Mechanisms of Phthalate Ester Toxicity in the Female Reproductive System

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Phthalates are high-production-volume synthetic chemicals with ubiquitous human exposures because of their use in plastics and other common consumer products. Recent epidemiologic evidence suggests that women have a unique exposure profile to phthalates, which raises concern about the potential health hazards posed by such exposures. Research in our laboratory examines how phthalates interact with the female reproductive system in animal models to provide insights into the potential health effects of these chemicals in women. Here we review our work and the work of others studying these mechanisms and propose a model for the ovarian action of di-(2-ethylhexyl) phthalate (DEHP). *In vivo*, DEHP (2 g/kg) causes decreased serum estradiol levels, prolonged estrous cycles, and no ovulations in adult, cycling rats. *In vitro*, monoethylhexyl phthalate (MEHP; the active metabolite of DEHP) decreases granulosa cell aromatase RNA message and protein levels in a dose-dependent manner. MEHP is unique among the phthalates in its suppression of aromatase and in its ability to activate peroxisome proliferator-activated receptors (PPARs). We hypothesize that MEHP activates the PPARs to suppress aromatase in the granulosa cell. MEHP-, PPARα-, and PPARγ-specific ligands all similarly decreased estradiol production and RNA message levels of aromatase *in vitro*. Our model shows that MEHP acts on the granulosa cell by decreasing cAMP stimulated by follicle stimulating hormone and by activating the PPARs, which leads to decreased aromatase transcription. Thus, the environmental contaminant DEHP, through its metabolite MEHP, acts through a receptor-mediated signaling pathway to suppress estradiol production in the ovary, leading to anovulation. *Key words:* aromatase, diethylhexyl phthalate, estradiol, female reproductive toxicity, granulosa cells, monoethylhexyl phthalate, phthalates, ovary, peroxisome proliferator, proliferator-activated receptors. *Environ Health Perspect* 111:139–145 (2003). [Online 28 October 2002]
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Phthalates are high-production-volume synthetic chemicals with ubiquitous human exposures because of their use in plastics and other common consumer products. Globally, more than 18 billion pounds of phthalates are used each year, primarily as plasticizers in flexible polyvinyl chloride (PVC) products (Blount et al. 2000a). Di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer for PVC, and the production volume of DEHP alone in 1999 was estimated to be 2 million tons [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2000]. Phthalates are found in most PVC products including vinyl upholstery, tablecloths, shower curtains, raincoats, and soft-squeeze children's toys. In the United States and Canada, DEHP is no longer used to manufacture children's products intended for mouthing, such as pacifiers (CERHR 2000), but it may still be found in larger toys, especially those made in other countries. Phthalates are used as inert ingredients in many sprays including pesticides and many consumer products such as cosmetics and wood finishes (Blount et al. 2000b). Phthalates are also used as adhesives, defoaming agents, solvents, and lubricants [National Toxicology Program (NTP) 1998]. DEHP is approved for use in medical devices such as tubing, blood bags, and dialysis equipment and is also used to manufacture the 500 million pairs of disposable medical examination and sterile surgical vinyl gloves produced annually (NTP 1998). Phthalates are used to impart flexibility to plastics, but they leach from plastic products into the environment over time. Given their high production volume, common use, and widespread environmental contamination, humans are exposed to these compounds through ingestion, inhalation, and dermal exposure on a daily basis.

The Agency for Toxic Substances and Disease Registry (ATSDR) estimates that the maximum daily exposure to DEHP for the general population is about 2 mg/day. However, occupational and medical exposures can reach much higher levels (ATSDR 1993). For instance, exposure to DEHP from blood transfusions can be as high as 250–300 mg, equivalent to a dose of 3.5–4.3 mg/kg for an adult weighing 70 kg (CERHR 2000). Phthalates are diesters of *o*-phthalic acid with various side chain lengths, and the toxicity of the different congeners vary (Woodward 1988). The diester forms of these chemicals are rapidly hydrolyzed by esterases in the gut, liver, and blood into the monoester forms, which are considered the ultimate toxicants (Figure 1). A recent study by the Centers for Disease Control and Prevention (CDC) measured phthalate monoesters in human urine, creatinine adjusted to account for variations in urine volume (Blount et al. 2000b). MEHP, monoethyl phthalate (MEP), monobenzyl phthalate (MBzP), and monobutyl phthalate (MBP) were detected in the urine of more than 75% of subjects tested (Blount et al. 2000b). The highest levels of MEHP in this study (67 ppb, 192 μg/g creatinine) are consistent with previous studies in occupationally exposed individuals (Blount et al. 2000b). The CDC study also found that women of reproductive age had significantly higher urinary levels of MBP (46.9 μg/g creatinine) than other sex/age groups (31.4 μg/g creatinine; $p = 0.003$) (Blount et al. 2000b). These higher levels may be due to the fact that dibutyl phthalate (DBP) is used in many beauty products including perfume, lotion, and nail polish. Thus, women have a unique exposure profile, which raises a concern about the potential health hazards posed by such exposures.

Limited studies in human populations suggest an association between phthalate exposure and adverse reproductive health outcomes. For example, chronic occupational exposure to high levels of phthalates is associated with decreased rates of pregnancy and higher rates of miscarriage in female factory workers (Aldyreva et al. 1975). Higher urinary phthalate levels correlated with pregnancy complications such as anemia, toxemia, and preeclampsia in women living near a plastics manufacturer (Tabacova 1999). Although occupational exposure is limited to a select population, women have exposures to phthalates in beauty and consumer products on a daily basis. During pregnancy and delivery, both the mother and fetus may be exposed to DEHP through medical devices. Exposure to the fetus *in utero* is a concern because some phthalates, including DEHP and DBP, are developmental toxicants (Mylchreest et al. 1998; Tyl et al. 1988). The Food and Drug

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Administration (FDA) recently issued a report acknowledging that PVC medical devices may be a concern to some critically ill infants (FDA 2001). Puerto Rican girls with premature breast development (thelarche) had higher levels of blood phthalates compared to other girls, suggesting an association between phthalate exposure and abnormal reproductive development (Colón et al. 2000).

These studies are suggestive of human health effects, but more epidemiologic data are needed in human populations, along with a better mechanistic understanding of the reproductive health effects of phthalates. Research efforts in our laboratory examine how phthalates interact with the female reproductive system in animal models to provide insights into the potential health effects of these chemicals in women. Here, we review our work and the work of others studying these mechanisms and propose a model for phthalate activity and implications of this model for human health.

Reproductive and Developmental Toxicity in Animal Models

Initial reproductive and developmental toxicity studies on phthalates in the female rodent involved *in vivo* exposure during organogenesis. Kaul et al. (1982) reviewed the toxic effects of DEHP on the pregnant rodent and fetus. Developmental toxicity of DEHP includes reduced implantations, increased resorptions, decreased fetal body weight, and increased malformations. These effects are dose dependent, with a maximal no effect level of DEHP on mouse fetuses of 70 mg/kg/day. Tomita et al. (1986) showed that effects of DEHP in mice depend on dose and timing of exposure. Exposure on gestational days 7 and 8 led to a high incidence of death and malformations, but exposure on other days had fewer effects (Tomita et al. 1986). *In utero* exposure to DEHP causes embryotoxic and teratogenic effects in mice and rats (Shiota and Nishimura 1982; Tyl et al. 1988).

Several of the structurally related phthalate esters, including DEHP and DBP, impair fertility of both sexes in rodents. Based on fertility studies in mice, DEHP is the most potent reproductive toxicant among the phthalates, followed by dihexyl phthalate (DHP), dipentyl phthalate (DPP), DBP, and dipropyl phthalate (DPrP) (Heindel et al. 1989). Phthalates with very short (diethyl) or very long (dioctyl) side chains are not reproductive toxicants (Heindel et al. 1989). In the male rodent, DEHP causes seminiferous tubule atrophy and decreases testis weight, sperm production, and testicular zinc levels (Foster et al. 1980). These testicular effects can lead to infertility, which has been confirmed in breeding studies (Lamb et al. 1987). Like DEHP, DBP is also a testicular toxicant and causes reproductive tract malformations in male rats after *in utero* exposure (Arcadi et al. 1998; Gray et al. 1999; Moore et al. 2001; Mylchreest et al. 1998, 1999; Parks et al. 2000). Among the structurally related phthalates, DEHP, DBP, DPP, and DHP cause testicular atrophy and are both female and male reproductive toxicants in rodents (Heindel and Powell 1992). Given the well-characterized testicular toxicity in the male, the ovary was considered a likely target for toxicity in the female.

Pathogenesis Studies in the Female Rodent

Our earliest studies determined the female reproductive toxicity of DEHP; it is the most potent reproductive toxicant among the phthalates. Initial studies *in vivo* demonstrated that the ovary was a target site for DEHP and that decreased estradiol production was a primary functional alteration of DEHP exposure. We determined that adult, reproductively cycling Sprague-Dawley rats dosed *in vivo* with 2 g/kg DEHP had decreased serum estradiol levels, prolonged estrous cycles, and no ovulations (Davis et al. 1994a). As a result of no ovulations, there was an absence of corpora lutea, and follicles became cystic. The site of estradiol production in the ovary is the preovulatory follicle. Morphometric analysis of preovulatory follicles determined that granulosa cells in DEHP-treated rats were significantly smaller than control cells. The functional decrease in estradiol and the morphologically smaller follicles suggested that the granulosa cells of preovulatory follicles were the target cells of DEHP in the ovary. The pathogenesis of the lesion could then be explained by the fact that DEHP significantly suppressed estradiol production in ovarian

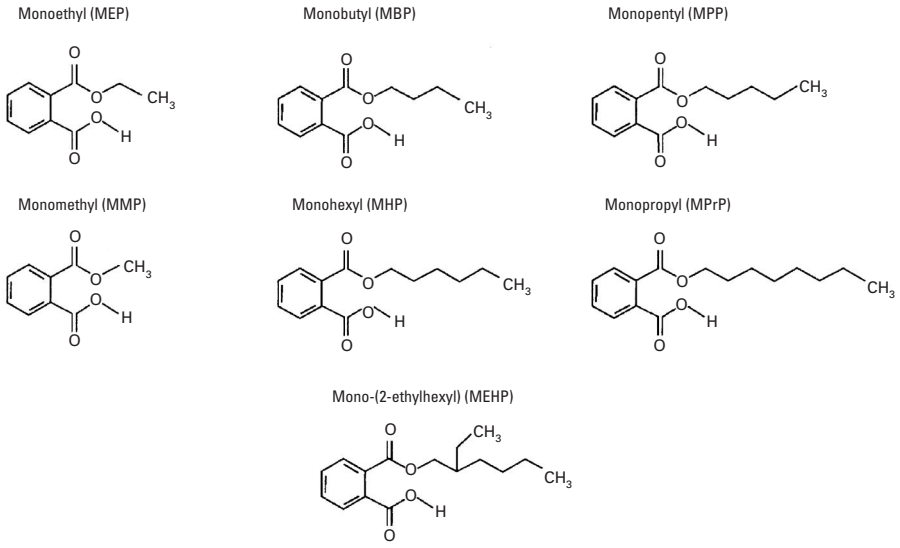

Figure 1. Structurally related phthalate monoesters. Diesters of *o*-phthalic acid are quickly metabolized *in vivo* to their active metabolites, the monesters. The length and structure of the side chain is important for toxicity.

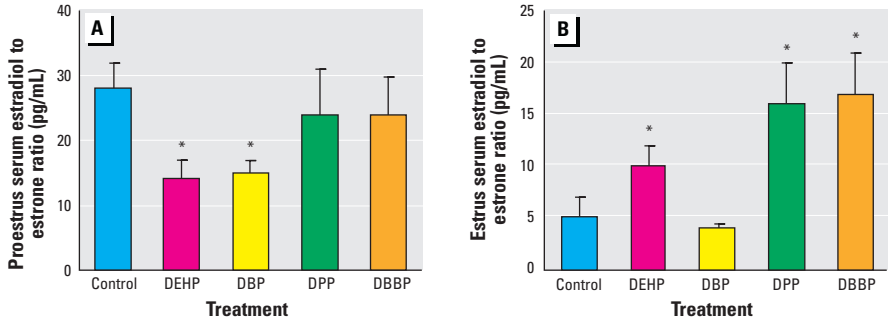

Figure 2. Phthalate effects on serum estradiol and estrone levels at (*A*) proestrus and (*B*) estrus. Adult 90-day-old female Sprague-Dawley rats (n = 12 per group) were treated with corn oil vehicle or 1,000 mg/kg of DEHP, DBP, DPP, or DBBP in corn oil given daily by gavage beginning at vaginal metestrus. Rats were killed at vaginal proestrus (n = 6 per treatment) or estrus (n = 6 per treatment) 8 or 9 days after dosing began, following methodology described by Davis et al. (1994a).
*Significantly different compared to control, $p < 0.05$.

granulosa cells, and the lowered estradiol was insufficient to trigger an ovulatory surge of luteinizing hormone (LH). Indeed, LH surges in DEHP-treated rats were not detected as long as the rats were treated with DEHP. However, DEHP-treated rats ovulated if stimulated with an LH-like compound, evidence that suppressed estradiol was the primary toxicity in the ovary (Davis et al. 1994a). These results were consistent with another study finding decreased estradiol production in cultures of minced ovaries from DEHP-treated rats in estrus (Laskey and Berman 1993).

Because the primary functional alteration of DEHP in adult female rats is suppression of estradiol levels, structurally related phthalates were tested for their effects on estradiol. *In vivo* studies examined serum estradiol and estrone, the primary metabolite of estradiol, to determine whether metabolism contributed to decreased serum estradiol levels, as well as to decreased estradiol production. Female rats were treated with 0 or 1,000 mg/kg of either DEHP, DBP, DPP, or dibutyl benzyl phthalate (DBBP) for 8–10 days over their estrous cycle under the same experimental conditions that were used to examine *in vivo* effects of DEHP (Davis et al. 1994a). At proestrus, when estradiol levels are normally rising, the ratio of estradiol to estrone was significantly decreased in rats treated with 1,000 mg/kg of either DEHP or DBP (Figure 2A). This was due to a significant decrease in estradiol levels and slightly increased estrone levels in DEHP-treated rats, whereas DBP-treated rats had significantly increased estrone levels and only slightly lower levels of estradiol. DBP-treated rats also had polycystic ovaries similar to DEHP-treated rats. There was no effect of DPP or DBBP on serum estradiol or estrone levels measured during proestrus. However, estradiol to estrone ratios were increased in DPP- and DBBP-treated animals sacrificed at estrus (Figure 2B), although these rats ovulated. Metabolites of the structurally related phthalates were also tested *in vitro* for their effect on the granulosa cell, the primary source of estradiol in the female rat. MEHP, the active metabolite of DEHP, was the only phthalate that significantly decreased estradiol production in rat granulosa cells *in vitro* (Figure 3) (Lovekamp and Davis 2001). Other monoesters including monobutyl, monoethyl, monohexyl, monomethyl, monopropyl, and monopentyl phthalate, had no effect on granulosa cell estradiol production (Lovekamp and Davis 2001). The differences in the *in vivo* and *in vitro* studies suggest that different phthalates have different hormonal effects in the female rodent and likely different organ toxicity.

If only DEHP affected granulosa cell production of estradiol, but both DEHP and DBP caused cystic ovaries and altered estradiol to estrone ratios, the question then was how did DBP alter estradiol levels *in vivo* without affecting the ovary? Part of the answer was revealed when Fan et al. (1988) reported that in the liver, DBP and DEHP induce 17β-hydroxysteroid dehydrogenase type IV (17β-HSD IV), the enzyme that metabolizes estradiol to estrone. These data suggested that the altered estrogen ratios in DBP-, DBBP-, or DPP-treated rats could be due to induction of estradiol metabolizing enzymes in the liver. Furthermore, the action in different tissues was consistent with the potency of the phthalates. For example, DEHP, the most potent reproductive toxicant, altered both granulosa cell estradiol production and metabolism, whereas DBP altered only estradiol metabolism. These studies show that phthalates display multiple effects in the female, and the key to understanding their toxicity lies in understanding their specific cellular and molecular effects.

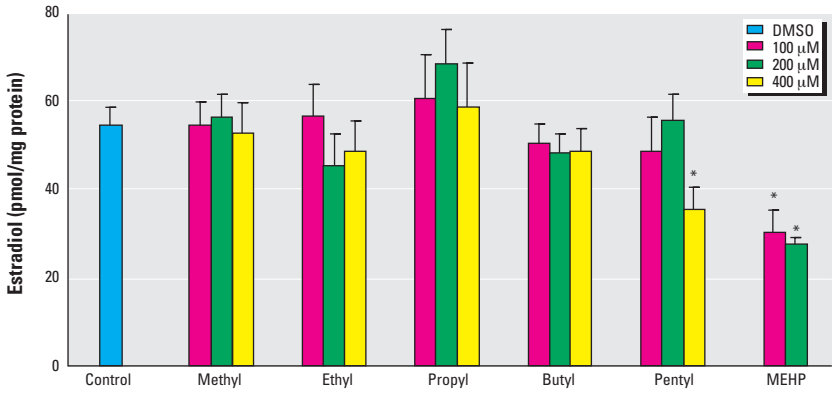

Figure 3. Structurally related phthalate monoesters tested *in vitro* for their effect on granulosa cell estradiol production. Granulosa cells were cultured from rats stimulated *in vivo* with diethylstilbestrol. Cells were supplied with FSH and testosterone and cultured for 48 hr before estradiol was measured in medium. Estradiol was normalized to cell protein. Bars represent mean and SE in three separate experiments.
*Significantly different compared to control, $p < 0.05$.

MEHP Decreases Estradiol and Aromatase in Granulosa Cells

To determine the molecular mechanism by which DEHP/MEHP suppressed estradiol in the granulosa cell, the effects of MEHP on steroid hormone synthesis were determined in primary cultures of rat granulosa cells. Primary cultures of granulosa cells serve as a useful system to study hormone production *in vitro* because these cells respond to follicle stimulating hormone (FSH) by inducing steroidogenic enzymes and differentiation (Erickson 1983). The hormonal effects of FSH in primary culture bear striking similarities to normal biochemical events *in vivo* (Erickson 1983; Hsueh et al. 1984). *In vivo*, ovarian hormone production is controlled by the pituitary hormones FSH and LH, which stimulate granulosa and thecal cells, respectively (Liu and Hsueh 1986). The gonadotropins FSH and LH bind to specific G-protein–coupled receptor sites to stimulate adenylate cyclase and the accumulation of cAMP, which activates enzymes involved in steroidogenesis (Hsueh et al. 1984). Cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc) in both granulosa and thecal cells. The stimulation of P450scc involves enzyme activation by FSH-stimulated cAMP as well as intramitochondrial movement of cholesterol to the enzyme (Hsueh et al. 1984). Pregnenolone is the key steroidogenic intermediate common to all classes of steroid hormones. It is converted to progesterone by 3β-hydroxysteroid dehydrogenase-isomerase. Progesterone is then converted to androstenedione by the actions of the 17α-hydroxylase and 17,20-lyase enzyme complex, which is present in thecal cells but not in granulosa cells. Androstenedione is converted to testosterone by 17β-HSD III. Finally, testosterone diffuses into the granulosa cell, where it is converted to estradiol by the aromatase enzyme (Richards 1980). Aromatase is stimulated both by FSH action on granulosa cells and by thecal androgens, the substrates for aromatase (Daniel and Armstrong 1980; Hillier and De Zwart 1981).

In rat ovarian granulosa cell cultures, MEHP (100 μM) inhibited FSH-stimulated cAMP and progesterone production (Treinen et al. 1990). The decrease in progesterone was prevented by stimulators of cAMP or by pregnenolone, the precursor of progesterone. However, MEHP decreased estradiol production *in vitro* when granulosa cells were stimulated either with FSH or a nondegradable analogue of cAMP, 8br-cAMP (Davis et al. 1994b). Therefore, the effect on estradiol was separate from the effect on FSH-stimulated cAMP and progesterone production in the granulosa cell.

Because the granulosa cell is devoid of the 17α-hydroxylase and 17,20-lyase enzyme

complex and requires androgens to produce estradiol, the post-cAMP effect on estradiol was localized to the aromatase enzyme. MEHP decreased the maximum activity of aromatase without acting as an enzyme inhibitor (Davis et al. 1994b). Thus, MEHP altered the levels or availability of aromatase in the granulosa cell either by decreasing synthesis or increasing degradation of the enzyme. Recent studies demonstrated that MEHP decreased aromatase RNA message and protein levels in a dose-dependent manner (Lovekamp and Davis 2001). In contrast, MBP had no effect on granulosa cell aromatase message or protein levels, consistent with earlier studies demonstrating that MBP had no effect on granulosa cell estradiol production. Furthermore, MEHP was specific for aromatase and did not decrease transcript levels of the cholesterol side-chain cleavage enzyme (P450scc) in the granulosa cell. Treatment with an exogenous form of cAMP for the last 24 hr of culture increased transcript levels of P450scc above baseline. However, the decrease in aromatase after MEHP treatment could not be rescued by adding 8-br-cAMP (Lovekamp and Davis 2001). These observations provide molecular evidence supporting previous observations that MEHP suppressed aromatase and estradiol independent of FSH-stimulated cAMP (Davis et al. 1994b).

PPAR Activation in Granulosa Cells

Given that MEHP suppressed aromatase transcripts independent of cAMP-related pathways, we examined alternative pathways that could be responsible for the suppression of aromatase by MEHP. Recent studies suggest a link between MEHP activity as a peroxisome proliferator and its toxic effect on the granulosa cell (Mu et al. 2000, 2001; Rubin et al. 2000). Peroxisome proliferators exert their effects by activating the peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily. PPAR acts as a heterodimer with retinoid X Receptor (RXR) to regulate transcription, and once activated, PPAR regulates the transcription of genes containing specific peroxisome proliferator response elements within DNA (Corton et al. 2000). There are three known isoforms of PPAR, and all have been detected in the rat ovary (Braissant et al. 1996). PPARγ is the predominant isoform in the preovulatory granulosa cell (Komar et al. 2001). PPARγ ligands such as troglitazone and 15d-PGJ2 inhibit aromatase expression level and activity in human breast adipose stromal cells (Rubin et al. 2000). The PPARγ ligand troglitazone also inhibits aromatase activity and mRNA level in cultured human ovarian granulosa cells, and PPARγ and RXR ligands act synergistically to inhibit aromatase in these cells (Mu et al. 2000, 2001). Maloney and Waxman (1999) showed that MEHP activates mouse and human PPARα and γ in a COS-1 cell-based transient *trans*-activation assay.

Based on these data, we hypothesized that MEHP decreases aromatase through activation of PPARα and PPARγ in the rat granulosa cell. To test this hypothesis, we first compared MEHP-, PPARα-, and PPARγ-specific ligands and found that all similarly decrease estradiol production and RNA message levels of aromatase in rat ovarian granulosa cells (Lovekamp and Davis 2001; Lovekamp-Swan et al. In press). To determine which receptor pathway was activated by MEHP, the PPARγ pathway was inactivated by a PPARγ-selective antagonist and MEHP (50 μM) was added to the cells. Under these conditions, aromatase was only partially suppressed. Additional experiments suggest that aromatase is not completely suppressed because MEHP still activates PPARα. Studies are currently in progress to examine further the effects of PPAR activation by MEHP.

Proposed Model in the Granulosa Cell

We propose that DEHP/MEHP mimics the effects of fatty acids on the granulosa cell, beginning with the evidence that DEHP and MEHP are ligands for fatty acid binding protein (FABP) (Kanda et al. 1990). MEHP first acts at the membrane to inhibit FSH-stimulated cAMP production, possibly by activating an inhibitory G-protein (G_i) (Figure 4; Lovekamp-Swan. Unpublished data). As MEHP diffuses into the cell, it also activates PPARs. Although MEHP activates PPAR receptors (Corton et al. 2000), there is no evidence that MEHP directly binds to PPAR. Thus, activation of this receptor by MEHP occurs either through release of endogenous fatty acids from FABPs or through a yet unidentified intermediate factor. Activation of both PPARα and PPARγ by MEHP in the granulosa cell results in decreased transcription of aromatase, the primary mechanism of its female reproductive toxicity. Activation of both PPARα and PPARγ also increases mRNA for FABP in the granulosa cell (Lovekamp-Swan et al. In press), creating more binding proteins for PPAR activators. Decreased estradiol synthesis and increased estradiol metabolism contribute to suppressed estradiol levels after MEHP treatment.

PPARγ is a key regulator of cell differentiation, and understanding that MEHP activates PPARγ provides further insight into the female reproductive toxicity of MEHP. We hypothesize that by activating PPARγ, MEHP disrupts the critical timing of the growth and differentiation of the ovarian follicle. Normally, granulosa cells of a preovulatory follicle respond to basal levels of FSH by increasing cAMP and protein kinase A, which stimulate increased expression of aromatase. Sufficient estradiol production during the preovulatory stage is critical for stimulating the ovulatory surge of LH. Aromatase is rapidly shut down after the LH surge, both by increased degradation of

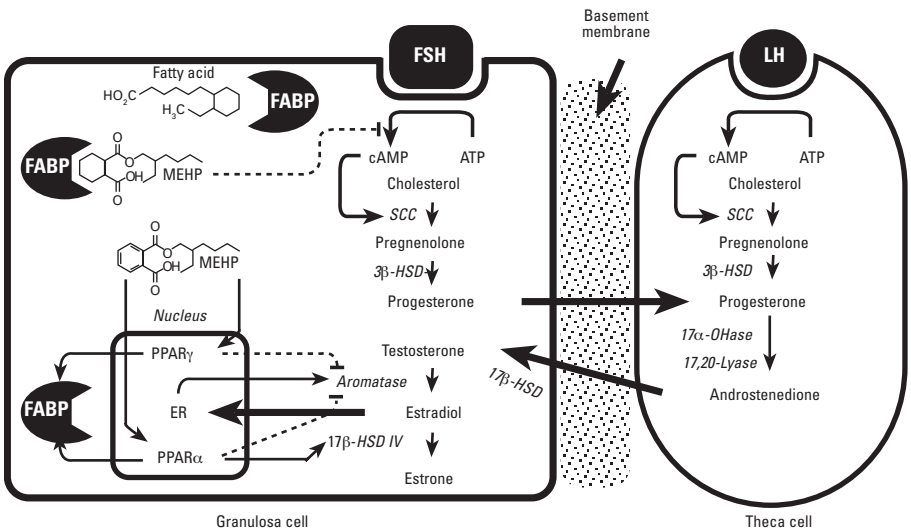

Figure 4. Proposed model of MEHP action in the granulosa cell. MEHP interferes with two points of the steroid hormone pathway. Abbreviations: ER, estrogen receptor; 17α-OHase, 17α-hydroxylase; SCC, P450 side chain cleavage enzyme. First, MEHP suppresses FSH-stimulated cAMP, possibly by inhibiting binding of FSH to its receptor or altering activation of adenylate cyclase (Grasso et al. 1993). MEHP also activates PPARs, possibly by release of fatty acids, endogenous activators of PPAR. The activation of either PPARα or PPARγ decreases aromatase mRNA. PPARα activation also causes an increase in the transcript level of 17β-HSD IV, which metabolizes estradiol to estrone. Finally, both PPARα and γ increase levels of FABP in the cell, which is able to transport MEHP and fatty acids through the cell, delivering these ligands to the PPAR receptors.

aromatase mRNA and inhibition of transcription (Fitzpatrick et al. 1997). PPARγ activation also causes decreased transcription of the aromatase gene and increased turnover of its mRNA (Mu et al. 2001) and is likely part of the program of LH-induced luteinization. Although aromatase is shut down during the process of luteinization, genes such as P450scc are constitutively expressed at high levels (Gonzalez-Robayna et al. 1999). Likewise, MEHP suppresses aromatase levels without altering levels of P450scc (Lovekamp and Davis 2001). Thus, by activating PPARγ, MEHP advances granulosa cell differentiation to a postovulatory phenotype without ovulation actually occurring.

PPAR-mediated effects of MEHP in the granulosa cell are consistent with those observed in the liver of rodents. As in the granulosa cell, the induction of 17β-HSD IV by MEHP in the liver depends on PPARα (Corton et al. 1997; Fan et al. 1998). Increased conversion of estradiol to estrone in both the liver and the granulosa cell contribute to decreased serum estradiol levels after *in vivo* DEHP treatment. Peroxisome proliferators such as DEHP also activate expression of FABP in the liver (Kaikaus et al. 1993; Poirier et al. 2001). In fact, it has been suggested that FABP acts as a gateway for PPAR agonists, directly interacting with PPAR in the nucleus (Wolfrum et al. 2001). The PPAR-mediated effects of DEHP/ MEHP depend on tissue distribution of the PPAR isoforms and the PPAR-responsive genes in each tissue. Further research will uncover the normal roles of the PPAR receptors in the ovary as well as their role in ovarian toxicity of environmental chemicals such as DEHP.

What Does Mechanism Tell Us about Health Effects?

Based on this model, one could predict the effects of DEHP in different tissues based on PPARα and PPARγ distribution and known PPAR-mediated effects. To date, most of what is known about the effects of PPARα and γ comes from studies in the major tissues of expression, the liver and adipose, respectively. PPARα mediates the effects of peroxisome proliferators in the liver, including induction of peroxisomal and microsomal enzymes for oxidation of fatty acids. PPARα is responsible for the hepatocarcinogenic effect of DEHP, as was shown by PPARα-deficient mice, which did not develop liver tumors when treated with DEHP (Ward et al. 1998). It is hypothesized that PPARα-mediated activation of metabolizing enzymes leads to oxidative stress and free radical production. Although this response is generally thought to contribute to the carcinogenic effect of DEHP in the liver, it may be causing more generalized toxicity in organs such as the ovary. In a microarray study using ovaries from animals treated with DEHP *in vivo*, a select number of altered genes suggest an oxidative stress response, including the aryl hydrocarbon receptor, CYP1B1, and epoxide hydrolase (Lovekamp-Swan. Unpublished data). Activation of metabolizing enzymes by PPARα also raises concern that exposure to DEHP may increase susceptibility to other toxicants requiring metabolic activation. PPARγ regulates lipid and glucose homeostasis and is responsible for the antidiabetic actions of thiazolidinediones such as troglitazone. PPARγ plays a critical role in adipocyte differentiation, and it is likely that DEHP/MEHP activates PPARγ in tissues other than the liver to alter normal pathways of differentiation (Ward et al. 1998). Alteration of differentiation pathways would help explain the teratogenic effects of DEHP because development is a critical time for differentiation.

Although all PPAR isoforms are detected in the ovary (Braissant et al. 1996), their physiological functions are not completely understood. PPARs work together to exert some effects (aromatase, FABP), but they also have isoform-specific effects (17β-HSD IV). It is important to understand why some cells express only one PPAR isoform, while others, such as the granulosa cell, express multiple isoforms, and whether the different PPAR isoforms interact with each other. Because PPARγ is highly expressed in adipocytes, MEHP may have effects here that have not previously been explored. For example, could the premature breast development in Puerto Rican girls with high blood phthalate levels (Colón et al. 2000) be explained by increased adipocyte differentiation in the breast after MEHP exposure?

PPARγ is expressed in both human and rodent ovary (Komar et al. 2001; Lambe and Tugwood 1996), and MEHP stimulates the transcriptional activity of both human and rodent PPARγ (Maloney and Waxman 1999). Studies in our laboratory have shown that in granulosa cells, MEHP is similar in action to another PPARγ activator, troglitazone. Thiazolidinediones, such as troglitazone, improve insulin sensitivity and have been used as treatments for type 2 diabetes and polycystic ovarian syndrome (PCOS). Not only does troglitazone inhibit estradiol production in granulosa cells (Mu et al. 2000), it also inhibits androgen production in ovarian theca cells (Schoppee et al. 2002). This is consistent with the finding that PCOS patients treated with troglitazone (200–400 mg) had significantly decreased plasma concentrations of androgens and estrogens after 3 months (Dunaif et al. 1996). Because PCOS is characterized by hyperandrogenism, suppression of androgen production by troglitazone is effective in the treatment of this disease. It is not known how the suppression of aromatase and estradiol production by troglitazone relates to the treatment of PCOS. Although troglitazone is now off the market due to its hepatotoxicity, other thiazolidinediones that activate PPARγ are currently used as antidiabetic agents. It would be useful to study whether any adverse effects on fertility have occurred due to suppressed estradiol levels. Humans may not be susceptible to the PPARα-mediated carcinogenic effects of DEHP in the liver due to a low level of PPARα expression (Palmer et al. 1998) and to species differences in PPARα responsiveness (Mukherjee et al. 1994). However, recent studies in mice lacking the PPARα receptor show that the fetotoxicity, teratogenicity, and testicular toxicity of DEHP occur at least partly independently of this isoform (Tickner et al. 2001; Ward et al. 1998). Further study is needed to compare the extent of PPARα versus PPARγ expression and activation in humans and rodents.

The structurally related phthalates present a challenge to toxicologists faced with identifying endocrine disruptors in the environment. While DEHP acts through a PPAR-mediated pathway to exert its effects on the female reproductive system, other phthalates such as DBP may activate only liver enzymes to alter steroid metabolism. It is clear that steroid hormones can be altered by several different mechanisms, including both receptor-mediated and non-receptor–mediated events. In recent years, the focus on environmental endocrine disruptors has led to the development of screening assays to identify such compounds. One such assay is designed to detect activity on the estrogen receptor. However, this assay would not identify compounds such as MEHP that work through a different receptor pathway to affect the endocrine system. Zacharewski et al. (1998) tested the estrogenic activity of eight commercial phthalate esters and found that only selected phthalates (DBP, BBP, DHP) exhibited weak estrogen receptor-mediated activity in some *in vitro* assays at high concentrations. However, none of the phthalates tested elicited classic *in vivo* estrogenic responses. Therefore, we conclude that phthalates are not acting via the estrogen receptor, but in fact act via PPAR. Although screening assays are valuable tools, it is important to remember that endocrine disruptors represent a vast array of compounds with different mechanisms of action.

How Is This Mechanism Relevant to Humans?

Because micromolar doses of phthalates are used for *in vitro* reproductive toxicity studies, the question of dose relevance often arises. The activation of PPARs by MEHP helps explain why such a dose is necessary. Unlike ligands of nuclear receptors, which bind in nanomolar concentrations, the endogenous PPAR ligands,

fatty acids, mediate their effects at micromolar concentrations (Willson et al. 2001; Xu et al. 1999). Natural PPAR ligands are lipophilic carboxylic acids, and the binding site of PPAR can recognize a range of lipophilic ligands, through relatively nonspecific hydrophobic interactions (Willson et al. 2001; Xu et al. 1999). The question is whether humans are exposed to phthalates at high enough concentrations to elicit a PPAR response.

Based on recent studies by the CDC, exposure to phthalate esters is widespread and occurs at higher levels than previously anticipated (Blount et al. 2000b). People at risk for reproductive toxicity of DEHP are likely to include those exposed occupationally as well as those exposed during medical procedures such as dialysis or blood transfusion. Of particular concern is DEHP exposure in newborns, who receive among the highest doses in the population from blood transfusions, extracorporeal membrane oxygenation, and respiratory therapy (Tickner et al. 2001). Because phthalates can cross the placenta, exposure to the developing fetus during critical points in development is also a concern, especially if DEHP is altering receptor-mediated differentiation pathways. It is clear that the environmental contaminant DEHP, through its metabolite MEHP, acts through a receptor-mediated signaling pathway to alter estradiol production in the ovary. The pathways leading to ovarian hormone production are similar in rodents and humans, and it is reasonable to assume that MEHP would suppress aromatase in the human ovary. It is possible that MEHP has effects throughout a woman's life span, from *in utero* development to puberty, reproductive years, and beyond. Because women represent a unique phthalate exposure group, further research is needed to determine levels of exposure among women in particular. By identifying the molecular mechanism of MEHP, we hope to identify molecular biomarkers of exposure. Future mechanistic toxicology and epidemiology studies will determine to what extent human exposure to phthalates impairs ovarian function and hence, women's health.

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