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

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Estrogen sulftotransferase (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, such as ABC1C. The objective of this study was to investigate the regulation of EST and ABC1C during human chorionic gonadotropin (hCG)-induced ovulation/luteinization. The transcripts for EST and ABC1C were cloned by 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 ABC1C mRNA after hCG treatment ($P < 0.05$). In granulosa cells, a significant increase in EST transcript was observed 30–39 h after hCG. Similarly, ABC1C transcript levels were induced in granulosa cells 12–39 h after hCG. In contrast, no significant changes in either EST or ABC1C were detected in theca interna samples after hCG. The increase in EST and ABC1C 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 ABC1C expression in preovulatory follicles. The present study demonstrates that follicular luteinization is accompanied not only by a decrease in 17β-estradiol biosynthesis but also by an increase in expression of genes responsible for estrogen inactivation and elimination from granulosa cells, such as EST and ABC1C, respectively. (Endocrinology 147: 4222–4233, 2006)

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). Sulfoconjugates have been classified into two different groups depending on whether they are membrane bound or soluble. More than 44 soluble or cytosolic sulfoconjugates have been identified in mammals, with substrates ranging from endogenous compounds such as hormones and neurotransmitters to drugs and xenobiotics (2). The current nomenclature classifies the cytosolic sulfoconjugates into five SULT families, sharing less than 40% similarity with each other. The SULT1 family members act on phenolic drugs and catecholamines (SULT1A), thyroid hormones (SULT1B), xenobiotics (SULT1C), and estrogenic steroids (SULT1E) (3).

Estrogen sulftotransferase (EST, encoded by SULT1E1) is a 30- to 36-kDa protein that catalyzes the sulfoconjugation of estrogens at the 3-hydroxyl position. It uses $3'$-phosphoadenosine-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-sulfonate (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 (ABC) 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 ABC superfamily (ABC1C) 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.

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Abbreviations: ABC, ATP-binding cassette; E1-S, estrone-3-sulfonate; E2-S, 17β-estradiol-sulfonate; EST, estrogen sulftotransferase; hCG, human chorionic gonadotropin; MRP1, multidrug resistance protein 1; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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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 LH and is characterized by numerous physical and biochemical changes, including the decreased production of E2 (14–16). This loss in E2 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 MRPI, respectively, during this period. In the present study, the equine preovulatory follicle is used as a 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 and proteins 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 [α-32P]dCTP was purchased from PerkinElmer Canada, Inc. (Woodbridge, Ontario, 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-kb DNA ladder, and synthetic oligonucleotides were purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada). The QIAGEN OneStep RT-PCR System was obtained from QIAGEN, Inc. (Mississauga, Ontario, Canada). The Expand High Fidelity DNA Polymerase was purchased from Roche Diagnostics (Laval, Québec, Canada). Biotrans nylon membranes (pore size, 0.2 mm) were obtained from ICN Pharmaceuticals, Inc. (Montreal, Québec, Canada), and all electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA). The hCG was obtained from The Buttlar 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 dianinobenzidine 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-bp RT-PCR product (Fig. 1Aa) was initially cloned from pooled equine ovarian RNA samples isolated from a preovulatory follicle and an ovulatory follicle (36 h after 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 RNA and oligonucleotide primers designed by sequence alignments of known EST species homologs (primers 1 and 2; Fig. 1B). After agarose gel electrophoresis, the RT-PCR product was excised and ligated into the

![Fig. 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 indicate UTRs; open boxes designate the open reading frame (ORF). Lengths of the deduced transcript and its structural elements are indicated in base pairs. 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.](https://academic.oup.com/endo/article-abstract/147/9/4222/2528327)
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, 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 homologs (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 using oligonucleotide primers that bound to the anchor sequence in conjunction with EST-specific forward primers. The product of the second PCR (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).

Because of the large size of the equine ABCC1 transcript, it was cloned using a multistep cloning strategy (Fig. 1C). A 981-bp RT-PCR product (Fig. 1Ca) was initially cloned from pooled equine ovarian RNA samples isolated from a preovulatory follicle and an ovulatory follicle (36 h after hCG treatment). RT-PCR was performed using the OneStep RT-PCR System (Qiagen) as directed by the manufacturer, using 500 ng RNA and oligonucleotide primers designed by sequence alignments of known ABCC1 species homologs (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 (primer 4; Fig. 1Cb). The second RT-PCR also required the design of a sense oligonucleotide from sequence alignments of species homologs (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 homologs (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 homologs (primers 8 and 10). These yielded 1359-bp (Fig. 1Cc) and 1029-bp fragments, respectively. Finally, a sixth RT-PCR was done using equine-specific sense and antisense primers (primers 11 and 12; Fig. 1D) and yielded a 1228-bp fragment that was then sequenced (Fig. 1C).

Equine tissues and RNA extraction

Testicular tissues were obtained from the Large Animal Hospital of the Faculté de Médecine Vétérinaire (Université de Montréal) after 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 preovulatory follicles reached 35 mm in diameter during estrus, the theca interna. Ovariectomies were also performed on mares 8–10 h after estrus to obtain corpora lutea (n = 3 mares). After ovariectomies, corpora lutea were stored at 4°C for 1–3 days and then homogenized using a polycarbonate micropestle. Total RNA was isolated from follicles with TRIzol reagent (Invitrogen Canada), according to the 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 semiquantitative analysis of EST, ABCC1, and rpL7a mRNA levels in equine tissues. Reactions were performed according to the manufacturer’s directions, using touchdown PCR (22). Expand Long Template (Roche) was used as the DNA polymerase. The primer 5′-AGAGGACGAGCAAGCACTTCC-3′ and antisense 5′-GTTTCTCCCTCCAGAAGTTTG-3′ primers specific for equine EST, sense 5′-AGCGAGGAGCAGCAGAGC-3′ and antisense 5′-GAGTCCACGGTCCACGTCC-3′ primers specific for equine ABCC1, and sense 5′-ACGAGGACATCGACGCAAAAGC-3′ and antisense 5′-GCTCCTTGTCTCAGGATTTG-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. After PCR amplification, samples were electrophoresed on 2% Tris-acetate/EDTA-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 MRPI 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 dithiothreitol) containing 1.0% Tween. The sonicate was centrifuged at 16,000 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) using a peptide fragment encompassing amino acids Glu1262-Arg1277, whereas the equine-specific anti-MRP1 antibody was generated (New England Peptide) using a peptide fragment encompassing amino acids Glu1262-Arg1277. Membranes were incubated with either the polyclonal anti-EST antibody (1:1000) or anti-MRP1 antibody (1:1000), and immunoreactive proteins were visualized on Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) by autoradiography 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, Baie d’Urfé, Québec, Canada).

Immunohistochemical localization of EST

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories), as previously described (21). Briefly, formalin-fixed tissues were paraffin embedded, and 3-μm-thick sections were prepared and deparaffinized 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. Membranes were rinsed in PBS and then incubated with polyclonal rabbit anti-EST antibody (1:1000 dilution) or anti-MRP1 antibody (1:1000), and immunoreactive proteins were visualized on Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) by autoradiography after incubation with the horseradish peroxidase H reagents for 45 min at room temperature. Sections were washed in PBS for 10 min and incubated with avidin DH-biotinylated horse.
Characterization of equine EST and ABCC1 cDNAs

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 after 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 method, using chemical ionization. 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 homologs. 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’-untranslated region (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 an 891-bp open reading frame (GenBank accession no. 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) homologs. The equine EST has 81.0% identity at the amino acid level (Fig. 2A) and an 85.1% identity at the nucleic acid level when compared with 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. ~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 after 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 before treatment with hCG (0 h) but were clearly induced at 36 h after hCG (Fig. 4A). The EST transcript expression returned to basal levels in the corpus luteum at d 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 after 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 before hCG treatment with an increase observable 12–36 h after hCG (Fig. 4D). The ABCC1 transcript expression returned to basal levels in the corpus luteum at d 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 after hCG (P < 0.05; Fig. 4F). Again, no change in rpL7a transcript was observed after gonadotropin treatment (Fig. 4E).

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
### A EST

| species | sequence | alignment |
|---------|----------|-----------|
| equine | MMDSSKFDSSSYPGRIGMOLYKDFVPEW | -N.-EL.YYEK.EEV-..-KY.-N.-Q.-..-..-.V.-..Y.-..-..-.V.-..I.F.-..-NL | 80 |
| human  | -N.-EL.YYEK.EEV-..-KY.-N.-Q.-..-..-.V.-..Y.-..-..-.V.-..I.F.-..-NL | 88 |
| bovine | -N.-EL.YYEK.EEV-..-KY.-N.-Q.-..-..-.V.-..Y.-..-..-.V.-..I.F.-..-NL | 89 |

Identical residues are indicated by a printed period, and 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 MRP1

| species | sequence | alignment |
|---------|----------|-----------|
| equine | MALRGFCASGSDPLWEMTNTWNTS | -N.-EL.YYEK.EEV-..-KY.-N.-Q.-..-..-.V.-..Y.-..-..-.V.-..I.F.-..-NL | 80 |
| human  | -N.-EL.YYEK.EEV-..-KY.-N.-Q.-..-..-.V.-..Y.-..-..-.V.-..I.F.-..-NL | 88 |
| bovine | -N.-EL.YYEK.EEV-..-KY.-N.-Q.-..-..-.V.-..Y.-..-..-.V.-..I.F.-..-NL | 89 |

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).

Fig. 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; GenBank accession no. P49888) and bovine (bov; CAA39806) ESTs. Identical residues are indicated by a printed period, and 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 (N_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).
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 insignificant, transient induction of both EST and ABCC1 mRNAs in theca interna cells (Fig. 5, Ab and Bb, 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 after 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. 6, B–E). Follicles isolated before hCG treatment (0 h) showed 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 MRP1 accumulation (Fig. 7, C and D) was observed. Control sections of follicles isolated at 39 h after hCG showed no staining when anti-EST antibody was omitted (Fig. 7E).

Measurements 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. Sulfonconjugated E1 and E2 as well as their unconjugated counterparts were quantified. The absolute levels of sulfonconjugated estrogens and the ratio of sulfonconjugated estrogens to free ones were shown to significantly change after hCG (Fig. 8). Results demonstrate that levels of E1-S and E2-S had a tendency to increase 12–24 h after hCG and returned to basal levels at 39 h yet were only significantly increased at either 24 and 33 h after hCG for E1-S or 12 and 33 h after hCG for E2-S (Fig. 8, A and B, 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 after 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 after hCG (Fig. 8C), whereas the relative levels of E2-S to E2 were significantly increased 24–36 h after hCG (Fig. 8D).
This study demonstrates for the first time that the process of follicular luteinization induced by hCG is accompanied by the colocalized expression and up-regulation 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 E2 biosynthesis are down-regulated, 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 ab-
FIG. 5. Regulation of EST and ABCC1 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 and 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 after hCG (P < 0.05). Insets show results of either EST (A) or ABCC1 (B) and rpl7a mRNA levels from one sample per time point.
sent, levels of EST transcript were observed in granulosa cells of preovulatory follicles before hCG, with an up-regulation 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 pregnant mare serum gonadotropin-primed immature rats as a model demonstrated that treatment with hCG resulted in an up-regulation 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 h after hCG), they show an induction occurring approximately 6 h before ovulation, as depicted in the present study. The inflammatory cytokine IL-1β has been shown to up-regulate 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 d before 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 before hCG with a significant increase in E1-S and E2-S being observed at 24 and 12 h after 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 after hCG, most likely because of the decreased estrogen biosynthesis, as previously described (15, 17, 18, 29). Nonetheless, the ratio of sulfoconjugated estrogens to free estrogens significantly increases...
FIG. 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 two bands of predicted MRP1 size ($M_r = 170,000$ and 190,000 when glycosylated). 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 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 and 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).

FIG. 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 and 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 after hCG ($P < 0.05$).
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-hydroxy group of estrogenic steroids; they do not, however, sulfonate either the 16α- or 17β-hydroxy 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 CxxGxxK 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 Gly262 and Asn269, which have been shown to be required for estrogen sulfocojugation in the guinea pig (49). As opposed to EST, MRP1 has many substrates. However, it has previously been shown to transport E1-S, in a reduced-glutathione-dependent manner, in membrane vesicles from transfected HeLa cells (50). Interestingly, the modifier subunit of glutamate cysteine ligase, the enzyme responsible for reduced glutathione synthesis, has been demonstrated to increase in preovulatory follicles after 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 colocalized expression and up-regulation in granulosa cells of luteinizing preovulatory follicles. By means of sulfocojugation, 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 up-regulation of 17β-hydroxysteroid dehydrogenase type 4, an enzyme responsible for E2 oxidation (52). Future studies will be required to investigate the necessity of inactivating estrogens during the periovulatory period.

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

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