, and sections were incubated overnight at 4 C. Control sections were incubated with PBS. After rinsing in PBS for 10 min, a biotinylated goat anti-rabbit antibody (1:222 dilution; Vector Laboratories, Inc.) was applied, and sections were incubated for 45 min at room temperature. Sections were washed in PBS for 10 min and incubated with avidin DH-biotinylated horseradish peroxidase H reagents for 45 min at room temperature. After washing with PBS for 10 min, the reaction was revealed using diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with Gill's hematoxylin stain and mounted.

Measurement of E1-S, E2-S, E1, and E2 concentrations in follicular fluid

The follicular fluid present in equine preovulatory follicles isolated between 0 and 39 h post-hCG was analyzed for E1, E2, E1-S and E2-S content by a gas chromatographic mass spectrometric method developed to measure steroid hormone levels in serum (25). Briefly, steroids were extracted from follicular fluid by liquid-liquid and solid-phase extraction. Derivatization reactions were performed to improve chromatographic and detection response of the steroids. Unconjugated steroids were quantified by means of a sensitive gas chromatographic/mass spectrometric (GC/MS) method, using chemical ionization (CI). The lower limit of quantification for the steroid measurements was evaluated at 0.075 ng/ml for both sulfoconjugated estrogens and 0.008 ng/ml and 0.002 ng/ml for E1 and E2, respectively.

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG administration on levels of EST and ABCC1 mRNA, and follicular fluid hormones. EST and ABCC1 mRNA levels were normalized with the control gene rpL7a before analysis. When

ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple comparisons of individual means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC).

RESULTS

Characterization of equine EST and ABCC1 cDNAs

To clone the equine EST transcript, RT-PCR was performed on ovarian RNA using oligonucleotide primers designed by sequence alignment of known EST species homologues. The resulting cDNA fragment (Fig. 1Aa) was sequenced and found to be highly homologous to EST transcripts identified thus far. A combination of RT-PCR and 3'-RACE reactions yielded cDNA products corresponding to all remaining coding regions, as well as the partial 5'-UTR and complete 3'-UTR (Fig. 1, Ab and Ac). A final RT-PCR was performed that encompassed the entire coding region, and upon sequencing confirmed that all RT-PCR products isolated thus far were from the same transcript. The deduced EST 1330-bp primary transcript encoded a 891-bp open reading frame (GenBank accession number DQ418452), which predicted a protein of 296 amino acid residues. This 296-amino acid protein is highly conserved when compared with human (P49888) and bovine (CAA39806) homologues. The equine EST has 81.0% identity at the amino acid level (Fig. 2A) and a 85.1% identity at the nucleic acid level when compared to human EST. All putative domains and conserved amino acids, as found in the P-loop motif, appear to be present in the equine enzyme (Fig. 2A).

The ABCC1 transcript was also cloned using ovarian RNA and oligonucleotide primers designed by sequence alignment of known ABCC1 species homologues. Many RT-PCR reactions were performed in order to obtain the entire coding region (Fig.1C, a-f) and all RT-PCR products had sufficient overlap to confirm they originated from the same transcript. The deduced ABCC1 4629-bp primary transcript encoded a 4596-bp open reading frame (Genbank accession number DQ418453), which encoded a protein of 1531 amino acid residues (Fig. 2B). This 1531-amino acid protein is highly conserved when compared with human (NP_004987) and bovine (NP_776648) homologues. The equine ABCC1 has 89.7% identity at the amino acid level (Fig. 2B) and a 88.2% identity

at the nucleic acid level when compared to human ABCC1. All putative transmembrane domains and conserved amino acids from the nucleotide-binding domain, as well as the N-linked glycosylation sites appear to be present in the equine enzyme (Fig. 2B).

Tissue distribution of equine EST and ABCC1 mRNA

To study the tissue distribution of equine EST and ABCC1, various equine tissues were obtained and the expression of both transcripts was examined by RT-PCR/Southern blot. Results showed that the EST and ABCC1 transcripts were expressed in many of the tissues studied (Fig. 3). Levels of EST mRNA were highest in brain, lung, liver, uterus and testis; moderate in adrenal, skin and skeletal muscle; low in heart, thymus, kidney, stomach, and a preovulatory follicle isolated 36 h after hCG (i.e. approximately 3-6 h before ovulation); and very low in spleen (Fig. 3A). Levels of ABCC1 transcript were highest in the preovulatory follicle isolated 36 h post-hCG; moderate in heart, testes and skeletal muscle; low in lung, thymus, kidney, spleen, and skin; and very low or absent in brain, liver, adrenal, stomach, and uterus (Fig. 3B). However, levels of the control gene rpL7a remained relatively constant in all tissues studied (Fig. 3C).

Regulation of EST and ABCC1 transcripts in preovulatory follicles and corpora lutea

The regulation of EST and ABCC1 mRNAs was studied in preovulatory follicles by RT-PCR/Southern blot, using follicles isolated during estrus at 0, 12, 24, and 36 h after the administration of an ovulatory dose of hCG. Total RNA was extracted from the follicle wall (theca interna with attached granulosa cells), as well as from three corpora lutea obtained on d 8 of the estrous cycle. Levels of equine EST mRNA were low, almost absent in equine preovulatory follicles prior to treatment with hCG (0 h), but were clearly induced at 36 h post-hCG (Fig. 4A). The EST transcript expression returned to basal levels in the corpus luteum at day 8 of the cycle (Fig. 4A). When results from multiple follicles and corpora lutea were expressed as ratios of EST to rpL7a, a significant increase in EST transcript was detected in follicles at 36 h post hCG ($P < 0.05$; Fig. 4C). No change in rpL7a transcript was detected after gonadotropin treatment (Fig. 4B). When ABCC1 mRNA was examined in the same follicles, levels

were low prior to hCG treatment with an increase observable 12-36 h post hCG (Fig 4D). The ABCC1 transcript expression returned to basal levels in the corpus luteum at day 8 of the cycle (Fig. 4D). These results were then expressed as ratios of ABCC1 to rpL7a and a significant increase in ABCC1 transcript was detected in follicles isolated between 12 and 36 h post-hCG ($P < 0.05$; Fig. 4F). Again, no change in rpL7a transcript was observed after gonadotropin treatment (Fig. 4E).

In order to determine which cell type in the equine follicle wall was responsible for the expression of the EST and ABCC1 transcripts, granulosa and theca interna cells were isolated from follicles obtained between 0 and 39 h post hCG (Fig. 5). Results indicated that granulosa cells were the predominant site of both EST and ABCC1 transcript regulation. In granulosa cells, the increase in EST became significant between 30 and 39 h post hCG ($P < 0.05$; Fig. 5Aa), whereas ABCC1 mRNA expression was significantly increased 12-39 h post hCG ($P < 0.05$; Fig. 5Ba). Results demonstrated a slight, yet unsignificant, transient induction of both EST and ABCC1 mRNAs in theca interna cells (Fig. 5Ab and 5Bb, respectively).

Expression of EST and MRP1 proteins in equine preovulatory follicles

The gonadotropin-dependent induction of EST and ABCC1 was studied at the protein level by immunohistochemistry in follicles at 0 and 39 h post hCG. As shown, the antibody raised in rabbit recognized the equine EST protein from equine liver cell extracts, with a clear band appearing at approximately 35 kDa (Fig. 6A). Immunohistochemical results demonstrated a dramatic increase in EST production after hCG treatment (Fig. 6B-E). Follicles isolated prior to hCG treatment (0 h) show a very compact granulosa cell layer and very light staining (Fig. 6B). The administration of hCG caused the granulosa cell layer to expand and an increase in EST accumulation (Fig. 6C-E) was observed. Control sections of follicles isolated at 39 h post hCG showed no staining when anti-EST antibody was omitted (Fig 6G). The pattern in testes demonstrated EST expression in Leydig cells only (Fig. 6F).

The anti-MRP1 antibody raised in rabbit was shown to recognize the equine MRP1 protein from an equine preovulatory follicle cell extract, with two clear bands appearing at approximately 170 and 190 kDa (Fig. 7A). Immunohistochemical results demonstrated an increase in MRP1 production after hCG treatment (Fig. 7C-D). Follicles isolated prior to hCG treatment (0 h) show a very compact granulosa cell layer and light staining (Fig. 7B). It is difficult to tell whether the staining observed in the antral granulosa cells is due to the localization of the MRP1 protein or to the "edge effect" observed when using this technique (Fig. 7B). The administration of hCG caused the granulosa cell layer to expand and an increase in MRP1 accumulation (Fig. 7C-D) was observed. Control sections of follicles isolated at 39 h post hCG showed no staining when anti-MRP1 antibody was omitted (Fig 7E).

Mesurements of E1, E2, E1-S and E2-S in the follicular fluid

Follicular fluid was obtained from the same follicles described above, isolated between 0 and 36 h after hCG. Sulfoconjugated estrone and 17β-estradiol, as well as their unconjugated counterparts were quantified. The absolute levels of sulfoconjugated estrogens and the ratio of sulfoconjugated estrogens to free ones were shown to significantly change after hCG (Fig. 8). Results demonstrate that levels of estrone-3-sulfate (E1-S) and E2-S had a tendency to increase 12-24 h post-hCG and returned to basal levels at 39 h, yet were only significantly increased at either 24 and 33 h post-hCG for E1-S or 12 and 33 h post-hCG for E2-S (Fig. 8A and 8B, respectively). Concentrations of E1 had a tendency to increase after hCG and gradually decrease thereafter. However, none of these changes were significant (data not shown). Levels of E2 in follicular fluid decreased gradually after hCG, this change being significant at 39 h post-hCG (data not shown). When relative levels of E1-S to E1 were analyzed, they were shown to increase significantly at 30 and 36 h post-hCG (Fig. 8C), whereas the relative levels of E2-S to E2 were significantly increased 24-36 h post-hCG (Fig. 8D).

DISCUSSION

This study demonstrates for the first time that the process of follicular luteinization induced by hCG is accompanied by the co-localized expression and

upregulation of EST and ABCC1 in granulosa cells, proteins responsible for estrogen sulfoconjugation and export, respectively. The process of follicular luteinization has previously been associated with dramatic changes in steroid production; enzymes responsible for 17β-estradiol biosynthesis are downregulated, whereas those responsible for enhanced progesterone biosynthesis are induced (15, 17, 26, 27). Similar changes were also observed in the equine model (18, 28, 29). However, only one study examining EST expression in the ovary during the periovulatory period has been published and it was limited to the examination of EST transcript regulation by Northern blot and in situ hybridization in immature rat preovulatory follicles (30). Also, there has been no report on the regulation of ABCC1 during the periovulatory period. Previous investigations of the expression of ABCC1 in the ovary have primarily been limited to its detection in mice by branched DNA analysis, as well as its detection in human primary ovarian carcinomas by real-time RT-PCR (31, 32).

This study presents, for the first time, the cloning of equine EST and ABCC1 and identifies the luteinizing preovulatory follicle as a site of EST and ABCC1 expression. High levels of EST transcript were also identified in the brain, lung, liver, uterus and testes. This is consistent with human studies that have demonstrated a wide pattern of EST expression by quantitative PCR and immunohistochemistry (33). Very low levels of EST have also been detected in the human spleen (33). The expression of EST protein in equine Leydig cells is observed in the present study, as previously reported in other species (33, 34). Indeed, disruption of the EST gene in the male mouse leads to structural and functional lesions in the male reproductive system, suggesting a protective role for the sulfoconjugation of estrogens (13). A different pattern of expression is seen for ABCC1. Highest levels were detected in the equine preovulatory follicle 36 h after hCG, with a marked expression also occurring in the testes and skeletal muscle. Few studies depicting ABCC1 tissue distribution have been published, however, it has been shown to be highly expressed in the testes, lung, kidney, heart and skeletal muscle of the mouse, and similarly to the present study, very low levels of ABCC1 were detected in the liver (35).

The regulation of ABCC1 in the ovary has not been addressed and there is little information available from any tissue on the mechanisms involved in the regulation of EST and ABCC1. This study identifies hCG as a potential transcriptional modulator of both EST and ABCC1 expression in granulosa cells of the preovulatory follicle. Low, almost absent, levels of EST transcript were observed in granulosa cells of preovulatory follicles prior to hCG, with an upregulation of EST mRNA occurring 30-39 h after gonadotropin treatment. The increase in EST transcript expression was reflected in immunohistochemical results obtained from follicles isolated before and 39 h after hCG. A previous study using PMSG-primed immature rats as a model, demonstrated that treatment with hCG resulted in an upregulation of EST transcript visible in the 6 h post-hCG samples, with a return to almost basal levels at 12 h (30). Although the samples of preovulatory follicles used in the rodent study were different (0, 6 and 12 hours post-hCG), they show an induction occurring approximately 6 h prior to ovulation, as depicted in the present study. The inflammatory cytokine interleukin-1β (IL-1β) has been shown to upregulate EST transcript expression and activity in vascular smooth muscle cells derived from human aortas (36). Interestingly, IL-1β has been shown to be induced after LH treatment in cultured human granulosa cells (37) and in granulosa cells obtained 6 h after gonadotropin treatment in the mare (38). The molecular regulation of ABCC1 has been studied and shown to involve many transcriptional elements and transcription factors, including Sp1, p53 and AP-1 (39-41). However, some discrepancies are discernable concerning p53 activation or repression (42).

Results from the present study suggest that the induction of EST and ABCC1 may contribute to the increase in sulfoconjugated estrogens observed during the periovulatory period. Indeed, a peak in plasma E1-S levels has been demonstrated to occur approximately 1 day prior to ovulation in a number of species, including the marmoset and the mare (43, 44). The present study establishes that such an increase in sulfoconjugated estrogens also occurs in the follicular fluid of mares. The results presented herein depict the presence of sulfoconjugated steroids prior to hCG with a significant increase in E1-S and E2-S being observed at 24 and 12 h post-hCG, respectively. These findings suggest that the steroid sulfoconjugates present in the

follicular fluid at these early times, before EST mRNA levels increase, may also originate from peripheral circulation. Also, a decrease in sulfoconjugated steroids is observed at 36 h post-hCG, most likely due to the decreased estrogen biosynthesis, as previously described (15, 17, 18, 29). Nonetheless, the ratio of sulfoconjugated estrogens to free estrogens significantly increases after hCG treatment, supporting the argument that sulfotransferase levels are elevated to blunt the active estrogen levels at a time in the cycle when estrogen is no longer needed. Indeed, ESTs purified from numerous tissues of several species have been shown to be specific for the 3-hydroxyl group of estrogenic steroids, they do not, however, sulfonate either the 16α- or 17β-hydroxyl group of phenolic steroids (45-47). The high homology between ESTs from different species suggests that this activity may be conserved in the mare. Indeed, the GxxGxxK structure found in the P-loop motif and involved in PAPS binding is highly conserved in all sulfotransferases (48), including the mare, as are amino acids Gly^{262} and Asn^{269}, which have been shown to be required for estrogen sulfoconjugation in the guiney pig (49). As opposed to EST, MRP1 has many substrates. However, it has previously been shown to transport estrone-3-sulfate, in a reduced glutathione (GSH)-dependent manner, in membrane vesicles from transfected HeLa cells (50). Interestingly, the modifier subunit of glutamate cysteine ligase, the enzyme responsible for GSH synthesis, has been demonstrated to increase in preovulatory follicles following gonadotropin treatment in the rat (51). The precise role of EST and ABCC1/MRP1 during follicular luteinization/ovulation is intriguing and remains to be investigated.

In summary, this is the first study to clone the equine EST and ABCC1 cDNAs, and to examine their co-localized expression and upregulation in granulosa cells of luteinizing preovulatory follicles. By means of sulfoconjugation, EST changes a steroid's properties, thereby dictating the biological potency of estrogens and in concert with a transporter like ABCC1, can act as a local regulator of estrogen activity in mammals. The concept of estrogen inactivation during the process of follicular luteinization/ovulation is evolving, and results from the present study complement those previously reporting the hCG-dependent upregulation of 17β-hydroxysteroid dehydrogenase type 4, an enzyme responsible for 17β-estradiol oxidation (52). Future

studies will be required to investigate the necessity of inactivating estrogens during the periovulatory period.

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LEGENDS

Figure 1. Cloning of equine EST and ABCC1. A and C, Cloned equine cDNA fragments for EST and ABCC1, respectively. Each fragment is schematically represented, with its identity indicated on the right and its position in the deduced transcript sequence indicated in parentheses. Lines indicated untranslated regions (UTRs); open boxes designate the open reading frame (ORF). Lengths of the deduced transcript and its structural elements are indicated in base pairs (bp). Arrows indicate the position and orientation of the oligonucleotides employed in the cloning processes, with numbers indicating their identity. B and D, Oligonucleotides used in the various cloning procedures for EST and ABCC1, respectively.

Figure 2. Deduced primary structure of the equine EST and MRP1 proteins. A, comparison with known human and bovine EST. The predicted amino acid sequence of the equine EST protein is aligned with human (hum; GeneBank accession number P49888) and bovine (CAA39806) ESTs. Identical residues are indicated by a printed period, numbers on the right refer to the last amino acid residue on that line. *Bold underlined* residues are highly conserved among sulfotransferases, residues with an *asterisk* are involved in estrogen sulfoconjugation, and *boxed* residues are part of the conserved P-loop motif (49). B, the predicted amino acid sequence of equine MRP1. Numbers on the right refer to the last amino acid residue on that line and percentages presented below represent homology to human (NP_004987) and bovine (NP_776648) MRP1s. *Bold single underlined* residues are known as Walker A sequences, whereas *bold double underlined* residues are part of the Walker B sequences (35). *Bold triple underlined* amino acids are part of the active transport family signature (35). Residues with an *asterisk* are putative N-linked glycosylation sites (53), whereas *boxed* residues represent transmembrane domains (54).

Figure 3. Expression of EST and ABCC1 mRNA in equine tissues. Total RNA was extracted from various equine tissues, and samples (100 ng) were analyzed for EST, ABCC1 and rpL7a (control gene) content by a semi-quantitative RT-PCR/Southern

blotting techniques, as described in *Materials and Methods*. A, Expression of EST mRNA in equine tissues. B, Expression of ABCC1 mRNA in equine tissues. C, Expression of rpL7a mRNA in equine tissues. The number of PCR cycles for each gene was within the linear range of amplification, and they represented 26, 20 and 18 cycles for EST, ABCC1 and rpL7a, respectively. Numbers on the *right* indicate the size of the PCR fragment.

Figure 4. Regulation of EST and ABCC1 transcript by hCG in equine preovulatory follicles. Preparations of follicle wall were obtained from preovulatory follicles isolated between 0-36 h after hCG and of corpora lutea (CL) isolated on d 8 of the estrous cycle. Samples (100 ng) of total RNA were analyzed for EST, ABCC1 and rpL7a content by a semi-quantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. A, Regulation of EST mRNA in equine follicles (one follicle per time point). B, Constitutive expression of rpL7a mRNA in the same follicles used in A. Numbers on the *right* indicate the size of the PCR fragment. C, Relative changes in intensity of EST signal was normalized with the control gene rpL7a (five or six distinct follicles, i.e. animals per time point and three different corpora lutea). *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$). D, Regulation of ABCC1 mRNA in equine follicles (one follicle per time point). E, Constitutive expression of rpL7a mRNA in the same follicles used in D. Numbers on the *right* indicate the size of the PCR fragment. F, Relative changes in intensity of ABCC1 signal was normalized with the control gene rpL7a (mean ± SEM; five or six distinct follicles, i.e. animals per time point and three different corpora lutea). *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$).

Figure 5. Regulation of EST and ABCC1 mRNA in equine granulosa and theca interna cells. Preperations of granulosa cells (a) and theca interna (b) were isolated from equine preovulatory follicles between 0-39 h after hCG treatment, and samples (100 ng) of total RNA were analyzed for EST (A), ABCC1 (B) and rpL7a content by a semiquantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. The EST and ABCC1 signal was normalized with the control gene rpL7a, and results are

presented as a ratio of either EST to rpL7a or ABCC1 to rpL7a (mean ± SEM; n = 4 samples; *i.e.* mares per time point). *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$). *Insets* show results of either EST (A) or ABCC1 (B) and rpL7a mRNA levels from one sample per time point.

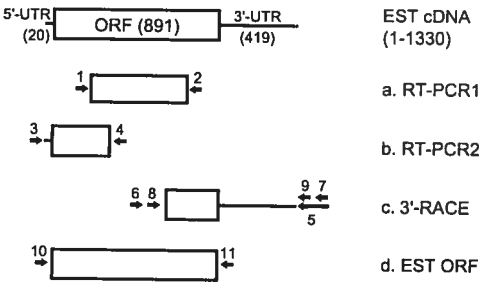
Figure 6. Immunohistochemical localization of EST in equine preovulatory follicles and EST primary antibody specificity. A, Protein extracts were prepared from equine liver and anti-EST antibody was shown to recognize a single band of predicted EST size (M_r = 35,000). Immunohistochemistry was performed on formalin-fixed sections of preovulatory follicles isolated 0 and 39 h after hCG treatment and of equine testicular tissues, as described in *Materials and Methods*. Results show relatively weak EST staining in granulosa (GC) and theca interna (TI) cells of a preovulatory follicle obtained 0 h after hCG administration (B), but a marked increase in signal intensity in both cell types of distinct follicles isolated 39 h after hCG treatment (C-E). F, Intense EST immunoreactivity is observed in Leydig cells (L) of equine testicular sections. G, Control staining from the follicular tissue presented in D was negative when the primary antibody was omitted. Magnification, ×200 (B-D, and F) and ×400 (E and G).

Figure 7. Immunohistochemical localization of MRP1 in equine preovulatory follicles and MRP1 primary antibody specificity. A, Protein extracts were prepared from equine preovulatory follicles and anti-MRP1 antibody was shown to recognize a two bands of predicted MRP1 size (M_r = 170,000 and 190,000 when glycosylated (55)). Immunohistochemistry was performed on formalin-fixed sections of preovulatory follicles isolated 0 and 39 h after hCG treatment, as described in *Materials and Methods*. Results show relatively some MRP1 staining in granulosa (GC) of a preovulatory follicle obtained 0 h after hCG administration (B), but a marked increase in signal intensity was observed mainly in granulosa cells of follicles isolated 39 h after hCG treatment (C-D). E, Control staining from the follicular tissue presented in D was negative when the primary antibody was omitted. Magnification, ×200 (B and C) and ×400 (D and E).

Figure 8. Absolute and relative concentrations of sulfoconjugated estrogens in follicular fluid. E1-S (A), E2-S (B), E1-S/E1 (C) and E2-S/E2 (D) were measured in follicular fluid of preovulatory follicles isolated between 0-36 h after hCG treatment (mean ± SEM; n = 4-6 samples per time point [i.e. mares]). Bars marked with *asterisks* are significantly different from 0 h post-hCG ($P < 0.05$).

FIGURE 1

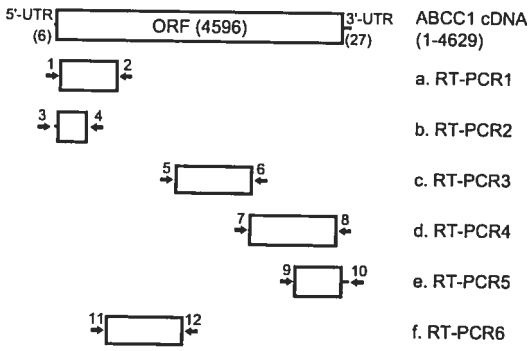
A



B

1. 5'-GGTGATGTGGAAAAGTGCAA-3'
2. 5'-TGGATTGTTCTTCATCTCCTGG-3'
3. 5'-CCCAGTTGCTTTCACAGGATCA-3'
4. 5'-GCATTCCAAGTAAGGTACTCG-3'
5. 5'-GTACCGGATCCTCTAGAGAGCTCGTCGACCT CGAGGAATTCAAGCTTTTTTTTTTTTTTTTTTT-3'
6. 5'-GGGAAGGAAACCATCAGAGG-3'
7. 5'-GTACCGGATCCTCTAGAGAGCTC-3'
8. 5'-AGCTTGTGGACAAGATTGTAC-3'
9. 5'-GTCGACCTCGAGGAATTCAAGCTT-3'
10. 5'-CTCGGATCCGCCGCCGAGATGGTGGATTCTT CCAAATTTGAC-3'
11. 5'-GAGTCTAGATTAGATCTCAGTTTGTAATTTCAA TG-3'

C



D

1. 5'-ATGGCGCTCCGGGGCTTCTGC-3'
2. 5'-ATGAGGAAGTAGGGCCCAAAGG-3'
3. 5'-GCCCGCCGCCGCCCGCGCC-3'
4. 5'-GCATGATCCCTGAGGACTGGACT-3'
5. 5'-GTGGCCGTGGTGGGCCAGG-3'
6. 5'-CCATGAACATCTTGATGACC-3'
7. 5'-GAGCTGGACACGGTGGACTCG-3'
8. 5'-ACACCAAGCCGGCGTCTTTGG-3'
9. 5'-GTGGGCAACTGCATCGTCCTGT-3'
10. 5'-CTGGCATATAGGCCCTGCAGT-3'
11. 5'-CCAAGGTGGATGTGAACGAGG-3'
12. 5'-ACCTTCTCCATCTCGGCCAAG-3'

FIGURE 2

A. EST

```
equ   MMDSSKFDSSSYFGRIHGILMYKDFVEFWD DVEAFEARPDDLVIATYPKSGTTWLSEIVD MIYKEGDVEKCKEDAIFNRVPYLECRKEDV  90
hum   -.N-.EL.YYEK.EEV.........KY.. N....Q.................V....Y ..............V....I.F......NL  88
bov   -.S...PSF.D...KLG..P...K.I.QFH N..E.........V.............IC ...NN.........V..........ST.H.  89

equ   MNGVKQLKQMASPRIMKTHLSADLLPGSFW EKNCKMIYLCRNAKGVVVSFYYFFLMVAGH PHPGSFQEFVEKFMDGEVPYGSWYRHAKSW  180
hum   .......DE.N....V....PPE...A... ..D..I........D.A.............. .N....P.......Q.Q.......K.V...  178
bov   .K.....NE......V.S..PVK...V... .....I...S....D....Y.FLI...TAI .D.D...D..............FE.T...  179

equ   WEKRKKPHVLFLFYEDMQEDIKREVIKLMQ FLGRKPSEELVDKIVQHTSFHEMKNNPSTN YTMLPDEIMNQKISPFMRKGIVGDWKNHFT  270
hum   ...G.S.R........LK...RK.....IH ..E.........R.IH....Q......... ..T.........L.........T........  268
bov   ...S.N.Q.........K.N.RK..M..LE .....A.D......IK....Q......... ..T....V....V.....D.........  269

equ   VALNEKFDTHYEQEMKGSTLKLQTEI                                                                  296
hum   ........K....Q..E....FR...      81,0%                                                       294
bov   ........M....Q.......FR.K.      74,9%                                                       295
```

B. MRP1

```
MALRGFCSADGSDPLWEWNVTWNTS NPDFTKQFQNTVLVWVPCAYLWVCF PLYFLHLSRHDRGYIQMTHLNKAKT ALGFLLWIVCWADLFYSFWERSWGR  100
FVAPVLLVSPTLLGVTMLLATFLIQ LERRKGVQSSGIMLTFWLIALLCAL AILRSKIMAALKEDADVDVFRDVTF YIYSSLVLIELVLSCFSDRSPLFSE  200
TINDPNPCPESSASFLSRITFWWIT GMMVQGYRQPLESADLWSLNKEDMS EQVVPILVKNWKKECAKARKQPVKV VYSSKDPARPKGSSKVDVNEEAEAL  300
IVKSPAKERDPSLFKVLYKTFGPYF LMSFLFKALHDLMMFAGPEILKLLI NFVNDQQAPDRQGYFYTALLFISAC LQTLVLHQYFHICFVSGMRVKTAVI  400
GAVYRKALVITNSARKSSTVGEIVN LMSVDAQRFMDLATYLNMVWSAPLQ VILALYLLWLNLGPSVLAGVAVMIL MVPVNAVMAMETKTYQVAHMKSKDN  500
RIKLMNEILNGIKVLKLYAWELAFK DKVLAIRQEELKVLEKSAYLGAVGT FTWVCTPFLVALSTFAVYVTIDENN ILDAQKAFVSLALFNILRFPLNILP  600
MVISSIVQASVSLKRLRIFLSHEEL EPDSIERRPGKDGGGANSITVKNAT FTWARGEPPTLSGITFSVPEGSLVA VVGQVGCGKSSLLSALLAEMEKVEG  700
HVAIKGSVAYVPQQAWIQNDSLQEN ILFGRQLQERYYKAVVEACALLPDL EILPSGDLTEIGEKGVNLSGGQKQR VSLARAVYCDSDIYLFDDPLSAVDA  800
HVGKHIFENVIGPKGMLKNKTRLLV THGISYLPQVDVIIVMSGGKISEMG SYQELLARDGDFAEFLRTYASAEQE QDEQDNGSAGIGGPGKEGKQMENGM  900
LVTDAVGKQMQRQLSNPSTYSSDIG RHCNSTAELQKDEAKKEEAWKLVEA DKAQTGQVKLSVYWGYMRAIGLFLS FLSILLFISNHVASLASNYWLSLWT  1000
DDPVVNGTQEHTTVRLSVYGGLGIL QGLSVFGYSMAVSLGGVLASRRLHV DLLHHVLRSPMSFFERTPSGNLVNR FSKELDTVDSMIPQVIKMFMGSLCN  1100
VIGACIVILLATPIAAIVIPPLGLI YFFVQRFYVASSRQLKRLESVSRSP VYSHFNQTLLGVSVIRAFEEQDRFI HQSDLKVDDNQKAYYPSIVANRWLA  1200
VRLECVGNCIVLFASLSAVISRHSL SAGLVGLSVSYSLQVTTYLNWLVRM SSEMETNVVAVERLKEYSEIEKEAP WRIQEMTPPSDWPQVGRVEFRNYGL  1300
RYREDLDLVLKRINVTINGGEKVGI VGRTGAGKSSLTLGLFRINEPAEGE IIIDDVNIAKIGLHDLRFKITIIPQ DPVLFSGSLRMNLDPFSKYSDEEVW  1400
TALELAHLKDFVSSLPDKLNHECAE GGENLSVGQRQLLCLARALLRKTKI LVLDEATAAVDLETDNLIQSTIRTQ FDDCTVLTIAHRLNTIMDYMRVIVL  1500
DKGEILECGSPSDLLQQKGLFYTMA KDAGLV                                                                  1531

89,7% Homologous to Human ABCC1
90,5% Homologous to Bovine ABCC1
```

FIGURE 3

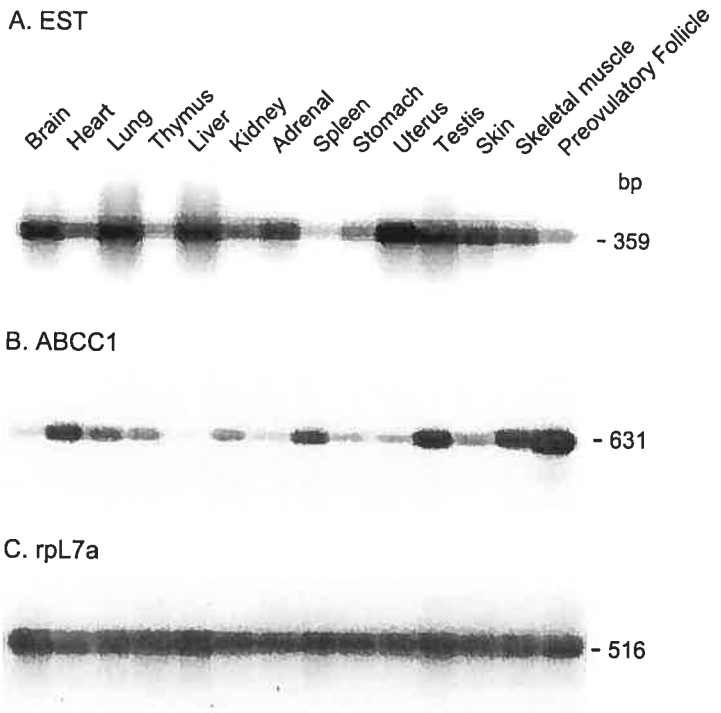

A. EST
Brain
Heart
Lung
Thymus
Liver
Kidney
Adrenal
Spleen
Stomach
Uterus
Testis
Skin
Skeletal muscle
Preovulatory Follicle
bp
- 359
B. ABCC1
- 631
C. rpL7a
- 516

FIGURE 4

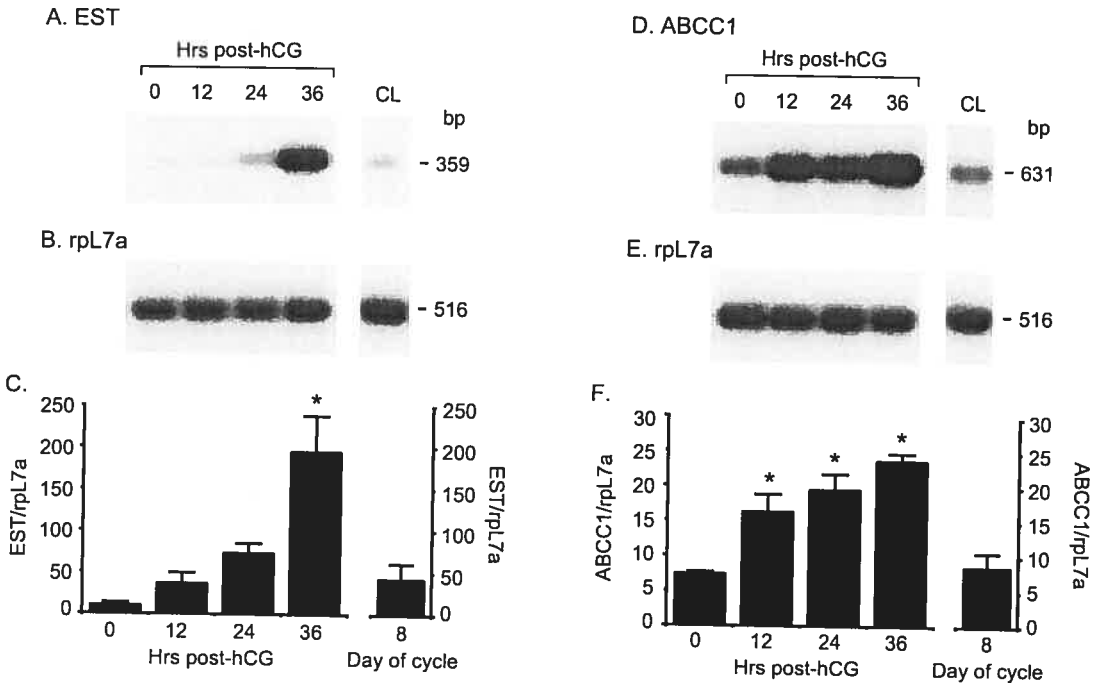
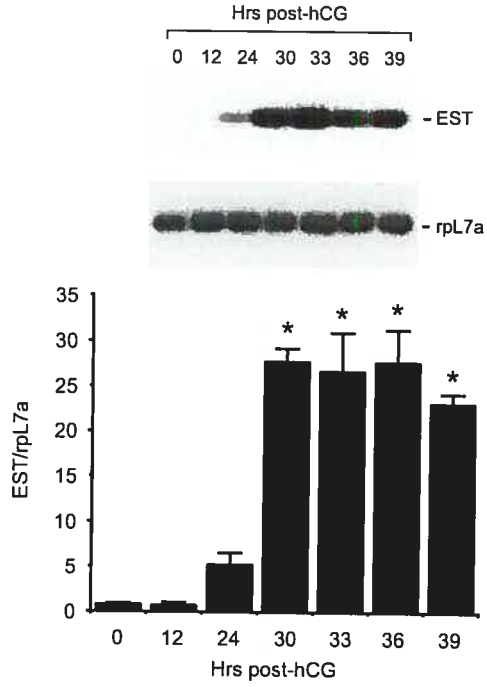

A. EST
Hrs post-hCG
0 12 24 36
CL
bp
- 359
B. rpL7a
- 516
C.
EST/rpL7a
250
200
150
100
50
0
0 12 24 36
Hrs post-hCG
8
Day of cycle
D. ABCC1
Hrs post-hCG
0 12 24 36
CL
bp
- 631
E. rpL7a
- 516
F.
ABCC1/rpL7a
30
25
20
15
10
5
0
0 12 24 36
Hrs post-hCG
8
Day of cycle

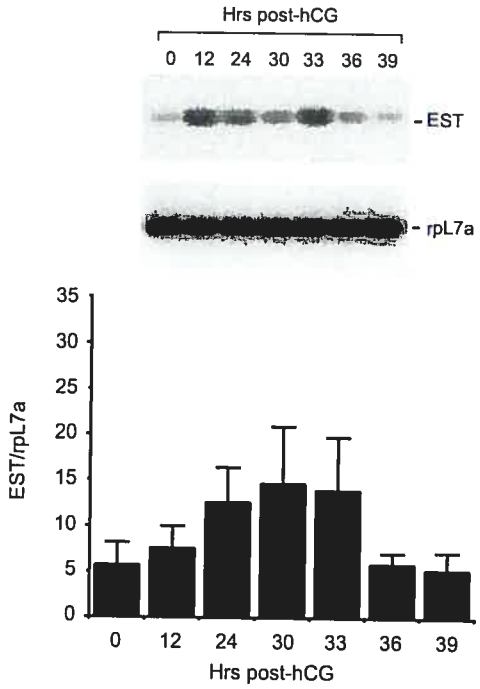
FIGURE 5

A. EST

a. Granulosa cells

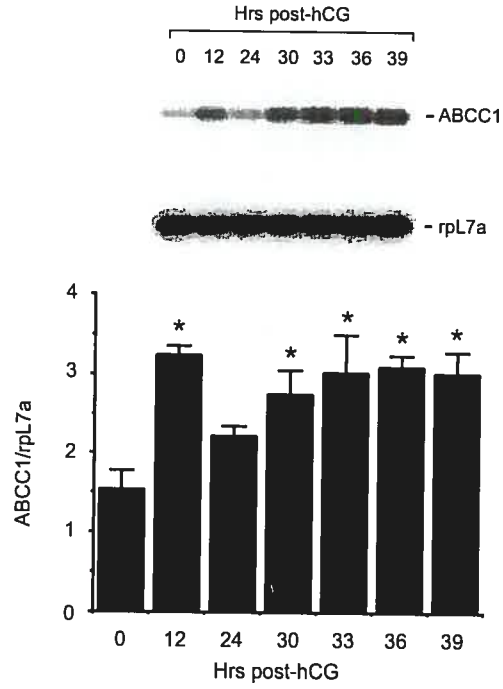


b. Theca interna



B. ABCC1

a. Granulosa cells



b. Theca interna

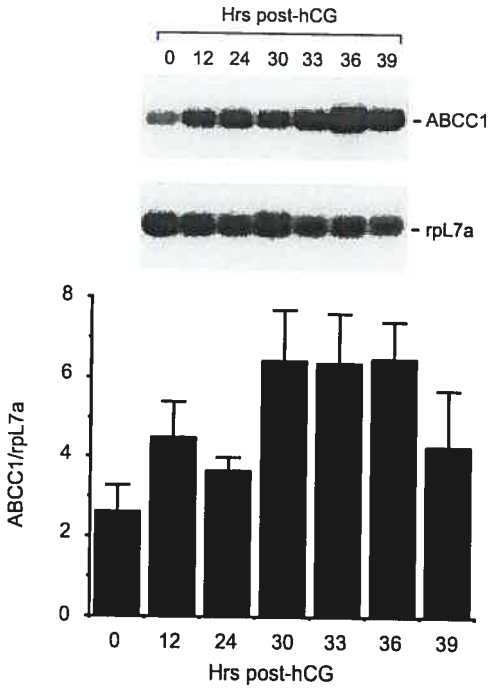


FIGURE 6

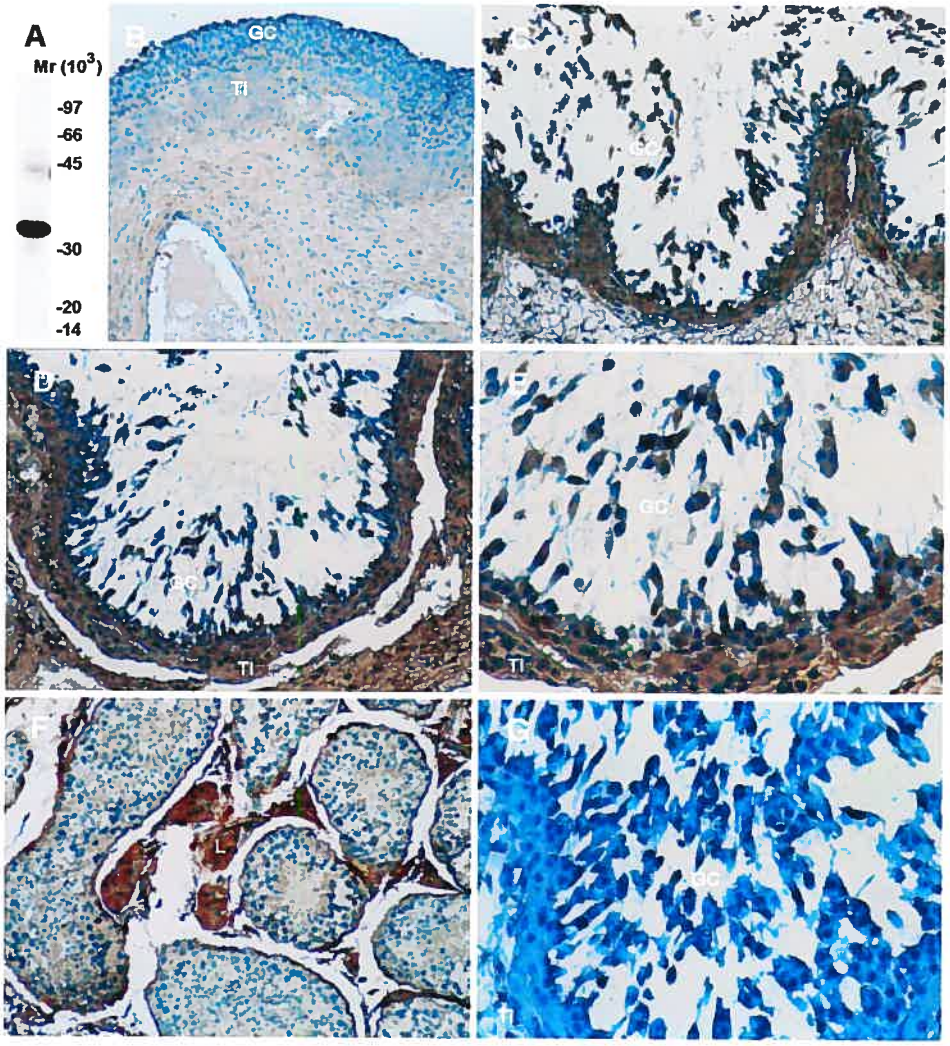


FIGURE 7

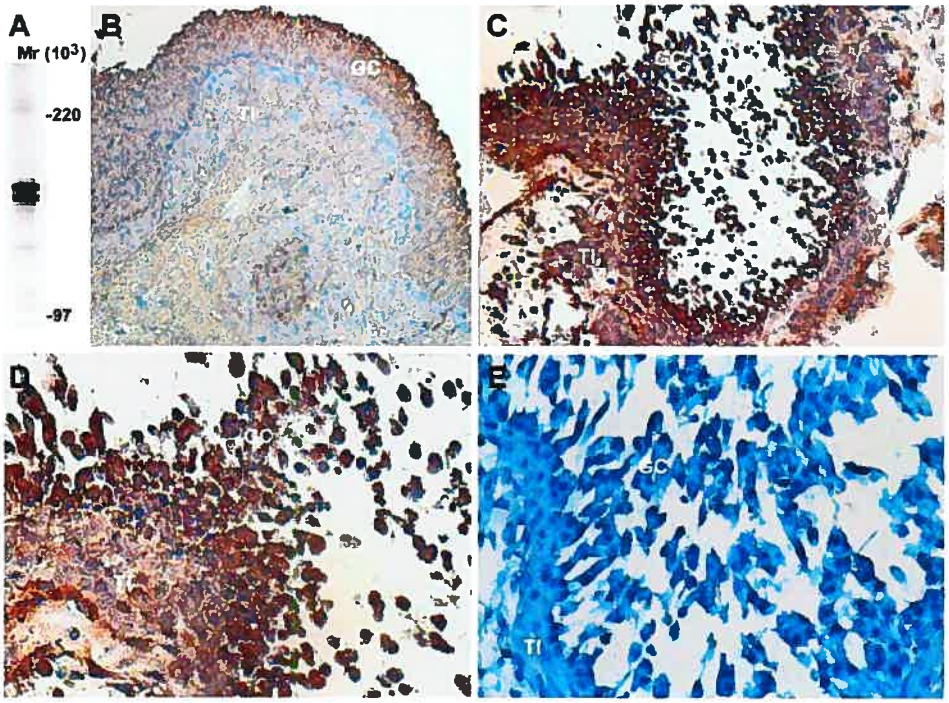
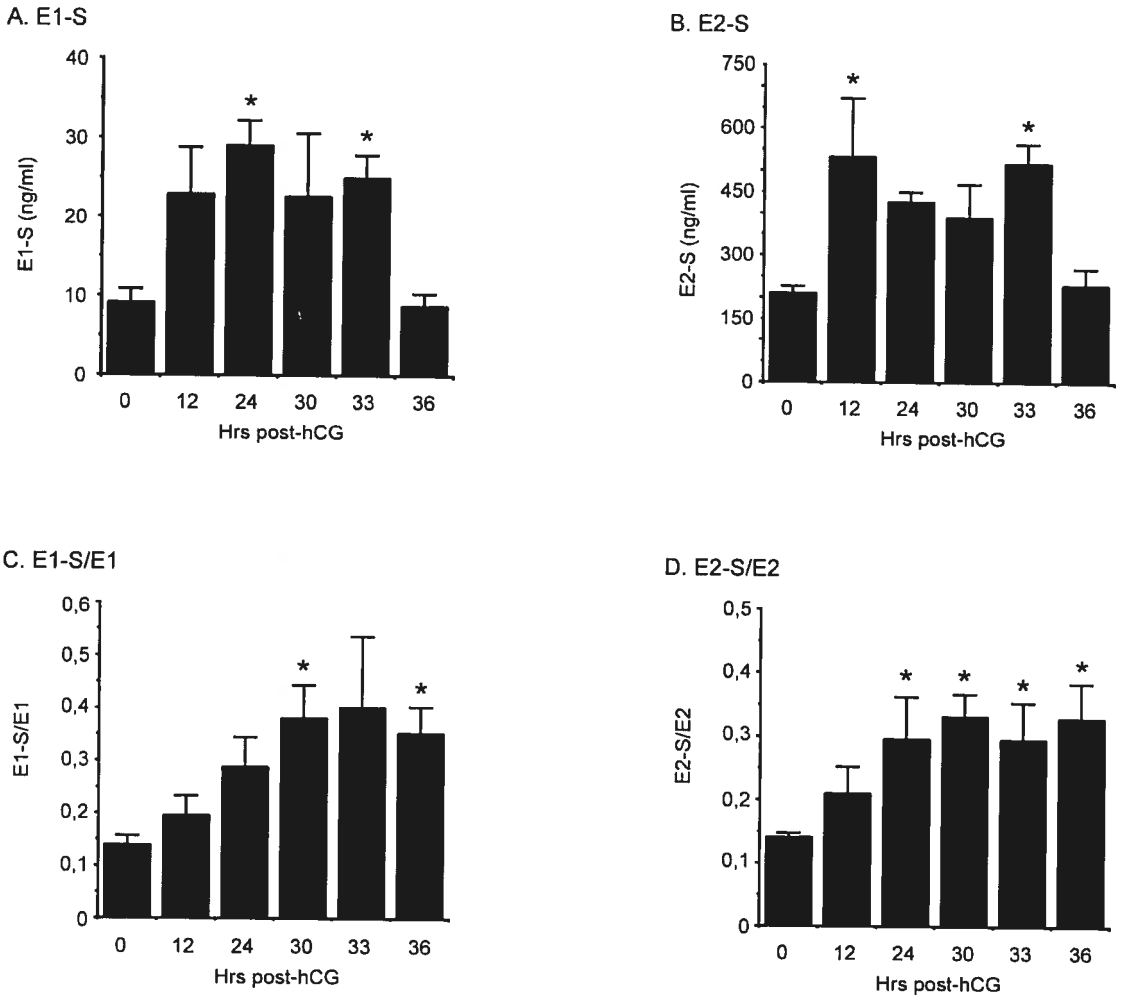


FIGURE 8


A. E1-S
E1-S (ng/ml)
40
30
20
10
0
0 12 24 30 33 36
Hrs post-hCG
B. E2-S
E2-S (ng/ml)
750
600
450
300
150
0
0 12 24 30 33 36
Hrs post-hCG
C. E1-S/E1
E1-S/E1
0,6
0,5
0,4
0,3
0,2
0,1
0
0 12 24 30 33 36
Hrs post-hCG
D. E2-S/E2
E2-S/E2
0,5
0,4
0,3
0,2
0,1
0
0 12 24 30 33 36
Hrs post-hCG

SUMMARY OF ARTICLE FIVE

Title: Human chorionic gonadotropin-dependent induction of an equine aldo-keto reductase (AKR1C23) with 20α-hydroxysteroid dehydrogenase activity during follicular luteinization in vivo. **Kristy A. Brown**, Derek Boerboom, Nadine Bouchard, Monique Doré, Jacques G. Lussier and Jean Sirois *Accepted for publication in Journal of Molecular Endocrinology (February 2006).*

Thesis author's contribution to this work: As the primary author, I was responsible for all aspects of this article. Secondary authorship credits reflect contributions to AKR1C23 cDNA cloning. Whereas tertiary and quaternary authorship credits reflect technical support for Southern blotting and immunohistochemistry, respectively. Fifth and sixth authorships reflect co-directorship and directorship, respectively.

Summary:

- The equine AKR1C23 cDNA was characterized by a combination of RT-PCR, 5'- and 3'-RACE reactions in pooled RNA from equine preovulatory follicles.
- Sequence analysis of the AKR1C23 transcript revealed an open reading frame that encoded a 322-amino acid protein, more than 80% identical to the human orthologue. Residues involved in substrate and cofactor binding, as well as other amino acids conserved among AKRs are also present in the equine protein.
- The regulation of the equine AKR1C23 was examined in equine preovulatory follicles by semi-quantitative RT-PCR in samples obtained at different times after hCG. Results from Southern blot analyses revealed low levels of AKR1C23 mRNA expression prior to hCG, with a dramatic increase observable from 12 to 39 h post-hCG occurring selectively in the granulosa cell layer.
- The localization of the equine AKR1C23 protein inside the preovulatory follicle by immunohistochemistry revealed that AKR1C23 expression is low in follicles obtained prior to hCG treatment with higher levels being detected in both theca and granulosa cells 39 h after hCG.

- The reduction of progesterone catalyzed by AKR1C23 was tested using NADPH, and revealed a K_m of 3.12 μM and a V_{max} of 0.86 pmol/min/10 μg of protein.
- The concentration of the progesterone metabolite, 20α-DHP, in the follicular fluid of the preovulatory follicles of hCG-treated revealed a significant increase occurring 30 to 36 h post-hCG.

Work's contribution to the advancement of science:

- This work characterizes, for the first time, the primary structure of the equine AKR1C23 cDNA and protein.
- It demonstrates the gonadotropin-dependent induction of the AKR1C23 transcript in the granulosa cells of preovulatory follicles occurring between 12 and 39 h post-hCG, as well as the hCG-dependent increase in AKR1C23 protein in granulosa cells.
- This study is also the first to demonstrate the 20α-HSD activity of the AKR1C23 protein and to correlate its expression with the 20α-DHP concentrations from the follicular fluid.
- Overall, it supports the hypothesis that enzymes involved in progesterone metabolism play a role in regulating levels of active progestins during the ovulatory process.

Human Chorionic Gonadotropin-Dependent Induction of an Equine Aldo-Keto Reductase (AKR1C23) with 20α-Hydroxysteroid Dehydrogenase Activity during Follicular Luteinization In Vivo

Kristy A. Brown, Derek Boerboom, Nadine Bouchard, Monique Doré, Jacques G. Lussier and Jean Sirois§

Centre de recherche en reproduction animale et Département de biomédecine vétérinaire (K.A.B., D.B., N.B., J.G.L. and J.S.) and Département de pathologie et microbiologie (M.D.), Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada J2S 7C6

Abbreviated title: Induction of AKR1C23 in preovulatory follicles

Key Words: Aldo-keto reductase, 20α-HSD, granulosa cells, preovulatory follicles, luteinization, ovary, horse

The nucleotide sequence reported in this paper has been submitted to GenBank with accession number AY955082.

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ABSTRACT

Aldo-keto reductases (AKRs) are multi-functional enzymes capable of acting on a wide variety of substrates, including sex steroids. AKRs having 20α-hydroxysteroid dehydrogenase (20α-HSD) activity can reduce progesterone to 20α-hydroxy-4-pregnen-3-one (20α-DHP), a metabolite with lower affinity for the progesterone receptor. The objective of the study was to investigate the regulation of equine AKR1C23 during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The equine AKR1C23 cDNA was cloned and shown to encode a 322-amino acid protein that is conserved (71-81% identity) when compared to mammalian orthologs. RT-PCR/Southern blot analyses were performed to study the regulation of AKR1C23 transcripts in equine preovulatory follicles isolated between 0-39 h after hCG treatment (ovulation occurring 39-42 h post-hCG). Results showed the presence of low AKR1C23 expression before hCG treatment, but a marked increase was observed in follicles obtained after 12 h post-hCG ($P < 0.05$). Analyses of isolated preparations of granulosa and theca interna cells identified low mRNA expression in both cell types prior to hCG treatment, with granulosa cells clearly being the predominant site of follicular AKR1C23 mRNA induction. A specific polyclonal antibody was raised against a fragment of the equine protein and immunoblot analyses showed an increase in AKR1C23 protein in granulosa cell extracts when comparing follicles isolated at 36 h post-hCG versus those collected prior to treatment, in keeping with mRNA results. Immunohistochemical data confirmed the induction of the enzyme in follicular cells after hCG treatment. The enzyme was tested for 20α-HSD activity and was shown to exhibit a K_M of 3.12 μM, and a V_{max} of 0.86 pmol/min/10μg protein towards progesterone. The levels of 20α-DHP measured in follicular fluid reflected this activity. Collectively, these results demonstrate for the first time that the gonadotropin-dependent induction of follicular luteinization is accompanied by an increase in AKR1C23 expression. Considering the 20α-HSD activity of AKR1C23, its regulated expression in luteinizing preovulatory follicles may provide a biochemical basis for the increase in ovarian 20α-DHP observed during gonadotropin-induced luteinization/ovulation.

INTRODUCTION

Enzymes capable of performing oxidoreductase activities are widespread. One superfamily comprises the aldo-keto reductases (AKRs), characterized as being monomeric, nicotinamide cofactor-dependent, cytosolic proteins of approximately 320 amino acids. These AKRs lack a Rossman-fold motif required by short-chain dehydrogenase/reductase (SDR) family members for NAD(P)H binding (el-Kabbani O *et al.*, 1995, Hoog SS *et al.*, 1994, Jornvall H *et al.*, 1995, Wilson DK *et al.*, 1992, Wilson DK *et al.*, 1995). They are found in a wide variety of organisms, from bacteria to mammals, and metabolize a wide variety of substrates, including aliphatic and aromatic aldehydes, isoflavinoids, monosaccharides, steroids, prostaglandins and polycyclic aromatic hydrocarbons. Fourteen families of AKRs are currently known (AKR1-14), encompassing over 100 proteins, with the AKR1 family being the largest and containing the aldehyde reductases, the aldose reductases, the hydroxysteroid dehydrogenases and the 5β-reductases. Isoforms of the AKR1C family have been demonstrated to share at least 84% amino acid sequence identity, and to exhibit 3α-, 17β- and 20α-hydroxysteroid dehydrogenase activities (Penning TM *et al.*, 2000). The current AKR1C3 has, in the past, also been named human liver 3α-HSD type II, 17β-HSD type V, dihydrodiol dehydrogenase (DD) type X, and prostaglandin F synthase, thereby, contributing to the confusion regarding nomenclature (Penning TM *et al.*, 1996).

Aldo-keto reductases having 20α-HSD activity convert progesterone to 20α-hydroxy-4-pregnen-3-one (20α-DHP), a steroid considered inactive due to its lower affinity for the progesterone receptor (PR) (Ogle TF and Beyer BK, 1982). However, levels of 20α-DHP have been demonstrated to increase after the coitus-induced preovulatory surge in luteinizing hormone (LH) in the rabbit, as well as after human chorionic gonadotropin (hCG) treatment in both rats and rabbits and in cultured rat granulosa cells (Lacy WR *et al.*, 1993, Lau IF *et al.*, 1978, Nordenstrom K and Johanson C, 1985). Injection of equine chorionic gonadotropin (eCG) to immature rats with subsequent hCG treatment resulted in an increased production of progesterone and testosterone, as well as an increase in 20α-DHP levels surpassing those of progesterone

prior to ovulation (Bauminger S *et al.*, 1977). 20α-DHP has been shown to induce ovulation when administered to immature rats after eCG treatment at doses 3 times that required by progesterone, an effect not seen with 20α-DHP's 5α-reduced metabolites (Gilles PA and Karavolas HJ, 1981). 20α-DHP has also been shown to induce a positive feedback effect on LH serum concentrations in an estrogen primed eugonadal woman (Leyendecker G *et al.*, 1976) and has been shown to prolong the preovulatory LH discharge in the rabbit (Hilliard J *et al.*, 1967). When monolayer cultures of rat pituitaries were exposed to 20α-DHP, a negative feedback effect was observed on the basal secretion of FSH, whereas this progesterone metabolite increased the effect of gonadotropin-releasing hormone on LH secretion (Tang LK and Spies HG, 1975).

In mammals, follicular luteinization is triggered by a surge in LH released by the anterior pituitary. At this time, a vast number of biological and structural changes occur: the steroidogenic enzymes responsible for 17β-estradiol production are down-regulated, whereas those contributing to progesterone synthesis, a steroid required for the establishment of pregnancy, are induced. The regulation of genes responsible for progesterone synthesis and action have been studied in great detail during the periovulatory period (Boerboom D *et al.*, 2003, Boerboom D and Sirois J, 2001, Natraj U and Richards JS, 1993, Park OK and Mayo KE, 1991, Sugawara T *et al.*, 1997). However, no attempt has been made to study the regulation of proteins having progesterone-metabolizing activities, such as 20α-HSD, during the luteinization process. In the present study, the equine preovulatory follicle was used as a model to investigate the regulation of a novel aldo-keto-reductase, AKR1C23, during hCG-induced ovulation/luteinization. The specific objectives were to clone equine AKR1C23 cDNA and determine the expression of its mRNA and protein in preovulatory follicles after hCG treatment.

MATERIALS AND METHODS

Materials

The Prime-a-Gene labeling system, pGEM-T Easy Vector System I, and the Access RT-PCR system were purchased from Promega Corp. (Madison, WI). The [α-^{32}P]dCTP was purchased from PerkinElmer Canada, Inc. (Woodbridge, ON, Canada), and the QuickHyb hybridization solution was obtained from Stratagene Cloning Systems (La Jolla, CA). The TRIzol total RNA isolation reagent, SuperScript II reverse transcriptase, 1-kilobase DNA ladder, synthetic oligonucleotides, 5'-rapid amplification of cDNA ends (RACE) system (Version 2.0), pcDNA3.1+ vector, and LipofectAMINE PLUS were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). The Qiagen OneStep Reverse Transcription-Polymerase Chain Reaction (RT-PCR) System, the pQE-30 vector and the Ni-NTA Superflow beads were obtained from Qiagen, Inc. (Mississauga, ON, Canada). The pGEX-2T vector, protease-deficient *E. coli* BL-21, and glutathione-Sepharose beads were obtained from Amersham Pharmacia Biotech (Baie d'Urfé, PQ, Canada). The Expand High Fidelity DNA Polymerase was purchased from Roche Diagnostics (Laval, PQ, Canada). Biotrans nylon membranes (pore size, 0.2 mm) were obtained from ICN Pharmaceuticals, Inc. (Montréal, PQ, Canada), and all electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA). The hCG was obtained from The Buttler Co. (Columbus, OH). The Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA). The diaminobenzidine tetrahydrochloride, β-nicotinamide adenine dinucleotide phosphate (NADPH), and progesterone were purchased from Sigma Chemical Co. (St. Louis, MO).

Cloning of the equine AKR1C23 cDNA

The equine AKR1C23 transcript was isolated in fragments using a multistep cloning strategy (Fig. 1). A 402-base pair (bp) reverse transcription-PCR product (RT-PCR) (Fig. 1Aa) was initially cloned from pooled equine ovarian RNA samples isolated from preovulatory follicles isolated before (0 h) and after (36 h) hCG treatment. Ovarian tissues were isolated and RNA was extracted as previously described (Kerban A *et al.*, 1999). RT-PCR was performed using the Access RT-PCR kit (Promega) as directed by the manufacturer, using 500 ng of RNA and oligonucleotide primers

designed by sequence alignments of known AKR species homologues (Fig. 1B; primers 1 and 2). Following agarose gel electrophoresis, the RT-PCR product was excised and ligated into the PGEM-T Easy plasmid vector (Promega), and proper recombinant plasmids were identified from transformed bacterial colonies using standard techniques (Sambrook J *et al.*, 1989). Sequencing of the insert was performed by the Service de Séquençage de l'Université Laval (Québec, PQ, Canada) using vector-based T7 and SP6 oligonucleotide primers. Sequences obtained from the initial RT-PCR product served as the basis for design of specific oligonucleotides for 5'- and 3'-Rapid Amplification of cDNA Ends (RACE) procedures. The 3'-RACE was performed as previously described (Boerboom D *et al.*, 2000), except 5 µg of pooled ovarian tissue RNA (as described above) was used as a template for the initial RT reaction (Fig. 1Ab). Briefly, an RT reaction was performed using a poly-dT oligonucleotide with anchor sequences at its 5' end (Fig. 1B; primer 3). This was followed by nested PCR reactions using oligonucleotide primers that bound to the anchor sequence in conjunction with AKR1C23-specific forward primers (Fig. 1B; primers 4, 5, 6, and 7). The product of the second PCR reaction was isolated and sequenced as described above. 5'-RACE was performed using the 5'-RACE System, Version 2.0 kit (Invitrogen) as directed by the manufacturer, using 5 µg of pooled ovarian tissue RNA (as described above) and AKR1C23-specific primers for reverse transcription and PCR (Fig. 1B; primers 8, 10, and 12), along with forward primers supplied with the kit (Fig. 1B; primers 9 and 11). The longest product obtained (Fig. 1Ac) was isolated and sequenced as described above. A clone encompassing the entire coding region was isolated by RT-PCR (Fig 1Ad), incorporating KpnI and XhoI restriction sites for subcloning into the eukaryotic expression vector pcDNA 3.1+ (Invitrogen), and found to correspond with the deduced primary AKR1C23 transcript reported herein (Fig. 1A). AKR1C23 nomenclature was attributed after the sequence was submitted to the aldo-keto reductase superfamily homepage (www.med.upenn.edu/akr) (Hyndman D *et al.*, 2003).

Equine tissues and RNA extraction

Testicular tissues were obtained from the Large Animal Hospital of the Faculté de médecine vétérinaire (Université de Montréal) following a routine castration, whereas

other nonovarian tissues were collected at a local slaughterhouse. Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares as previously described (Sirois J and Doré M, 1997). Briefly, when preovulatory follicles reached 35 mm in diameter during estrus, the ovulatory process was induced by injection of hCG (2500 IU, iv) and ovariectomies were performed via colpotomy using an ovariotome at 0, 12, 24, 30, 33, 36, or 39 h post hCG (n = 4-6 mares / time point; ovulation occurring 39-42 h post-hCG) (Sirois J and Doré M, 1997). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna. Ovariectomies were also performed on day 8 of the estrous cycle (day 0, day of ovulation) to obtain corpora lutea (n = 3 mares) (Sirois J and Doré M, 1997). All animal procedures were approved by the institutional animal use and care committee. Total RNA was isolated from tissues with TRIzol reagent (Invitrogen Canada Inc.), according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada).

Semiquantitative RT-PCR and Southern analysis

The Access RT-PCR System (Promega Corp.) was used for semi-quantitative analysis of AKR1C23 and ribosomal protein L7a (rpL7a) mRNA levels in equine tissues. Reactions were performed according to the manufacturer's directions, using sense (5' – GAAGCAACAAACAATGGATCCC - 3') and antisense (5' – CCACCTGGTTGCAGAC AGGC - 3') primers specific for equine AKR1C23, and sense (5' – ACAGGACATCCAG CCCAAACG - 3') and antisense (5' - GCTCCTTTGTCTTCCGAGTTG - 3') primers specific for the equine control gene rpL7a. These reactions resulted in the production of AKR1C23 and rpL7a DNA fragments of 587 and 516 bp, respectively. Each reaction was performed using 100 ng total RNA, and cycling conditions were one cycle of 48 C for 45 min and 94 C for 2 min, followed by a variable number of cycles of 94 C for 30 sec, 55 C for 1 min, and 68 C for 2 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification and was 21 cycles for AKR1C23, and 18 cycles for rpL7a. Following PCR amplification, samples were electrophoresed on 2% TAE-

153 agarose gels, transferred to nylon membranes, and hybridized with corresponding
154 radiolabeled AKR1C23 and rpL7a cDNA fragments using Prime-a-Gene labelling
155 system (Promega) and QuickHyb hybridization solution (Stratagene). Membranes were
156 exposed to a phosphor screen, and signals were quantified on a Storm imaging system
157 using the ImageQuant software version 1.1 (Molecular Dynamics, Amersham
158 Biosciences, Sunnyvale, CA).
159
160 *Production of an anti-equine AKR1C23 antibody*
161 A pair of sense (5' - GATGGATCCGATCCCAAAGGTTGGCGTGT - 3') and
162 antisense (5' - CAGAATTCCCCTGCGGTTAAAGTTGGACAC - 3') primers that
163 incorporated a *BamHI* and an *EcoRI* restriction site, respectively, were designed from
164 the equine AKR1C23 open reading frame to generate a fragment (ΔAKR1C23) spanning
165 the region from Asp^{2} to Arg^{171}. The fragment was amplified by PCR using the Expand
166 High Fidelity polymerase (Roche Molecular Biochemicals) and following the
167 manufacturer's protocol. The fragment was isolated after electrophoresis, digested with
168 *BamHI* and *EcoRI*, subcloned into pGEX-2T in frame with the GST coding region
169 (Amersham Pharmacia Biotech), and sequenced to confirm its identity. Protease-
170 deficient *E. coli* BL-21 (Amersham Pharmacia Biotech) were transformed with the
171 ΔAKR1C23/pGEX-2T construct, expression of the recombinant ΔAKR1C23/GST
172 fusion protein was induced with isopropyl-1-thio-β-D-galactopyranoside (Fisher
173 Scientific, Montréal, Canada), and bacterial protein extracts were obtained after
174 sonication and centrifugation. The ΔAKR1C23/GST fusion protein was purified by
175 affinity on glutathione-Sepharose beads (Amersham Pharmacia Biotech), digested with
176 thrombin to release the ΔAKR1C23, resolved by one-dimensional SDS-PAGE,
177 transferred to nitrocellulose, and stained with Ponceau S Red (Brûlé S *et al.*, 2000). The
178 ΔAKR1C23 band (M_r = 19,200) was cut and used to immunize rabbits as previously
179 described (Brûlé S *et al.*, 2000).
180
181 To demonstrate the specificity of the AKR1C23 antibody, the coding region of
182 the AKR1C23 was subcloned into the mammalian expression vector pcDNA3.1+
183 (Invitrogen) and transient transfections were performed using the HEK293 cell line as

previously described (Filion F *et al.*, 2001). Briefly, HEK293 cells were seeded in 75 cm^2 plates and transfected using 6 μg/plate of AKR1C23/pcDNA3.1 constructs and 36 μg of LipofectAMINE PLUS (Invitrogen) in 1.7 ml of MEM, in accordance with the manufacturer's protocol. Three hours after transfection, cells were incubated in fresh culture media for 24 h, collected, and protein was extracted and analyzed by immunoblot.

Cell extracts and immunoblot analysis

Ovarian cell extracts were prepared as previously described (Filion F *et al.*, 2001). Briefly, tissues were homogenized and sonicated on ice in TED buffer [20 mm Tris (pH 8.0), 50 mm EDTA, and 0.1 mm diethyldithiocarbamic acid) containing 1.0% Tween. The sonicates were centrifuged at 16,000 x g for 15 min at 4 C. The recovered supernatant (whole cell extract) was stored at -80 C until electrophoretic analyses were performed. Protein concentration was determined by the method of Bradford (Bradford MM, 1976) (Bio-Rad protein assay). Samples (50 μg proteins) were resolved by one-dimensional SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Filion F *et al.*, 2001). Membranes were incubated with the polyclonal anti-AKR1C23 antibody (1:4,000), and immunoreactive proteins were visualized on Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) after incubation with the horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:10,000 dilution) and the enhanced chemiluminescence system (ECL Plus), following the manufacturer's protocol (Amersham Pharmacia Biotech).

Immunohistochemical localization of AKR1C23

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories, Inc.), as previously described (Sirois J and Doré M, 1997). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 μm-thick sections were prepared and deparaffined through a graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with diluted normal goat serum for 20 min at room temperature. The anti-AKR1C23 antibody was diluted in PBS

(1:1000 dilution) and applied, and sections were incubated overnight at 4 C. Control sections were incubated with PBS. After rinsing in PBS for 10 min, a biotinylated goat antirabbit antibody (1:222 dilution; Vector Laboratories, Inc.) was applied, and sections were incubated for 45 min at room temperature. Sections were washed in PBS for 10 min and incubated with avidin DH-biotinylated horseradish peroxidase H reagents for 45 min at room temperature. After washing with PBS for 10 min, the reaction was revealed using diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with Gill's hematoxylin stain and mounted.

AKR1C23 expression, in vitro enzyme activity and measurement of 20α-DHP concentration in follicular fluid

A pair of sense (5' - CTCGGTACCATGGATCCCAAAGGTTGGCGT - 3') and antisense (5' - TGGCTGCAGTTAATAATCATCAGAAAATGG - 3') primers that incorporated a *KpnI* and a *PstI* restriction site, respectively, were designed from the equine AKR1C23 open reading frame to generate a full-lenth AKR1C23 protein. The cDNA was amplified by PCR using the Expand High Fidelity polymerase (Roche Molecular Biochemicals) and following the manufacturer's protocol. The fragment was isolated after electrophoresis, digested with *KpnI* and *PstI*, subcloned into pQE-30 in frame with the His-tag coding region (Qiagen, Inc.), and sequenced to confirm its identity. *E. coli* M15 (Qiagen, Inc) were transformed with the AKR1C23/pQE-30 construct, expression of the His-tagged AKR1C23 fusion protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Fisher Scientific, Montréal, Canada), and bacterial protein extracts were obtained after lysis and centrifugation according to manufacturer's recommendations. The His-AKR1C23 fusion protein was purified by affinity on Ni-NTA resin (Qiagen, Inc), and concentrated using a 30-kDa cutoff Amicon centrifugal filter device (Millipore corp.). The 20α-HSD activity was examined by following the decrease in NADPH absorbance at 340 nm on a Beckman Coulter DU800 spectrophotometer (Madore E *et al.*, 2003). Ten μg of purified AKR1C23 protein was assayed at 37 C in 50 mM Tris-HCl (pH 7.5) with saturating NADPH (100 μM) at various progesterone concentrations. The follicular fluid present in equine preovulatory follicles isolated between 0 and 39 h post-hCG was analyzed for 20α-DHP content by a

246 gas chromatographic mass spectrometric method developed to measure steroid hormone
247 levels in rat and monkey serum (Bérubé R *et al.*, 2000). Briefly, 20α-DHP was
248 extracted from follicular fluid by liquid-liquid and solid-phase extraction. Derivatization
249 reactions were performed to improve chromatographic and detection response of the
250 steroids. Unconjugated steroids were quantified by means of a sensitive gas
251 chromatographic/mass spectrometric (GC/MS) method, using chemical ionization (CI).
252
253 *Statistical analysis*
254 One-way ANOVA was used to test the effect of time after hCG administration
255 on levels of AKR1C23 mRNA and on 20α-DHP concentration. AKR1C23 mRNA
256 levels were normalized with the control gene rpL7a before analysis. When ANOVAs
257 indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple
258 comparisons of individual means ($P < 0.05$). Statistical analyses were performed using
259 JMP software (SAS Institute, Inc., Cary, NC).
260
261
262 **RESULTS**
263 *Characterization of equine AKR1C23*
264 To clone the equine AKR1C23 transcript, RT-PCR was performed on ovarian
265 RNA using oligonucleotide primers designed by sequence alignment of known AKR1C
266 species homologs. The resulting cDNA fragment (Fig. 1Aa) was sequenced and found
267 to be highly homologous to 20α-HSD transcripts identified thus far. A combination of
268 5'- and 3'-RACE reactions yielded cDNA products corresponding to all remaining
269 coding regions, as well as 3'- and 5'-untranslated regions (Fig. 1, Ab and Ac). A RT-
270 PCR product was generated to extend the length of the open reading frame, thereby
271 confirming that all three products were derived from the same transcript (Fig. 1Ae). The
272 deduced 1562-bp primary transcript encoded a 969-bp open reading frame (Fig. 1A,
273 GenBank accession number AY955082), which predicted a protein of 322 amino acid
274 residues.
275

276 The predicted protein is highly conserved, with a single amino acid deletion,
277 when compared with human (AKR1C1) (Blouin K *et al.*, 2005), macaque (AKR1C1)
278 (Higaki Y *et al.*, 2002), rabbit (AKR1C5) (Lacy WR *et al.*, 1993), cow (Madore E *et al.*,
279 2003) and rat (AKR1C8) (Albarracin CT *et al.*, 1994) proteins suspected of having 20α-
280 HSD activity. The equine AKR1C23 has 80.8% identity at the amino acid level and a
281 85.3% identity at the nucleic acid level when compared to human AKR1C1 (Fig. 2). All
282 putative conserved amino acids implicated in AKR1C function appear to be present in
283 the equine enzyme (Fig. 2).
284
285 *Tissue distribution of equine AKR1C23 mRNA*
286 To study the tissue distribution of equine AKR1C23, various equine tissues were
287 obtained and the expression of AKR1C23 was examined by RT-PCR/Southern blot.
288 Results showed that the AKR1C23 transcript was expressed in many of the tissues
289 studied (Fig. 3A). Levels of AKR1C23 mRNA were highest in a preovulatory follicle
290 isolated 36 h after hCG (i.e. approximately 3-6 h before ovulation) and testis; moderate
291 in liver, skeletal muscle, heart , kidney and lung; low in brain, stomach and uterus; and
292 very low in thymus, adrenal, spleen and skin. However, levels of the control gene rpL7a
293 remained relatively constant in all tissues studied (Fig. 3B).
294
295 *Regulation of AKR1C23 transcript in preovulatory follicles and corpora lutea*
296 The regulation of AKR1C23 mRNA in preovulatory follicles was studied by RT-
297 PCR/Southern blot, using follicles isolated during estrus at 0, 12, 24, and 36 h after the
298 administration of an ovulatory dose of hCG. Total RNA was extracted from the follicle
299 wall (theca interna with attached granulosa cells), as well as from three corpora lutea
300 obtained on day 8 of the estrous cycle. Levels of equine AKR1C23 mRNA were low in
301 equine preovulatory follicles prior to treatment with hCG (0 h), but were clearly induced
302 from 12 h to 36 h post hCG (Fig. 4A). The AKR1C23 mRNA expression returned to
303 basal levels in the corpus luteum at day 8 of the cycle (Fig. 4A). When results from
304 multiple follicles and corpora lutea were expressed as ratios of AKR1C23 to rpL7a, a
305 significant increase in AKR1C23 transcript was detected in follicles, reaching a plateau

306 from 12 to 36 h post hCG ($P< 0.05$; Fig. 4C). No change in rpL7a transcript was
307 detected after gonadotropin treatment (Fig. 4B).
308
309 In order to determine which cell type in the equine follicle wall was responsible
310 for the expression of the AKR1C23 transcript, granulosa and theca interna cells were
311 isolated from follicles obtained between 0 and 39 h post hCG (Fig. 5). Results indicated
312 that granulosa cells were the predominant site of AKR1C23 expression and that this cell
313 type contributed more importantly to the increase in transcript observed in intact follicle
314 wall preparations. In granulosa cells, this increase was significant between 12 and 39 h
315 post hCG ($P< 0.05$; Fig. 5A). Results demonstrated a slight yet significant induction of
316 AKR1C23 mRNA in theca interna cells at 39 h post hCG ($P< 0.05$; Fig. 5B).
317
318 *Expression of AKR1C23 protein in equine preovulatory follicles*
319 The hCG-dependent induction of AKR1C23 was studied at the protein level by
320 immunoblotting and immunohistochemistry in follicles at 0 and 36 or 39 h post hCG.
321 The specificity of the antibody was confirmed, as it recognized the equine AKR1C23
322 protein overexpressed in HEK293 cells (M_r = 37,000; Fig. 6A). Immunoblotting
323 analyses were performed on protein extracts from granulosa cells and theca interna at 0
324 and 36 h post-hCG. An increase in levels of immunoreactive AKR1C23 was observed
325 at 36 h post hCG in granulosa cells (Fig. 6B). However, little or no signal was detected
326 in theca interna samples (Fig. 6C). Immunohistochemical results demonstrated a
327 marked change in AKR1C23 staining after hCG treatment (Fig. 7). Follicles isolated
328 prior to hCG treatment (0 h) show a very compact granulosa cell layer and light staining
329 (Fig. 7A). Although some darker staining is apparent in the periantral granulosa cells,
330 this is likely due to the "edge effect" observed when using this technique (Fig. 7A). The
331 administration of hCG caused the granulosa cell layer to expand and an increase in
332 AKR1C23 accumulation (Fig. 7B- D) was observed in follicles isolated 39 h post hCG.
333 Control sections of follicles isolated at 39 h post hCG showed no staining when
334 AKR1C23 antibody was omitted (Fig 7F). The pattern of expression in testes
335 demonstrated AKR1C23 expression in Leydig cells only (Fig. 7E).
336

20α-hydroxysteroid dehydrogenase activity and concentration of 20α-DHP in follicular fluid

The AKR1C23 recombinant protein was overexpressed in *M15 E.coli* cells and affinity-purified using a Ni-NTA column. Activity assays were done by monitoring the absorbance at 340 nm, at a temperature of 37 C, and values were corrected for the background signal in the absence of substrate. Enzyme functionality was confirmed via its ability to reduce phenanthrenequinone (data not shown). The use of various concentrations of progesterone established the 20α-HSD activity of the AKR1C23 enzyme. The Lineweaver-Burke plot for AKR1C23 reflected a K_M of 3.12 μM and a Vmax of 0.86 pmol/min/10 μg protein towards progesterone (Fig. 8A). When follicular fluid was examined, a significant increase in 20α-DHP was observed 30-36 h post-hCG (Fig. 8B).

DISCUSSION

This study demonstrates for the first time that the process of follicular luteinization induced by hCG is accompanied by an increase in expression of AKR1C23, an aldo-keto reductase with many potential activities, including the conversion of progesterone to 20α-DHP. The process of luteinization/ovulation has previously been associated with dramatic changes in levels of steroidogenic enzymes in the different cellular compartments of the preovulatory follicle. Notably, a marked decrease or loss in the cytochrome P450 17α-hydroxylase/17,20-lyase and aromatase, which are key enzymes involved in androgen and estrogen biosynthesis, and the increase in steroidogenic acute regulatory protein and cytochrome P450 cholesterol side-chain cleavage expression, which contribute to enhanced progesterone synthesis (Fortune JE, 1994, Richards JS, 1994, Ronen-Fuhrmann T *et al.,* 1998, Sandhoff TW *et al.*, 1998). Such changes have also been observed during hCG-induced luteinization/ovulation in the mare (Boerboom D *et al.,* 1999, Boerboom D and Sirois J, 2001, Kerban A *et al.*, 1999). However, there has been no report on the regulation of enzymes with 20α-HSD activity during the periovulatory period. Previous investigations of the expression of 20α-HSD in the ovary have primarily been limited to the examination of its regulation

368 during luteolysis prior to parturition by Northern blot analysis and by tissue distribution
369 analyses by *in situ* hybridization (Albarracin CT *et al.*, 1994, Nishizawa M *et al.*, 2000,
370 Pelletier G *et al.*, 2003). Collectively, these studies revealed that the 20α-HSD gene
371 (AKR1C8) was highly expressed in the rat corpus luteum at the end of gestation, and
372 that the human 20α-HSD (AKR1C1) promoter was functional in porcine luteinized
373 granulosa cells in culture.
374
375 Results from the present study suggest that the induction of a progesterone-
376 metabolizing enzyme such as AKR1C23 may provide the biochemical basis for the
377 increase in 20α-DHP observed during the periovulatory period. Indeed, an increase in
378 20α-DHP levels has been demonstrated to occur in a number of species, including the
379 rat and rabbit (Lacy WR *et al.*, 1993, Lau IF *et al.*, 1978, Nordenstrom K and Johanson
380 C, 1985). This study establishes that such an increase in 20α-DHP also occurs in mares.
381 However, the precise role of AKR1C23 during follicular luteinization/ovulation is
382 intriguing and remains to be investigated. Its purpose as a progesterone-metabolizing
383 enzyme remains perplexing since progesterone appears to be required for ovulation, as
384 demonstrated by the anovulatory phenotype of progesterone receptor-mutant mice
385 (Lydon JP *et al.*, 1995). AKR1C23's physiological importance may involve its ability to
386 produce 20α-DHP. This metabolite has been shown to promote ovulation and
387 gonadotropin secretion, and has been shown to increase sexual receptivity when
388 metabolized further by 5α-reductase in the brain (Frye CA and Leadbetter EA, 1994).
389
390 The multifunctional nature of the AKR protein led investigators to question the
391 predominant role of the enzyme under physiological conditions. However, the
392 difficulties involved in substrate manipulation, product analysis and enzyme stability,
393 make it hard to have a comparative view of all possible AKR activities under similar
394 conditions. In this study, we demonstrate that the AKR1C23 enzyme does convert
395 progesterone to 20α-DHP with an affinity that approaches that previously reported for
396 the human enzyme (Zhang Y *et al.*, 2000). AKRs of this type, like AKR1C3 and
397 AKR1C7, have also been shown to harbour prostaglandin F synthase-like activity, where
398 this enzyme converts PGD_2 into 9α,11β PGF_2, a $PGF_{2\alpha}$ isomer as well as converting

399 PGH_2 into $PGF_{2\alpha}$ (Desmond JC *et al.*, 2003, Watanabe K *et al.*, 1986). Considering that
400 the levels of $PGF_{2\alpha}$ have been demonstrated to increase following hCG treatment in
401 equine preovulatory follicles (Sirois J and Doré M, 1997), it is not unreasonable to think
402 that AKR1C23 may contribute to this increase in $PGF_{2\alpha}$. The concept of a protein
403 having many enzymatic activities working in concert has previously been described
404 (Madore E *et al.*, 2003), where an aldose reductase (AKR1B5) was shown to have both
405 20α-HSD and PGFS activities in cultures of bovine uterine endometrial cells. It was
406 speculated that these concerted activities may lead to termination of the estrous cycle
407 (Madore E *et al.*, 2003). Other possible activities of AKR1C23 such as 17β-HSD type
408 V and 3α-HSD type II, which convert androstenedione to testosterone and 5α-
409 dihydrotestosterone to androstanediol, respectively, have yet to be examined and their
410 role, if any, during follicular luteinization needs to be elucidated.
411
412 The molecular control of AKR1C expression remains largely uncharacterized.
413 Moreover, because of confusion regarding nomenclature, difficulties lie in identifying
414 precisely which AKR is being studied in previous publications. Many reports addressed
415 the regulation of AKRs speculated to have 20α-HSD activity in various tissues,
416 including the ovary (Albarracin CT *et al.*, 1994, Lacy WR *et al.*, 1993, Pelletier G *et al.*,
417 2003, Stocco CO *et al.*, 2000, Strauss JF, 3rd and Stambaugh RL, 1974). However,
418 most ovarian studies remained largely at the level of the corpus luteum. Early
419 investigations on the luteolytic effects of $PGF_{2\alpha}$ showed that 20α-HSD activity is
420 induced 150-fold in the rat ovary (Strauss JF, 3rd and Stambaugh RL, 1974), this being
421 consistent with more recent findings that demonstrate that rat 20α-HSD (AKR1C8) is
422 induced by $PGF_{2\alpha}$ (Stocco CO *et al.*, 2000). The transcription factor Nur77 was shown to
423 play a key role in the $PGF_{2\alpha}$-dependent induction of 20α-HSD (AKR1C8) in rat luteal
424 cells (Stocco CO *et al.*, 2000). The present study identifies high/ovulatory levels of
425 gonadotropins as a physiological regulator of AKR1C23 in the preovulatory follicle,
426 with the predominant regulation observed in granulosa cells. Interestingly, LH has
427 previously been shown to upregulate Nur77 in rat granulosa cells (Park JI *et al.*, 2003),
428 thus providing a potential trans-activating factor for follicular AKR1C23 gene
429 expression. Conversely, previous investigations suggest that prolactin is a repressor of

20α-HSD expression, since it was shown to reduce 20α-HSD (AKR1C8) protein level during corpus luteum regression in vivo, and in rat luteal cells in vitro (Albarracin CT *et al.*, 1994).

In summary, this study is the first to characterize the primary structure of AKR1C23, to investigate the expression of the AKR1C23 gene in a developmental series of preovulatory follicles, and to identify ovulatory levels of gonadotropins as a positive regulator of AKR1C23 expression. The deduced primary structure of AKR1C23 is highly conserved when compared to species homologs with all putative conserved amino acids implicated in NADPH and substrate binding present (Jez JM *et al.*, 1997). Although the precise molecular control of AKR1C23 induction in preovulatory follicles remains to be elucidated, it is interesting to note that the luteinization/ovulatory process is accompanied by an induction of $PGF_{2\alpha}$ and Nur77 (Park JI *et al.*, 2003, Sirois J and Doré M, 1997). Given their putative role in 20α-HSD expression (Stocco CO *et al.*, 2000, Strauss JF, 3rd and Stambaugh RL, 1974), it will be interesting to determine whether they are intermediates in the gonadotropin-dependent induction of the AKR1C23 in follicular cells. The precise physiological significance of AKR1C23 during ovulation and its role in regulating the bioactivity of progestins prior to follicular rupture should also remain the focus of future investigations.

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667 **LEGENDS FOR FIGURES**

668

669 Fig. 1. Cloning of equine AKR1C23. A, Cloned cDNA fragments. Each fragment is
670 schematically represented, with its identity indicated on the right and its position in the
671 deduced transcript sequence indicated in parentheses. Lines indicate untranslated
672 regions (UTRs); open boxes designate the open reading frame (ORF). Lengths of the
673 deduced transcript and its structural elements are indicated in base pairs (bp). Arrows
674 indicate the position and orientation of the oligonucleotides employed in the cloning
675 processes, with numbers indicating their identity. B, Oligonucleotides used in the
676 various cloning procedures. Oligonucleotides 9 and 11 are components of the 5'-RACE
677 kit (Invitrogen).

678

679 Fig. 2. Deduced primary structure of the equine AKR1C23 protein and comparison with
680 known 20α-HSDs and bovine PGFS. The predicted amino acid sequence of the equine
681 AKR1C23 protein is aligned with human (hum; AKR1C1; GeneBank accession number
682 NP_001344), macaque (mcq; AKR1C1; Q95JH7), rabbit (rab; AKR1C5; P80508), and
683 rat (AKR1C8; NP_612519) 20α-HSDs, as well as cow PGFS-like2 (cow; N/A;
684 AAN11329). Identical residues are indicated by a printed period, numbers on the right
685 refer to the last amino acid residue on that line. *Bold underlined* residues are highly
686 conserved among AKRs, *boxed* residues are thought to be involved in NADPH cofactor
687 binding, and residues with an *asterisk* are involved in substrate binding (Jez JM *et al.*,
688 1997).

689

690 Fig. 3. Expression of AKR1C23 mRNA in equine tissues. Total RNA was extracted
691 from various equine tissues, and samples (100 ng) were analyzed for AKR1C23 and
692 rpL7a (control gene) content by a semi-quantitative RT-PCR/Southern blotting
693 techniques, as described in *Materials and Methods*. A, Expression of AKR1C23 mRNA
694 in equine tissues. B, Expression of rpL7a mRNA in equine tissues. The number of PCR
695 cycles for each gene was within the linear range of amplification, and they represented
696 21 and 18 cycles for AKR1C23 and rpL7a, respectively. Numbers on the *right* indicate
697 the size of the PCR fragment.

Fig. 4. Regulation of AKR1C23 transcript by hCG in equine preovulatory follicles. Preparations of follicle wall were obtained from preovulatory follicles isolated between 0-36 h after hCG, and corpora lutea (CL) were isolated on d 8 of the estrous cycle. Samples (100 ng) of total RNA were analyzed for AKR1C23 and rpL7a content by a semi-quantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. A, Regulation of AKR1C23 mRNA in equine follicles (one representative follicle per time point). B, Constitutive expression of rpL7a mRNA in the same follicles. Numbers on the *right* indicate the size of the PCR fragment. C, Relative levels of intensity of AKR1C23 signal, normalized to the control gene rpL7a (n = 5-6 distinct follicles [i.e. mares] per time point and n = 3 different corpora lutea). *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$).

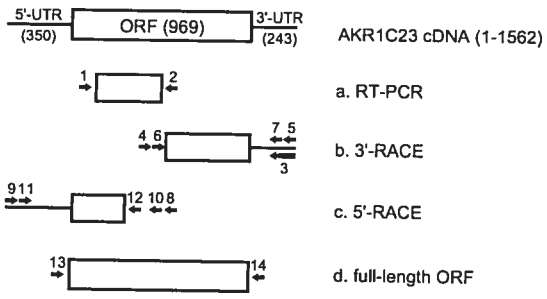
Fig. 5. Regulation of AKR1C23 mRNA in equine granulosa and theca interna cells. Preperations of granulosa cells (A) and theca interna (B) were isolated from equine preovulatory follicles between 0-39 h after hCG treatment, and samples (100 ng) of total RNA were analyzed for AKR1C23 and rpL7a content by a semiquantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. The AKR1C23 signal was normalized with the control gene rpL7a, and results are presented as a ratio of AKR1C23 to rpL7a (mean ± SEM; n = 4 samples [*i.e.* mares] per time point). *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$). *Insets* show representative results for AKR1C23 and rpL7a mRNA levels from one sample per time point.

Fig. 6. AKR1C23 primary antibody specificity and regulation of AKR1C23 protein by hCG in equine granulosa and theca interna. Protein extracts were prepared from transiently transfected and mock-transfected (*control*) HEK293 cells (A), as well as from granulosa cells (B) and theca interna (C) isolated 0 and 36 h after hCG treatment (n = 2 samples [*i.e.* mares] per time point) and were analyzed by one-dimensional SDS-PAGE and immunoblotting using a specific polyclonal antibody raised against a fragment of the equine AKR1C23 protein, as described in *Materials and Methods*.

729 Results from protein extracts (50 μg/lane) are shown. Markers on the *right* indicate the
730 migration of the molecular weight (M_r) standards (A) and expected position of
731 AKR1C23 protein (B and C).
732
733 Fig. 7. Immunohistochemical localization of AKR1C23 in equine preovulatory follicles.
734 Immunohistochemistry was performed on formalin-fixed sections of preovulatory
735 follicles isolated at 0 and 39 h after hCG treatment and of equine testicular tissues, as
736 described in *Materials and Methods*. Results show relatively weak, but detectable,
737 AKR1C23 staining in granulosa (GC) and theca interna (TI) cells of a preovulatory
738 follicle obtained 0 h after hCG administration (A), but a marked increase in signal
739 intensity in both cell types of distinct follicles isolated 39 h after hCG treatment (B-D).
740 E, Intense AKR1C23 immunoreactivity is observed in Leydig (L) cells of equine
741 testicular sections. F, Control staining from the follicular tissue presented in D was
742 negative when the primary antibody was omitted. Magnification, ×200 (A-C, and E)
743 and ×400 (D and F).
744
745 Fig. 8. Enzyme kinetics of AKR1C23 and steroid levels in follicular fluid. Lineweaver-
746 Burke plot of 20α-HSD activity of equine AKR1C23 (A). Various concentrations of
747 progesterone were assayed at 37C by monitoring absorbance at 340 nm. Concentrations
748 of 20α-DHP (B) were measured in follicular fluid of preovulatory follicles isolated
749 between 0-36 h after hCG treatment (n = 4-6 samples per time point [i.e. mares]). Data
750 were transformed to logarithms before analysis as heterogeneity of variance was
751 observed. Bars marked with *asterisks* are significantly different from 0 h post-hCG ($P < 0.05$).
752
753
754

FIGURE 1

A



B

1. 5'-GGACTGGCCATCCGAAGCAAGAT-3'
2. 5'-CGGATGACATTCTACCTGGTTGCA-3'
3. 5'-GTACCGGATCCTCTAGAGAGCTCGTCGACCTCGAGG AATTCAAGCTTTTTTTTTTTTTTTTTTTT-3'
4. 5'-TGGGCTACTTCTCTTCGACTGC -3'
5. 5'-GTACCGGATCCTCTAGAGAGCTC-3'
6. 5'-CGGAGTGTCCAACTTTAACCGC-3'
7. 5'-GTCGACCTCGAGGAATTCAAGCTT-3'
8. 5'-CCCTGGCTTGACAGCCACAGG-3'
9. 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'
10. 5'-GGCAGGCCGGACCAATTGCAG-3'
11. 5'-GGCCACGCGTCGACTAGTAC-3'
12. 5'-TGCAGTCGAAGAGAAGTAGCCCA-3'
13. 5'-GTGGGTACCGCCGCCACAATGGATCCCAAAGGTTGGC-3'
14. 5'-GGTCTCGAGTTAATAATCATCAGAAAATGGATACTCA-3'

FIGURE 2

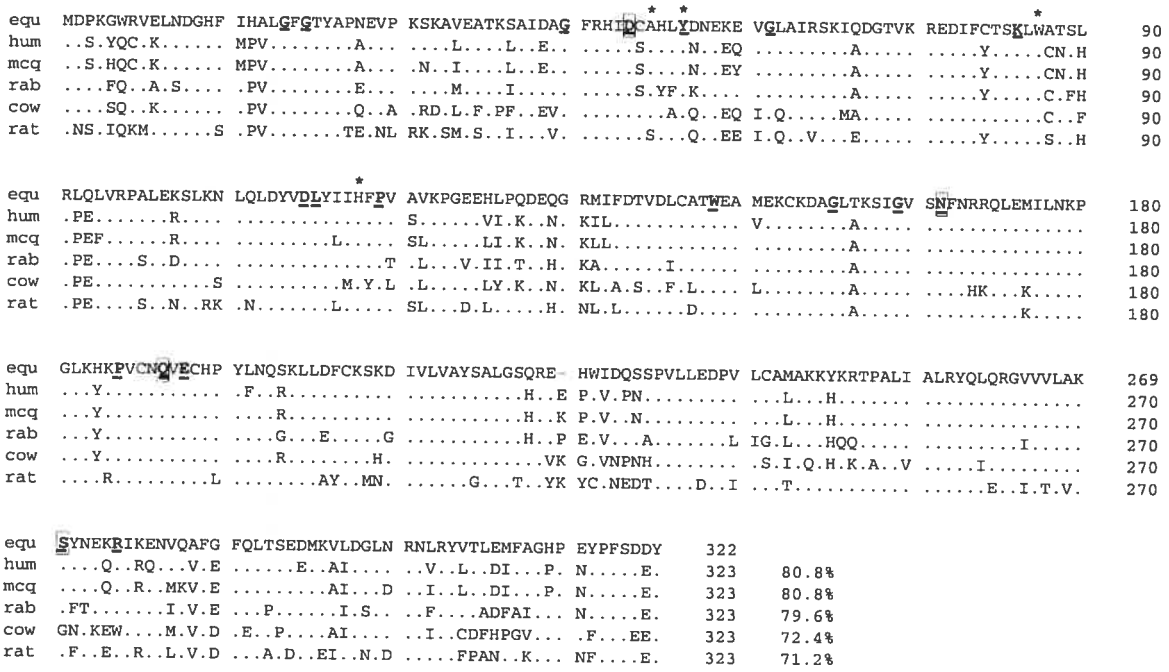

equ MDPKGWRVELNDGHF IHALGFGTYAPNEVP KSKAVEATKSAIDAG FRHIDCAHLYDNEKE VGLAIRSKIQDGTVK REDIFCTSKLWATSL 90
hum ..S.YQC.K..... MPV.....A... ....L....L..E.. .....S....N..EQ .....A..... .....Y.....CN.H 90
mcq ..S.HQC.K..... MPV.....A... .N..I....L..E.. .....S....N..EY .....A..... .....Y.....CN.H 90
rab ....FQ..A.S.... .PV.....E... ....M....I..... .....S.YF.K.... .....A..... .....Y.....C.FH 90
cow ....SQ..K..... .PV.....Q..A .RD.L.F.PF..EV. .....A.Q..EQ I.Q.....MA..... .....C..F 90
rat .NS.IQKM.....S .PV.....TE.NL RK.SM.S..I...V. .....S...Q..EE I.Q..V...E..... .....Y.....S..H 90
equ RLQLVRPALEKSLKN LQLDYVDLYIIHFPV AVKPGEEHLPQDEQG RMIFDTVDLCATWEA MEKCKDAGLTKSIGV SNFNRRQLEMILNKP 180
hum .PE.....R.... ..... S.....VI.K..N. KIL..... V.....A..... 180
mcq .PEF.....R.... .....L..... SL.....LI.K..N. KLL..... .....A..... 180
rab .PE....S..D.... .....T .L...V.II.T..H. KA.....I..... .....A..... 180
cow .PE.....S .....M.Y.L .L.....LY.K..N. KL.A.S..F.L.... L.....A..... ....HK...K..... 180
rat .PE....S..N..RK .N.....L..... SL...D.L.....H. NL.L.....D.... .....A..... .....K..... 180
equ GLKHKPVCNQVECHP YLNQSKLLDFCKSKD IVLVAYSALGSQRE- HWIDQSSPVLLEDPV LCAMAKKYKRTPALI ALRYQLQRGVVVLAK 269
hum ...Y..... .F..R..... .....H..E P.V.PN..... ...L...H..... 270
mcq ...Y..... ....R..... .....H..K P.V..N..... ...L...H..... 270
rab ...Y..... ....G...E.....G .....H..P E.V...A.....L IG.L...HQQ..... .....I..... 270
cow ...Y..... ....R.....H. .....VK G.VNPNH..... .S.I.Q.H.K.A..V .....I..... 270
rat ....R.....L .....AY..MN. .....G...T..YK YC.NEDT....D..I ...T..... .....E..I.T.V. 270
equ SYNEKRIKENVQAFG FQLTSEDMKVLDGLN RNLRYVTLEMFAGHP EYPFSDDY 322
hum ....Q..RQ...V.E .....E..AI.... ..V..L..DI...P. N.....E. 323 80.8%
mcq ....Q..R..MKV.E .....AI...D ..I..L..DI...P. N.....E. 323 80.8%
rab .FT.....I.V.E ...P.....I.S.. ..F....ADFAI... N.....E. 323 79.6%
cow GN.KEW....M.V.D .E..P....AI.... ..I..CDFHPGV... .F...EE. 323 72.4%
rat .F..E..R..L.V.D ...A.D..EI..N.D .....FPAN..K... NF....E. 323 71.2%

FIGURE 3

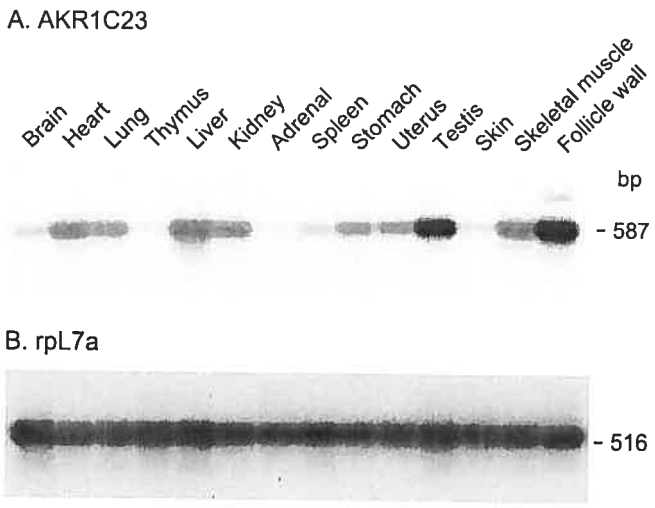

A. AKR1C23
Brain
Heart
Lung
Thymus
Liver
Kidney
Adrenal
Spleen
Stomach
Uterus
Testis
Skin
Skeletal muscle
Follicle wall
bp
- 587
B. rpL7a
- 516

FIGURE 4

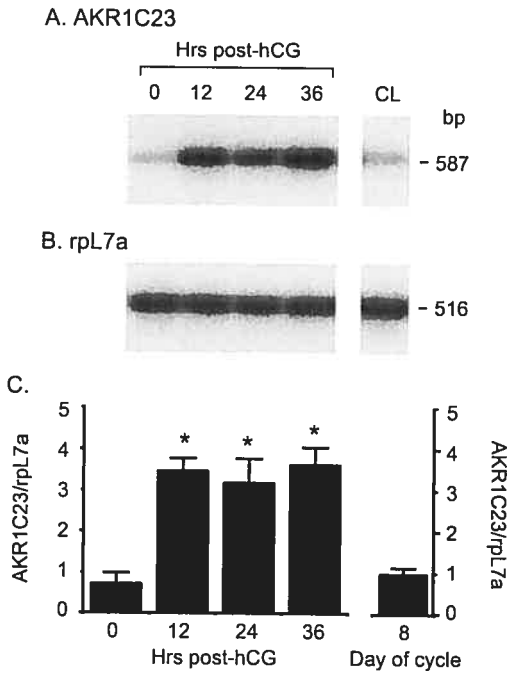

A. AKR1C23
Hrs post-hCG
0 12 24 36
CL
bp
587
B. rpL7a
516
C.
AKR1C23/rpL7a
5
4
3
2
1
0
*
*
*
0 12 24 36
Hrs post-hCG
8
Day of cycle

FIGURE 5

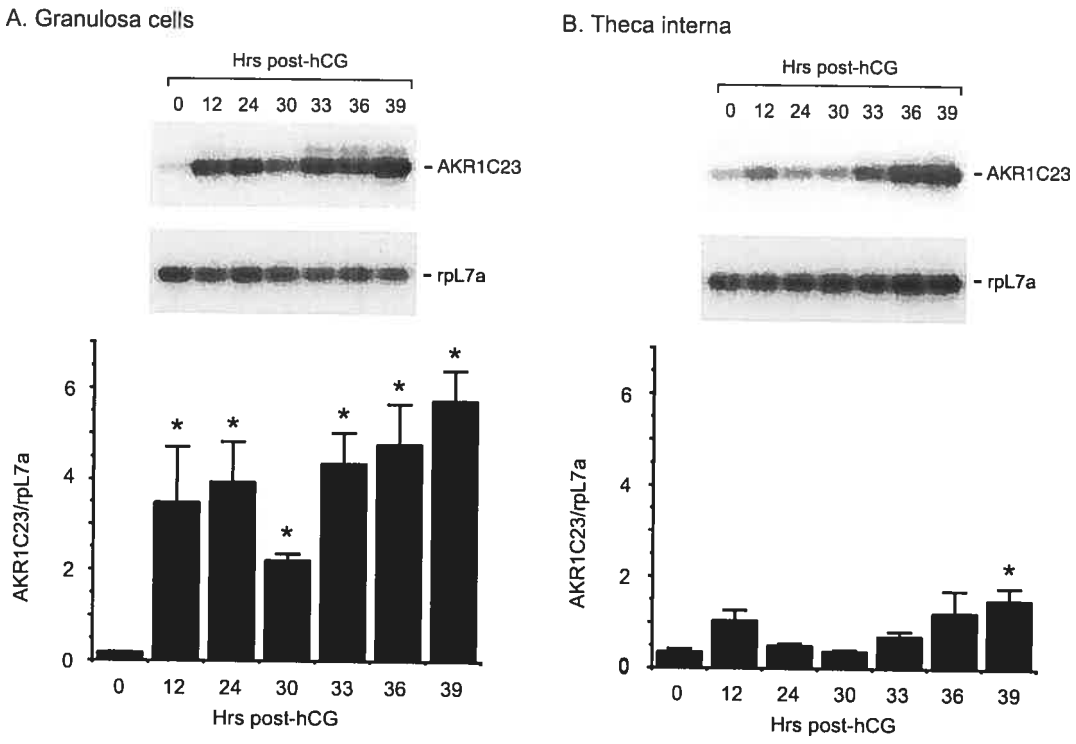

A. Granulosa cells
Hrs post-hCG
0 12 24 30 33 36 39
- AKR1C23
- rpL7a
AKR1C23/rpL7a
0
2
4
6
0 12 24 30 33 36 39
Hrs post-hCG
B. Theca interna
Hrs post-hCG
0 12 24 30 33 36 39
- AKR1C23
- rpL7a
AKR1C23/rpL7a
0
2
4
6
0 12 24 30 33 36 39
Hrs post-hCG

FIGURE 6

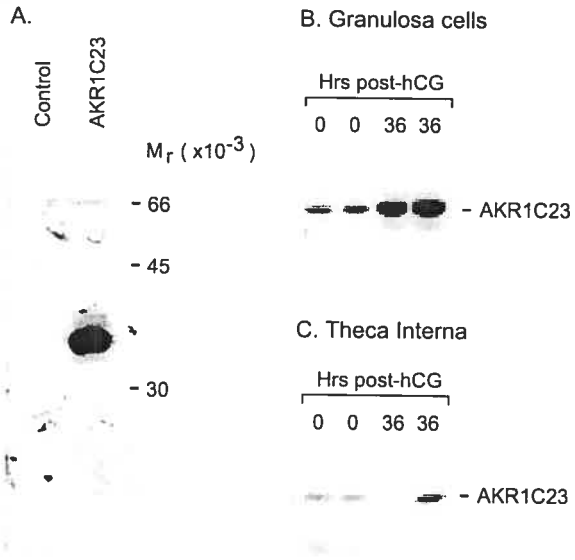

A.
Control
AKR1C23
M_r (x10^{-3})
- 66
- 45
- 30
B. Granulosa cells
Hrs post-hCG
0 0 36 36
- AKR1C23
C. Theca Interna
Hrs post-hCG
0 0 36 36
- AKR1C23

FIGURE 7

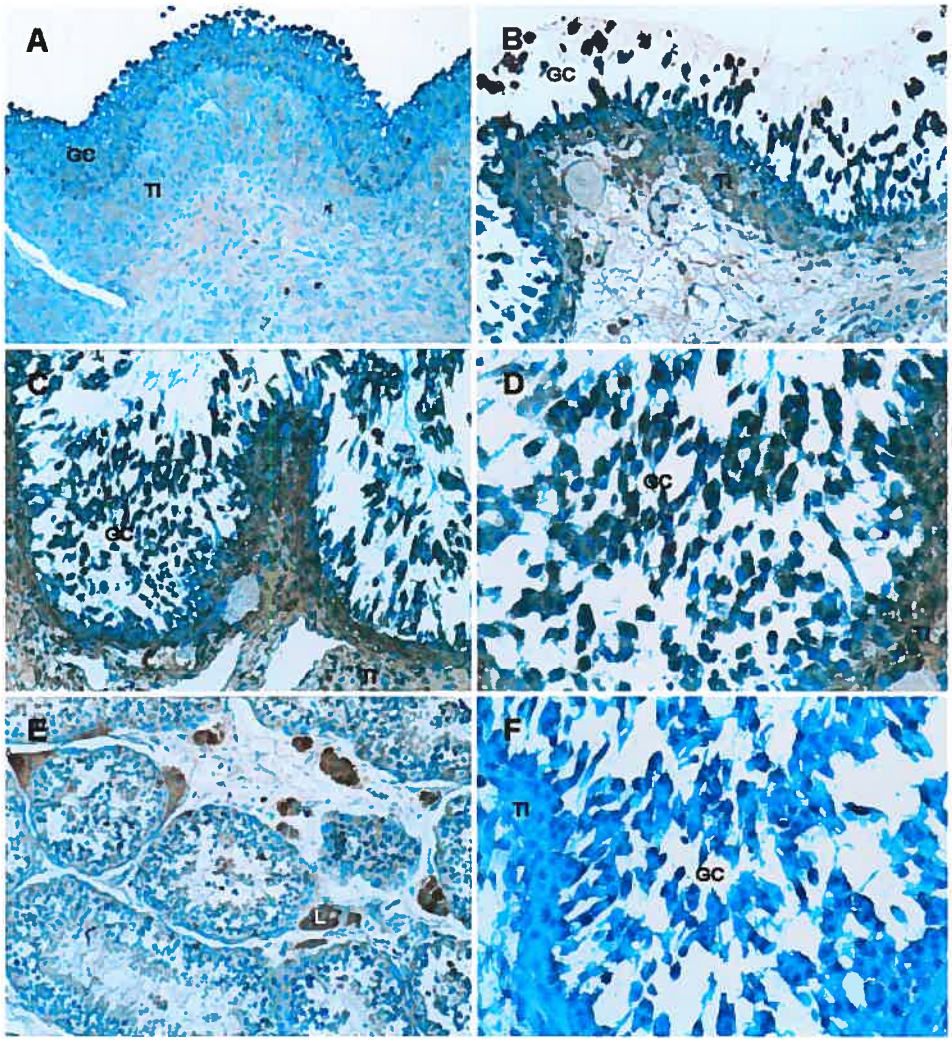
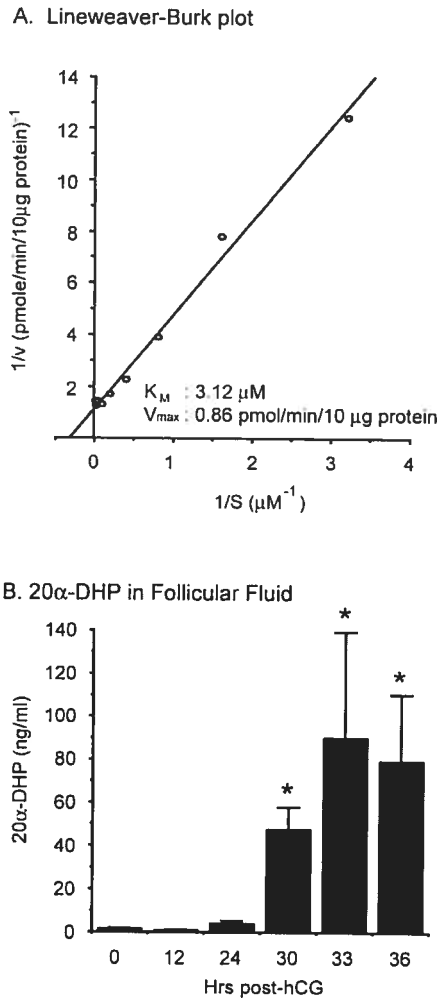


FIGURE 8


A. Lineweaver-Burk plot
1/v (pmole/min/10µg protein)$^{-1}$
14
12
10
8
6
4
2
K_M : 3.12 µM
V_{max} : 0.86 pmol/min/10 µg protein
0
1
2
3
4
1/S (µM^{-1})
B. 20α-DHP in Follicular Fluid
20α-DHP (ng/ml)
140
120
100
80
60
40
20
0
*
*
*
0
12
24
30
33
36
Hrs post-hCG

GENERAL DISCUSSION

The overall hypothesis of this thesis was that the change in steroid concentrations observed during the ovulatory process is a result of the programmed modulation of the expression of specific genes responsible for the activation, inactivation and bioavailability of steroids. To test this hypothesis, we undertook a series of cloning and mRNA regulation studies, supported at times by protein analyses, activity assays and steroid concentrations from the follicular fluid, and thereby elucidating some of the gene regulation events induced by gonadotropins in the equine ovary. The primary objective of this thesis was to establish the luteinizing preovulatory follicle as a site where genes responsible for steroid activation, inactivation and bioavailability were tightly regulated. The sum of the data argue for the validity of the hypothesis. The conclusions drawn from these investigations have been grouped into five discussion sections.

The gonadotropin-dependent downregulation of 17βHSD1 mRNA provides an additional molecular basis for the decrease in 17β-estradiol biosynthesis observed during the process of luteinization

The first paper in this thesis was aimed at characterizing the transcriptional regulation of an enzyme involved in the last step of 17β-estradiol biosynthesis, namely 17βHSD1. To achieve this aim, the primary structure of the equine 17βHSD1 gene, cDNA and protein needed to be identified. Difficulties in obtaining the 3'-end of the 17βHSD1 cDNA, partly due to its high GC content as well as its relatively short 3'-UTR, led to the screening of an equine genomic library. The genomic structure and the exon/intron boundaries of the equine 17βHSD1 gene were found to be highly conserved when compared to mammalian orthologues, although certain differences in intron and exon lengths had been reported for many species (131). Notably, a slight variation in the lengths of introns 3 and 4, which measure 131 and 443 bp, respectively in the mare, have been shown to range between 80 and 198 bp for intron 3, and between 377 and 530 bp for intron 4 in other species. The length of exon 6 is itself highly variable at the 3'-end, which results in cDNAs from different species having different

sized open reading frames, and consequently leading to different sized proteins with highly variable carboxy-termini. Indeed, the 308-amino acid equine 17βHSD1 protein is 20 amino acids shorter than the human protein (GenBank accession number NP_000404), which is 16 amino acids shorter than both rodent models (rat: AAH86365 and mouse: CAA61770). These differences are inconsequential, however, as the catalytic domain for the human protein has been shown to be located at approximately two thirds of the distance from the amino-terminus, with amino acids His^{222}, Glu^{283} and Tyr^{156} being involved substrate-binding, and Val^{189} which has been shown to be important in cofactor binding (420).

In the present study, total RNA was extracted from equine follicles isolated between 0 and 39 h post hCG and served to study the gonadotropin-dependent regulation of 17βHSD1 mRNA during the ovulatory process. RT-PCR/Southern blot analyses demonstrated a very high level of 17βHSD1 expression occurring selectively in the granulosa cells of preovulatory follicles obtained prior to hCG treatment. Gonadotropin treatment resulted in a rapid downregulation of 17βHSD1 transcript expression visible in the first sample studied, namely a follicle isolated at 12 h post-hCG. Importantly, a similar regulatory process has also been demonstrated for promoter II-derived CYP19A1 mRNA (43). Considering that both these enzymes are involved in the final steps of 17β-estradiol biosynthesis, it is not unreasonable to think that they may be regulated through the same mechanisms.

Approximately 1.4 kb of the equine 17βHSD1 promoter was cloned and several putative *cis*-acting elements were identified. This first paper focuses on 200 bp of the proximal promoter located –222/-22 (+1 representing the transcription start site), which includes putative binding sites for AP-2, NFκB, C/EBP, Sp1 and GATA. This promoter fragment was analyzed by EMSA using nuclear extracts from granulosa cells isolated prior to hCG treatment and 30 h after hCG. A single protein/DNA complex was observed with a noticeable decrease in promoter binding occurring in samples after hCG treatment, indicating that the gonadotropin-dependent downregulation of

17βHSD1 transcript expression may be due to a decrease in transcription factor binding. Competitors for Sp1 decreased nuclear extract binding, whereas competitors of NFκB and GATA completely abolished complex formation, suggesting that these three elements may be involved in the transcriptional regulation of the 17βHSD1 gene. Because an appropriate cell system is not available for studying non-luteinized equine granulosa cells, it was impossible to further the study of the promoter system. Previous work describing the human 17βHSD1 proximal promoter in choriocarcinoma cells had also identified AP-2, Sp1 and GATA response elements, however, the NFκB response element was not described (146). In these cells, the Sp1 binding element was found to be necessary for the complete functioning of the 17βHSD1 promoter. Sp1 has also been shown to be involved in the transcriptional activation of the LDL receptor gene by LH in porcine granulosa-luteal cells (421), and that Sp1 and SF-1 are required and function cooperatively to transactivate the bovine CYP11A1 promoter in bovine luteal cells (422, 423). Competitors for AP-2 did not affect nuclear extract binding to the –222/-22 fragment of the equine 17βHSD1 promoter. However, these studies were performed using extracts from equine granulosa cells obtained prior to hCG treatment, and AP-2 has been shown to act on the 17βHSD1 promoter by binding a site near that of Sp1, and preventing its binding and resultant activation (146). Therefore, it may interesting to analyze the components involved in promoter binding after hCG treatment, if any, at a latter date. The most noticeable effect on equine protein/DNA complex formation was the use of a NFκB/GATA competitor. This resulted in the complete loss of complex formation, and mutations in either site of the competitor reestablished complex formation, suggesting that both sites are involved. GATA-3 was shown to silence the human 17βHSD1 gene in choriocarcinoma cells (146), therefore it or other GATAs may be involved in suppressing 17βHSD1 promoter activity during follicular luteinization. Complex interactions seem to govern NFκB transcriptional regulation. In cultured human granulosa cells, NFκB was shown to be responsible for upregulating CYP19A1 promoter II activity, whereas PPARγ and RXR appeared to interfere with NFκB promoter binding (101). The mRNA regulation for 17βHSD1 is again reminiscent of what is seen for CYP19A1.

It can be said that the ovulatory stimulus clearly modulates 17βHSD1 in the mare. A demonstration that this enzyme can alter the bioavailability of steroids would place these findings into the context of a test of the hypothesis. Even though the activity of the equine 17βHSD1 protein has not been tested, it can be speculated that it will convert estrone to the more biologically active 17β-estradiol. This is based on comparison with human and non-human primate, where this enzyme selectively catalyzes the reduction of estrogens (134, 137, 148, 424). Therefore, its gonadotropin-dependent downregulation in granulosa cells provides an additional molecular basis for the decrease in 17β-estradiol production during the process of follicular luteinization/ovulation.

The high level of SLCO2B1 and STS mRNA expression and their gonadotropin-dependent downregulation in preovulatory follicles is suggestive of a putative alternative pathway for 17β-estradiol biosynthesis

The second paper of this thesis focuses on the presence and regulation of a system employing sulfoconjugated steroid precursors for the production of active estrogens, namely by the influx transporter OATP2B1, encoded by the SLCO2B1 gene, and by the protein responsible for the hydrolysis of the steroid sulfoconjugates, STS. In order to study their regulation, the primary structures of the SLCO/OATP2B1 and STS cDNAs and proteins were characterized. The 3.4-kb cDNA for SLCO2B1 encoded a 709-amino acid protein that contained all of the putative conserved amino acids included in the OATP superfamily signature motif as well as the twelve transmembrane domains identified in species homologues. Members of the OATP superfamily are known for their wide tissue distribution as well as their wide substrate specificities. Although many OATPs mediate the import of sulfoconjugated steroids, the only one that will selectively transport sulfoconjugated steroids is OATP2B1 (365). Therefore, its characterization during the ovulatory process provides new insights into the capture and subsequent use of circulating steroids by steroidogenic cells. Once the sulfoconjugated steroids are transported into the cell, they remain inactive as long as they are

sulfonated. Only an enzyme with the activity of steroid sulfatase can catalyze the hydrolysis of the sulfonate group, thereby releasing a potentially active steroid that can either bind directly to its cognate receptor, as is the case for E2-S, or can be acted upon by steroidogenic enzymes to yield biologically active steroids. In the present study, a fragment of the STS cDNA was also identified, and it was sufficiently homologous to species orthologues to allow its positive identification as equine STS.

Again, total RNA extracted from equine preovulatory follicles isolated between 0 and 39 h post-hCG served to study the gonadotropin-dependent regulation of SLCO2B1 and STS transcript expression during the ovulatory process. RT-PCR/Southern blot analyses demonstrated high levels of SLCO2B1 and STS transcript expression in both granulosa cells and theca interna of preovulatory follicles obtained prior to hCG. This co-localized expression in the two compartments of the preovulatory follicle supports the concept of production of follicular estrogens from circulating steroid sulfoconjugates. While there are no known studies reporting the transport of sulfoconjugated pregnenolone by OATP2B1, there is one article describing the inhibition of E1-S uptake by pregnenolone sulfonate (383). Whether this indicates that sulfoconjugated pregnenolone competes for transport by OATP2B1 is unclear, but the possibility that this occurs allows the speculation that pregnenolone could be imported into thecal cells, where STS is abundantly expressed and serve as a substrate for CYP17A1, which is also abundantly expressed prior to hCG (43). The presence of the OATP/STS system in the theca interna may also be important for directing an estrogenic signal by the granulosa cells. Many species have been shown to express estrogen receptors in the theca interna (425-431) and the role of estrogens in this cell type of the mare needs to be further explored. Nonetheless, it can be speculated that 17β-estradiol may play a role in maintaining the theca interna in the early stages of follicular luteinization. The presence of the OATP/STS system in granulosa cells prior to hCG is supported by the presence of enzymes like 3βHSD/KSI, CYP19A1 and 17βHSD1.

In humans, DHEA-S has been shown to be quantitatively the most abundant form of circulating steroid hormone (266, 267), with E1-S being the most abundant form of circulating estrogen (269-271). Whether this is also the case in the mare remains to be elucidated. However, circulating DHEA-S could be imported and hydrolyzed by OATP2B1 and STS in the granulosa cells and then be used to produce active estrogens via the actions of 3βHSD/KSI, CYP19A1 and 17βHSD1 (30, 43).

The results reported in the second article of this thesis further demonstrate the gonadotropin-dependent downregulation of SLCO2B1 and STS in preovulatory follicles. SLCO2B1 mRNA is shown to be rapidly and dramatically dowregulated following hCG treatment in the granulosa cells. This regulation is reminiscent of that observed for the promoter II-derived CYP19A1 mRNA (43), as well as the 17βHSD1 transcript (article 1). These data thereby support a role for SLCO2B1 as a putative contributor to active estrogen production in equine preovulatory follicles. The regulation of STS mRNA is slightly different, being significantly downregulated in granulosa cell samples somewhat later, between 24-39 h post-hCG. In isolated preparations of theca interna, the SLCO2B1 transcript is also downregulated. The decrease is more gradual than what was observed in granulosa cells, with a significant decrease being observed in samples 30-39 h post hCG, but a more dramatic drop observed in 36-39 h post-hCG samples. The loss of the SLCO2B1 transcript in theca interna may not be as a consequence of transcriptional downregulation, but rather due to a putative degenerative process of the equine theca interna. This compartment is believed to be undergoing apoptosis near the time of ovulation and therefore does not contribute to the formation of the corpus luteum (432). Interestingly, equine CYP17A1, the classical marker of thecal cells, also displays this pattern of dramatic downregulation during gonadotropin-dependant follicular luteinization/ovulation (43).

These studies were limited to the characterization of the gonadotropin-dependent regulation of SLCO2B1 and STS at the transcriptional level because no specific antibody was available to detect protein expression and no adequate cell system could sustain functional studies. However, the equine SLCO2B1 and STS transcripts have

been shown to be expressed in the placenta, as well characterized organ for OATP/STS action in humans (378, 383, 433). The second paper provides further support for the hypothesis by demonstrating the time-dependent and programmed downregulation of the expression of SLCO2B1 and STS, two factors that would normally increase follicular estrogen synthesis. Their downregulation dramatically reduces the bioavailability of estrogens to follicular cells.

The induction of 17βHSD4 involves oxidative 17βHSDs in the luteinization process and raises the possibility that estrogens may need to be inactivated during this process

The decrease in 17β-estradiol has mainly been attributed to the downregulation of biosynthetic enzymes. However, the third paper in this thesis addresses the regulation of another 17βHSD, namely 17βHSD4, an oxidative enzyme that has been shown to catalyze the conversion of 17β-estradiol to estrone. To characterize the transcriptional regulation of 17βHSD4 during the process of equine follicular luteinization/ovulation, the cDNA for 17βHSD4 was identified. It was shown to encode a 735-amino acid protein that was highly conserved when compared to mammalian orthologues. The equine protein retains the three domain morphology of other 17βHSD4s, namely a N-terminal SDR domain, a central hydratase domain and a carboxy-terminal SCP2-like domain as well as the peroxisomal targeting signal (167, 189, 192). The 17βHSD4 proteins identified thus far have been shown to possess a putative peroxisomal protease cleavage site, Ala-[Ala/Pro]-Ser, located around position 320 (167). The equine 17βHSD4 protein also has this putative cleavage site located at positions Ala^{316}-Ala^{317}-Ser^{318}. The extent of processing has been shown to differ depending on the tissue examined (167). As demonstrated in the third paper of this thesis, it is interesting that the predominant form of the 17βHSD4 protein with potential estrogen-inactivating activity, was the 80-kDa full length protein. It has been shown that both the truncated 32-kDa form and the full length 17βHSD4 protein conserve their affinity for 17β-estradiol, namely K_m of 0.3 and 0.4 μM, respectively.

The gonadotropin-dependent regulation of the 17βHSD4 transcript was examined during the ovulatory process using total RNA extracted from equine preovulatory follicles obtained between 0 and 39 h post-hCG. Results from RT-PCR/Southern blot analyses as well as Western blot and immunohistochemistry, demonstrate a change in steady-state levels of 17βHSD4 after hCG treatment. Detectable levels of 17βHSD4 mRNA and protein were present in both the theca and granulosa cell compartments of the preovulatory follicle prior to gonadotropin-treatment. However, the expression of 17βHSD4 was significantly induced in samples collected between 24 and 39 h post-hCG, with the predominant contribution originating from the granulosa cell layer. Interestingly, gonadotropin treatment resulted in the time-dependent decrease in 17βHSD4 processing, suggesting that this full length protein may be important during the process of follicular luteinization/ovulation. The precise role of the 17βHSD4 protein in the overall steroidogenic economy of the follicle remains intriguing. Its gonadotropin-dependent induction during luteinization may be involved in the inactivation of 17β-estradiol to estrone. It is also the only 17βHSD protein to be localized to the peroxisomes and has been shown to catalyze the β-oxidation of fatty acids (167). More importantly, it also possesses a SCP2-like domain that has been shown to catalyze the transfer of cholesterol between membranes in vitro (167, 189), an activity shared by the protein StAR which is also upregulated in granulosa cells during the ovulatory process in the mare (41). Thereby, the upregulation of 17βHSD4 may also contribute to bringing about the increased cholesterol supply for the high level of progesterone biosynthesis that occurs during follicular luteinization.

The gonadotropin-dependent induction of EST and ABCC1 provides new insights on the role of phase II conjugation enzymes and their contribution towards the inactivation of estrogens during follicular luteinization/ovulation

The upregulation of a multifunctional protein such as 17βHSD4 leads to the question of what might be its actual role. Does it contribute significantly to reducing

levels of 17β-estradiol or is this function secondary to the β-oxidation of fatty acids? The fourth article in this thesis deals with an enzyme whose function cannot be questioned, EST selectively catalyzes the sulfoconjugation of estrogens at their 3-hydroxyl position (315-317). Once sulfonated, estrogens no longer bind to their cognate receptors and can no longer diffuse freely across the lipid bilayer (264). They will then either remain in the cell to be acted upon by STS or leave the cell via an active transport system. Member C1 of the ABC family (ABBC1) has been shown to encode the protein MRP1, capable of transporting E1-S out of the cell (357). In the present study, the primary structures for equine EST and ABCC1 cDNAs and proteins were characterized. The equine EST cDNA encodes a 296-amino acid protein that is one and two amino acids longer that its bovine (GenBank accession number CAA39806) and human (P49888) orthologues, respectively. The amino acids known to be important in EST substrate-binding were conserved in the equine protein, namely the conserved P-loop motif involved in PAPS binding as well as other amino acids shown to be conserved among sulfotransferases (263). The equine ABCC1 cDNA encodes a putative 1531-amino acid protein that was highly homologous to human and bovine orthologues, with almost 90% identity at the amino acid level when compared to the human protein. The putative 17 transmembrane domains were conserved in the equine protein (434), as well both Walker domains and the amino acids of the active transport family signature present in the nucleotide binding domain (435). The putative glycosylation sites are also present (436) and differential glycosylation states may account for the two different molecular weight proteins identified by Western blot in equine preovulatory follicle protein extracts, as the amino acid content accounts for the 170-kDa form and MRP1 has previously been shown to migrate at approximately 190-kDa when glycosylated (437).

Total RNA extracted from equine preovulatory follicles isolated between 0 and 39 h post-hCG served to study the gonadotropin-dependent regulation of EST and ABCC1 transcript expression during the ovulatory process. RT-PCR/Southern blot analyses demonstrated barely detectable levels of EST mRNA and slightly more abundant ABCC1 transcripts in preovulatory follicles prior to hCG treatment. The extremely low

staining for EST protein in immunohistochemical analyses of preovulatory follicles prior to gonadotropin treatment supports the mRNA abundance data. After hCG treatment, both EST and ABCC1 transcripts and protein abundance were upregulated in granulosa cells. The EST mRNA was dramatically upregulated at 30 h post-hCG and remained high thereafter. The induction of ABCC1 mRNA, however, occurred faster than that of EST, becoming significant in follicles obtained between 12 and 39 h post-hCG.

The concentrations of sulfoconjugated estrogens in the follicular fluid were also significantly upregulated during the ovulatory process. Levels of E2-S significantly increased at 12 and 33 h post-hCG, whereas concentrations of E1-S were significantly induced at 24 and 33 h post-hCG. The increases in sulfoconjugated steroids at 12 and 24 h after gonadotropin treatment occur more rapidly than the induction of EST. It can be speculated that this discrepancy may be due to the EST protein having a high rate of activity, and it being sufficient for a noticeable increase in sulfoconjugated estrogens even at low levels of expression. Alternatively, the presence of other enzymes capable of sulfoconjugating estrogens, such as isoforms of the EST protein or other hydroxysteroid sulfotransferases, may contribute the overall sulfoconjugated steroid pool (256, 292, 438). This notwithstanding, the simultaneous increase in E1-S and E2-S concentrations at 30 h post-hCG may be attributable, however, to a sulfotransferase being more specific to estrogens, like EST (315-317).

The transport of E1-S by MRP1 has been shown to be dependent on the presence GSH (357). As mentioned in article 4, the enzyme responsible GSH synthesis, the modifier subunit of glutamate cysteine ligase, has been shown to be upregulated in preovulatory follicles following gonadotropin treatment in the rat (439), thereby adding strength to the hypothesis that MRP1 is involved in sulfoconjugated estrogen export during the ovulatory process.

This article presents evidence for the involvement of estrogen inactivation during the ovulatory process consistent with the hypothesis of programmed modulation of

inactivation and bioavailability of steroids as a result of the gonadotropin stimulus. Previous work has shown that no mitotic figures are present in preovulatory follicles near the time of ovulation in the mare (9), suggesting that the equine preovulatory follicle undergoes terminal differentiation in response to LH. The elimination of active estrogens would support this view as 17β-estradiol has been shown to be mitogenic by increasing cell cycle progression (51-53).

The induction of AKR1C23 suggests a potential role for local progesterone metabolism during gonadotropin-dependent follicular luteinization

To further explore the concept of steroid inactivation during the ovulatory process, the fifth paper addresses the regulation of an aldo-keto reductase with potential progesterone-inactivating capacities, namely AKR1C23. Although the nomenclature AKR1C23 is specific to the mare, it was cloned using oligonucleotides designed from highly conserved regions of mammalian 17βHSD5s. The resultant primary structures for the AKR1C23 cDNA and protein were characterized and found to be more highly homologous to published sequences of mammalian 20αHSDs. The extensive confusion still present regarding AKRs renders comparison of specific sequences difficult as they have frequently been published under a given name, often without appropriately being characterized. The difficulties involved in substrate and protein manipulation confound efforts to account for the numerous potential AKR1C activities (440-442). For example, it is practically impossible to test for 20αHSD and prostaglandin F synthase activities in cell culture because of the rapid metabolism of both substrates, and similarly, the 17βHSD5 activity has been shown to be highly labile in humans (199) as well as in the mare (data not shown). These catabolic considerations preclude accurate *in vitro* analysis.

The gonadotropin-dependent regulation of the AKR1C23 transcript was examined during the ovulatory process using total RNA extracted from equine preovulatory follicles obtained between 0 and 39 h post-hCG. Results from RT-PCR/Southern blot analyses demonstrated that very low levels of AKR1C23 mRNA were present in

preovulatory follicles prior to hCG treatment. A dramatic increase in transcript abundance was observed in isolated preparation of granulosa cells from preovulatory follicles obtained 12 h post-hCG. Once activated, the expression remained high thereafter. Low levels of AKR1C23 were detected in samples of theca interna, with a slight increase observed at 39 h post-hCG, most likely due to granulosa cell contamination. The increase in AKR1C23 transcript was reflected at the protein level by Western blotting and immunohistochemistry of preovulatory follicle samples isolated before and after hCG.

The purified equine AKR1C23 protein was shown to possess 20αHSD activity, and to convert progesterone to 20α-DHP. The kinetic constants were not different from those previously reported for human 20αHSD (244) and considering its regulation during the ovulatory process, the change in follicular fluid concentrations of 20αDHP reflected a pattern of expression consistent with this enzyme metabolizing progesterone. The biological significance of these results is not known, as progesterone has been shown to be necessary for luteal formation (29), however, one can speculate about possible roles for this enzyme and its product. Since 20α-DHP has been shown to promote ovulation and gonadotropin secretion, and has been shown to increase sexual receptivity when metabolized further by 5α-reductase in the brain (443), it is possible that it is not the inactivation of progesterone that is important during the ovulatory process, but rather the production of its metabolite, 20α-DHP. Its positive feedback effect on gonadotropin secretion may also account for the prolonged LH secretion seen in the mare (3, 14).

The metabolism of progesterone in the preovulatory follicle may also serve to regulate the local effects of progesterone, as well as produce a reservoir of progesterone precursors for action in other tissues, including the uterus. The enzyme 17βHSD2 has been shown to catalyze the conversion of 20α-DHP to progesterone and is highly expressed in the glandular epithelium isolated from the secretory phase endometrium (152). The potential prostaglandin F synthase activity of the AKR1C23 should also be

considered, since prostaglandins have been shown to be important in mediating the inflammatory aspect of ovulation (15). The results of the fifth article also address the hypothesis because they demonstrated programmed upregulation of the expression of factors that increase inactivation and reduce bioavailability of progestagens to the follicle.

CONCLUSION

In conclusion, the results presented herein represent significant advancements in our understanding of the genes regulated during follicular luteinization/ovulation. Notably, they introduce sulfoconjugated steroids as putative precursors to 17β-estradiol production prior to luteinization and illustrate the molecular mechanisms that underly the decrease in estrogen biosynthesis, as well as suggest the involvement of steroid inactivating enzymes in preovulatory follicles during the ovulatory process. These results can thereby be integrated into a model describing the regulation of steroid activation, inactivation and bioavailability in preovulatory follicles (figure 1).

In preovulatory follicles prior to hCG treatment (0 h), the biosynthetic machinery necessary for 17β-estradiol production is present. Large amounts of StAR can be detected, which is responsible for importing cholesterol into the mitochondria where it can be acted upon by CYP11A1 producing pregnenolone. CYP17A1 is also expressed prior to hCG and has the potential to convert pregnenolone to DHEA. This weak androgen can diffuse to the granulosa cell layer where the successive actions of the enzymes 3βHSD/KSI and CYP19A1 will convert it to estrone. The enzyme 17βHSD1 then catalyzes the conversion of estrone to 17β-estradiol. Circulating DHEA-S and E1-S can enter the theca interna and granulosa cell compartments via the action of OATP2B1, and subsequently be acted upon by STS, which will hydrolyze their sulfonate groups, thereby allowing them to also be utilized by steroidogenic enzymes. The mitogenic 17β-estradiol can act locally in granulosa cells through its estrogen receptors or in other cells, including those of the theca interna.

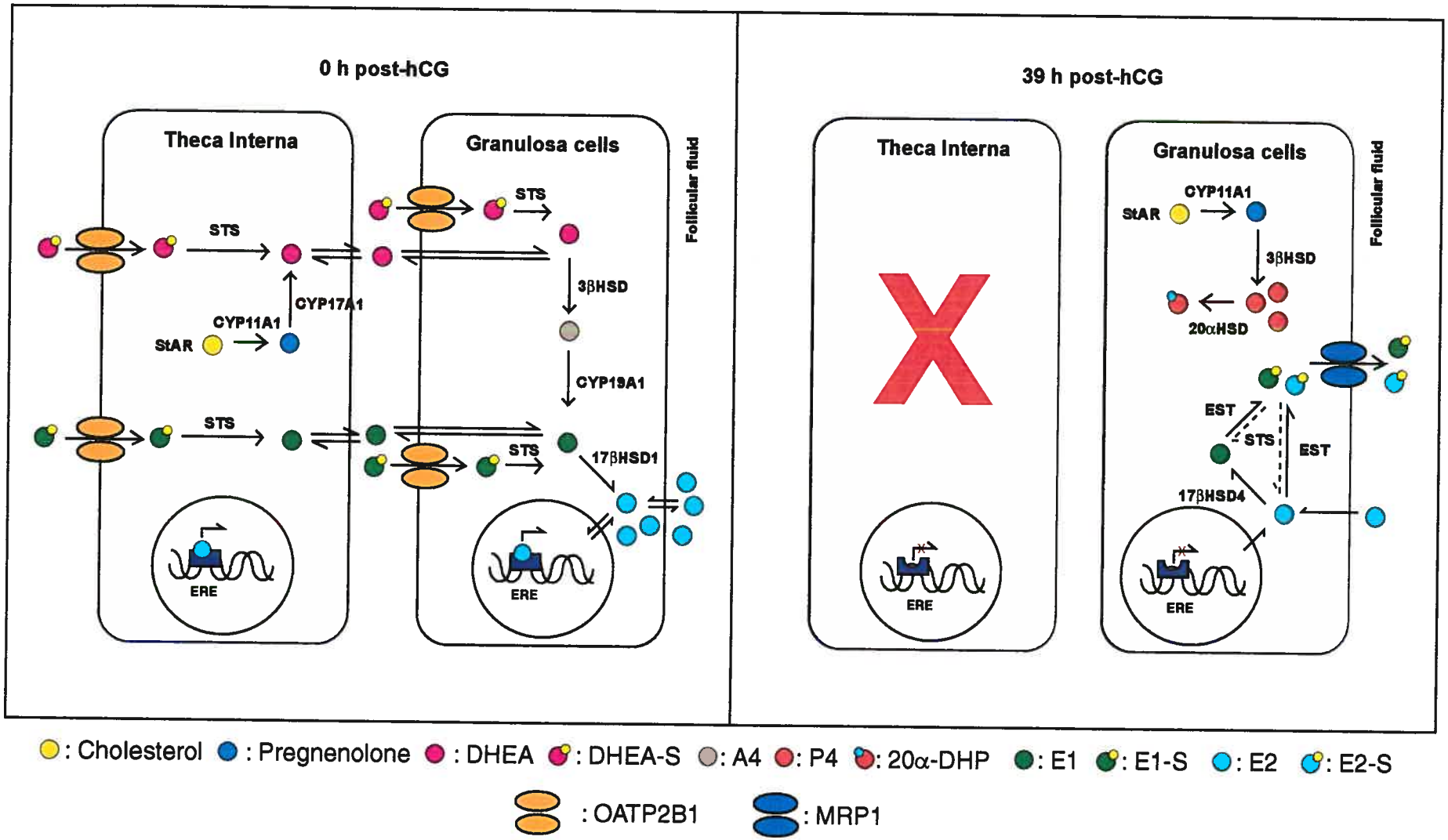


FIGURE 1: Model describing the regulation of steroid activation, inactivation and bioavailability during the ovulatory process in the mare.

After hCG, around the time of ovulation, most enzymes of the theca interna are completely silenced, reflecting its degeneration. Granulosa cells acquire StAR and CYP11A1, thereby permitting the import of cholesterol to the mitochondria and its conversion to pregnenolone. The enzyme 3βHSD/KSI is not regulated by hCG and its expression remains high after gonadotropin treatment, allowing it to convert pregnenolone to progesterone, a steroid required for gestation. The enzyme AKR1C23 can modulate levels of active progestagens by converting progesterone to 20α-DHP. The 17βHSD4 enzyme is induced after hCG, conferring 17β-estradiol oxidative potential to granulosa cells. Estrogens can also be inactivated by EST via the addition of a highly charged sulfonate group and eliminated from the cell by MRP1. Elimination of 17β-estradiol from the cells prevents further estrogen receptor-mediated cell division.

Prospective studies

Results described in this thesis have resulted in the development of novel concepts and open the door to new and exciting experiments regarding steroid activation, inactivation and bioavailability during the ovulatory process.

Although a pseudogene for 17βHSD1 has been identified in a number of other species and shown to be located approximately 4.3 kb upstream of the active gene (131), its presence has not been characterized in the mare. It will be interesting to look upstream of the isolated equine 17βHSD1 genomic sequence to determine whether it exists. Studies employing equine samples obtained at earlier time points (e.g. 1 h, 3 h, and 6 h post-hCG) would also allow precise monitoring of the regulation of genes like 17βHSD1 and SLCO2B1. Promoter activity assays for 17βHSD1 in equine granulosa cells are impractical, as primary equine granulosa cells are not easily cultured and granulosa cell lines have already luteinized. More efforts will be needed to develop such a culture system in the mare. Nonetheless, co-transfecting putative transcription factors with the 17βHSD1 promoter can give insight as to their promoter-activating potential.

Functional *in vitro* assays for OATP2B1 and STS in granulosa cells would also be difficult, as SLCO2B1 transcript is lost during luteinization. However, transfecting cells with OATP2B1 and following E1-S import and hydrolysis by pulse chase techniques would provide interesting functional information. EST and MRP1 functionality could also be investigated by pulsing radiolabeled E1 to granulosa cells and quantifying E1-S in the culture medium. The use of multiple siRNA knockdowns would confirm the identity of the proteins involved in this process. The study of glucoronidation as a means for estrogen inactivation during the ovulatory process is likewise of considerable interest as proteins like MRP1 have been shown to transport glucoronidated estrogens. Also, the inactivation of estrogens provide the impetus for equine granulosa cells to exit to cell cycle. Examining factors involved in cell cycle progression, such as cyclin D2, may provide additional support for this hypothesis.

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