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An Investigation of the Single and Combined Phthalate Metabolite Effects on Human Chorionic Gonadotropin Expression in Placental Cells

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Background: Observational studies have reported associations between maternal phthalate levels and adverse outcomes at birth and in the health of the child. Effects on placental function have been suggested as a biologic basis for these findings.

Objective: We evaluated the effects of phthalates on placental function *in vitro* by measuring relevant candidate genes and proteins.

Materials and Methods: Human trophoblast progenitor cells were isolated at 7–14 wk of pregnancy (two female and three male concepti), and villous cytotrophoblast cells (vCTBs) were isolated at 15–20 wk (three female and four male concepti). Cells were cultured *in vitro* with four phthalate metabolites and their combination at concentrations based on levels found previously in the urine of pregnant women: mono-*n*-butyl (MnBP, 200 nM), monobenzyl (MBzP, 3 μM), mono-2-ethylhexyl (MEHP, 700 nM), and monoethyl (MEP, 1.5 μM) phthalates. mRNA levels of *CGA*, *CGB*, *PPARG*, *CYP19A1*, *CYP11A1, PTGS2, EREG*, and the intracellular β subunit of human chorionic gonadotropin (hCGβ) and peroxisome proliferator activated receptor γ (PPARγ) were measured in the cellular extracts, and protein levels for four forms of secreted hCG were measured in the conditioned media.

Results: Previously reported associations between maternal phthalates and placental gene expression were reproduced experimentally: MnBP with *CGA*, MBzP with *CYP11A1*, and MEHP with *PTGS2*. *CGB* and hCGβ were up-regulated by MBzP. In some cases, there were marked, even opposite, differences in response by sex of the cells. There was evidence of agonism in female cells and antagonism in male cells of PPARγ by simultaneous exposure to multiple phthalates.

Conclusions: Concentrations of MnBP, MBzP and MEHP similar to those found in the urine of pregnant women consistently altered hCG and PPARγ expression in primary placental cells. These findings provide evidence for the molecular basis by which phthalates may alter placental function, and they provide a preliminary mechanistic hypothesis for opposite responses by sex. https://doi.org/10.1289/EHP1539

Introduction

Phthalates are a class of synthetic, endocrine-disrupting chemicals that are detected in all pregnant women in the United States (Woodruff et al. 2011) Prenatal maternal exposure to phthalates has been associated with short-term outcomes in pregnancy, such as the duration of labor (Adibi et al. 2009; Ferguson et al. 2014; Latini et al. 2003), the risk of preeclampsia (Cantonwine et al. 2016), and fetal sex differentiation as determined at birth (Swan et al. 2015). Long-term outcomes in the children may include negative trends in behavior, IQ, attention, and social communication [11 studies of children 0 to 12 y old reviewed by Ejaredar et al. (2015)].

The role of the placenta in these associations has been partially identified. Placental prostaglandin production, which might be a target of phthalates, is highest at the end of pregnancy and is required for the induction of labor (Tetz et al. 2015). Phthalates may alter circulating levels of antiangiogenic molecules that are produced by the placenta and are indicators of preeclampsia risk (Ferguson et al. 2015). We have identified a potential role of sex-specific placental hormone production in contributing to the effect of phthalate exposures on a neonatal marker predicting the future reproductive health of the child (Adibi et al. 2015a). Clarifying the role of the placenta in these associations will improve temporal and spatial precision in estimating the short- and long-term health consequences of prenatal exposure.

It is not possible to directly observe the critical time points in early pregnancy when mRNA and protein expression in the placenta and in the embryo/fetus are vulnerable to perturbation by tissue and circulating levels of maternal phthalates. Owing to genetic, anatomic, and physiologic differences in placental–fetal biology between species (Maltepe et al. 2010; Rawn and Cross 2008), animal models alone are insufficient to identify causal relationships in human pregnancy. As such, we believe a combination of experimental and observational models that are human-specific and informative of early pregnancy relationships is best suited to deliver these necessary insights.

Another motivation to work simultaneously in experimental and observational systems is the need to overcome a source of intractable confounding: between-person differences in placental metabolism. This is a special problem in phthalate epidemiology because the urinary biomarkers used to assess exposure are also partially products of placental metabolism (Hakkola et al. 1998). At the present time, we have no methods to assess or control for this type of bias. Experimental methods (and statistical methods applied to observational data) may drastically reduce this type of bias and give greater confidence and reproducibility regarding the unconfounded effects of the chemicals on gene and protein expression.

We selected a list of candidate genes to determine if the types of phthalate effects on gene expression that have been identified in other species and in other tissue types were also targets in the human placenta (Adibi et al. 2010). In a companion to this manuscript, we have reported sex-specific associations of eight of these

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candidate mRNAs measured in term placental tissue biopsies with concentrations of six maternal urinary phthalates (Adibi et al. 2017). Here, we report the experimental follow-up study to those associations using models of undifferentiated and differentiated trophoblasts (Tbs) isolated from first- and second-trimester placentas. In the placental tissue, chorionic gonadotropin α (*CGA*) was the most strongly associated with the highest number of phthalate metabolites (Adibi et al. 2017). *CGA* encodes an α subunit of glycoprotein hormones including placenta-specific gonadotropin, called human chorionic gonadotropin (hCG), which is a primary end point in the present study. Here, we follow up on this finding by reporting phthalate effects on mRNAs of both α- and β-subunits of hCG (encoded by *CGA* and by *CGB* and its homologues, respectively), on protein levels of intracellular and secreted hCG (dimer of α- and β-subunits), and on the mRNA and intracellular levels of a regulatory factor of hCG, peroxisome proliferation activated receptor γ (*PPARG*/PPARγ). In this analysis, we compared the molecular response of human Tbs to phthalate exposure according to fetal sex differences, application of single phthalate metabolites versus mixtures, cell type, and consistency between mRNA and protein effects.

Materials and Methods

Study Subjects

Women undergoing elective pregnancy terminations between 7 and 20 wk gestation at the Women's Options Center at the University of California, San Francisco between 2012 and 2013 donated tissue anonymously. Donation was restricted to women without fetal anomalies. Written informed consents were obtained from all subjects. The University of California, San Francisco Committee on Human Research approved the tissue collection.

Cell Culture

The trophoblast progenitor cell (TBPC) represents an undifferentiated, multipotent population derived from the mesoderm of the chorion at gestational weeks 7.0–14.9. Derivation and maintenance conditions are described elsewhere (Genbacev et al. 2011; Genbacev et al. 2016). TBPCs were cultured in six-well plates that had been incubated with 0.5% gelatin for 30 min. They were plated at 250,000 cells/well. Details on the cell-culture medium are described elsewhere (Genbacev et al. 2016). The villous cytotrophoblasts (vCTBs) were isolated and purified from placentas obtained at 15 wk 4 d to 20 wk 1 d of gestation using microdissection, enzymatic digestion, and cell culture techniques described elsewhere (Hunkapiller and Fisher 2008). Cells were plated on Matrigel® in 24-well plates at 500,000 cells/well, dosed in duplicate, and cultured in serum-free medium (Hunkapiller and Fisher 2008). The vCTBs were sampled from placentas obtained from three female and four male placentas, and the TBPCs were sampled from two female and three male placentas (these cells are referred to hereafter as female and male cells, respectively). Details on experiments and replicates are in the Supplemental Material (see Tables S5 and S6). Single phthalate metabolite doses were designed to mimic maternal urinary concentrations measured in a birth cohort study (Adibi et al. 2017): 200 nM mono-*n*-butyl phthalate (MnBP; TCI America), 3 μM monobenzyl phthalate (MBzP; TCI), 700 nM mono-2-ethylhexyl phthalate (MEHP; Wako), and 1.5 μM monoethyl phthalate (MEP; Sigma-Aldrich). Specifically, we chose urinary concentrations that in our previous study were associated with *CGA* mRNA levels and where the association differed in male versus female placentas [Figures 1A, B, D in Adibi et al. (2017)]. In addition, we combined the four abovementioned concentrations of single phthalates into a mixture dose. Vehicle control cells for the single metabolite doses received 0.1% dimethyl sulfoxide (DMSO), and controls for the phthalate mixture received 0.4% DMSO; both of these doses are below the DMSO concentration that affects Tb differentiation (Thirkill and Douglas 1997). The self-renewing TBPC cultures were grown for 72 h, which is the point at which they reach 90% confluency. The vCTB cultures were maintained for 40 h from the time of dosing. vCTBs are nonrenewing; therefore, 40 h is optimal to evaluate functional changes in hCG secretion while cells are metabolically active. In all experiments, cell morphology and cell density were assessed and documented by phase contrast microscopy.

Gene Expression in Cultured Cells

The genes selected for this study were measured previously in placental tissue biopsies and were found to be correlated with maternal urinary phthalates (Adibi et al. 2010, 2017), with the exception of *CGB* and *EREG*. *CGB* was selected because it encodes a β subunit of human chorionic gonadotropin (hCG), a placental hormone that we hypothesize to be a target of phthalate exposure. The *CGB* primer set amplifies *CGB*, *CGB3*, *CGB5*, *CGB7*, and *CGB8* (TaqMan™, ThermoFisher Scientific). *EREG* is an ovarian target of luteinizing hormone (LH), a gonadotropin that binds to the same receptor as hCG (Park et al. 2004). *In vivo*, hCG has been found to stimulate *EREG*, which is why we also selected *EREG* for this study (Huber et al. 2007). RNA was isolated using an RNeasy Plus Mini Kit (Qiagen) and measured on a NanoDrop™ spectrophotometer (ThermoFisher Scientific). Reverse transcription was carried out using an iScript™ cDNA Synthesis Kit (BioRad). A TaqMan™ assay for *RPS4Y1* was used to assign sex to all control cells. The Ct (amplification cycle at which the mRNA concentration was detectable) for female cells was either >35 cycles, or amplification was nondetectable. For males, amplification of *RPS4Y1* was detected at ≤25 cycles. *RPS4Y1*, a Y-linked gene, was selected for this purpose after reviewing three independently generated transcriptome data sets of placental cells in culture and placental biopsies to determine which of the Y-linked genes showed the greatest discrimination in males and females (J. Adibi, unpublished data, 2013). TaqMan™ gene expression assays (see Table S4) were used for mRNA quantitation, and 10 ng of cDNA was loaded in a 10-μL reaction volume and run in triplicate on a 7900HT quantitative polymerase chain reaction (PCR) instrument (Applied Biosystems). *RN18S1* RNA was used as an internal control based on the validated convention of the laboratory where the experiments were designed and conducted (Winn et al. 2007).

hCG in Conditioned Media

Conditioned media from each well from both cell types were aliquoted and stored at −20°C. Aliquots were shipped to the University of Helsinki (Finland), where they were analyzed for a panel of hCG subunits and variants including intact hCG, hCGα, hCGβ, and the hyperglycosylated form of hCG (hCG-h). Intact hCG was measured using time-resolved immunofluorometric assays (IFMAs) (DELFIA®, Perkin-Elmer Wallace), and the other forms were measured using in-house assays with monoclonal antibodies (Alfthan et al. 1992; Lee et al. 2013). The lower limit of quantitation was 0.5 pmol for intact hCG, 5 pmol for hCGα, 1 pmol for hCGβ, and 10.0 pmol for hCG-h. The intact hCG assay measures only the hCG αβ dimer, irrespective of whether it is hyperglycosylated or not. The hCGβ assay measures only free hCGβ subunit, including hyperglycosylated hCGβ. The hCGα assay measures the free α subunit. The hyperglycosylated hCG assay (hCG-h) measures hyperglycosylated intact hCG and hyperglycosylated hCGβ. The hCGα subunit is a gonadotropin

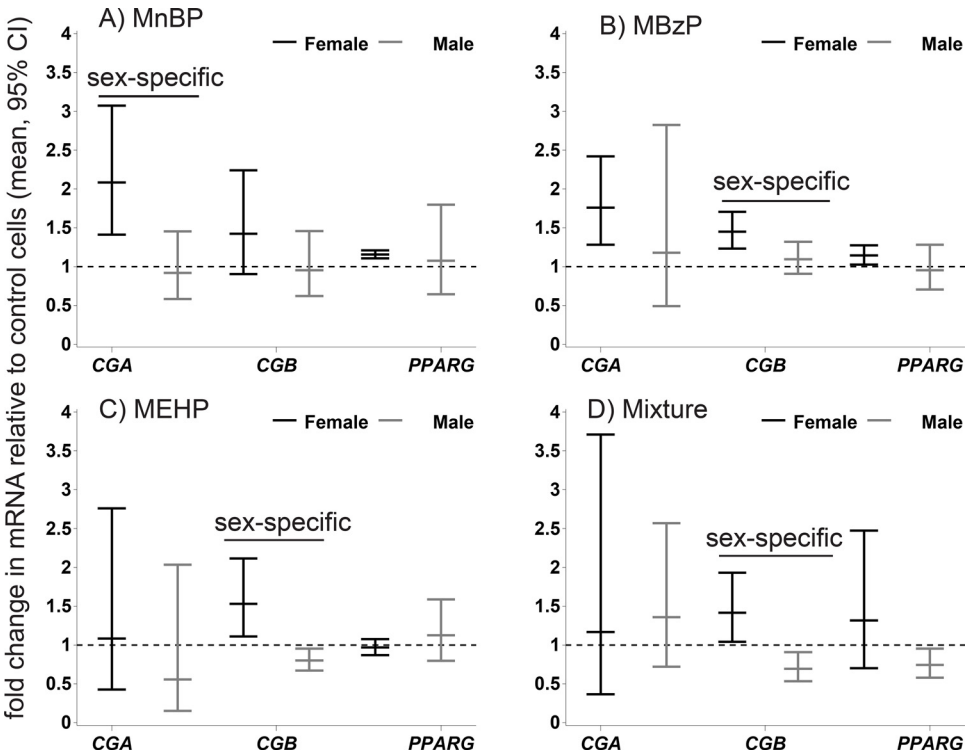

Figure 1. Phthalate effects on mRNAs. The effects are expressed as mean relative fold change ($2^{\Delta\Delta ct}$) and 95% confidence intervals (CIs) compared with DMSO-treated control cells in undifferentiated trophoblasts (TBPCs). (*A*) 200 nM MnBP; (*B*) 3 μM MBzP; (*C*) 700 nM MEHP; (*D*) mixture of all four metabolites (MnBP, MBzP, MEHP, MEP). The black lines indicate female-specific effects, and the gray lines indicate male-specific effects. Overall effects that were significant ($p \leq 0.05$) are indicated by a line and marked "sex-specific" if the phthalate effect differed in male and female cells. CG, chorionic gonadotropin; DMSO, dimethyl sulfoxide; MBzP, monobenzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEP, monoethyl phthalate; MnBP, mono-*n*-butyl phthalate; PPARG, peroxisome proliferator activated receptor gamma.

subunit that is shared by all glycoprotein hormones, that is to say, hCG, TSH, LH, and FSH. hCGβ lacks hCG activity but may play a role in Tb invasion (Lee et al. 2013).

Quantitative Western Blots

Protein was purified from the same vCTB lysate used for RNA isolation according to the manufacturer's instructions (AllPrep® RNA/Protein Kit and RNeasy Plus, Qiagen) and was additionally cleaned up by acetone precipitation. Protein lysate was mixed with four volumes of ice-cold acetone and incubated at −20°C for 30 min. After centrifuging at 12,000 × *g* for 10 min, the supernatant was discarded, and the pellet was air-dried. The pellet was then resuspended in 5% sodium dodecyl sulfate (SDS). The protein concentration was measured using a Direct Detect® Spectrometer (EMD Millipore). The protein was denatured at 95°C for 5 min in 4 × protein loading buffer (Li-Cor) supplemented with 5% β-mercaptoethanol (Bio-Rad) and separated using Any kD™ Precast Protein Gels (Bio-Rad). The proteins were transferred to nitrocellulose membrane using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). After blocking in phosphate-buffered saline (PBS) blocking buffer (Li-Cor) at room temperature (20–22°C) for 1 h, the membrane was incubated with primary antibodies in blocking buffer at 4°C overnight. The membrane was then washed and incubated with secondary antibodies at room temperature (20–22°C) for 50 min. The reactive proteins were detected using an Odyssey CLx (Li-Cor) imaging system, a method that has been optimized and validated for quantitation (Wang et al. 2007). The Western blot bands were quantified using Image Studio (version 5.0; LI-COR). The primary antibodies were anti-hCG (1:2,000, Dako, A0231), anti-PPAR-γ (1:1,000, Cell Signaling, 24,355), and anti-β-Actin (1:5,000, Santa Cruz Biotechnology, sc4778). The secondary antibodies were IRDye 800CW donkey anti-rabbit IgG (H + L) (Li-Cor) and IRDye 680LT donkey anti-mouse IgG (H + L) (Li-Cor). The hCGβ antibody yielded four bands in the Western blot, of which we quantified three: 42 kDa, 23 kDa, and 21 kDa. The 42-kDa band gave the strongest signal, and the 23- and 21-kDa bands were closest in size to the predicted band. The goal was to determine which band had the highest correlation with *CGB* mRNA and secreted hCG protein as validation of the Dako antibody and as further insight into possible sex differences in hCG synthetic machinery. The 42-kDa band was 4 log units higher on average than the 23-kDa band and strongly correlated with β-actin in the same sample ($r = 0.61$, $p < 0.0001$). The 21-kDa band was only detected in 57% of female cells and 43% of male cells, and it was not analyzed further (χ^2 $p = 0.02$). Results for the 23-kDa band are presented (Figure 3F). The 23-kDa band has been used for hCG quantitation in previous studies (Racca et al. 2011). The PPARγ antibody gave a single band at 51 kDa, consistent with previous studies (Senol-Cosar et al. 2016) (Figure 3G).

Statistical Analysis

For the RNA analysis, mean Ct $\log_2$ values (amplification cycle at which the mRNA in the sample was detected; i.e., higher Ct values indicate lower mRNA concentration in the sample) of the technical replicates ($n = 2$) were calculated for each sample. Because of the significant variability in baseline gene and protein expression between biologic replicates and because it is established that hCG also differs significantly by day of gestation (Wald et al. 2003), we chose to analyze the data using multivariate mixed effects models. This method allows us to quantitate differences in gene and protein expression between the treated and

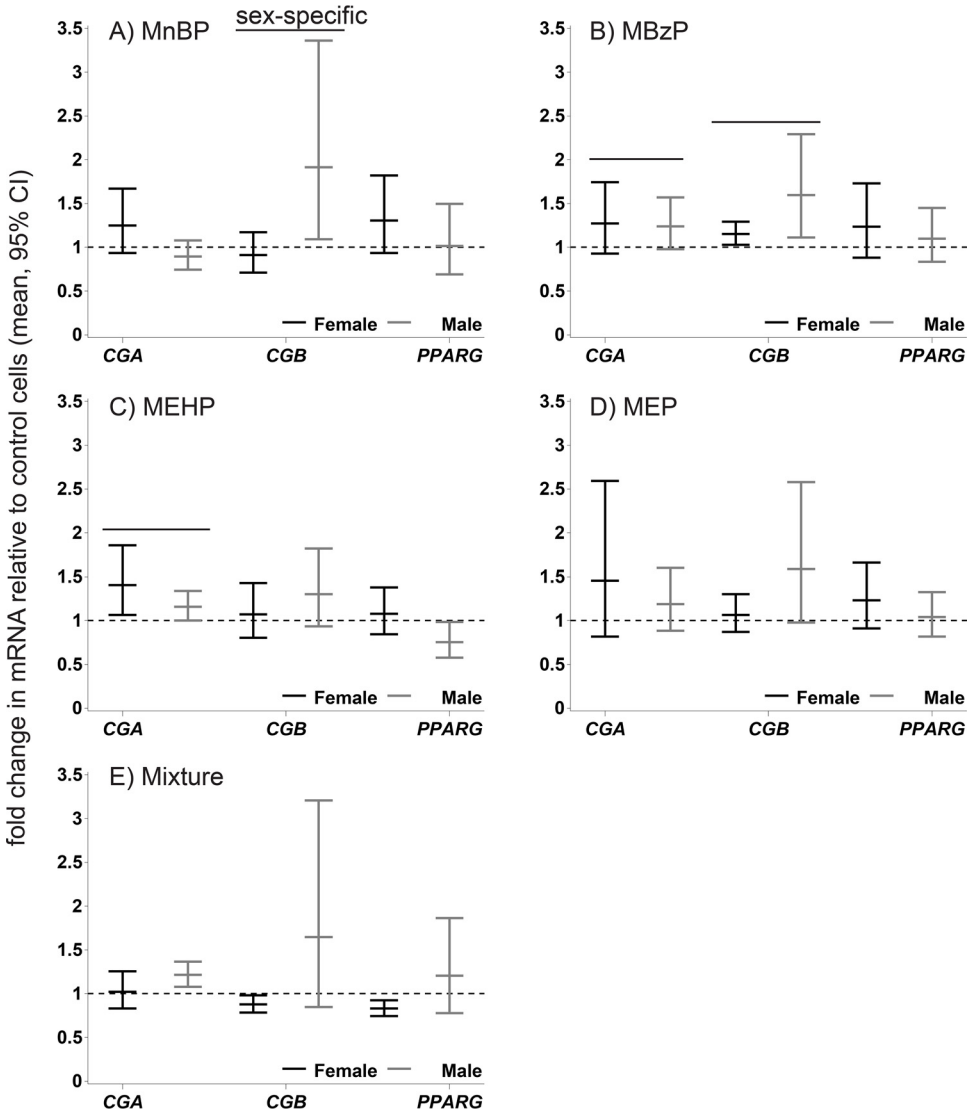

Figure 2. Phthalate effects on mRNAs. The effects are expressed as mean relative fold change ($2^{\Delta\Delta ct}$) and 95% confidence intervals (CIs) compared with DMSO-treated control cells in differentiated cytotrophoblasts (vCTBs). (*A*) 200 nM MnBP; (*B*) 3 μM MBzP; (*C*) 700 nM MEHP; (*D*) 1.5 μM MEP; (*E*) mixture of all four metabolites. The black lines indicate female-specific effects, and the gray lines indicate male-specific effects. Overall effects that were significant ($p \leq 0.05$) are indicated by a line and marked "sex-specific" if the phthalate effect differed in male and female cells. CG, chorionic gonadotropin; DMSO, dimethyl sulfoxide; MBzP, monobenzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEP, monoethyl phthalate; MnBP, mono-*n*-butyl phthalate; PPARG, peroxisome proliferator activated receptor gamma.

control cells after adjustment for the clustering of values within each biologic replicate (random effect) and adjustment for fixed effects (sex, gestational age, etc.). In this case, the random effect or experiment number is a proxy for unmeasured biologic variability at baseline (i.e., genetic and epigenetic variability, maternal health, preprocedure exposures). We included as model covariates gestational age at the time of pregnancy termination, *RN18S* mRNA levels in the sample, sex of the cells, phthalate dose, and institution at which the experiment was conducted. We report the population marginal means and their 95% confidence intervals (CIs) for all treatment groups by sex. For plotting the changes, we used the β coefficient (equivalent to the ΔΔCt) and its 95% CIs after transformation to the linear scale ($2^{\Delta\Delta ct}$). Sex-specific parameters were calculated, using the Estimate statement in SAS (SAS Institute Inc.), from a model that included a term for phthalate dose by sex. We used two models to estimate all dose effects: the first included the 0.1% DMSO vehicle control samples and the single-metabolite dose groups (also with 0.1% DMSO), and the second included 0.4% DMSO and the phthalate-mixture dose group (also with 0.4% DMSO). Dose group was treated as a categorical variable and interacted on sex.

We applied the same strategy in the analysis of intracellular protein and secreted hCG levels. We report all results for protein on a log scale; a one $\log_e$ unit difference is equivalent to a 2.7-fold change on a linear scale. For ease of comparison and interpretation, we report log unit differences as fold changes (the difference between the 2 log values transformed to the linear scale). In the analysis of the Western blot data, β-actin was a covariate to control for total protein in the sample. Intracellular hCGβ and PPARγ were modeled as $\log_e$-transformed intensity values, and secreted hCG variants were modeled as log-transformed concentrations. To estimate subunit-specific effects, we calculated ratios of the α, β, and hyperglycosylated hCG to intact hCG. This calculation was performed to normalize for overall hCG production, which serves as an indicator of quantity and viability of the cells. We used Spearman rank correlations to quantitate the relationship

Table 1. Mean log intensities of protein (95% CI) measurements of intracellular hCG-β and PPARγ protein expression in vCTBs treated with phthalate metabolites compared with DMSO-treated control cells.

Protein	Metabolite	Females	Males	Overall	*p*-Value	Sex-specific *p*-value
hCGβ	0.1% DMSO	3.43 (1.40, 5.47)	4.51 (3.08, 5.94)	3.91 (2.83, 5.43)	Reference	Reference
hCGβ	MnBP	4.63 (4.12, 5.13)	5.00 (3.90, 6.11)	4.81 (4.11, 5.52)	0.08	0.38
hCGβ	MBzP	3.94 (2.64, 5.23)	5.43 (4.16, 6.71)	4.68 (3.57, 5.79)	0.04*	0.46
hCGβ	MEHP	4.50 (3.84, 5.16)	4.39 (3.14, 5.64)	4.44 (3.57, 5.32)	0.35	0.19
hCGβ	MEP	4.18 (2.88, 5.48)	4.72 (3.45, 5.99)	4.44 (3.23, 5.65)	0.35	0.59
hCGβ	0.4% DMSO	4.00 (2.41, 5.60)	4.93 (3.28, 6.57)	4.47 (3.45, 5.48)	Reference	Reference
hCGβ	Mixture	4.41 (3.81, 5.00)	5.21 (3.90, 6.52)	4.81 (4.11, 5.50)	0.34	0.85
PPARγ	0.1% DMSO	5.80 (4.50, 7.10)	5.39 (3.11, 7.68)	5.60 (4.32, 6.87)	Reference	Reference
PPARγ	MnBP	6.05 (4.76, 7.34)	5.99 (4.31, 7.67)	6.02 (4.95, 7.08)	0.01*	0.29
PPARγ	MBzP	6.34 (5.57, 7.10)	6.03 (3.95, 8.10)	6.18 (5.12, 7.24)	0.01*	0.81
PPARγ	MEHP	6.16 (5.01, 7.31)	5.53 (3.71, 7.34)	5.84 (4.81, 6.87)	0.37	0.65
PPARγ	MEP	5.79 (5.14, 6.45)	5.27 (4.15, 6.39)	5.59 (4.83, 6.34)	0.98	0.87
PPARγ	0.4% DMSO	6.36 (5.36, 7.37)	5.94 (4.82, 7.07)	6.11 (5.39, 6.83)	Reference	Reference
PPARγ	Mixture	6.87 (5.95, 7.79)	5.51 (4.67, 6.34)	6.23 (5.45, 7.01)	0.38	0.01*

Note: *p*-Values are reported for the overall and sex-specific effects of the phthalate dose on secreted hCG. $*p \leq 0.05$. Means were estimated by using a mixed effects model with a random intercept for experiment, allowing for control for between- versus within-placenta variability in protein expression. The final sample included three female and three male biologic replicates. Dose groups: MnBP, 200 nM; MBzP, 3 μM; MEHP, 700 nM; MEP, 1.5 μM. The mixture includes all 4 concentrations. CI, confidence interval; DMSO, dimethyl sulfoxide; hCG, human chorionic gonadotropin; MBzP, monobenzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEP, monoethyl phthalate; MnBP, mono-*n*-butyl phthalate; PPARγ, peroxisome proliferator activated receptor gamma; vCTB, villous cytotrophoblast cells.

between mRNAs and intracellular and secreted proteins that were normalized for internal controls. In all mixed effects models, we estimated empirical standard errors that are more robust when the assumption of equal covariance across experiments cannot be confirmed (Verbeke and Molenberghs 2000). Analyses were performed using SAS (version 9.3; SAS Institute Inc.).

Results

The intracellular and secreted hCG protein levels from the TBPCs were primarily below the level of detection and were not analyzed (data not shown). All mRNA and protein end points were well above the detection limit in the vCTBs. Differences in cellular morphology and cell density were not observed in treated versus control cells. We present the sex-specific means (Ct values, protein intensities, and secreted protein concentrations) of cells treated with single metabolites and with the combined metabolites.

mRNA

MnBP stimulated the *CGA* mRNA expression in female TBPCs (2.1-fold; 95% CI: 1.4, 3.1) and vCTBs (1.3-fold; 95% CI: −0.9, 1.7) (Figures 1A, 2A; see also Tables S1 and S2), whereas an opposite and weaker effect was observed in male cells (TBPCs −0.9-fold; 95% CI: −0.6, 1.5; vCTBs −0.9-fold; 95% CI: −0.7, 1.1). This was the only sex-specific effect on mRNA that was common to both cell types (Figures 1, 2). In the TBPCs, the phthalate mixture increased the *CGB* levels in female cells 1.4-fold (95% CI: 1.0, 1.9) and decreased the levels in male cells 0.7-fold (95% CI: −0.5, −0.9). In the female cells, the mixture's effect on *CGB* resembled the average effect of the single metabolites (1.4-fold vs. 1.5-fold in the mixture-dosed cells). In the male cells, the effect of the mixture was similar to that of MEHP, which significantly reduced *CGB* (−0.8-fold by MEHP alone vs. −0.7-fold in the mixture-dosed cells). Conversely, in the female vCTBs, the mixture lowered *CGB* mRNA −0.9-fold (95% CI: −0.8, −1.0) and increased *CGB* in the male vCTBs 1.7-fold (95% CI: 0.9, 3.2) relative to the controls. There was inadequate biologic replication for the MEP-dosed TBPCs (see Table S5, results not shown).

In the vCTBs, we analyzed three genes that are targets of phthalates in other cell types (Howdeshell et al. 2007; Lovekamp and Davis 2001; Schlezinger et al. 2004) and that are important to placental function: *CYP19A1* and *CYP11A1* are involved in steroidogenesis and in xenobiotic metabolism, respectively (Hakkola et al. 1998; Miller 1998), and *PTGS2* (COX-2) is involved in prostaglandin production by the placenta (Challis et al. 2005). *CYP19A1* and *CYP11A1* were up-regulated by phthalates in both sexes. MBzP increased *CYP11A1* levels in the female cells 1.4-fold (95% CI: 1.2, 1.6) compared with the control cells (see Figure S1). *PTGS2* levels were increased in female cells by MBzP 1.2-fold (95% CI: −0.9, 1.6) and decreased in male cells −0.7-fold (95% CI: −0.5, 1.0). In male and female cells combined, MEHP lowered *PTGS2* −0.7-fold (95% CI −0.5, −1.0, $p = 0.02$).

Intracellular Proteins

MnBP and MBzP increased hCGβ (2.5-fold and 2.2-fold, respectively) and PPARγ (1.5-fold and 1.8-fold, respectively) (Table 1, Figure 3). The mixture dose had a stronger sex-specific effect on PPARγ than the single metabolites. PPARγ was increased in female cells [1.7-fold (95% CI: 1.1, 2.4)] and was decreased in male vCTBs [−0.6-fold (95% CI: −0.4, 1.1)].

Secreted hCG Isoforms and Subunits

Phthalate effects on secreted hCG levels in the conditioned media differed by metabolite (Table 2, Figure 4). MEP had the strongest effect, with a −0.66-fold decrease (95% CI: −0.59, −0.74) in hCG-h secreted from female cells. In male cells, MEHP increased hCGα secretion 1.21-fold (95% CI: 1.11, 1.33). The mixture significantly suppressed hCGβ (−0.93-fold; 95% CI: −0.88, −0.99) and hCG-h (−0.86-fold 95% CI: −0.75, −0.98) levels in cells of both sexes.

To evaluate specific effects of phthalates on the levels of hCG subunits, we used ratios of the subunits to intact hCG as our end points (see Figure S2). Here, intact hCG serves as an indicator of overall hCG production. We did not detect sex-specific effects. MnBP significantly up-regulated %hCGβ (1.07-fold; 95% CI: 1.00, 1.15), and MEHP significantly down-regulated %hCGβ (−0.89-fold; 95% CI: −0.82, −0.96). MEP up-regulated %hCGα (1.22-fold; 95% CI: 1.06, 1.42) and %hCGβ (1.17-fold; 95% CI: 1.07, 1.28). The mixture did not significantly alter the hCG subunits.

Correlations between mRNA and Intracellular and Secreted Protein

Even though it is only a snapshot, we can evaluate these correlations as indicators of potential sex differences in the underlying *in vitro* mechanisms of hCG synthesis and secretion. A direct and

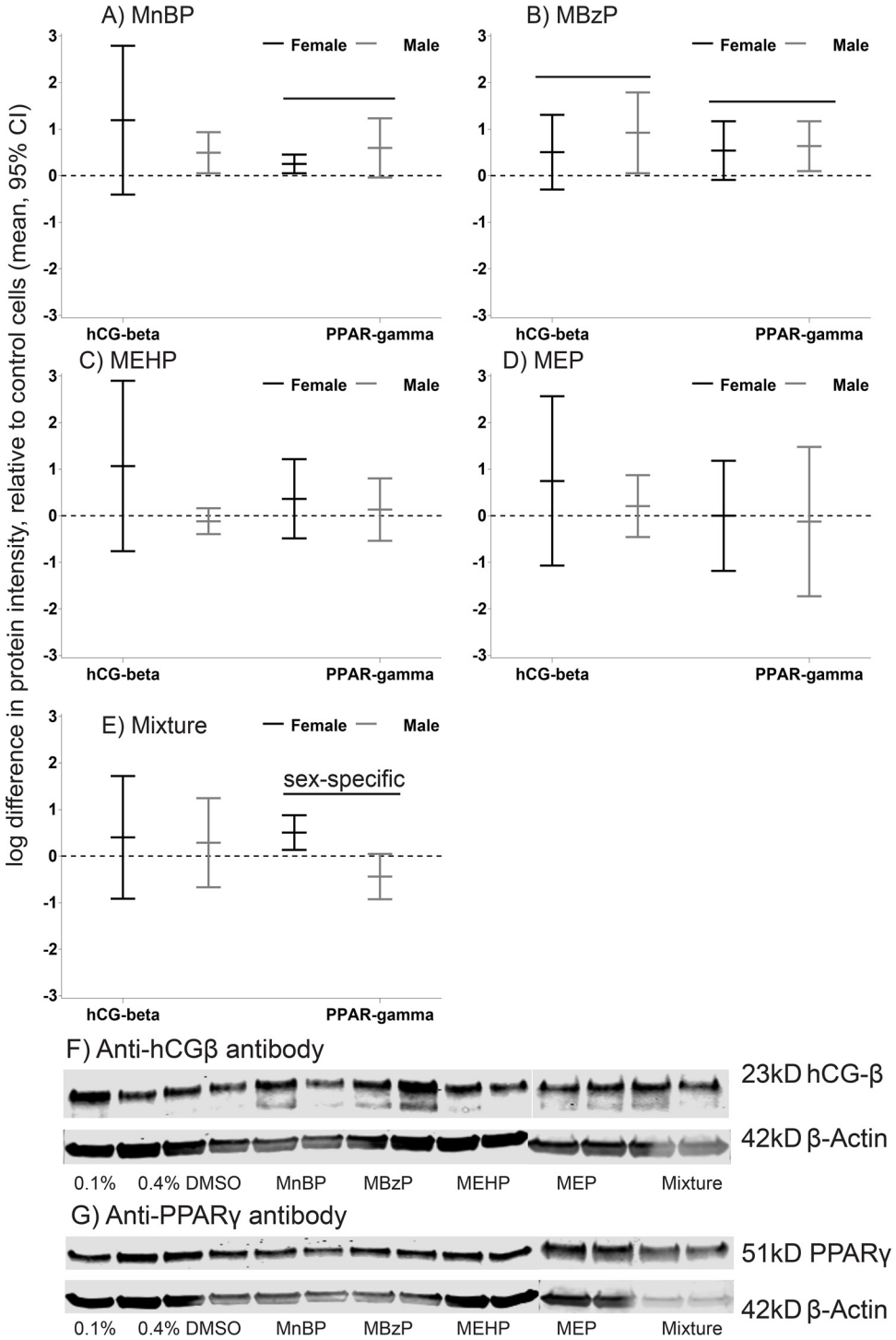

Figure 3. Phthalate effects on intracellular hCGβ and PPARγ levels in differentiated cytotrophoblasts (vCTBs). The effects are expressed as the mean natural log difference in protein intensity and 95% confidence intervals (CIs) compared with DMSO-treated control cells. (*A*) 200 nM MnBP; (*B*) 3 μM MBzP; (*C*) 700 nM MEHP; (*D*) 1.5 μM MEP; (*E*) mixture of all four metabolites. The black lines indicate female-specific effects, and the gray lines indicate male-specific effects. Overall effects that were significant ($p \leq 0.05$) are indicated by a line and marked "sex-specific" if the phthalate effect differed in male and female cells. Examples of (*F*) hCGβ and (*G*) PPARγ Western blots. Each dose group was assayed in duplicate (two lanes). This represents a single experiment conducted on cells isolated from a female placenta at 15.6 wk gestation. DMSO, dimethyl sulfoxide; hCGβ, human chorionic gonadotropin β; MBzP, monobenzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEP, monoethyl phthalate; MnBP, mono-*n*-butyl phthalate; PPARγ, peroxisome proliferator activated receptor gamma.

positive correlation between *CGB* mRNA and intracellular hCGβ was only observed in females (23 kDa $r = 0.58$, $p < 0.0001$; Figure 5; see also Table S3). This finding is similar to measurements obtained by others who used this antibody, but without consideration of the sex of the placental cells (Uhlén et al. 2015). In males, there was no correlation between levels of

Table 2. Mean log concentrations (95% CI) of hCG in the conditioned media of vCTBs treated with four phthalate metabolites, a mixture of the metabolites, and with DMSO.

Protein	Metabolite	Females	Males	Overall	*p*-Value	Sex-specific *p*-value
hCGα	0.1% DMSO	7.06 (6.77, 7.35)	6.74 (6.54, 6.94)	6.90 (6.72, 7.07)	Reference	Reference
hCGα	MnBP	7.04 (6.81, 7.28)	6.73 (6.55, 6.91)	6.88 (6.73, 7.04)	0.71	0.95
hCGα	MBzP	7.10 (6.75, 7.45)	6.83 (6.65, 7.02)	6.97 (6.77, 7.16)	0.05*	0.49
hCGα	MEHP	7.16 (6.83, 7.48)	6.94 (6.72, 7.15)	7.04 (6.84, 7.24)	0.001*	0.18
hCGα	MEP	7.05 (6.78, 7.33)	6.84 (6.63, 7.04)	6.94 (6.77, 7.12)	0.29	0.26
hCGα	0.4% DMSO	7.05 (6.73, 7.37)	6.87 (6.59, 7.15)	6.96 (6.76, 7.15)	Reference	Reference
hCGα	Mixture	7.03 (6.64, 7.42)	6.88 (6.57, 7.19)	6.95 (6.73, 7.18)	0.94	0.58
hCGβ	0.1% DMSO	5.14 (4.00, 6.29)	5.62 (4.88, 6.36)	5.38 (4.73, 6.03)	Reference	Reference
hCGβ	MnBP	5.14 (4.10, 6.17)	5.61 (4.82, 6.40)	5.37 (4.73, 6.00)	0.79	0.98
hCGβ	MBzP	5.19 (4.10, 6.27)	5.72 (4.96, 6.49)	5.45 (4.82, 6.09)	0.01*	0.26
hCGβ	MEHP	5.03 (3.86, 6.21)	5.58 (4.82, 6.34)	5.30 (4.63, 5.97)	0.08	0.33
hCGβ	MEP	5.06 (3.90, 6.22)	5.68 (4.88, 6.47)	5.37 (4.69, 6.04)	0.88	0.27
hCGβ	0.4% DMSO	5.19 (3.50, 6.88)	5.94 (4.84, 7.04)	5.56 (4.64, 6.48)	Reference	Reference
hCGβ	Mixture	5.11 (3.44, 6.78)	5.87 (4.77, 6.97)	5.49 (4.57, 6.41)	0.03	0.87
hCG-h	0.1% DMSO	4.47 (3.68, 5.27)	4.65 (4.06, 5.23)	4.55 (4.08, 5.02)	Reference	Reference
hCG-h	MnBP	4.33 (3.44, 5.22)	4.53 (3.91, 5.15)	4.42 (3.91, 4.94)	0.02*	0.79
hCG-h	MBzP	4.40 (3.46, 5.33)	4.66 (4.04, 5.28)	4.53 (3.99, 5.07)	0.69	0.37
hCG-h	MEHP	4.40 (3.36, 5.44)	4.53 (3.92, 5.15)	4.47 (3.87, 5.06)	0.34	0.78
hCG-h	MEP	4.06 (3.19, 4.93)	4.52 (3.92, 5.11)	4.28 (3.78, 4.79)	0.001*	0.01*
hCG-h	0.4% DMSO	4.47 (2.97, 5.98)	4.84 (4.04, 5.64)	4.66 (3.88, 5.44)	Reference	Reference
hCG-h	Mixture	4.36 (2.93, 5.79)	4.69 (3.88, 5.49)	4.52 (3.76, 5.28)	0.01*	0.55
Intact hCG	0.1% DMSO	5.15 (4.48, 5.83)	5.22 (4.76, 5.68)	5.18 (4.78, 5.57)	Reference	Reference
Intact hCG	MnBP	5.05 (4.48, 5.62)	5.16 (4.72, 5.61)	5.10 (4.75, 5.46)	0.21	0.74
Intact hCG	MBzP	5.15 (4.44, 5.86)	5.30 (4.87, 5.72)	5.23 (4.83, 5.62)	0.13	0.25
Intact hCG	MEHP	5.20 (4.52, 5.87)	5.25 (4.80, 5.70)	5.22 (4.81, 5.63)	0.11	0.66
Intact hCG	MEP	4.85 (4.31, 5.38)	5.19 (4.72, 5.67)	5.01 (4.67, 5.36)	0.04*	0.02*
Intact hCG	0.4% DMSO	5.20 (4.27, 6.12)	5.38 (4.78, 5.97)	5.29 (4.77, 5.80)	Reference	Reference
Intact hCG	0.1% DMSO	5.14 (4.15, 6.13)	5.33 (4.76, 5.91)	5.24 (4.71, 5.76)	0.21	0.79

Note: *p*-Values are reported for the overall and sex-specific effects of the phthalate dose on secreted hCG. $^*p \leq 0.05$. Means were estimated by using a mixed effects model with a random intercept for experiment, allowing for control for between- versus within-placenta variability in hCG secretion. The final sample included three female and four male biologic replicates. Dose groups: MnBP, 200 nM; MBzP, 3 μM; MEHP, 700 nM; MEP, 1.5 μM. The mixture includes all 4 concentrations. CI, confidence interval; DMSO, dimethyl sulfoxide; hCG, human chorionic gonadotropin; MBzP, monobenzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEP, monoethyl phthalate; MnBP, mono-*n*-butyl phthalate; PPARγ, peroxisome proliferator activated receptor gamma; vCTB, villous cytotrophoblast cells.

mRNAs for hCG and the intracellular protein levels. Correlations that were common to males and females were the negative correlation between *CGA* and secreted hCGα and the positive correlation between *CGB* and secreted hCGβ. *PPARG* mRNA was positively correlated with its encoded protein only in the male ($r = 0.49$, $p = 0.002$) but not the female vCTBs ($r = -0.05$, $p = 0.74$). These are univariate correlations and do not take into account clustering within placentas or sources of variability in the correlations other than the sex of the cells.

Discussion

Using experimental methods, we generated data that reproduced, in cell models, observed relationships between prenatal exposure to MnBP, MBzP, and MEHP and genes essential to placental gonadotropin synthesis (*CGA*), placental progesterone synthesis (*CYP11A1*), and placental prostaglandin production (*PTGS2*). To further evaluate the biological relevance of these changes in mRNA, we measured two corresponding proteins: hCG and PPARγ. MnBP and MBzP changed hCGβ, and MEHP changed hCGα, in ways that were consistent with mRNA effects. To maximize the two-way translational value of our findings to human pregnancy, in the present study, we used primary human placental cells and dosed them with phthalate metabolites at concentrations found in the urine of pregnant women exposed to environmental levels of phthalates. Compared with working with homogenous or immortalized cell lines or high doses of phthalates, this approach presents unique experimental and statistical challenges, yet it produces results with greater translational significance to pregnancy.

In two birth cohort studies, we previously reported that maternal urinary phthalates were associated with higher levels of mRNA and protein in female placentas/fetuses and with lower levels in male placentas/fetuses (Adibi et al. 2015a, 2017). In the present study, we explored this further by studying mRNA and protein effects in tandem and by including a transcription factor that regulates hCG in the placenta that is also activated by phthalates—PPARγ (Fournier et al. 2011; Handschuh et al. 2007; Hurst and Waxman 2003). It has been hypothesized that PPARγ may be the mechanism by which phthalates can exert endocrine-disrupting effects (Desvergne et al. 2009; Lovekamp-Swan and Davis 2003).

Unexpectedly, we observed sex-specific relationships between PPARγ and hCG. *PPARG* mRNA and PPARγ protein were positively correlated with hCGβ in male but not female cells. hCG synthesis also differed by sex. In female cells, *CGB* mRNA was positively correlated with intracellular and secreted hCGβ, as expected. In male cells, *CGB* mRNA was not correlated with intracellular hCGβ. hCGα and hCGβ are subunits of intact hCG but may also have unique functions independent of classical LH/hCG-receptor (Blithe and Nisula 1987; Blithe et al. 1991; Hussa 1980, 1982; Lee et al. 2013). hCGα and hCGβ were highly positively correlated at the mRNA level in both sexes, but not at the protein level. In female cells, the subunits were inversely correlated, and they were not correlated in male cells. hCG subunit variation may be relevant to sex-specific hCG regulation or to other types of posttranscriptional regulation of hCG.

PPARγ may be a key intermediary between phthalate exposure and placental hCG levels, explaining why hormonal effects are opposite in direction for males and females. The effects of the mixture dose on PPARγ were opposite in males and females. The

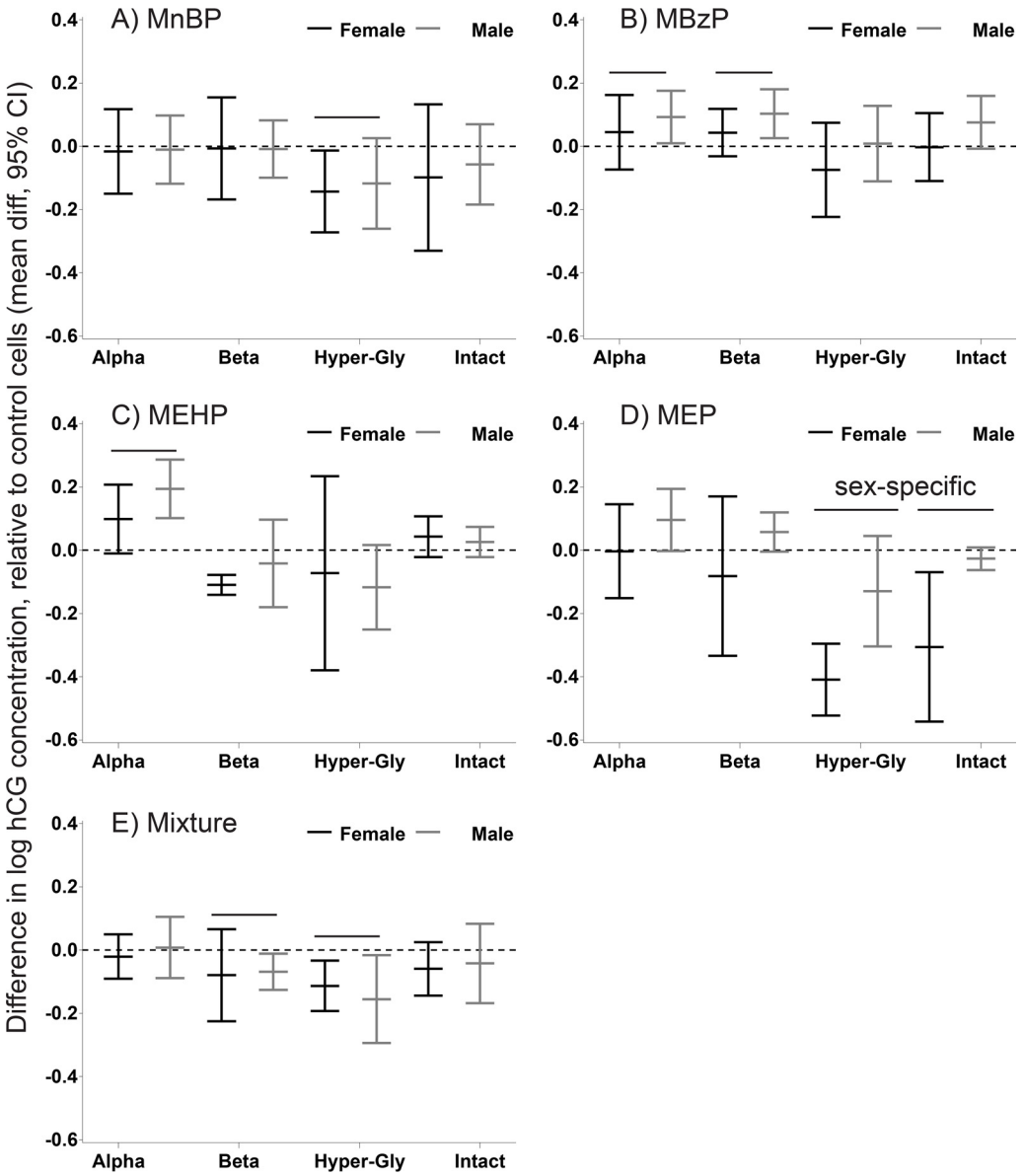

Figure 4. Phthalate effects on secreted hCG forms in the conditioned media of differentiated trophoblasts (villous cytotrphoblasts, vCTBs). The effects are expressed as the difference in mean natural log concentration and 95% confidence intervals (CIs) compared with DMSO-treated control cells. (*A*) 200 nM MnBP; (*B*) 3 μM MBzP; (*C*) 700 nM MEHP; (*D*) 1.5 μM MEP; (*E*) mixture of all four metabolites. The black lines indicate female-specific effects, and the gray lines indicate male-specific effects. Overall effects that were significant ($p \leq 0.05$) are indicated by a line and marked "sex-specific" if the phthalate effect differed in male and female cells. DMSO, dimethyl sulfoxide; hCG human chorionic gonadotropin; MBzP, monobenzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEP, monoethyl phthalate; MnBP, mono-*n*-butyl phthalate.

sex difference in the correlation of *PPARG* with hCGβ and hCGα could explain the opposite effects of phthalates on hCG. These are important and novel insights that give rise to testable hypotheses that can be further studied using biomarkers in human pregnancy and *in vitro* by using experimental techniques. Additional levels of complexity in this relationship should be considered, such as the epigenetic regulation of *PPARG* (Lendvai et al. 2016), mitochondrial expression of *PPARG* in the placenta (Calabuig-Navarro et al. 2016), and sex-specific mitochondrial dysfunction in response to maternal exposures (Muralimanoharan et al. 2015).

We based our experimental doses on phthalate concentrations in maternal urine that were correlated with placental tissue *CGA* mRNA to compare the two sets of results and to evaluate reproducibility (Adibi et al. 2017). In three cases, the sex-specific associations between phthalates and placental mRNA expression were supported by the *in vitro* replication. For *CYP11A1*, the association with MBzP was stronger in magnitude than the *in vitro* effect. *PTGS2* was down-regulated by MEHP in male placentas in both studies. Discrepancy in results between the two study designs may indicate that isolated trophoblast cells, cultured in the absence of fetal tissue and signals from the fetal pituitary/adrenal/gonadal cells, exhibit weaker or even a reversal of hCG sex differences measured *in vivo*. We observed heterogeneity in the direction and magnitude of the hCG effects by phthalate metabolite, by sex, by hCG subunit, and by differentiated versus undifferentiated Tbs. We offer these as testable hypotheses to be pursued in future studies *in vitro* and in human populations. hCG is an essential hormone for pregnancy maintenance and is correlated with many obstetric outcomes (Filicori et al. 2005; Yaron et al. 2002b), yet it has not been considered in studies of

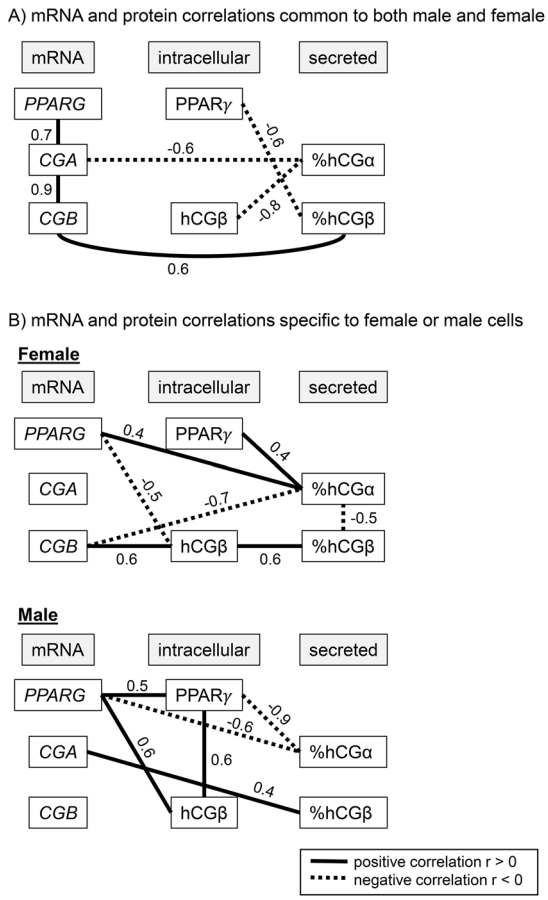

Figure 5. Spearman rank correlations between levels of mRNAs, intracellular proteins, and secreted proteins in female and male differentiated trophoblasts (villous cytotrphoblasts, vCTBs). (*A*) Correlations that are common to male and female vCTBs; (*B*) Correlations detected only in female and in male cells ($p \leq 0.05$). Positive correlations are drawn as solid black lines, and negative correlations are drawn as dotted lines. hCG, human chorionic gonadotropin; PPAR, peroxisome proliferator activated receptor.

fetal endocrine disruption to the same degree as androgens, estrogens, and progesterone. In both sexes, there is evidence that hCG can act as a potent gonadotropin at different points in development. In females, hCG is used to stimulate ovulation for the purpose of *in vitro* fertilization (Yen et al. 2014). In males, hCG has been used to induce virilization and penile growth in prepubertal males with hypogonadotrophic hypogonadism (Bistritzer et al. 1989) and to induce spermatogenesis in adult life. In normal pregnancy, hCG binds to the luteinizing hormone/chorionic gonadotropin (LH/CGR) receptor in the male fetus during the first trimester, stimulating testicular steroidogenesis and thereby indirectly guiding genital differentiation (Huhtaniemi et al. 1977). If the LH/CGR receptor is inactive because of mutation of its gene, males are born with defective genital masculinization (XY, disorder of sexual differentiation) (Kremer et al. 1995). Women with 20% lower circulating hCG had an increased chance of giving birth to cryptorchid boys (Chedane et al. 2014). Taken together, these findings support the idea that disruption of hCG production and function by phthalates or by other endocrine-disrupting chemicals during pregnancy may have effects on fetal sex differentiation.

There are not likely to be real-life situations where a person would only be exposed to one phthalate metabolite at a time; therefore, we evaluated and compared the effects of phthalate mixtures and of single phthalates. We used a nonbalanced approach (i.e., nonequivalent doses) in the design of our doses to accurately reflect real-life exposures during pregnancy (Evans et al. 2012). We detected three cases of a significant effect of the mixture. In the case of *CGB* mRNA in undifferentiated trophoblasts, the mixture effect was analogous to the single-metabolite effects. We interpret this to mean that there was a common mechanism that was not overwhelmed by the phthalate concentrations used. In the case of intracellular PPARγ in the differentiated trophoblasts, the mixture effect was stronger than the single-metabolite effects, sex-specific, and opposite in direction. The mechanism of PPARγ activation may differ in the case of multiple versus single phthalates. In the case of the female cells, the different metabolites may have synergized to increase the strength of the positive effect on PPARγ (i.e., agonism). In the case of the male cells, the metabolites may have competed for or antagonized (or both) a common mechanism to cause the down-regulation of PPARγ. This latter scenario may also apply to secreted hCGβ, where the negative effect of the mixture was a reversal of the positive effect of MBzP. Comparisons between the effects of single metabolites versus mixtures are critical in establishing which metabolites are more biologically potent and should be prioritized in efforts to reduce risks to the placenta and fetus.

Sex and gestational variation in hCG have been previously established at the population level in analyses of hCG biomarker data (Adibi et al. 2015b; Bremme and Eneroth 1983; Buckberry et al. 2014; Clements et al. 1976; Cowans et al. 2009; Nagy et al. 1994a, 1994b; Steier et al. 1999; Yaron et al. 2002a). This type of variation is generally not considered when analyzing *in vitro* experimental data. We observed in our primary tissue cultures that the sex and gestational-age variation in mRNA and secreted protein levels that were present at baseline persisted and rendered our biologic replicates less comparable. For this reason, we controlled for these variables in the data analysis using basic multivariate statistical techniques.

There are noteworthy caveats in making comparisons between observational and experimental findings. In the *in vitro* study, our controls received no phthalates, whereas in the observational studies, we compared pregnancies with lower but not zero exposure because all subjects were environmentally exposed. Similarly, all of the pregnant women were exposed to a phthalate mixture even though we estimated associations with single metabolites to which we compared the results of the present study (Adibi et al. 2017). Phthalate concentrations in placental tissue are most likely lower than the urine concentrations modeled here [phthalates are not measured in blood owing to a short half-life and to a high risk of phthalate contamination in the sampling process (Calafat et al. 2015)]. In a small pilot study, we estimated that MnBP, MBzP, and MEP were higher, but within an order of magnitude, in urine than in placental tissue by 14-, 28-, and 8-fold respectively. MEHP was 4-fold higher in placental tissue; therefore, we may have slightly underestimated the true exposure to the placenta in this study (J. Adibi and N. Snyder, unpublished data, 2017); this may increase the translational value over previously published studies that dosed with concentrations of MEHP that are 1–3 orders of magnitude higher than urinary levels (Meruvu et al. 2016; Tetz et al. 2013; Wang et al. 2016).

These relationships and the relatively small effect sizes are supported by studies conducted by other investigators. In a study of immature rat Leydig cells, *CYP11A1* mRNA was 30–40% higher than in controls at 50–500 nM MnBP (Li et al. 2016), similar to the effect we found in male placental cells (33%) in the present study. In an immortalized first-trimester cell line (sex not specified), *PTGS*, the gene that encodes the COX-2 protein, increased approximately 2- to 3-fold at a 90-μM MEHP dose (129-fold as high as the dose used in our study) (Tetz et al.

2013). In another study, the effects of MEHP on the COX-2 protein were not detected within the dose range that we used, but only at higher doses (Wang et al. 2016). We measured a significant reduction in *PTGS2* by MEHP. Results cannot be easily compared owing to the large differences in dose and to a high likelihood of different mechanisms at low versus high doses. In a review of 35 published studies that reported associations of prenatal phthalate exposures with obstetric outcomes, the authors indicated that knowledge of a mechanism and of ways to measure specific biologic intermediaries in human pregnancy are lacking (Marie et al. 2015). Our findings address this gap by offering biologic insight into correlations of prenatal phthalate exposure with placental end points.

Conclusion

In conclusion, we moved one step beyond an observational association by showing sex-specific relationships between MnBP and placental *CGA*, and between MBzP and placental *CYP11A1* using experimental methods with primary cells. The finding that MnBP can alter chorionic gonadotropin α (*CGA*) was extended to other phthalate metabolites (MBzP, MEHP, and MEP) and to intracellular and secreted hCG and its subunits. The sex-specific effects of phthalates may be challenging to reproduce *in vitro* in the absence of the fetus. However, our results support the hypothesis that hCG is altered by low concentrations of single and combined phthalates, which is relevant for environmental exposure to phthalates. We are the first to report sex differences in hCG transcription and translation, which we believe to be partially regulated by PPARγ. This finding provides a testable hypothesis to better understand why the hormonal effects of phthalates are opposite in direction between males and females. In future studies, it will be important to quantify the functional significance (for cells, for organs, and for the future child) of small perturbations in hCG by ubiquitous phthalate exposures.

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