ABSTRACT

During the secretory phase of the human menstrual cycle, the endometrium is minimally responsive to the estrogens secreted from the ovaries. Conjugation of β-estradiol (E₂) with sulfate is thought to be an important mechanism in the regulation of the levels of active E₂ in endometrial tissue. Estrogen sulfation is reportedly increased during the secretory phase in response to the high levels of progesterone secreted by the ovaries. Estrogen sulfotransferase (hEST), a distinct form of human cytosolic sulfotransferase (ST) with an affinity for E₂ and estrone at low nanomolar concentrations, has recently been cloned and expressed in mammalian cells and in bacteria (J Steroid Biochem Mol Biol 52:529, 1995). At least two other forms of human cytosolic ST, dehydroepiandrosterone ST (hDHEA-ST) and the phenol-sulfating form of phenol-ST (hP-PST), also conjugate estrogens but at micromolar concentrations. This report describes the specific induction of hEST in human Ishikawa endometrial adenocarcinoma cells by progesterone as a model for the increases in estrogen sulfation observed in women during the secretory phase of the menstrual cycle.

The metabolism of steroids in hormone responsive tissues is an important mechanism in the modulation of steroid activity. One of the mechanisms for the inactivation of steroids is via sulfate conjugation. The addition of a sulfate moiety to a steroid decreases the binding of the steroid to its receptor, effectively lowering the concentration of the bioactive steroid. Sulfation also increases the aqueous solubility of the steroid, thereby facilitating its excretion. The conjugation of steroids with sulfate is catalyzed by a family of enzymes termed the sulfotransferases (STs). Four forms of cytosolic ST have been identified in human tissues by purification and cloning studies (4–8), and three of these enzymes have been reported to conjugate estrogens and hydroxysteroids (8–10). Dehydroepiandrosterone ST (hDHEA-ST) conjugates the 3-hydroxy position of both hydroxy-steroids such as DHEA and pregnenolone, as well as many bile acids (10, 11). hDHEA-ST also conjugates estrogens and testosterone; however, the enzyme does not conjugate cortisol or dexamethasone (10). The phenol-sulfating form of phenol ST (hP-PST) conjugates only the 3-phenolic hydroxyl of estrogens and does not sulfate the aliphatic hydroxyl groups of hydroxysteroids such as DHEA, androstenedione, testosterone, and cortisol (10, 12). Recently, the cDNA for human liver estrogen ST (hEST) has been cloned (4, 8), and the active enzyme has been expressed in bacteria and purified to homogeneity (8). hEST differs from hDHEA-ST and hP-PST in that hEST sulfates E₂ and estrone (E₁) with Kₘ of...
approximately 5 nM (8). The high affinity of hEST for the sulfation of E₁ and E₂ suggests that it may have a role in the regulation of the levels of active estrogens at concentrations at which estrogens interact with the ER. Therefore, increases in hEST activity induced by the increases in progesterone concentrations in secretory endometrium may be involved in decreasing the responsiveness of this tissue to estrogens.

This report describes the specific induction of hEST activity, protein, and mRNA in human Ishikawa endometrial adenocarcinoma cells in response to progesterone treatment. The Ishikawa endometrial cell line was isolated from a well differentiated endometrial adenocarcinoma (13). The cells possess both ER and progesterone receptors and are capable of responding to both progesterone and estrogens (14-17). Because of their hormone responsiveness, Ishikawa cells have been used as a model system for the investigation of the function and regulation of estrogen and progesterone-stimulated processes in the endometrium (17). The characteristics and specificity of hEST induction in Ishikawa cells by progesterone may provide a model system for the regulation and role of estrogen sulfation during the menstrual cycle in normal human endometrium.

Materials and Methods

Materials

- p-Nitrophenol, E₂, DHEA, dopamine, progesterone, testosterone, pregnenolone, cortisol, medroxyprogesterone acetate and DEAE-Sepharose CL-6B were obtained from Sigma Chemical Co. (St. Louis, MO). Minoxidil was a gift from The Upjohn Co. (Kalamazoo, MI). [1,2,6,7-³H]DHEA (79 Ci/mmol), [6,7-³H]-E₂ (45 Ci/mmol), [2,4,6,7-³H]-E₁ (96 Ci/mmol), [7-³H]pregnenolone (25 Ci/mmol), [7-³H]testosterone (27.7 Ci/mmol), [1,2,3,4,5,6,7,8-²H]-cortisol (54.4 Ci/mmol) and [³³S]-3'-phosphoadenosine 5'-phosphosulfate (PAPS) (2 Ci/mmol) were purchased from New England Nuclear (Boston, MA). RU-486 was a gift from Roussel Uclaf (Romainville, France). All other chemicals were of reagent grade quality.

Cell culture

The Ishikawa cells were a generous gift from Dr. E. Gurfide, Mt. Sinai Medical Center. Ishikawa cells were maintained in MEM with 7% FBS at 37 °C in an humidified atmosphere with 5% CO₂. For progesterone induction of hEST activity, cells were passed into 100-mm tissue culture plates in 7% FBS/MEM. After 24 h, the medium was removed and replaced with serum-free MEM for 4 h to quiesce the cells. The medium was then replaced with fresh MEM and progesterone (or another test compound) was added from an ethanol stock solution to the appropriate final concentration. Control cells received a similar concentration of ethanol; the final concentration of ethanol in the medium was consistently less than 0.1%. The medium was replaced every 24 h with medium containing fresh drugs. For the RU-486 experiments, progesterone and RU-486 were applied simultaneously. Cytosol was prepared at 48 h post-induction except in the time course experiments. Ishikawa cell cytosols were prepared from confluent plates of cells by rinsing twice with FBS, pH 7.4, then scraping the cells from the plate into triethanolamine buffer (10 mM triethanolamine, pH 7.5, 10% glycerol, 1.5 mM dithiothreitol, 10 μg/μl phenylmethylsulfonylfluoride). After cell disruption on ice with a motor-driven Teflon-glass homogenizer, the homogenate was centrifuged at 100,000 × g for 1 h to obtain the cell cytosolic fraction.

Sulfation assays

Cell cytosols were assayed for estrogen sulfation activity at a concentration of 20 nM E₁, using [³H]-E₁, and nonradioabeled PAPS (20 μM). [³H]-E₁-sulfate was resolved from [³H]-E₁ using an alkaline-chloroform extraction procedure (18). E₂ and DHEA sulfation activities were assayed in the same manner, using 20 nM [³H]-E₂ or 3 μM [³H]DHEA as substrates. Sulfation of p-nitrophenol (2 μM) and dopamine (10 μM) was assayed as described previously (12) using the barium precipitation procedure of Foldes and Meek (19) with [³³P]-PAPS as the sulfonate donor.

The ability of control and progesterone-treated Ishikawa cells to sulfate 20 nM E₁ added to the medium was tested. Quiesced Ishikawa cells were induced for 48 h with 10 μM progesterone, then 20 nM [³H]-E₁ was added to the cells in fresh medium. Aliquots (50 μl) of the medium were removed at different time points and alkalized with 150 μl of 250 mM Tris-HCl, pH 8.7. The medium was then extracted with 3 ml of chloroform and the [³H]-E₁ sulfate in the aqueous phase determined by scintillation spectroscopy. The identity of the [³H]-E₁-sulfate was verified by TLC with commercial standards as well as with enzymatically synthesized [³H]-E₁-sulfate (10).

Immunoblot analysis

For immunoblot analysis of the ST proteins present in cytosol from both progesterone-induced and uninduced Ishikawa cells, cytosolic proteins were resolved by SDS-PAGE in a 12.5% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane using the buffer system of Towbin et al. (20). The membrane was blocked with 5% nonfat dry milk for 1 h. Primary antibody incubations were carried out overnight at 4°C. The three different specific rabbit anti-hST sera (anti-hDHEA-ST (21), anti-hEST (12), anti-hEST (6)) were used as primary antibodies at a 1:15,000 dilution. Goat antirabbit IgG horseradish peroxidase conjugate was used as the secondary antibody at a 1:20,000 dilution. Immunoconjugates were visualized by chemiluminescence using a Lumiglo Chemiluminescent Substrate kit.

Northern blot analysis

Total RNA was prepared from plates of cells using a guanidinium thiocyanate extraction method (RNAzol). Poly(A) RNA was isolated using a Poly A-tract kit (Promega). Poly(A) RNA was resolved by electrophoresis in a 1% agarose gel containing 6% formaldehyde, visualized by brief staining in ethidium bromide, transferred to nitrocellulose paper, and UV irradiated. To prepare the hybridization probe, hEST cDNA was labeled with [³²P]-DCTP by random priming using a Pharmacia Oligolabelling kit and denatured by boiling. Prehybridization and hybridization were performed at 68°C using Quickhyb (Stratagene). Filters were washed twice at room temperature with 2 X SSC/0.1% SDS, then three times at 60°C with 1 X SSC/0.1% SDS, for 15 min each. Autoradiography was performed at 70°C with an intensifying screen or by phosphorescence imaging with a Bio-Rad GS-250 Molecular Imageer.

Results

The induction of estrogen sulfation activity by progesterone was investigated in the Ishikawa human adenocarcinoma endometrial cell line. Ishikawa cells possess both progesterone and estrogen receptors and are responsive to both hormones (14-17). Progesterone treatment of quiesced Ishikawa cells produced a 3- to 11-fold increase in the sulfation of E₁ assayed at a concentration of 20 nM in cytosol prepared from progesterone-treated cells as compared to control cells. Figure 1 shows that the largest increases in E₁ sulfation activity were obtained with the addition of 10 μM progesterone to the culture medium. Maximal levels of E₁
Fig. 1. Concentration curve and time course for the induction of hEST activity in Ishikawa cells by progesterone. A, Quiesced Ishikawa cells in 100-mm plates were treated with increasing concentrations of progesterone for 48 h. Cytosol was prepared from the different plates and hEST activity was assayed with 20 nM E1. hEST activity is expressed as the increase in activity as compared with control cells treated only with ethanol. Each bar represents the average activity of two separate plates. B, Quiesced Ishikawa cells in 100-mm plates were treated with increasing concentrations of several other steroids. Cytosol was prepared at different times and hEST activity was assayed with 20 nM E1. hEST activity is expressed as the increase in activity as compared with control cells treated only with ethanol. Each bar represents the average activity of four plates treated with each progesterone concentration.

Sulfation activity were observed 48–72 h after progesterone treatment.

To determine whether the induction of E1 sulfation activity was specifically elicited by progesterone, Ishikawa cells were treated with different concentrations of several other steroids and the changes in cytosolic E1 sulfation activity monitored. Figure 2 shows that progesterone treatment significantly increased E1 sulfation activity whereas incubation of the cells with 10 nM or 10 μM testosterone, E2, cortisol, or dexamethasone had no effect on E1 sulfation activity. To investigate the possibility that progesterone metabolism may be responsible for the relatively high levels of progesterone required for hEST induction, medroxyprogesterone, which is metabolized much more slowly, was tested for its ability to induce hEST activity. Figure 3 shows that medroxyprogesterone induced hEST activity approximately 6-fold with maximal induction occurring between 10 nM and 10 μM.

To further investigate the specificity of the induction of E1 sulfation activity, the effect of the antiprogestin, RU-486, on the induction of E1 sulfation activity was tested. Figure 4 shows that coincubation of RU-486 and progesterone with the Ishikawa cells resulted in a dose-dependent decrease in the induction of E1 sulfation activity. RU-486 had no effect on the basal E1 sulfation activity in these cells but prevented the stimulation of E1 sulfation caused by progesterone.

Four human cytosolic STs have been identified in human tissues, and three of these enzymes have been reported to sulfate estrogens. The changes in the sulfation of specific substrates for the different forms of human ST were assayed in cytosol from progesterone-treated and control Ishikawa cells to determine which of the ST activities were being affected by the progesterone treatment. Table 1 shows that progesterone treatment significantly increased the levels of E1 sulfation at 20 nM. E1 sulfation assayed at 20 nM is highly specific for hEST activity (8). The sulfation of either p-nitrophenol or dopamine was not affected. At low concentrations, dopamine is a selective substrate for hM-PST and p-nitro-
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Fig. 3. Induction of hEST activity in Ishikawa cells by medroxyprogesterone acetate. Quiesced Ishikawa cells were treated with various concentrations of medroxyprogesterone acetate for 48 h. Cytosol was prepared from the cells and assayed for hEST activity with 20 nM E, to determine whether induction had occurred. Each value represents the average and SD of three separate plates.

Fig. 4. Effect of RU-486 on the induction of hEST activity by progesterone. Quiesced Ishikawa cells were treated with 10 μM progesterone, RU-486 (0.1 μM, 1.0 μM, 10 μM) or a combination of 10 μM progesterone and RU486 applied simultaneously. Cytosol was prepared at 48 h post induction and assayed for hEST activity with 20 nM E, to determine whether induction had occurred. Each value is the average of two experiments, and the difference in the activity of the two experiments is less than 7%.

Table 1. ST activities in control vs. progesterone-induced Ishikawa cell cytosols

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>Progesterone</th>
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<tbody>
<tr>
<td>Estrone</td>
<td>0.109 ± 0.12</td>
<td>16.9 ± 2.1</td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td>4.1 ± 0.6</td>
<td>ND</td>
</tr>
<tr>
<td>Dopamine</td>
<td>15.5 ± 1.0</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>DHEA</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

DHEA sulfation was detectable in either the progesterone-treated or the control Ishikawa cells. These results indicate that progesterone specifically induces hEST activity in Ishikawa cells and does not significantly alter the activity of the two forms of PST also present in these cells. Immunoblot analysis of progesterone-treated and control Ishikawa cells shows an increase in hEST protein in the progesterone-treated cells as compared with control cells (Fig. 5). Immunoblot analysis of these cells with specific rabbit anti-hPST antibodies did not detect significant differences in the expression of either hP-PST or hM-PST (data not shown).

Northern blot analysis of RNA isolated from progesterone-treated and control Ishikawa cells was performed. Figure 6 shows that [32P]-labeled hEST cDNA hybridized to a band of approximately 1300 nucleotides in poly(A) RNA isolated from both progesterone-treated and control Ishikawa cells. The level of hybridization was greater in poly(A) RNA isolated from the progesterone-treated cells than in the control cells. Poly(A) RNA isolated after a 16 h treatment with 10 μM progesterone possessed approximately 5-fold more hEST message than did control cells.

The effect of progesterone treatment on the ability of Ishikawa cells to sulfate E, was investigated to determine...
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Fig. 6. Northern blot analysis of RNA from control and progesterone-treated Ishikawa cells. Total RNA was isolated from quiesced Ishikawa cells 5 and 16 h after treatment with 10 μM progesterone or ethanol. Poly(A) RNA was isolated from total RNA obtained from the different treated cells using a Poly AT-tract kit. Poly(A) RNA (10 μg) was resolved by electrophoresis in a formaldehyde-urea agarose gel and transferred to a nitrocellulose membrane. Upper panel, the membrane was probed with [32P]-labeled hEST cDNA (1 x 10^6 cpm/μg) and washed as described previously (10). The presence of bound probe was visualized by phosphorescence imaging. Lanes A and C contain poly(A) RNA from control cells isolated at 5 and 16 h after treatment, respectively. Lanes B and D contain RNA from progesterone-treated cells isolated at 5 and 16 h after treatment, respectively. Lower panel. To determine the loading and transfer efficiency, the membrane was stripped by boiling in water until no radioactivity was detectable on the membrane. The membrane was then probed with [32P]-labeled β-actin (0.5 x 10^6 cpm/μg), washed, and exposed to autoradiography film.

Fig. 7. Sulfation of estrone by control and progesterone-treated Ishikawa cells. Quiesced cells in 35-mm plates were treated with 10 μM progesterone or ethanol for 48 h. At 48 h, 20 nM [3H]E1 was added to the plates. Medium was sampled at various time points. E1-sulfate formation was determined by alkaline chloroform extraction and scintillation spectroscopy. Each point represents the average ± SD of three separate plates.

Whether the increase in cytosolic hEST activity was reflected in an increase in the sulfation of E1 added to the medium of intact cells. Figure 7 shows that the progesterone-treated cells secreted E1-sulfate into the medium approximately 4-fold more rapidly than did control cells. The increase in cytosolic hEST activity in parallel cultures of Ishikawa cells treated with progesterone was also approximately 4-fold.

Discussion

Estrogen and progesterone regulation of endometrial function is an important aspect of the menstrual cycle. Studies using human endometrial tissue slices or fragments have indicated that there is an increase in estrogen sulfation in secretory endometrium as compared to proliferative endometrium in response to progesterone treatment (23, 24). This report demonstrates the specific induction of human EST by progesterone in human Ishikawa endometrial adenocarcinoma cells as a model system for the increases in estrogen sulfation in human endometrium. Progesterone increased both hEST activity and immunoreactive protein but did not affect the activity of other forms of cytosolic ST. Although previous studies of estrogen sulfation indicated the presence of a high affinity EST activity in endometrial tissues and endometrial tumor cell lines, only recently has a human EST been cloned, expressed, purified, and kinetically characterized (4, 8). Expressed hEST has a K_m for both E1 and E2 of approximately 5 nM (8). This value is similar to the K_m for E2 sulfation reported by Tseng and Liu (24) using endometrial slices and by Grosso and Way (25) for an EST activity in RL 95–2 endometrial carcinoma cells.

The affinity of hEST for E2 and E1 is such that the enzyme functions efficiently in the range of estrogen concentrations normally found in human endometrial tissues. The rapid intracellular sulfation of E2 and E1 in secretory endometrium elicited by progesterone would enhance the decrease in estrogen responsiveness associated with a decrease in ER levels.
caused by progesterone in these cells. Also, sulfation may be more important in decreasing the activity of estrogens than the oxidation of E₂ to E₁. Hata et al. (3) have reported that at low nanomolar (1–10 nM) estrogen concentrations, sulfation is the major pathway for estrogen metabolism in Ishikawa cells, whereas the oxidation of E₂ to E₁ by 17β-hydroxysteroid dehydrogenase becomes important at higher concentrations. The Kₘ of 17β-hydroxysteroid dehydrogenase for E₂ in endometrial tissues is approximately 1 μM. The induction of hEST by progesterone in Ishikawa cells and the kinetic properties of this enzyme strongly suggest a physiological role for this enzyme in regulating E₂ and E₁ activity in the endometrium.

Ishikawa cells also possess significant levels of both hP-PST and hM-PST activity but no detectable hDHEA-ST activity. Although hP-PST is capable of conjugating both E₂ and E₁, the affinity of hP-PST for estrogens is at least 400 fold lower than that of hEST. Maximal sulfation of E₂ and E₁ by hP-PST occurs at concentrations of 6 μM and 20 μM, respectively (10). It has been reported that partially purified human liver M-PST activity does not sulfate estrogens (9). Also, cloned hM-PST expressed in bacteria was not capable of sulfating any of the steroids, including E₂ and E₁, tested as substrates (26). The lack of modulation of hP-PST and hM-PST activities by progesterone and the low affinity of hP-PST for estrogen sulfation does not support a physiological role for these enzymes in regulating estrogen levels in Ishikawa cells.

The induction of hEST activity by progesterone apparently involves the binding of the progesterone receptor. Only progesterone and medroxyprogesterone acetate increased hEST activity in Ishikawa cells and the presence of RU-486 inhibited the increase stimulated by progesterone. The differences in the concentrations of progesterone and medroxyprogesterone needed to induce hEST activity may be related to an increased metabolism of progesterone as compared to the more slowly metabolized medroxyprogesterone. The inability of RU-486 to decrease hEST activity in untreated Ishikawa cells suggests that there is a level of constitutive expression of hEST in these cells that is independent of progesterone stimulation. Whether significant levels of hEST occur in normal human proliferative endometrium in the relative absence of progesterone has not been reported. However, the availability of specific molecular and antibody probes for the individual human cytosolic STs will aid in these investigations.

The induction of hEST activity by progesterone in Ishikawa cells is the first demonstration of the regulation of a specific human ST by a steroid hormone. In rodent tissues, multiple forms of ST are involved with the conjugation of steroids, and these STs display a clear sexual dimorphism that is independent of progesterone expression. Whether significant levels of hEST occur in nor-

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