Influence of Estrogen and Xenoestrogens on Basolateral Uptake of Tetraethylammonium by Opossum Kidney Cells in Culture[5]

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ABSTRACT

The sex steroid hormone estrogen down-regulates renal organic cation (OC) transport in animals, and it may contribute to sex-related differences in xenobiotic accumulation and excretion. Also, the presence of various endocrine-disrupting chemicals, i.e., environmental chemicals that possess estrogenic activity (e.g., xenoestrogens) may down-regulate various transporters involved in renal accumulation and excretion of xenobiotics. The present study characterizes the mechanism by which long-term (6-day) incubation with physiological concentrations of 17β-estradiol (E2) or the xenoestrogens diethylstilbestrol (DES) and bisphenol A (BPA) regulate the basolateral transport of the OC tetraethylammonium (TEA) in opossum kidney (OK) cell renal cultures. Both 17β-E2 and the xenoestrogen DES produced a dose- and time-dependent inhibition of basolateral TEA uptake in OK cell cultures, whereas the weakly estrogenic BPA had no effect on TEA uptake. Treatment for 6 days with either 1 nM 17β-E2 or DES reduced TEA uptake by ~30% and 40%, respectively. These effects were blocked completely by the estrogen receptor antagonist ICI 182780 (Faslodex, fulvestrant), suggesting that these estrogens regulate OC transport through the estrogen receptor, which was detected (estrogen receptor α) in OK cell cultures by reverse transcription-polymerase chain reaction. The \( J_{\text{max}} \) value for TEA uptake in 17β-E2- and DES-treated OK cell cultures was ~40 to 50% lower than for ethanol-treated cultures, whereas \( K_{\text{m}} \) was unaffected. This reduction in transport capacity was correlated with a reduction in OC transporter OCT1 protein expression following treatment with both agents.

Renal excretion, accomplished largely by active proximal tubular secretion, is involved in the elimination of a diverse array of organic cations (OCs) from the systemic circulation, including clinically important therapeutics (e.g., cimetidine), endogenous metabolites (e.g., norepinephrine), and environmental toxins (e.g., nicotine). Uptake of OCs across the peritubular membrane (i.e., basolateral membrane) of renal proximal tubule cells is the initial step in tubular secretion, and this process is mediated primarily by the OC transporters OCT1 (SLC22A1) and OCT2 (SLC22A2) (Gründemann et al., 1994; Okuda et al., 1996). Depending on the prevailing set of electrical and chemical gradients, OCT-mediated transport can occur either via electrogenic facilitated diffusion (i.e., driven by the negative membrane potential) or electroneutral OC/OE exchange (Busch et al., 1996). The OC transporters in the SLC22A family (which also includes the transporters responsible for tubular secretion of organic anions) provide one line of defense against xenobiotic toxicity.

Sex steroid hormones have been implicated in regulation of renal OC transport, as manifested by gender differences in the transport of tetraethylammonium (TEA) across the peritubular membrane. For example, in rats TEA uptake is greater into renal cortical slices (representing peritubular transport) and basolateral membrane vesicles isolated from males than from females (Bowman and Hook, 1972; Urakami et al., 1999), and these observations correlate with higher mRNA and protein levels for OCT2, but not OCT1 or OCT3, in male versus female rat kidneys (Urakami et al., 1999).

ABBREVIATIONS: OC, organic cation; OCT, organic cation transporter; TEA, tetaethylammonium; BPA, bisphenol A; DES, diethylstilbestrol, 17β-E2, 17β estradiol; OK, opossum kidney; BSA, bovine serum albumin; WB, Waymouth’s buffer; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline-Tween 20; ER, estrogen receptor; RT, reverse transcription; PGR, polymerase chain reaction; bp, base pair(s); EtOH, ethanol.
Consistent with the gender differences observed for renal TEA transport, TEA uptake into renal cortical slices, and renal OCT2 mRNA and protein levels, were simultaneously elevated in both male and female rats treated with testosterone for several days, whereas reduced TEA uptake and OCT2 levels were observed following estradiol treatment (Urakami et al., 2000). Accordingly, the rat OCT2 promoter contains two androgen response elements, which are stimulated by physiological concentrations of testosterone to promote the transcription of rat OCT2 (Asaka et al., 2006). Consistent with the results from the rat, testosterone (and other steroid hormones) was shown to stimulate OCT2 mRNA expression and TEA transport across the basolateral membrane of Madin-Darby canine kidney cells, an immortal cell line derived from distal tubule cells (Shu et al., 2001).

Regulation of renal OC transport function by sex steroids raises the possibility that xenoestrogens (environmental contaminants with estrogen-like activity) may also affect the renal excretion of OCs. Xenoestrogens as environmental contaminants have sparked much debate as a public health concern, because significant levels are present in the environment (Degen and Bolt, 2000). The industrial chemical bisphenol A (BPA; used to make polycarbonate plastics and epoxy resins), naturally occurring plant estrogens (e.g., zearalenone), and the potent synthetic pharmaceutical estrogen diethylstilbestrol (DES) are some of the more prevalent environmental xenoestrogens of toxicological interest (Degen and Bolt, 2000; Juberg, 2000; Safe, 2000). Although the potential hazard to human health from exposure to environmental xenoestrogens has not been conclusively determined (Degen and Bolt, 2000; Juberg, 2000; Safe, 2000), significant reproductive and associated anomalies in wildlife have been attributed to their exposure to xenoestrogens (Juberg, 2000), raising a significant health concern for humans.

Much of the focus on xenoestrogens has been on the disruption of reproductive function (Degen and Bolt, 2000; Juberg, 2000; Safe, 2000). However, as noted above, sex hormones also have well documented physiological effects on nonreproductive tissues such as the kidney, which contain receptors for both testosterone and estrogen (DeVries et al., 1972; Stefani et al., 1994). Since manipulation of sex steroid hormone levels associated, for example, with estrogen administration has been shown to depress renal OC transport function (Urakami et al., 2000), the regulation by estrogen and/or xenoestrogens of OC transport could significantly influence the pharmacokinetics of xenobiotic elimination, thereby altering drug efficacy and/or the development of toxicity.

The objective of the present study was to determine whether the endogenous estrogen 17β-estradiol (17β-E2) and the synthetic xenoestrogens DES and BPA influence OC transport across the basolateral membrane of opossum kidney (OK) cell cultures. The localization of estrogen receptors in OK cells (Stock et al., 1992), along with the expression of OC transport function (McKinney et al., 1990; Yuan et al., 1991; Endo et al., 2000), makes this renal culture an appropriate model system for investigating estrogen regulation of OC transport. The data reported here demonstrate that the endogenous estrogen 17β-E2 and the synthetic pharmacological estrogen DES reduce TEA transport across the basolateral membrane of OK cells.

Materials and Methods

Materials. OK cells were a generous gift from Dr. Michael Gekle (Physiological Institute, University of Wuerzburg, Germany). [125I]TEA (55 mCi/mmol) was purchased from Amersham Biochemicals (St. Louis, MO). The estrogen receptor antagonist ICI 182780 (faslodex, fulvestrant) was purchased from Tocris Cookson Inc. (Ellisville, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and they were of the highest purity available.

Cell Culture. OK cell cultures (passages 72–88) were maintained at 37°C in humidified 5% CO2, 95% air atmosphere. OK cell cultures were grown and maintained in Dulbecco's modified Eagle’s medium/Ham’s F-12 containing 2 mM glutamine supplemented with 10 μg/ml bovine insulin, 1 mg/ml BSA fraction V, 1 mg/ml bovine Pederson fetuin, and 25 mM NaHCO3 without antibiotics as described by Leiderman et al. (1989). For transport measurement and Western blot analysis, cells maintained in serum-free medium were seeded onto Corning Transwell permeable supports (0.4-μm pore diameter, 4.7 or 44 cm2; Corning Life Sciences, Acton, MA) at a density of 4.5 × 104 cells and 4.0 × 104 cells, respectively. The medium was changed every other day. Five days after seeding, confluent monolayers of OK cell cultures were treated with the vehicle (100% ethanol, final concentration of <0.1% in the medium), 17β-E2, DES, or BPA. The times of exposure and concentrations used are described under Results and in the figure legends. In some cases, cells were treated with the estrogen receptor antagonist ICI 182780.

[125I]TEA Uptake Measurements. Before initiating transport, OK cell cultures were rinsed three times at room temperature with serum-free Dulbecco's modified Eagle's medium/Ham's F-12 containing 2 mg/ml BSA fraction V to remove 17β-E2, DES, or BPA from the extracellular space. Monolayers were then rinsed two times at room temperature in Waymouth's buffer (WB): 135 mM NaCl, 28 mM d-glucose, 5 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 0.8 mM MgSO4, and 13 mM HEPES-NaOH, pH 7.4. After removing WB from the apical and basal compartments, the apical side was replaced with fresh WB, and uptake was initiated with the addition of WB containing 2 μM [125I]TEA to the basal compartment. In some cases, unlabeled TEA was included in the transport reaction. Because initial experiments showed that the uptake of [125I]TEA across the basolateral membrane is linear for 10 min (at room temperature), 2-min uptakes were used to approximate the initial transport rate. After 2 min, WB was removed from the basal and apical compartments by vacuum aspiration, and both compartments were rinsed two times with ice-cold WB to “stop” transport. Permeable supports were removed from their housings and transferred to scintillation vials with scintillation cocktail added for counting radioactivity. Data were normalized to surface area (square centimeters).

Preparation of OK Cell Crude Membranes. Crude membranes were prepared from OK cells using a modification of the method described by our laboratory (Pelis et al., 2006). OK cells were rinsed twice with PBS and the confluent monolayer was scraped from either one 44-cm2 or six separate 4.7-cm2 permeable supports using a cell scraper. The cells were resuspended in 20 ml of PBS and pelleted by centrifugation at 230g for 10 min at 4°C. The cell pellet was resuspended in 1 ml of 10 mM NaHCO3 containing protease inhibitors, transferred to a glass homogenizer, and homogenized using 10 strokes with a Teflon pestle. Insoluble cellular material was removed by centrifugation at 100g for 5 min at 4°C. The supernatant was centrifuged for 30 min at 15,800g at 4°C, and the resulting pellet was resuspended (by vortexing) in 10 mM NaHCO3. Protein concentration was determined by the Bradford method. Crude membrane proteins were diluted to 3 to 5 μg/μl in Laemmli sample buffer.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. To examine the effect of 17β-E2, and DES on OCT1 and OCT2 protein expression, 50 μg of crude membrane protein from OK cell cultures that were treated with the vehicle, 17β-E2, or DES were
separated on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes using a semidyrid electroblotting unit (Invitrogen, Carlsbad, CA). Membranes were blocked in blocking buffer containing PBS-T (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 0.1 mM CaCl2, and 1 mM MgCl2, and 0.05% Tween 20, pH 7.3) and 5% nonfat dry milk. After blocking, the membranes were incubated overnight (12–14 h) at 4°C with either rabbit anti-rat OCT1 (final concentration of 1.5 μg/ml) or rabbit anti-rat OCT2 (final concentration of 1.5 μg/ml) antibodies (Alpha Diagnostics International, San Antonio, TX) diluted in blocking buffer. Membranes were then washed six times for 15 min in PBS-T before incubation for 1 h in horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce Chemical, Rockford, IL) diluted (final concentration of 3 μg/ml) in blocking buffer. Detection of immunoreactivity was performed using the West Femto Enhanced Chemiluminescence Detection system (Pierce Chemical). Relative immunoreactivity was determined using Image 1.34 s Analysis Software (National Institutes of Health, Bethesda, MD) as described previously (Pelis et al., 2006).

Cloning of Partial Sequences of Estrogen Receptor α from OK Cells. Degenerate sense and antisense oligonucleotide primers were designed on the basis of consensus sequences of the human, mouse, cow, quail (Coturnix japonica), crocodile (Crocodylus niloticus), and gray short-tailed opossum (Monodelphis domestica) orthologs of ERα. The beginning and latter halves of the ERα’s were targeted for RT-PCR resulting in primer set 1 (sense, 5’-TGAACCATGACCTCAGCAGCAA-3’ and antisense, 5’-CCTGCCCCATGCACCCTTC-3’) and primer set 2 (sense, 5’-CAGATGATCACTGGCCAAGAAGAGT-3’ and antisense, 5’-TGCTCCATBCCTTTGTTGCACA-3’), respectively. For first-strand synthesis, 0.5 μg of OK cell poly(A)⁺ RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase H⁻ at 37°C for 20 min. After incubation at 70°C for 15 min, ribonuclease (RNase) H was added, and the reactions were kept at 37°C for 20 min. The reverse transcriptase reaction (2 μl) was used directly for amplification. The PCR solution was assembled and heated at 94°C for 2 min before Pfu DNA polymerase was added. Subsequently, PCR was performed using the following conditions: 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min (36 cycles). The last cycle was terminated after an elongation time of 7 min. The ~700-bp (primer set 1) and ~500-bp (primer set 2) RT-PCR products were gel purified, and they were sequenced with a model 373A sequencing unit (Applied Biosystems, Foster City, CA) at the University of Arizona (Tucson, AZ) sequencing facility.

Data Analysis. All data are expressed as means ± standard errors, with calculations of standard errors based on the number of separate experiments conducted on cells at a different passage number. Significant differences among sample means were determined by Student’s t test. One-way analysis of variance was used to test the effect of multiple treatments, and it was followed by the Fisher’s least significant difference post hoc test for pairwise comparisons. All statistical analyses were performed with ProStat 3.81c (Poly Software International, Inc., Pearl River, NY), and differences were deemed significant when * P < 0.05.

Results

Effect of 17β-E₂, DES, and BPA on Basolateral TEA Uptake. 17β-E₂ and DES reduced TEA uptake across the basolateral membrane of OK cell cultures in a dose-dependent manner (Fig. 1A). Six-day treatment of OK cells with 1 nM 17β-E₂ or 100 μM DES reduced TEA uptake by 20 to 30%, compared with ethanol-treated controls. Maximal inhibition (50–60%) of basolateral TEA uptake was observed at 10 nM 17β-E₂ and 1 nM DES. In contrast, treatment of cells for 6 days with the purported environmental estrogen BPA had no effect on TEA uptake at concentrations as high as 100 μM (Fig. 1B). In addition to reducing TEA uptake in a dose-dependent manner, the response of basolateral TEA uptake to both 17β-E₂ and DES was time-dependent (Fig. 2). Four-hour treatment with 10 nM 17β-E₂ and 1 nM DES did not alter TEA uptake. However, uptake was reduced ~20 and 50 to 60% following treatment for 36 h and 6 days, respectively, with either 17β-E₂ or DES. The addition of 10 nM 17β-E₂ or 1 nM DES to the transport reaction (i.e., present in the transport buffer) did not alter TEA uptake, demonstrating that neither compound has direct nor acute effects on basolateral TEA transport (data not shown). In contrast to the reduction of basolateral TEA uptake, 6-day treatment with 100 nM 17β-E₂ had no effect on the basolateral uptake of the organic anion para-aminohippurate (2-min uptakes; 14 ± 6.1 versus 16 ± 5.2 fmol · cm⁻² for control and 17β-E₂, respectively; n = 5; P > 0.05, two-tailed t test). Because basolateral organic anion transport is influenced by both the transmembrane sodium gradient and generation of α-ketoglutarate from oxidative phosphorylation (Dantzler, 2002), these data suggest that long-term treatment with concentrations of 17β-E₂ used in the present study do not negatively impact cell viability or function.

Influence of Estrogen Receptor Antagonist ICI 182780 on Basolateral TEA Uptake. The aforementioned data reveal that both endogenous and synthetic estrogens reduce basolateral OC transport in OK cell cultures. To determine whether the response of basolateral TEA transport to 17β-E₂ and DES occurs through their binding to and
activation of an estrogen receptor, OK cells were treated with the estrogen receptor antagonist ICI 182780 (Fig. 3). ICI 182780 is an antagonist of estrogen receptors α and β, and it has been used clinically to treat metastatic breast cancers that require estrogen for growth (Wakeling and Bowler, 1992; Okazaki et al., 2002). ICI 182780 has also been demonstrated as an effective antagonist of several estrogen receptor subtypes in teleost fish (Hawkins and Thomas, 2004). When OK cell cultures were pretreated for 24 h with 50 nM ICI 182780 and then cotreated with 50 nM ICI 182780 and either 5 nM 17β-E2 or 1 nM DES for 6 days, the decrease in TEA uptake caused by 17β-E2 and DES was eliminated. Treatment of cells with 50 nM ICI 182780 alone was without effect on TEA transport. These observations are consistent with 17β-E2 and DES acting through an estrogen receptor to alter OC transport. Indeed, mRNA for ERα was identified in OK cell cultures (cells derived from the kidney of the North American opossum, Didelphis virginiana) using RT-PCR and degenerate primers (Supplemental Fig. 1A). Two separate 

\[
J = \frac{J_{\text{max}}[T^*]}{K_s + [T^*] + [S]} + C
\]

where \(J\) is the rate of [14C]TEA transport from a concentration of labeled substrate equal to \([T]\); \(J_{\text{max}}\) is the maximum rate of mediated substrate transport; \(K_s\) is the substrate concentration that results in half-maximal transport (Michaelis constant); \([S]\) is the concentration of unlabeled substrate in the transport reaction; and \(C\) is a constant that represents the component of radiolabeled substrate uptake that was not saturated (over the range of substrate concentrations tested), and presumably reflects the combined influence of diffusive flux, nonspecific binding, and/or incomplete rinsing of the cell layer. Treatment of OK cells for 6 days with 10 nM 17β-E2 reduced the \(J_{\text{max}}\) of basolateral TEA uptake ~40%, but it had no effect on the affinity (\(K_s\)) of basolateral transport for TEA (Table 1). Similarly, in a separate set of experiments, 6-day treatment with 1 nM DES reduced the \(J_{\text{max}}\) for basolateral TEA uptake by 50%, whereas \(K_s\) was not changed (Table 1). A reduced maximum rate of transport without a change in affinity is consistent with a reduced number of active transporters for TEA in the basolateral membrane of OK cells.

OCT1 and OCT2 Protein Expression following Treatment with 17β-E2 and DES. OCT1 and OCT2 mediate the peritubular uptake of a wide array of structurally diverse organic cations (Wright and Dantzler, 2004; Wright, 2005). The renal expression of OCT2, but not OCT1, is reduced in the rat following treatment for several days with estradiol (Urakami et al., 2000). Thus, Western blotting and densitometry were used to determine whether the decrease in the maximal rate of basolateral TEA transport that occurred in response to 17β-E2 and DES treatment of OK cells correlated

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(J_{\text{max}}) (pmol cm⁻² min⁻¹)</th>
<th>(K_s) (μM)</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>36.2 ± 4.35</td>
<td>21.6 ± 0.06</td>
</tr>
<tr>
<td>17β-E2</td>
<td>20.8 ± 1.12*</td>
<td>17.2 ± 1.96</td>
</tr>
<tr>
<td>Vehicle</td>
<td>12.1 ± 1.59</td>
<td>9.4 ± 0.43</td>
</tr>
<tr>
<td>DES</td>
<td>5.7 ± 1.27*</td>
<td>7.1 ± 0.81</td>
</tr>
</tbody>
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* Significantly different from control (vehicle); \(P < 0.05\), two-tailed t test.
with changes in either OCT1 and/or OCT2 protein expression. OCT1 and OCT2 from crude membranes migrated with an apparent molecular mass of 55 and 58 kDa, respectively, on Western blots (Fig. 4). Whereas OCT2 always migrated as a single band, occasionally a lower molecular mass product (<50 kDa) was observed for OCT1. OCT1 and OCT2 immunoreactivity was eliminated when the corresponding antibodies were preabsorbed with their respective antigen (i.e., purified rat OCT1 or rat OCT2 peptide) (data not shown). Because heterologous antibodies were used for detection of OCT1 and OCT2 in OK cells (anti-rat OCT1 and OCT2 antibodies), and the apparent molecular mass of OCT1 and OCT2 was similar (55 versus 58 kDa), we wanted to confirm that each antibody was indeed specific. When crude membranes were probed with both rat anti-rat OCT1 and OCT2 antibodies simultaneously, three bands were evident, i.e., a 55-kDa band and <50-kDa band that corresponded to OCT1 (Fig. 4) and a 58-kDa band that corresponded to OCT2 (Fig. 4), demonstrating that each rat antibody is specific for a different OK cell protein. The lower molecular mass product corresponding to OCT1 likely represents a proteolytic fragment, and not a partially glycosylated precursor, because preliminary studies suggested that neither OCT1 nor OCT2 in OK cells are N-glycosylated (data not shown). Treatment of OK cells with either 10 nM 17β-E2 or 1 nM DES significantly reduced OCT1 protein expression by 40%, but it did not alter OCT2 expression (Fig. 5). The reduced level of OCT1 protein approximates the lower maximum rate of basolateral TEA transport following similar treatment with 17β-E2 and DES. These data are consistent with 17β-E2 and DES influencing OCT1 protein expression to reduce basolateral TEA transport in OK cells.

**Discussion**

Gender differences in the renal transport of OCs have been demonstrated in several species, including humans (Wong et al., 1995), leading to speculation that sex steroid hormones regulate renal OC transport processes. Indeed, administration of a high concentration of 17β-E2 to male rats for 7 days reduced TEA uptake into renal slices, whereas treatment of male and female rats with high doses of testosterone had the opposite effect (Urakami et al., 2000). Although the aforementioned studies are consistent with regulation of OC transport by sex steroids, these studies do not provide direct evidence that “physiological” concentrations of either estrogen or testosterone are capable of directly altering renal OC transport. Hypothetically, rather than estrogen or testosterone, one, OC transport processes could be responsive to other hormones whose serum concentrations are gender-dependent or regulated by sex steroids. For example, growth hormone levels (mRNA) are higher in females than males (especially during proestrus, when circulating estrogen levels are elevated), and estrogen replacement in ovariectomized females increases growth hormone synthesis (Donahue et al., 2006). To directly assess whether 17β-E2 influences OC transport, the present study used cultured OK cells, a renal proximal tubule cell line derived from the opossum kidney. 17β-E2 produced a time- and concentration-dependent reduction of basolateral TEA uptake, with a concentration of 1 nM causing a significant decrease in transport activity. In healthy adult women, plasma levels of 17β-E2 can vary between low picomolar up to 1 to 2 nanomolar (Krejza et al., 2001). The alteration of basolateral TEA transport in OK cells by physiological concentrations of 17β-E2 provides support for the contention that gender differences in renal OC transport can be directly influenced by differences in circulating levels of sex steroids.

The effect of 17β-E2 on basolateral TEA uptake in OK cells led to the hypothesis that xenoestrogens, environmental chemicals with estrogen-like activity, may also influence renal OC transport. Accordingly, studies were conducted to test the effects on basolateral TEA uptake of the xenoestrogens DES and BPA. DES reduced basolateral TEA uptake in a time- and dose-dependent manner, and it proved to be a more potent inhibitor of TEA uptake than 17β-E2, because a 10-fold lower concentration (100 pM) was required to produce an effect similar to 17β-E2. BPA, which is weakly estrogenic (Kitamura et al., 2005), had no effect on TEA uptake at concentrations up to 100 μM, even though much lower concentrations (<1 μM) exhibit estrogenic effects in the human breast cancer cell line MCF-7 (Kitamura et al., 2005). Thus, cell type, species differences, or both may be contributing factors determining the sensitivity of cells to the estrogenic effects of BPA.

To further elucidate the mechanism by which 17β-E2 and DES diminish basolateral TEA uptake into OK cells, the kinetics of basolateral TEA transport was examined. The capacity for basolateral TEA uptake was reduced 40 to 50% by long-term treatment with 17β-E2 and DES, whereas the affinity of the uptake process(es) for TEA was unchanged. Changes to either the number of active transport proteins in the plasma membrane or to transport protein turnover number could potentially explain the observed changes in the capacity for TEA transport. Therefore, OCT1 and OCT2 protein expression in OK cells was examined, both of which transport TEA and both of which are expressed in renal proximal tubules in other mammalian species (Zhang et al., 1997; Zhang and Wright, 2002). The lower capacity (i.e., lower $J_{\text{max}}$) for basolateral TEA uptake following 17β-E2 and DES treatment was correlated with a 40% decrease in the expression of OCT1 protein, with no change in OCT2. These data suggest that 17β-E2 and DES alter OCT1 protein expression to reduce basolateral OC transport. In agreement with these results, Madin-Darby canine kidney cells were shown to express mRNA for only OCT2 (no OCT1 mRNA could be detected), and treatment of these cells for 72 h with 0.1 to 10 μM 17β-E2 had no effect on OCT2 mRNA levels or basolateral TEA transport (Shu et al., 2001). In contrast, treatment of male rats with 17β-E2 caused a parallel reduc-

**Fig. 4.** Western blot showing OCT1 and OCT2 protein from crude membranes prepared from OK cell cultures grown on permeable supports. Western blots were probed with rabbit anti-rat OCT1 antibody (OCT1), OCT2 antibody (OCT2), or both antibodies in combination (OCT1 and OCT2).
tion of basolateral TEA uptake into renal slices (a preparation that represents basolateral uptake) and renal OCT2 protein expression (Urakami et al., 2000). Although species differences may explain these conflicting results, renal OCT1 protein levels were not examined in the aforementioned study (because 17β-E2 failed to change renal OCT1 mRNA expression in rats; Urakami et al., 2000). In diabetic rats, OCT1 protein expression is reduced without a substantial change in mRNA levels (Grover et al., 2004). In rabbits, renal expression of OCT2 mRNA is much higher in male than female rabbits; yet, there are no gender differences in OCT2 protein expression or basolateral TEA transport (Groves et al., 2006). Importantly, these observations show that differences in mRNA levels are not a reliable estimate of protein expression, and more importantly, of protein function. Therefore, further investigation is required to determine whether 17β-E2 changes OCT1 protein expression in the kidney of rats. Regardless, the observation that 17β-E2 reduces OCT2 protein in the rat kidney but not in OK cells further supports the contention that the responsiveness of renal OC transport mechanisms to sex steroids is species-specific (Groves et al., 2006).

The effects on TEA uptake of both 17β-E2 and DES were time-dependent, taking ~3 days to significantly reduce uptake compared with controls, suggesting that both may operate through a genomic pathway to alter protein function. In this model, estrogen (or xenoestrogens) binds to its cytosolic receptor, and after translocation into the nucleus, it (they) alters transcription to ultimately change protein expression (i.e., OCT1 protein) and tissue-level function (i.e., basolateral TEA transport). OK cells were previously shown to express mRNA for an estrogen receptor, and functional expression was demonstrated with the estrogen receptor antagonist tamoxifen, which prevented the parathyroid hormone-induced stimulation of cAMP that occurred following prolonged treatment of OK cells with 17β-E2 (Stock et al., 1992). Indeed, using RT-PCR we were able to obtain a partial sequence of the ERα from the OK cell cultures. In the present study, treatment of OK cells with the estrogen receptor antagonist ICI 182780 (Schlattjan et al., 2005) prevented the reduction of basolateral TEA transport caused by 17β-E2 and DES, implying that both operate through an estrogen receptor to change OC transport function. Not surprising given its estrogenic activity, DES is a ligand of the human estrogen receptors α and β (Nikov et al., 2001).

The simplest explanation for the reduction of OCT1 protein expression following treatment with 17β-E2 and DES is that the OCT1 promoter contains an estrogen response element that, when activated, represses OCT1 transcription. The rat OCT2 promoter contains two androgen response elements, which are stimulated by physiological concentrations of testosterone to promote the transcription of rat OCT2 (Asaka et al., 2006). However, mRNA levels were not measured in the present study, and whether the opossum OCT1 promoter contains elements responsive to estrogen has not been determined. Regardless of whether OCT1 mRNA levels are reduced following either 17β-E2 or DES treatment, the possibility cannot be dismissed that alterations downstream from transcription, including post-translational modifications and/or changes in the stability of the transport protein in the plasma membrane, may have contributed to reduced levels of OCT1 protein.

Renal proximal tubular secretion is the primary means of removing many potentially toxic OCs from the systemic circulation, and the OCTs mediate the initial step in this process, i.e., uptake of OCs across the basolateral membrane. Therefore, the level of OCT expression in the renal proximal tubule could influence the renal excretion of OCs (i.e., removal of OCs from the circulation). The down-regulation of renal tubular OC uptake by estrogen and xenoestrogens may have many physiological, pharmacological, and toxicological implications. Some important issues that require further attention include how gender, pharmacological treatments that change circulating levels of sex steroids (e.g., estrogen replacement therapy), or exposure to environmental contaminants that are estrogenic may influence the pharmacokinetics of OC excretion.

In conclusion, long-term (3–6-day) treatment of OK cells with physiological concentrations of 17β-E2 and even lower concentrations of the xenoestrogen DES reduced TEA uptake.
across the basolateral membrane of OK cells in culture, and these effects were blocked by the specific estrogen receptor antagonist ICI 182780. In contrast to DES, high concentrations of the xenoestrogen BPA failed to alter TEA transport. Expression for both OCT1 and OCT2 protein were demonstrated in OK cells, and the reduction of basolateral TEA uptake induced by 17β-E2 and DES was correlated with lower levels of OCT1 protein. These results show that the endogenous sex steroid 17β-E2 and the xenoestrogen DES reduce renal basolateral OC transport through direct actions on renal tubule cells.

References


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