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Estrogenic Endocrine Disruptive Components Interfere With Calcium Handling and Differentiation of Human Trophoblast Cells

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Abstract During development, calcium (Ca) is actively transported by placental trophoblasts to meet fetal nutritional and the skeletal mineralization needs. Maternal exposure to estrogenic pesticides, such as 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) and methoxychlor (MTC), has been shown to result in reproductive disorders and/or abnormal fetal development. In this study, we have examined the effects of exposure of trophoblastic cells to MTC and DTT, in comparison to 17β-estradiol (E2) and diethylstilbestrol (DES), to test the hypothesis that cellular Ca handling is a target for these endocrine disruptive components. Treatment with DDT, MTC, DES, or E2 increased cellular Ca uptake, and the expression of trophoblast-specific human Ca binding protein (HCaBP) was down-regulated by both MTC and DDT. Treatment with MTC, DDT, and DES inhibited cell proliferation, induced apoptosis, and suppressed expression of several trophoblast differentiation marker genes. These effects were reversed by overexpression of metallothionein IIa, a gene highly responsive to cadmium and other metals. These results strongly suggest that trophoblast Ca handling functions are endocrinally modulated, and that their alteration by candidate endocrine disruptors, such as MTC and DDT, constitutes a possible pathway of the harmful effects of these components on fetal development. J. Cell. Biochem. 89: 755–770, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: Ca transport; DDT; endocrine disruption; methoxychlor; placenta; trophoblast; 17β-estradiol

Active calcium (Ca) transport across the placenta is critical for normal fetal growth, especially during the third trimester of human pregnancy, which corresponds to the skeletal mineralization period. Fetal Ca homeostasis is regulated by the placental trophoblast epithelium, which forms early during development and undergoes a highly regulated differentiation process [Lanford et al., 1991; Brunette and Leclerc, 1992; Kamath et al., 1992]. During the first half of human pregnancy, cytotrophoblasts undergo morphological differentiation into multinucleated syncytiotrophoblasts [Cross et al., 1994]. This differentiation is accompanied by the production of several pregnancy hormones, including the peptide hormones, human chorionic somatomammotropin (HCS), also known as placental lactogen (PL), and human choriogonadotropin (HCG), both of which stimulate progesterone synthesis from the ovary [Pepe and Albrecht, 1995; Musicki et al., 1997; Cronier et al., 1999]. At mid- to late gestation, the syncytiotrophoblasts acquire several enzymes of the progesterone and estrogen biosynthesis pathways, which transform fetal and/or maternal precursors into active hormones, marking the hormonal independence of the fetoplacental unit in regulating fetal development [Pepe and Albrecht, 1995; Cronier et al., 1999].

Normal cellular and functional trophoblast differentiation is critical to meet the nutritional and Ca needs of the fetus. Although the exact mechanism of trophoblast Ca transport is unclear, our previous studies have suggested

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that trophoblast intracellular Ca binding proteins (CaBP and HCaBP) may play a major role [Tuan, 1982; Tuan and Cavanaugh, 1986, 1988; Hershberger and Tuan, 1998]. In addition, several lines of evidence suggest that placental Ca transport is regulated by endocrine factors, including parathyroid hormone-related peptide (PTHrP) [Law et al., 1993; Kovacs and Kronenberg, 1997; Hershberger and Tuan, 1998; Kovacs et al., 2001], and steroid hormones, such as 1,25-dihydroxyvitamin D_3 and estrogen [Brunette, 1988; Krisinger et al., 1993, 1994; Jeung et al., 1994].

Estrogens play a central integrative role in placental maturation and growth [Pepe and Albrecht, 1995; Cronier et al., 1999]. In humans, despite the gradually rising levels of placental estrogens produced by the syncytiotrophoblasts from fetal precursors [Pepe and Albrecht, 1995], fetal exposure to estrogen is prevented in the first trimester by 17β-hydroxysteroid dehydrogenase type 2, which transforms E2 to the inactive form, estrone [Takeyama et al., 1998]. Murine fetuses exposed to 17β displayed increased calcification and length of long bones. Similarly, murine offsprings exposed during gestation to diethylstilbestrol (DES), a synthetic estrogen, were reported to have increased bone mass, but shortened long bones [Migliaccio et al., 1996]. These studies suggest that gestational exposure to estrogen or estrogen-like substances may have long-term deleterious consequences on Ca homeostasis in fetal development.

Several environmental pollutants with estrogenic activity, including polychlorinated biphenyls from industrial wastes, pesticides such as 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT), methoxychlor (MTC), and heavy metals such as cadmium (Cd), have been shown to accumulate in the placenta and cause developmental and growth abnormalities [Johnson et al., 1992; Bosse et al., 1996; Eroschenko et al., 1996]. Many of these compounds are also known to disrupt the endocrine system, and hence are collectively referred to as endocrine disrupting chemicals (EDC). Their accumulation in the placenta is suspected to specifically affect trophoblast Ca handling [Juberg et al., 1995]. DDT and/or MTC have been shown to inhibit placental Ca^{2+}-ATPase activity in humans [Treinen and Kulkarni, 1986]. Thus, the placental trophoblast is a potential target for the action of these environmentally derived toxic compounds, which could subsequently affect fetal growth. However, the mechanisms of action of these EDCs on trophoblast functions are not known.

In this study, JEG-3 cells were used to investigate the effect of EDCs on trophoblast function. These cells are derived from a human choriocarcinoma and display morphological and functional characteristics of human trophoblasts [Kohler and Bridson, 1971]. We examined the effects of MTC, a candidate EDC with estrogenic activity, in comparison to DDT, a banned pesticide as well as those of the synthetic estrogen, DES, and E2. Treatment of JEG-3 cells with either DDT or MTC increased cellular Ca uptake, mimicking the action of E2, and suppressed the expression of HCaBP as well as several trophoblast differentiation markers. These markers include cytochrome P450 side chain cleavage (P450SCC) and 3β-hydroxysteroid dehydrogenase 1 (3β-HSD-1), the placental enzymes involved in progesterone biosynthesis, which are known to be under estrogenic regulation [Beaudoin et al., 1997a,b], as well as the homeobox gene, *DLX4*, implicated in trophoblast differentiation [Quinn et al., 1997, 1998]. In addition, these components induced a reduction in cell proliferation and an increase in apoptosis [Quinn et al., 1997, 1998]. These effects were overcome by the overexpression of human metallothionein II (hMTIIa), a major cellular toxicant protective protein. Collectively, our results suggest that trophoblast Ca handling is under estrogenic modulation, and that exposure to DTT and MTC is able to interfere with estrogen signaling pathways and Ca handling in the placenta.

MATERIALS AND METHODS

Reagents

RPMI 1640, DMEM F-12, Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS), Ca^{2+}/Mg^{2+}-free Hanks buffered salt solution (HBSS) were obtained from BioWhittaker (Walkersville, MD), fetal bovine serum (FBS) from Atlanta Biological (Atlanta, GA), antibiotic–antimycotic solutions (10,000 U penicillin, 25 μg amphotericin B, 10,000 μg streptomycin) from Invitrogen (Carlsbad, CA), and cell dissociation solution from Sigma (St. Louis, MO). Total RNA prepared from human placenta was purchased from Clontech (Palo Alto, CA). E2, DES, and MTC, DDT, ethylene glycol-bis(β-amino-ethyl

ether)-tetraacetic acid (EGTA) were from Sigma. All hormone and EDC were dissolved in 95% ethanol, stored at −20°C and used as 10^{-2} or 10^{-3} M stock solutions. $^{45}CaCl_2$ (37 mBq) was from NEN (Boston, MA). All other chemicals used were of the purest grade available commercially.

Cell Culture

BeWo cells (ATCC) were grown in DMEM F-12 medium containing L-glutamine, 1.5 g/L sodium carbonate, and 10% FBS. JEG-3 cells (ATCC) were maintained in RPMI containing 25 mM HEPES, L-glutamin, antibiotic–antimycotic (10,000 U penicillin, 25 μg amphotericin B, 10,000 μg streptomycin), and 10% FBS. JEG-3 overexpressing hMTIIa (MT-JEG-3) were obtained by stable transfection of JEG-3 cells with an expression construct, pβActNeo/sMT-IIa (generously provided by Dr. J. Vicek, New York University Medical Center), which contains the full-length human MT-IIa cDNA [McAleer and Tuan, 2001]. MT-JEG-3 cells were maintained in RPMI containing 10% FBS and supplemented with 200 μg/ml of G418 sulfate (Sigma) to preserve stable, neomycin-resistant populations.

JEG-3 and MT-JEG-3, grown in complete medium as monolayer cultures, were transferred to medium containing 10% steroid-stripped serum (Atlanta Biological) prior to hormone treatment. Confluent cells were detached from flasks by treatment with cell dissociation solution and seeded into 8-well chamber slides, 6-well plates, 10 cm dishes, or T-75 flasks at a density of $1–3 \times 10^5$ cells/ml in medium containing steroid-stripped serum. Cells were then treated in serum–free medium with either 0.1% ethanol, 1 nM E2, 1 nM, 1 μM DES, 1–10 μM DDT, or 1–10 μM MTC for the indicated time period. Cells were harvested and processed for cell cycle, RNA, or protein analysis.

Cell Counts and Cell Cycle Analyses

JEG-3 or MT-JEG cells were seeded at 3×10^5 cells/ml onto 10 cm plates in complete medium containing steroid-stripped serum. Cells were then treated for 48, 72, or 96 h in serum–free medium with either 0.1% ethanol, 1 nM E2, 1 nM DES, 1 μM DDT, or 1 μM MTC. Cells were harvested, treated with 2 mg/ml type II collagenase (Sigma) prepared in RPMI, then processed for cell counts using a hemocytometer. Cells harvested at each time point were washed, fixed with ice cold 70% ethanol, and treated with 0.1 mg/ml RNAse A (Roche, Indianapolis, IN) and 20 μg/ml propidium iodide (Roche). Propidium iodide-stained DNA content of each cell was used as the parameter of cell–cycle profile using flow cytometry.

Ca Uptake

JEG3 and MT-JEG cells were treated with EDC before measurement of Ca uptake over specified time intervals. Briefly, culture medium was replaced with fresh serum-free RPMI 1640 medium, containing indicated concentrations of DDT, MTC, DES, E2, and plates incubated at 37°C in 5% CO_2 for 48 h before determination of uptake in Ca^{2+}/Mg^{2+}-free HBSS, pH 7.2–7.4 containing 0.5 mM $CaCl_2$ and 1.2 μCi/ml $^{45}CaCl_2$ tracer, over the indicated time intervals. Control cells were treated with 95% ethanol at 0.03%, final concentration.

For determination of uptake kinetics, cells were incubated in serum-free RPMI 1640 medium before measurement of uptake of graded concentrations of $CaCl_2$ (10–2,000 μM in HBSS) for 10 s, which reflects initial rates of uptake. In preliminary studies, it was determined that Ca uptake by these cells was linear up to about 40 s. At the end of the incubation period, the medium was aspirated and cells were washed thee times with ice-cold PBS, then lysed in 1% Triton X-100. Aliquots of cell lysates were used for protein determination and liquid scintillation counting to determine intracellular ^{45}Ca. Total cellular Ca uptake is expressed as nmole Ca/Mg protein after correction for non-specific uptake at time zero. Time course data were fitted to a simple hyperbola by non-linear least squares analysis using a SigmaPlot and SigmaStat statistics programs (SPSS, Chicago, IL). The initial rates of Ca uptake were obtained by determining the slopes of the linear portion of the graphs, which represent unidirectional influx.

Ca^{2+}-Activated ATPase Assay

JEG3 and MT-JEG cells were treated in serum-free RPMI 1640 medium, containing indicated concentrations of DDT, MTC, DES, or E2, for 48 h. Cells were then rinsed with Ca^{2+}/Mg^{2+}-free HBSS, and detergent-solubilized in 1% Triton X-100. The cell extract was used to measure membrane-bound Ca^{2+}-activated ATPase activity using the malachite green–ammonium molybdate assay [Tuan, 1985; Tuan

and Bigioni, 1990; Tuan et al., 1991]. The activity of Ca^{2+}-ATPase is defined as the difference between the activity in the presence of $CaCl_2$ and that in the presence of EGTA, and calculated as moles phosphate/min/mg.

Northern Blot Analysis

Total RNA was prepared from either freshly isolated cells or after storage at −80°C, with guanidine isothiocyanate, followed by phenol–chloroform extraction and isopropanol precipitation. RNA samples were fractionated by electrophoresis in 1% agarose-gel containing 6% formaldehyde, and 50% deionized formamide, then blotted onto nylon membranes (Boehringer Mannheim, Indianapolis, IN). Prehybridization and hybridization were done in a solution containing 0.5 M sodium phosphate (pH 7.0), 1% bovine serum albumin, and 5% sodium dodecyl sulfate (SDS). The probes used included: (1) random-primed [^{32}P]-labeled cDNAs for mouse placental CaBP (MCP) [Tuan and Kirwin, 1988], which cross-hybridized with HCaBP mRNA [Tuan et al., 1991; Lin et al., 1997], and for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Clontech), and (3) and (2) a [^{32}P]-labeled probe for 18S rRNA (Ambion, Austin, TX). Hybridization was carried out at 60°C (for MCP) or 68°C (for G3PDH and 18S rRNA), after which the blots were washed at the same temperature in the presence of 40 mM sodium phosphate buffer with 1% SDS. Autoradiographic signal intensities were determined by scanning densitometry (GS 300 Densitometer, Hoefer Scientific Instruments, San Francisco, CA), and normalized for RNA loading by comparison to those of the constitutively expressed 18S rRNA.

Reverse-Transcription-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was reverse transcribed and amplified using a single–step RT-PCR kit (Invitrogen) in a 50 µl reaction, using 1 µg of total RNA. Following RNA denaturation at 94°C for 2 min, reverse transcription reactions were performed at 55°C for 30 min, and terminated at 94°C for 2 min. PCR reactions were carried out for 30 cycles of amplification consisting of: 94°C for 30 s, 55–60°C for 30 s, and 72°C for 1 min. Specific primers for the genes examined include: human estrogen receptor α and β (hERα: ERβ); human progesterone receptor (hPR); the human homeobox genes *DLX4*, *P450 SCC*, *3β-HSD-1*, and *17β-HSD-1*; and G3PDH, and were designed based on corresponding cDNA sequences in the GenBank database. Primer sequence, position, and predicted size of the PCR products are presented in Table I.

Western Blot Analysis

Cells were harvested at various time points and washed with ice-cold PBS, and lysed in a buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.5 M NaCl, 1 mM Na_3VO_4, and 25% glycerol. Cell lysates (25–50 µg protein) were resolved by SDS–PAGE and transferred to nitrocellulose. After transfer, the filters were blocked for 1 h with 10% non-fat milk–PBS-0.1% Tween-20, incubated with a designated primary monoclonal or polyclonal antibodies, followed by a 1 h incubation with a

TABLE I. RT-PCR Primer Sequences and Predicted Size of Products

Name	Primer length	Sequence	Annealing T	Product size (bp)	Product positions
P450 SSC	21	5′ TCA AAG GCT ACC AGA CCT TTC 3′	56	630	82–754
	21	5′ ATG GCA TCA ATG AAT CGC TGG 3′			
3β-HSD1	21	5′ TTT CTG GGA CAG AGG ATC ATC 3′	56	322	131–453
	21	5′ GAG CTG GGT ACC TTT CAC ATT 3′			
17β-HSD2	21	5′ AGA CGT TGC AGC TGG ACG TAA 3′	57	500	1,150–1,650
	21	5′ GAA AGA CTT GCT TGC TGT GGG 3′			
DLX4	18	5′ AAC CGC TCT GTG CGG GCT 3′	58	381	36–517
	21	5′ GTC CTG TGA CCC TAG GAA GAA 3′			
ERα	21	5′ AAT TCA GAT AAT CGA CGC CAG 3′	54	344	457–801
	23	5′ GTG TTT CAA CAT TCT CCC TCC TC 3′			
ERβ	21	5′ TAG TGG TCC ATC GCC AGT TAT 3′	56	392	125–517
	19	5′ GGG AGC CAC ACT TCA CCA T 3′			
PR	21	5′ GAG AGG CAA CTT CTT TCA GTA 3′	54	406	2,342–2,748
	21	5′ AAA CCA ATT GCC TTG ATG AGC 3′			
G3PDH	20	5′ ACC ACA GTC CAT GCC ATC AC 3′	59	450	586–1,037
	20	5′ TCC ACC ACC CTG TTG CTG TA 3′			

horseradish peroxidase labeled goat anti-mouse antibody (diluted 1:2,500) or goat anti-rabbit antibody (diluted 1:2,500). Proteins were detected using enhanced chemiluminescence (ECL) (Amersham Pharmacia, Piscataway, NJ). The polyclonal antibodies to HCaBP were described previously [Tuan, 1985]. The ER and PR antibodies were obtained commercially (Santa Cruz Biotechnology, CA & Zymed Laboratories, CA, respectively).

Immunohistochemistry

Cells cultured in 8-well chamber slides (Nunc, Rochester, NY) were treated in serum free medium with, either 0.1% ethanol, 1 μM E2, 1 μM DES, 1–10 μM DTT, or 1–10 μM MTC for 48 h. Cells were then fixed with 4% paraformaldehyde in PBS, and immunostained for HCaBP by indirect immunofluorescence as described previously [Tuan, 1985; Tuan et al., 1991]. Non-immune antibodies were used instead of anti-HCaBP antibodies, as negative controls.

Statistical Analysis

Data was analyzed using the SPSS statistics programs. Comparisons between groups and within groups were done by analysis of variance (ANOVA) and differences with P values < 0.05 were considered significant.

RESULTS

Effects of Estrogen and EDCs on Ca Transport and HCaBP Expression in JEG-3 Cells

To investigate the effects of EDCs on Ca transport, we first examined Ca uptake by JEG-3 cells. The Ca uptake assay measured the net of cellular Ca influx and efflux over a 3 min period. Following 48 h treatment with 1 μM DDT, 1 μM MTC, JEG-3 cells exhibited a significant increase ($P < 0.05$) in cellular Ca uptake when compared to control cells over the indicated time interval with no apparent effect on initial uptake rates (Fig. 1). Similar results were observed in JEG-3 cells treated with 1 nM DES or 1 nM E2. However, none of the EDC treatments resulted in significant changes in the level of membrane Ca^{2+}-activated ATPase activity (data not shown).

Since we have previously observed that the trophoblast-specific human binding protein, HCaBP, plays a functional role in placental trophoblast Ca transport [Tuan, 1982; Tuan and Cavanaugh, 1986, 1988; Hershberger and Tuan, 1998], we next examined the effects of estrogens and EDCs on expression levels of HCaBP. Northern blot analyses of total RNA prepared from JEG-3 cells treated for 48 h with either 1 μM DDT or 1 μM MTC, also showed reduced

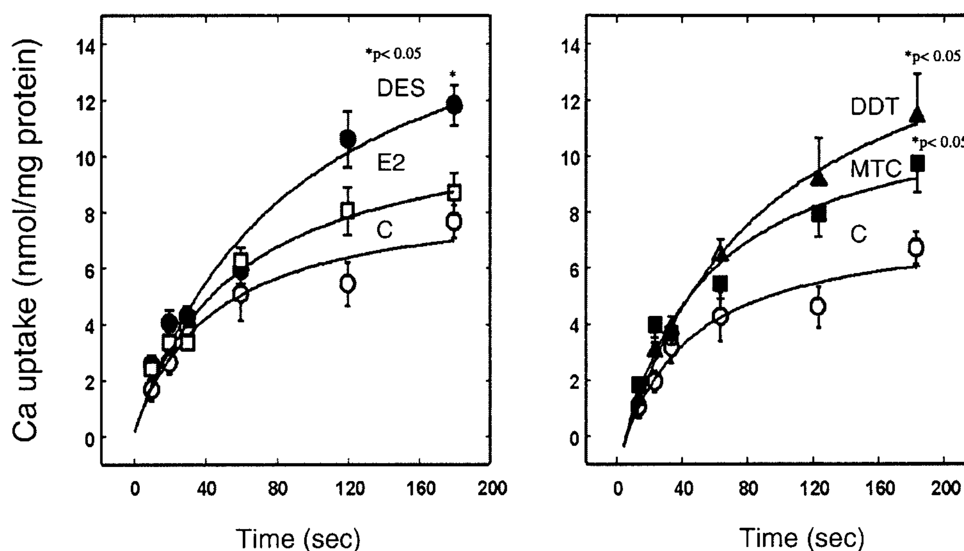

Fig. 1. Effect of endocrine disruptors on Ca uptake by JEG-3 cells cultured in monolayer. JEG-3 cells were incubated in serum-free RPMI 1640 medium with the indicated compounds for 48 h before measurement of Ca uptake in HBSS: 1 nM E2, 1 μM DES, 10 μM MTC, 10 μM DDT, or untreated control, C. All treatments enhance maximal level of Ca uptake. Values are means ± SEM of four experiments performed in duplicates.

levels of HCaBP mRNA transcript levels by 29 and 40%, respectively, when compared to control cells, after normalization of the signal to that of the 18S rRNA (Fig. 2B). HCaBP RNA expression was inhibited further, up to 45 and 90% respectively, when JEG-3 cells were treated with 10 μM MTC and DDT (data not shown), indicating the dose-dependent suppression of HCaBP by these components. Western blot analyses of cell extracts from JEG-3 cells treated with 1 nM E2 or 1 nM DES resulted in the inhibition of CaBP by 32 and 30%, respectively. Reduction in HCaBP levels in JEG-3 cells treated with 1 μM MTC or 1 μM DDT was estimated at 53 and 66%, respectively, when compared to control cells (Fig. 2A). CaBP protein was further inhibited when JEG-3 cells were treated with 10 μM DDT or 10 μM MTC, as detected by immunofluorescence (Fig. 3E,F). At the high doses of 100 μM MTC or 100 μM DDT, most of the cells detached or died by 48 h (data not shown). These results suggest that MTC and DTT effects on Ca handling may be mediated through inhibition of HCaBP expression at both the message and protein levels. These data also indicate that HCaBP expression is regulated by estrogen, and that MTC and DDT are able to interfere with this pathway.

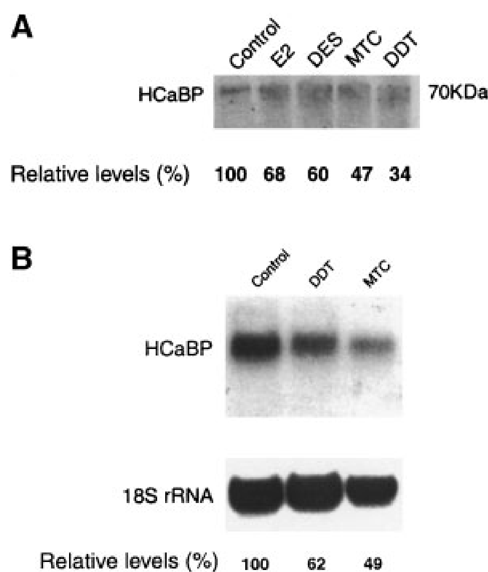

Fig. 2. Effect of endocrine disruptors on HCaBP expression in JEG-3 cells. **A**: Western blot analysis. Protein extracts (50 μg) isolated from untreated JEG-3 cells (control) and cells treated for 48 h with 1 nM E2, 1 nM DES, 1 μM DDT, or 1 μM MTC were electrophoretically separated on 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, then probed with rabbit polyclonal antibodies to HCaBP, and detected by ECL. The results show reduced levels of protein upon E2, DES, MTC, or DDT treatment. **B**: Northern blot analysis of HCaBP expression in JEG-3 cells. Total RNA isolated from untreated JEG-3 cells and cells treated for 48 h with 1 μM DDT or 1 μM MTC were hybridized, using a [^{32}P] labeled mouse placental CaBP cDNA probe (**top panel**) as well as a probe for 18S rRNA (**lower panel**). Both MTC and DDT treatments inhibited HCaBP mRNA expression.

Effect of Estrogen and EDCs on JEG-3 Cell Proliferation and Apoptosis

In initial studies, a range of concentrations of DDT, MTC, and 17β-estradiol were tested for JEG-3 treatment, ranging from 0.1 to 10 nM for E2 and DES, and 1 to 100 μM for MTC and DDT. These concentrations were selected to approximate and cover the range of the reported tissue levels of DDT and MTC found in exposed animals or humans [Saxena et al., 1983; Fein et al., 1984; Sager et al., 1987; Bouwman et al., 1991; van der Ven et al., 1992; Seiler et al., 1994; Rivero-Rodriguez et al., 1997] or in vitro effective doses [Jansen and Jongen, 1996; Kodavanti et al., 1996], and physiological estrogen concentrations [Khan-Dawood and Dawood, 1984; Williams and Chung, 1987], respectively. On the basis of cellular morphology, as well as [^{3}H]thymidine uptake and the level of lactate dehydrogenase levels in the culture media, treatment with 100 μM DDT or MTC for up to 48 h were clearly cytotoxic, whereas the lower concentrations of 1 and 10 μM did not affect these parameters (data not shown).

To examine the effects of E2 and EDCs on cell proliferation, JEG-3 cells were treated in serum–free medium for 48, 72, or 96 h with either 1 nM E2, 1 nM DES, or 1 μM MTC, then processed for cell counts and flow cytometry analysis. MTC treatment resulted in a 50% reduction in cell proliferation ($P < 0.05$), when compared to control, as assessed by cell counts (Fig. 4A). A similar effect was observed, with either 1 nM DES or 1 nM E2 treatment (Fig. 4A). Furthermore, MTC treatment increased the level of apoptosis by up to twofold ($P < 0.05$), when compared to control, untreated cells (Fig. 4B). However, no significant differences ($P > 0.05$) were observed in the cell cycle distribution (G0/G1, S, and G2/M) in all treatment groups, compared to untreated control cells (data not shown). These data indicate that JEG-3 cells show reduced proliferation and increased apoptosis following treatment with EDCs.

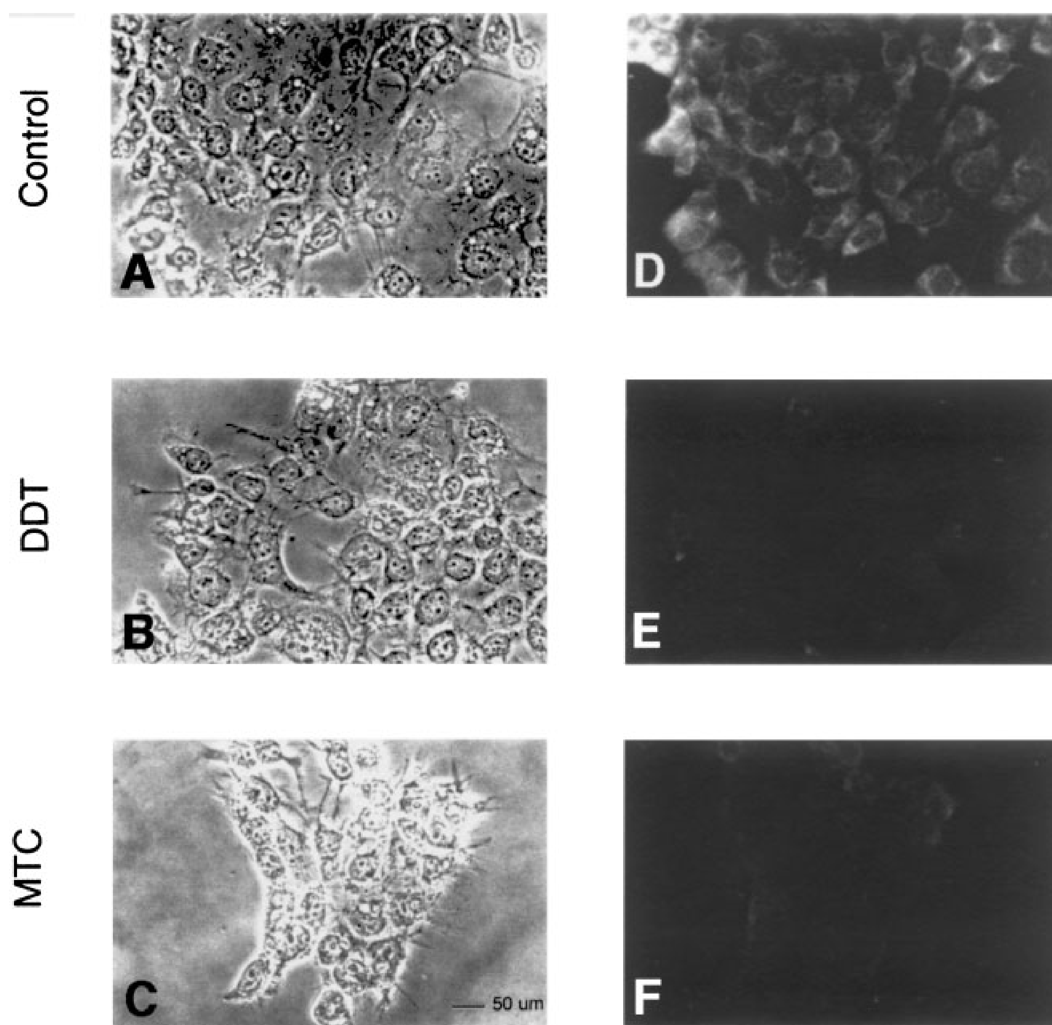

Fig. 3. Morphology and immunofluorescence analysis of HCaBP in JEG-3 cells treated with estrogenic compounds. **A**, **B**, **C**: Phase contrast optics; (**D**, **E**, **F**) epifluorescence optics of (A, B, C). A, D: Untreated control; (B, E) 10 μM DDT treatment; (C, F) 10 μM MTC treatment of JEG-3 cells for 48 h. Treatment with MTC or DDT resulted in cells exhibiting elongated cellular processes (B&C) and down-regulated HCaBP expression (E&F). Bar = 50 μm.

Effect of Metallothionein IIa on JEG-3 Cells EDC-Induced Cytotoxicity and Interference With Ca Uptake

We have previously shown that human metallothionein IIa (MTIIa), a major cellular toxicant protective protein, is able to reverse apoptosis induced by the heavy metal cadmium (Cd) and to block the interference by Cd on Ca handling in JEG-3 cells [McAleer and Tuan, 2001]. To investigate whether MTIIa was involved in the EDC effect on JEG-3 cells, we first tested the effects of varying MTIIa levels in JEG-3 cells on the alteration of Ca handling. JEG-3 overexpressing hMTIIa (MT-JEG-3) were treated for 48 h with either 1 nM E2, 1 nM DES, 1 μM DDT, or 1 μM MTC, and Ca uptake was then assessed and compared to that of the parental JEG-3 cells. In control cells, over a 3-min time course, Ca uptake kinetic was linear for up to about 40 s in both cell types, with the initial rate of Ca uptake by MT-JEG3 cells being 40% higher than that of JEG3 cells (11 vs. 6 nmol/mg protein/min) (Fig. 5A). The level of cellular Ca accumulation measured at 60 s in MT-JEG-3 cells was more than twofold higher ($P < 0.05$) than that of the parental cells (Fig. 5B).

Interestingly, upon treatment of MT-JEG-3 cells with estrogenic components, E2 and DES failed to have any effect on Ca accumulation, whereas a slight increase ($P < 0.05$) in Ca

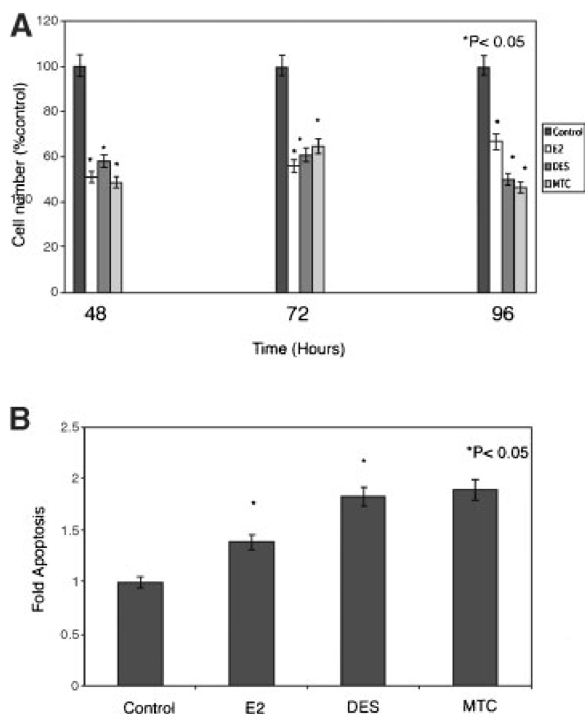

Fig. 4. Effect of estrogenic components on JEG-3 cell proliferation and apoptosis. **A**: JEG-3 cells were treated in serum free medium for 48, 72, or 96 h with either, 1 nM E2, 1 nM DES, or 1 μM MTC. At each time point, cells were counted using a hemocytometer. These treatments resulted in a 50% reduction in cell proliferation. Relative cell number represents the cell number in each treatment group divided by the number of cells in the control, expressed as a percentage of the control. Data presented are the mean of three independent experiments ± SEM. **B**: JEG-3 cells were treated in serum free medium for 72 h with either 1 nM E2, 1 nM DES, or 1 μM MTC. At each time point, cells were fixed in ethanol, stained with propidium iodide, and processed for flow cytometry. Apoptosis measurements from control cells were arbitrarily set at a value of 1. All treatments increased the level of apoptosis by 1.5 to 2-fold when compared to control untreated cells. Data presented are the mean of three independent experiments ± SEM.

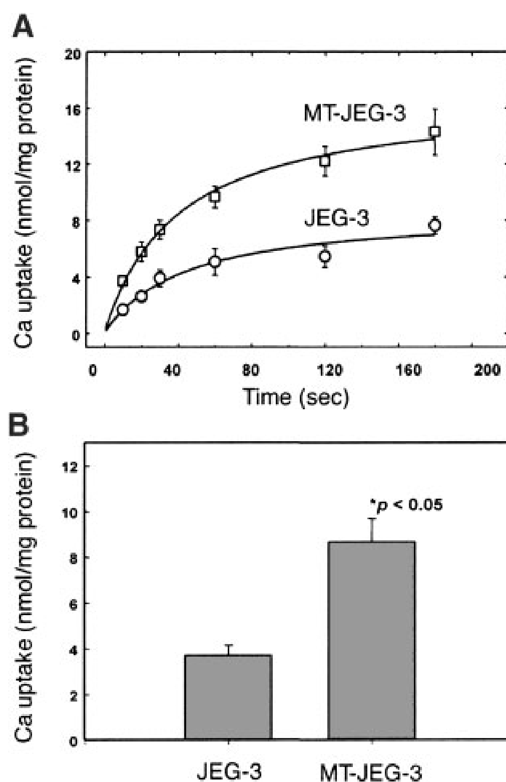

Fig. 5. Effect of overexpression of metallothionein on Ca handling by trophoblastic cells. **A**: Time course of Ca uptake by JEG3 and MT-JEG3 cells in 0.5 mM $CaCl_2$. In control cells, over a 3-min time course, Ca uptake kinetics were linear for up to about 40 s in both cell types, with the initial rate of Ca uptake by MT-JEG3 cells being 40% higher than that of JEG3 cells (11 vs. 6 nmol/mg protein/min). Values plotted are the mean ± SEM of 6–8 assays. **B**: Relative accumulation of Ca by JEG3 and MT-JEG3 cells. Ca accumulation was measured for 1 min, in 0.5 mM $CaCl_2$. The level of cellular Ca accumulation measured in MT-JEG-3 cells was more than two fold higher than that of the parental cells, JEG-3. Values are mean ± SEM from four separate experiments.

accumulation was observed with DDT and MTC (Fig. 6). In addition, treatment of MT-JEG-3 cells with either 1 nM E2, 1 nM DES, 1 μM MTC, or 1 μM DDT did not affect HCaBP levels, when compared to control cells, as shown by Western blot analyses (Fig. 7). Thus, exposure to some of these compounds resulted in JEG-3 cells being more susceptible to high cellular Ca accumulation; over-expression of MTIIa apparently rendered MT-JEG3 cells less sensitive to the effect of these compounds, indicating the protective effect of hMTIIa upon JEG-3 cells exposure to EDCs.

To test the functional involvement of hMTIIa in EDC-induced apoptosis of trophoblastic cells, MT-JEG-3 cells were treated, as described above, and then processed for cell counts and cell–cycle analyses. Treatment of MT-JEG-3 cells over a time course of 96 h with 1 nM E2, 1 nM DES, 1 μM DDT, or 1 μM MTC did not alter significantly ($P > 0.05$) cell proliferation when compared to control cells (Fig. 8). In addition, no significant differences ($P > 0.05$) were observed in cell death between different treatment groups compared to control untreated MT-JEG-3 (data not shown).

Effect of E2 and EDCs on the Expression of Trophoblast Differentiation Markers

To establish whether the effects of EDCs on Ca handling are mediated by changes in trophoblast differentiation, we examined the

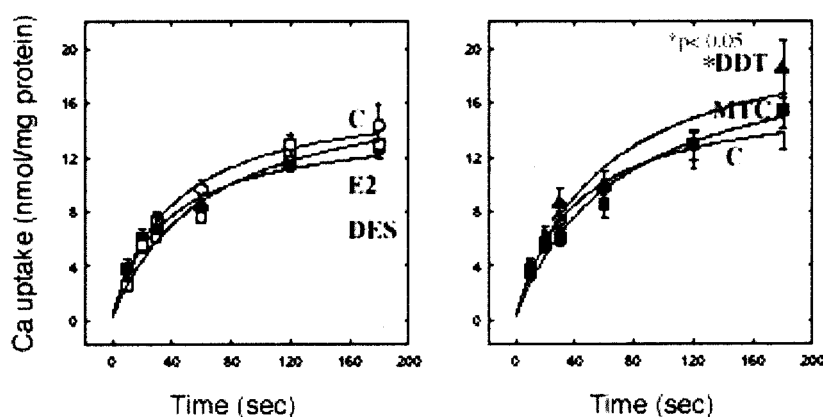

Fig. 6. Effect of EDCs on Ca uptake by MT-JEG-3 cells. Cells were cultured in serum-free RPMI 1640 medium with the following components for 48 h, before measurement of uptake in 0.5 mM $CaCl_2$: control, C (○), 1 nM E2 (□), 1 μM DES (●), 10 μM MTC (■), or 10 μM DDT (▲). Upon treatment of MT-JEG-3 cells with estrogenic components, E2 and DES failed to have any effect on Ca accumulation, whereas reduced accumulation of Ca was observed with DDT and MTC. Values plotted are means ± SEM of four experiments performed in duplicates.

expression profile of P450SCC and 3β-HSD-1. These enzymes have been shown to be both under the control of estrogens and Ca [Beaudoin et al., 1997a,b]. Total RNA was prepared from JEG-3 and MT-JEG-3 cells treated with either 1 nM E2, 1 nM DES, 1 μM MTC, or 1 μM DDT, and processed for RT-PCR analysis. In JEG-3 cells, expression of both P450SCC and 3β-HSD1 was upregulated by 30% upon treatment for 24 h with E2, but was inhibited by 33% when treated with DES, in comparison to control, untreated cells. However, both MTC and DDT up-regulated the expression of these genes by 5–18% when compared to control cells, suggesting that these components may interfere with progesterone synthesis by the trophoblast (Fig. 9A).

E2 has also been shown to regulate the expression of 17β-HSD-1, a placental enzyme involved in estrogen biosynthesis, by catalyzing the transformation of the inactive estrogen precursor, estrone, into the active form E2. Therefore, we examined the effect of DES, MTC, and DDT on the expression levels of 17β-HSD-1 by RT-PCR and densitometry analyses. E2 treated JEG-3 cells exhibited a 36% increase in 17β-HSD-1 levels, relative to untreated control cells. In contrast, MTC and DTT treatment suppressed 17β-HSD-1 expression by 28 and 30% respectively, whereas DES induced only a 5% reduction in 17β-HSD-1 expression level compared to control, untreated cells (Fig. 9A), indicating that DDT, MTC, and DES to a lower extent, can affect estrogen levels produced by the placental trophoblast. In these experiments, overexpression of hMTIIa was able to reverse the effect of DDT or MTC on the expression of 17β-HSD-1, but did not block their effect on *P450SCC* and *3β-HSD-1* gene expression (Fig. 9A). Similarly, inhibition of *P450SCC*, *3β-HSD-1*, and *17β-HSD-1* gene expression by DES

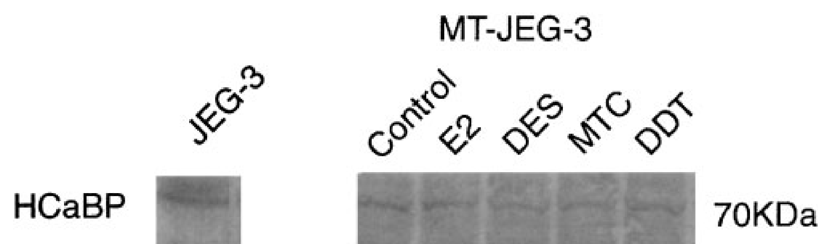

Fig. 7. Effect of EDCs on HCaBP levels in MT-JEG-3 cells. Cell extracts isolated from untreated MT-JEG-3 cells or MT-JEG-3 cells treated for 48 h with 1 nM E2, 1 nM DES, 1 μM MTC, or 1 μM DDT. Twenty five micrograms of protein were analyzed by Western blot using antibodies to HCaBP, and detected by ECL. E2, DES, MTC, or DDT treatment did not significantly alter HCaBP protein levels, when compared to untreated control cells.

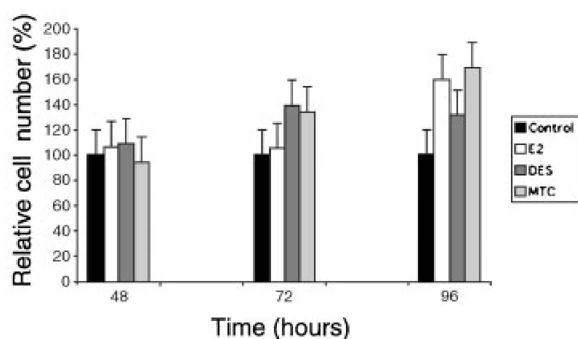

Fig. 8. Effect of EDCs on MT-JEG-3 proliferation. MT-JEG-3 cells were treated in serum free medium for 48, 72, or 96 h with either 1 nM E2, 1 nM DES, or 1 μM MTC. At each time point, cells were counted using a hemacytometer. E2, DES, MTC, or DDT treatment did not significantly alter cell proliferation when compared to control MT-JEG-3 cells. Relative cell number represents the cell number in each treatment group divided by the number of cells in the control, expressed as a percentage of the control. Data represent cell counts obtained from three independent experiments ± SEM.

was not rescued by MT, indicating that hMTIIa is not protective for progesterone and estrogen biosynthesis pathways.

Since both progesterone and estrogen biosynthesis in the trophoblast are markers of its functional differentiation, and in view of the known interaction between several markers of trophoblast differentiation and steroid receptors, we next assessed the levels of expression of *DLX4*, a homeobox gene implicated in trophoblast development and differentiation. While DLX4 was up regulated by 1 nM E2 treatment, both DES and MTC failed to increase its expression. In contrast, in JEG-3 expressing hMTIIa E2, DES, or MTC, treatments did not affect DLX4 expression (Fig. 9B). These results suggest that MTC and DDT alter expression of trophoblast differentiation genes, which could lead to the disruption of the hormonal regulation of fetal growth and development.

Expression of Steroid Receptors in Placenta and JEG-3 Cells

To establish whether the disruption of Ca handling in the trophoblast by DDT and MTC was due to their estrogenic activity and modulated via specific receptors, we examined the expression of members of the steroid receptor family in both JEG-3 and MT-JEG-3 cells and the human placenta. RT-PCR analyses, using primers specific to the human estrogen receptor

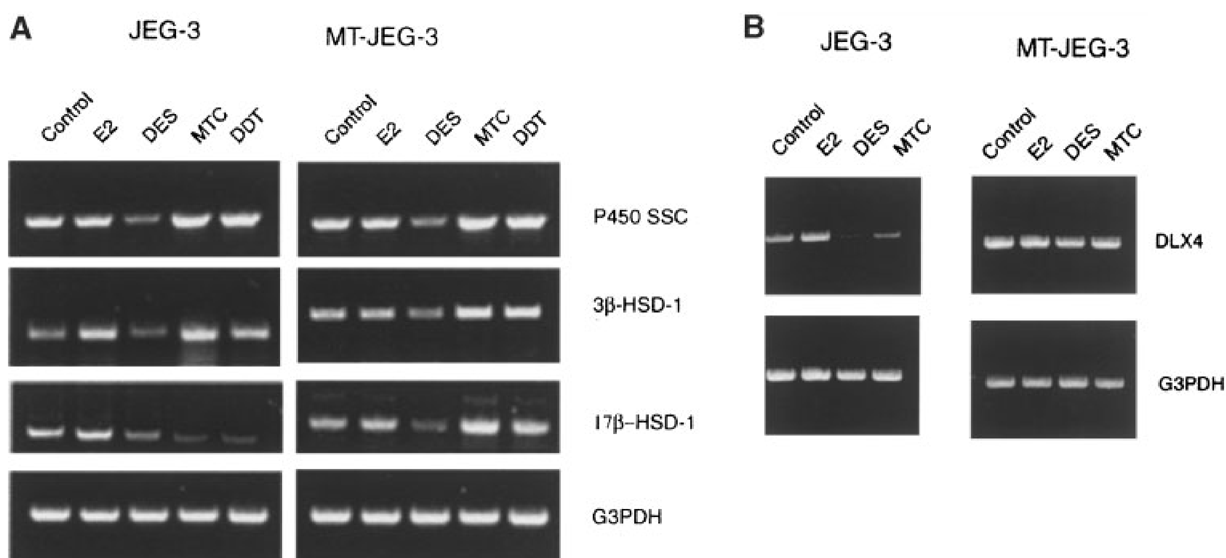

Fig. 9. Effect of EDCs on expression of markers of trophoblast differentiation in JEG-3 and MT-JEG-3 cells. **A**: Expression of P450SCC, 3β-HSD1, and 17β-HSD-1. JEG-3 and MT-JEG-3 cells were treated in serum free medium for 24 h with either 1 nM E2, 1 nM DES, 1 μM MTC, or 1 μM DDT, then gene expression analyses were performed, by RT-PCR. *P450SCC* and *3β-HSD1* gene expression was upregulated upon treatment with E2, MTC, and DDT, in JEG-3 (**left panel**) and MT-JEG-3 cells (**right panel**). MTC and DDT suppressed the expression of 17β-HSD-1 in JEG-3 but not in MT-JEG-3 cells. However, DES treatment inhibited *P450SCC*, *3β-HSD-1*, and *17β-HSD-1* gene expression, and overexpression of MT did not reverse this effect. G3PDH was used as a control for RNA loading. **B**: Expression of DLX-4. JEG-3 and MT-JEG-3 cells were treated as in (A) then *DLX-4* gene expression was examined by RT-PCR. DLX-4 expression was upregulated, upon treatment with E2 but inhibited by DES and MTC, in JEG-3 cells (**left panel**). In contrast, E2, DES, or MTC treatment did not alter DLX-4 expression in MT-JEG-3 cells (**right panel**). G3PDH was used as a control for RNA loading.

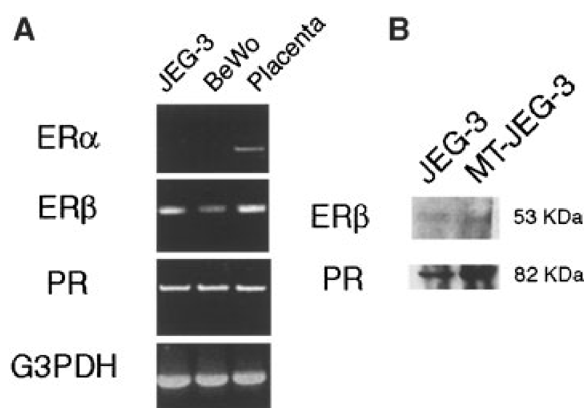

Fig. 10. Expression of estrogen and progesterone receptors in trophoblastic cells. **A**: RT-PCR. One microgram of total RNA prepared from JEG-3, BeWo cells, or from human placenta was examined for expression of ERα, ERβ, and PR. Human placenta is shown to express both ERα, ERβ, and PR, while the trophoblastic cell lines BeWO and JEG-3 cells express both ERβ and PR but not ERα. Also shown G3PDH used as a control for RNA loading. **B**: Western blot. Nuclear extracts prepared from JEG-3 and MT-JEG-3 cells were analyzes by Western blot using antibodies to human ER-β or human PR. The results showed that both ERβ and PR are expressed in JEG-3 and MT-JEG-3 cells, with ERβ levels being significantly lower than those of PR.

α and β (ERα, ERβ), and progesterone receptor (PR), showed that transcripts for all three genes were expressed in placental RNA (Fig. 10A). On the other hand, only ERβ and PR were expressed in JEG-3 cells as well as in BeWo cells, the original cell line from which JEG-3 cells were derived (Fig. 10A). In addition, Western blot analyses of nuclear extracts prepared from JEG-3 cells showed that PR protein (82 kDa) was present in JEG-3 cells (Fig. 10B), with ERβ protein (53 kDa) expressed at very low levels (Fig. 10B). These results suggest that E2 and EDC effects on trophoblast function may be mediated through classical steroid receptor signaling pathways.

DISCUSSION

We report here the cellular and molecular effects of endocrinal/estrogenic perturbation on trophoblast Ca handling, using the JEG-3 human choriocarcinoma cell line as an experimental model system. Treatment of JEG-3 cells with either DDT or MTC increased cellular Ca uptake, mimicking the action of E2, and suppressed the expression of HCaBP. In addition, these components down-regulated gene expression of several trophoblast differentiation markers and induced a reduction in cell proliferation and an increase in apoptosis. Interestingly, overexpression of MTIIa in JEG-3 cells reversed the effects induced by the endocrine disruptors. Collectively, our results suggest that trophoblast Ca handling is under estrogenic modulation, and that exposure to DTT and MTC interferes with estrogen signaling pathways and Ca handling in the placenta.

During fetal development, beginning from the second trimester of gestation, the human placenta actively translocates 100–150 mg of Ca/kg/day [Steichen et al., 1976; Tsang et al., 1976], and is functionally the key determinant of fetal Ca homeostasis. This function is carried out by the specialized placental trophoblast cells, that mediate the attachment of the embryo early during development and form a proper vascular connection for nutrient transport to allow pregnancy progression [Cross et al., 1994]. In humans, the level of placental estrogen, produced by syncytiotrophoblasts from fetally derived precursors, rises on the seventh week and continues throughout pregnancy. However, fetal exposure to estrogens is strictly regulated throughout development [Pepe and Albrecht, 1995; Bigsby et al., 1999] and gestational exposure to estrogen or estrogen-like substances may thus have long-term consequences on Ca homeostasis in fetal development.

Exposure of JEG-3 to DES or MTC was associated with a reduction in proliferation and an increase in apoptosis, indicating the toxic effects of these substances. It was recently reported that DES inhibits the proliferation of murine trophoblast stem cell lines and increases their differentiation into giant cells [Tremblay et al., 2001]. Earlier studies in mice have reported that pregnant mice injected with DES showed inhibition of trophoblast maturation and fetal blood vessel development, as well as increased coagulative necrosis [Scott and Adejokun, 1980]. There is some similarity between the DES/MTC effect and that of the heavy metal Cd on trophoblast cells. Cell proliferation of the choriocarcinoma cell line JAR was inhibited when treated with the heavy metal Cd, known for its toxicity to the placenta in a manner correlated with upregulation of MTIIa [Powlin et al., 1997]. High levels of expression of MTIIa and its localization to the nucleus have been reported in many tissues, especially in regions of high cellular proliferation, perhaps related to the requirement for high levels of Zn for several metalloenzymes

and transcription factors during certain stages of cell cycle [Cherian and Apostolova, 2000; Davis and Cousins, 2000; Miles et al., 2000]. We have shown recently that overexpression of human MTIIa was able to reverse Cd-associated inhibition of proliferation and induction of apoptotis in JEG-3 cells [McAleer and Tuan, 2001]. This protective effect was also observed in DES– and MTC–treated JEG-3 cells, supporting the hypothesis for a role for MT in protecting cells from DNA damage and apoptosis [Cherian and Apostolova, 2000; Davis and Cousins, 2000; Miles et al., 2000].

In individuals exposed to DDT and MTC, the highest amounts of these toxins are found in adipose tissues [van der Ven et al., 1992; Waliszewski et al., 2001], and are likely to be mobilized during pregnancy and deposited in the placenta [You et al., 1999; Waliszewski et al., 2000, 2001]. Earlier studies in mice showed increased bone mass, but shortened long bone, in murine offsprings exposed during gestation to DES, a synthetic estrogen [Migliaccio et al., 1995, 1996]. These findings, therefore, suggest that gestational exposure to estrogen or estrogen-like substances may have long-term consequences on developmental Ca homeostasis, perhaps by affecting placental function, and thus compromising fetal skeletal growth.

In this study, net cellular Ca accumulation (i.e., influx minus efflux) is measured by the Ca uptake assay, and net activity of the Ca^{2+}-activated ATPase is measured to assess activity of the Ca pump. Our data show that treatment with 10 μM DDT, 10 μM MTC, 1 μM DES, and 1 nM E2 resulted in significantly increased cellular Ca uptake in JEG-3 cells. However, MT overexpression in JEG-3 cells was able to block the effect of these components on Ca uptake. Since Ca^{2+}-ATPase activities are unchanged between untreated control and the experimental groups treated with DDT, MTC, or E2, the primary effect of these components is likely to be directly related to increased Ca uptake. These findings thus strongly suggest that the estrogenic activity of DDT and MTC may be responsible for their effects on trophoblastic cellular Ca handling.

The trophoblast-specific, high-Mr, human Ca-binding protein (HCaBP) has been previously shown to play a functional role in placental trophoblast Ca transport [Tuan, 1985; Tuan et al., 1991]. The dose-dependent reduction of HCaBP RNA and protein upon DDT and MTC treatment suggests that HCaBP expression is affected, which may be responsible for the imbalance of intracellular Ca buffering. Extracellular Ca entry occurs at the apical side of the cell via Ca channels and is distributed among several compartments, including HCaBP and intracellular Ca pools (mitochondria and the endoplasmic reticulum $[Ca^{2+}]$i). Ca then exits to the basal side of the cells via the action of the Ca^{2+}-ATPase pump. Since we observed an increase in Ca uptake in cells treated with MTC and E2, and given that the Ca^{2+}-activated ATPase activities remain functional and unchanged, the rate-limiting target is likely to be Ca entry. Previous studies showed that estrogen regulates voltage-gated Ca channel in the rat myometrium during gestation and enhances Ca uptake [Batra, 1986]. Functional components of placental Ca transport have been reported to be key elements for Ca entry (influx) and Ca exit (efflux). These include a voltage-gated L-type Ca channel [Yano et al., 1982], a H^{+}/Ca^{2+} exchanger [Brunette and Leclerc, 1992], and two isoforms of Ca-ATPase (HPMCA1 and HPMCA3) [Strehler et al., 1990]. Ca channels and Ca^{2+}-activated ATPase [Yano et al., 1982; Fisher et al., 1987; Howard et al., 1992; Meuris et al., 1994] are believed to be the key elements for Ca entry (influx) and Ca exit (efflux), respectively. It is noteworthy that several environmental estrogenic pollutants, including DDT, inhibit Ca influx via L-type Ca channels in smooth muscle cells [Ruehlmann et al., 1998]. How and whether EDCs also affect these components in the trophoblast cell remain to be examined.

Despite the established role of estrogens in maintaining pregnancy and coordinating other pregnancy-related hormones [Pepe and Albrecht, 1995; Albrecht and Pepe, 1999], there is no direct evidence for the expression of estrogen receptors in the placenta. In this study, we have shown the presence of both ERα and ERβ in the placenta, and demonstrated that JEG-3 cells express low levels of ERβ. The ability of E2 to regulate the expression of PR indicates that ER-mediated regulation of gene expression is functional in JEG-3 cells (data not shown). Moreover, the ability of E2 to upregulate the levels of 3β-HSD-1 demonstrates that ER signaling is functional in JEG-3 cells. Despite the fact that environmental estrogens such as DDT and MTC bind weakly (100 times lower affinity) to both ERα and ERβ, while

exhibiting estrogenic activity in vitro and in animal studies [Chen et al., 1997; Kuiper et al., 1998], several adverse effects of exposure to these compounds, related to the estrogen homeostasis and its functions during pregnancy, have been observed. These effects include interference with estrogen production via inhibition of 17β-HSD activity. In our study, we have observed that DES, MTC, and DDT are able to alter expression levels of P450 SCC, 3β-HSD-1, and 17β-HSD-1. Some gene-specific differences are seen in the effect of these components, in agreement with recent studies performed in the breast cancer MCF-7 cell line [Jorgensen et al., 2000]. Our observations strongly suggest that these components may interfere with progesterone and estrogen synthesis by the trophoblast, which could compromise cellular differentiation and function. Specifically, 17β-HSD-1 activity is crucial during the first trimester, for the inactivation of estrogens into estrone, to protect the fetus, indicating the role of the placental trophoblast in the redirection of the maternal endocrine system in favor of the fetus. In vivo studies suggest that transient changes in estrogen levels during development modulate bone turnover and osteoclastogenesis, likely participating in bone-cell imprinting during early phases of bone development [Migliaccio et al., 2000].

The human homeobox genes, *DLX4*, *HB24*, *MSX2*, and *MOX2*, play a central role in placental development [Knofler et al., 2000]. Regulation of homeobox genes by estrogen, their interaction with steroid receptors and interference of DES with their expression during development has been shown [Ma et al., 1998; Taylor et al., 1998; Block et al., 2000; Raman et al., 2000]. In mouse trophoblast cell lines, DES has been shown to inhibit the expression of a marker of trophoblast progenitor cells, and induce their differentiation into giant cells in vitro [Tremblay et al., 2001]. In this study, both DES and MTC down-regulated the level of DLX-4. Another member of this gene family, *DLX3*, has been shown to be involved in trophoblast differentiation [Morasso et al., 1999]. In addition, sequences located between +30 to +60 of the *DLX3* gene promoter have been shown to be responsible for the Ca^{+2}-dependent induction of DLX3 during keratinocyte differentiation [Park and Morasso, 1999]. This observation suggests that alteration of DLX-4 expression, in JEG-3 upon treatment with DES and MTC, may have resulted from the perturbation of cellular Ca handling. Such effects on trophoblast differentiation suggest that alteration of trophoblast differentiation may be mechanistically responsible for the disruption of placental function and fetal development by environmental estrogens.

In summary, using the JEG-3 cell model, our studies have clearly demonstrated that Ca handling by trophoblastic cells is likely to be under estrogenic regulation, and that DDT and MTC functionally perturb trophoblastic Ca transport, behaving like estrogen agonists. This perturbation may result from a combination of direct inhibition of the activities of cellular components involved in Ca transport, as well as interference with estrogen-regulated Ca transport pathway. Understanding the mechanisms underlying this regulation may help identify potential roles of EDCs in human neonatal morbidity and mortality associated with intrauterine growth retardation and low birth weight.

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