Exposure of Prepubertal Female Rats to Inhaled Di(2-ethylhexyl)phthalate Affects the Onset of Puberty and Postpubertal Reproductive Functions

Mingyue Ma,1 Tomoko Kondo, Susumu Ban, Tomohiro Umemura, Norie Kurahashi, Makoto Takeda, and Reiko Kishi

Department of Public Health, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan

Received April 24, 2006; accepted June 1, 2006

We evaluated the effects of inhaled di(2-ethylhexyl)phthalate (DEHP) on the onset of puberty and on postpubertal reproductive functions in prepubertal female rats. DEHP was administered by inhalation at doses of 0, 5, and 25 mg/m³ to groups of female rats for 6 h/day, 5 contiguous days/week from postnatal days (PNDs) 22 to 41 and to PND 84. The onset of puberty was determined by daily examination for vaginal opening (VO) and first estrous cycle. Reproductive function was evaluated by observing estrous cyclicity from PNDs 49 to 84. Upon completion of exposure, the rats were sacrificed at PND 42 and PNDs 85–88 during the diestrous stage. DEHP exposure advanced the age of VO and first estrous cycle, and serum cholesterol, luteinizing hormone, and estradiol levels were significantly elevated in the 25-mg/m³ DEHP group. Irregular estrous cycles were observed more frequently in DEHP-exposed rats, and serum cholesterol decreased in DEHP-exposed rats in adulthood; RT-PCR showed that the expression of aromatase mRNA, encoding a rate-limiting enzyme that catalyzes the conversion of testosterone to estradiol, was elevated in the 25-mg/m³ DEHP group. These data suggest that inhaled DEHP may advance the onset of puberty and alter postpubertal reproductive functions.

Key Words: DEHP; female; reproductive functions; inhalation.

Di(2-ethylhexyl)phthalate (DEHP) is used as a plasticizer for polyvinyl chloride (PVC) in the manufacture of a wide variety of consumer products. The release of DEHP directly into the atmosphere is believed to be the predominant mode of entry into the environment. Because of its low vapor pressure and poor water solubility, concentrations of DEHP in outdoor air and water are low (Kavlock et al., 2002). Indoor environments, however, are exposed to PVC in household products, floor coverings, wall coverings, and electronic devices (Wensing, 2005). Phthalate esters have been recognized as major indoor pollutants (Clausen et al., 2004; Wensing et al., 2005), and, second only to dietary exposure, indoor air is thought to be the most common medium through which the general population is exposed to DEHP (Kavlock et al., 2002). Children and adolescents are generally recognized as being particularly at risk for greater exposure to environmental contaminants because a child’s air intake relative to its body weight is high compared to adults (Chance and Harmsen, 1998). Koch et al. (2004) found that the level of total DEHP metabolites in children’s urine is significantly higher than in adult urine (parents and teachers) at a nursery school in Germany. There are only a few published studies about emissions of phthalates from different indoor materials. Uhde et al. (2001) reported that the concentration of DEHP from wallpaper was between 0.14 and 1 μg/m² in the room. However, for occupational exposure to DEHP, a TLV (Threshold Limit Value) -TWA (Time Weight Average) of 5 mg/m³ is recommended (ACGIH, 2001).

In recent years, DEHP has raised much public concern about its potential health hazards because of its reproductive effects in an animal model following oral exposure (Kavlock et al., 2002; Lovekamp-Swan and Davis, 2003). However, there are few studies on its reproductive toxicity following inhalation exposure; in this regard, Klimisch et al. (1992) assessed general toxicity and Kurahashi et al. (2005) studied male reproductive toxicity. By contrast, oral intake studies showed that DEHP can have testicular toxicity in male rodents (Akingbemi et al., 2001; Lamb et al., 1987), and it can also have ovarian toxicity in female rats (Davis et al., 1994a; Lovekamp-Swan and Davis, 2003). In vivo, DEHP decreases serum estradiol levels and prolongs estrous cycles in adult female rats (Davis et al., 1994a). In vitro, Mono(2-ethylhexyl)phthalate (MEHP) an active metabolite of DEHP, suppresses estradiol production by decreasing both mRNA and protein levels as well as the activity of aromatase (Davis et al., 1994b; Lovekamp and Davis, 2001). However, little is known about the in vivo effects of DEHP on the expression levels of steroidogenic enzymes.

Several studies in humans suggest an association between phthalate exposure and abnormal reproductive development in females. Exposure of female workers to high levels of phthalates was associated with a decreased pregnancy rate

1 For correspondence via fax: +81-11-706-7805. E-mail: mamingy@med.hokudai.ac.jp.

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DEHP EFFECTS ON REPRODUCTIVE FUNCTIONS

and a higher miscarriage rate (Aldyreva et al., 1975). Puerto Rican girls with premature breast development (thelarche) had higher levels of blood phthalates compared with normal girls (Colón et al., 2000). Endometriotic women showed significantly higher plasma DEHP concentrations than controls (Cobellis et al., 2003). However, there is no information on the effects of DEHP inhalation on the onset of puberty and postpubertal reproductive functions in female animal models. Therefore, the present study was designed to evaluate female reproductive toxicity upon exposure to high air doses of DEHP. We analyzed female hormone levels, cholesterol (the precursor for steroid biosynthesis) levels, and gene expression for all the enzymes involved in cholesterol transport and estradiol biosynthesis. The goals of the study were to investigate the effects of DEHP inhalation on the onset of puberty and to determine whether DEHP administered to prepubertal female rats affects reproductive functions in adulthood.

MATERIALS AND METHODS

Animals. Female Wistar-Imamichi strain rats, 21 days old, were obtained from the Institute for Animal Reproduction immediately after weaning (Sankyo Labo Service Corp, Tokyo, Japan). The animals were ranked by body weight and placed into treatment groups such that the mean body weight ± SD for all groups was similar in experiment 1. In experiment 2, the animals from the same litter were marked when they were purchased and then ranked by body weight and litter and placed into treatment groups to reduce the individual differences. Litters were equally distributed between the treatment groups. During the treatment and observation periods, animals were housed 5–6 per cage. The animals were maintained under controlled conditions of temperature (22 ± 0.5°C), humidity (40–50%), and light (12 h light/12 h dark) and provided water and food (rat pellets, Oriental Yeast Co., Ltd, Tokyo, Japan) ad libitum except during exposure. All animal experiments were performed under the Guidelines of the Animal Care and Use Committee of the Laboratory of Animal Experimentation at Hokkaido University.

Dosing solutions and procedures. DEHP (CAS No. 117-81-7), 99.0% pure, was purchased from Sigma-Aldrich Corp (St. Louis, MO). DEHP was continuously supplied by a special inhalation exposure device (SIS-G, Shibata Scientific Technology, Ltd, Tokyo, Japan). Exposure was carried out in stainless steel gas chambers. DEHP was administered to rats by inhalation at doses of 0, 5, and 25 mg/m³ for 6 h/day, 5 contiguous days/week. The DEHP vapor concentration in chambers was monitored daily with a gas chromatograph (GC-8APF, Shimadzu Corporation, Ltd, Kyoto, Japan).

Experimental design. According to the recommended TLV-TWA of 5 mg/m³ to DEHP by ACGIH (2001), the low dose was designed at 5 mg/m³ and high dose at 25 mg/m³. Two experiments were conducted. Three treatment groups (n = 10 per treatment) were included in experiment 1. Rats from postnatal days (PNDs) 22 to 84 were exposed to DEHP and were evaluated for changes in estrous cyclicity from PND 49 to 84. Detection of compound-related alterations in serum hormone concentrations could be best detected by sampling during the diestrous stage (Biegel et al., 1998), during which the animals were sacrificed at PNDs 85–88. These same treatment groups were used in experiment 2 (n = 12 per treatment), and food intake and water consumption were monitored daily. Rats were sacrificed on PND 42. In experiment 1, the mean concentrations of DEHP in the chamber were 4.10 ± 1.96 mg/m³ and 19.78 ± 3.69 mg/m³ for the two trials. In experiment 2, the mean concentrations of DEHP in the chamber were 5.21 ± 2.73 mg/m³ and 22.72 ± 7.59 mg/m³ for the two trials. Blood was collected by heart puncture under ether anesthesia, and rats were then dissected and lung, liver, kidney, ovary, and uterus weights were recorded in both experiments. In addition, serum was frozen at −20°C for subsequent measurement of follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol levels. Left ovaries were removed and immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction. The vagina, right ovary, and uterus were collected and fixed for histology.

Vaginal opening and estrous cyclicity. Between 9:00 A.M. and 10:00 A.M. each day (before DEHP exposure in the morning), body weight and vaginal opening (VO) were monitored for each rat. Beginning the day of VO, daily vaginal smears were collected and observed under a light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified cells to determine the age at the first estrous cycle and the long-term effects on estrous cyclicity. Similarly, the day of the first estrous cycle and the body weight on that day were recorded. After the first estrous cycle, vaginal smear monitoring was discontinued until PND 42. In experiment 2, vaginal smears were examined in the morning on PND 42 before necropsy. Vaginal smears were classified as diestrous (presence of leukocytes), proestrous (presence of nucleated epithelial cells), or estrous (presence of cornified cells) by Everett’s (1989) method. Regular estrous cycles were defined as having 4 day (including 2 day proestrous/estrus and 2 day diestrous) because female Wistar-Imamichi rats have a consistent 4-day estrous cycle at very high rates (Ishikawa and Hayashi, 1994). The number of cycles, days in estrus, and cycle length were recorded.

Serum hormone levels. Serum concentrations of FSH, LH, testosterone, and estradiol were measured by enzyme immunoassay (EIA). Commercial EIA kits were used for assays of FSH and LH (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK), testosterone (Cayman Chemical, Ann Arbor, MI), and estradiol (Endocrine Technologies, Inc, Newark, CA). Intra-assay and interassay coefficients of variations were, respectively, 7.4 and 11.4% for FSH, 10.9 and 7.6% for LH, 11.7 and 13.7% for testosterone, and 10.5 and 8.5% for estradiol. The sensitivities were 8.66 ng/ml, 0.1 ng/ml, 7.8 pg/ml, and 5 pg/ml for FSH, LH, testosterone, and estradiol, respectively.

Serum cholesterol levels. Serum concentration of total cholesterol was determined using an enzyme assay kit (BioVision Inc, Mountain View, CA). Total cholesterol was quantitated colorimetrically (570 nm) using a microplate reader (MTP-300, Corona Electric Corp, Hitachinaka, Japan).

Reverse transcription and quantitative RT-PCR. Total RNA was isolated from the left ovary using ISOGEN (Nippon Gene Co, Ltd., Tokyo, Japan) and the phenol-chloroform extraction method. The amount of total RNA was measured at 260 nm in a Smart Spec 3000 spectrophotometer (Bio-Rad, Tokyo, Japan), and its purity was estimated by the absorbance ratio 260/280 nm. cDNA was synthesized using 5 μg of total RNA and the First-Strand cDNA Synthesis kit (Amersham Biosciences UK, Ltd) according to the manufacturer’s instructions, and cDNA was stored at −20°C until the day of the experiment. Real-time RT-PCR was performed, and the results were analyzed on an ABI PRISM 7500 Sequence Detection System using SYBR Green PCR Master Mix reagent kits according to the manufacturer’s instructions for quantification of gene expression (Applied Biosystems, Foster City, CA). Amplicon sizes were 80–150 base pairs. The housekeeping gene encoding cyclophilin was used as a control. Cycling parameters were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. RT-PCR was performed in 96-well optical plates (Applied Biosystems) in triplicate. The primer sequences are listed in Table 1.

Statistical analysis. Data are expressed as means ± SDs or means ± SEMs. To approximate normal distributions, gene expression data for the hormone biosynthetic enzymes were logarithmically transformed. Differences were considered significant for p < 0.05. Differences among groups were analyzed by one-way analysis of variance followed by post hoc analysis by Dunn’s multiple comparisons test. Differences in body weight at the time of VO and first estrous cycle were analyzed by a general linear model, while adjusting for age because there was a confusing factor between body weight and age. The age at the first estrous cycle were analyzed by a general linear model, while adjusting for age because there was a confusing factor between body weight and age. The age at the first estrous cycle was evaluated for changes in estrous cyclicity and estradiol status. Statistical analyses were performed using SPSS Software for Windows, version 11.0 (SPSS Inc, Chicago, IL).
RESULTS

Weight and General Toxicity

None of the DEHP-exposed animals showed visible signs of toxicity in experiments 1 and 2. For animals exposed to 5 or 25 mg/m³ DEHP during experiment 1, mean body weight from exposure days 24 to 63 tended to be lower compared with unexposed animals and significantly reduced in the 25-mg/m³ DEHP group compared with controls (Fig. 1). In experiment 2, there were no significant changes in mean body weight (data not shown) and no statistically significant differences in daily food intake and water consumption. Furthermore, there were no significant differences in the absolute or relative weights of liver, kidney, lung, ovary, or uterus in experiments 1 or 2 (data not shown).

Pubertal Development

DEHP inhalation altered the age and body weight at which VO and first estrous cycle occurred in female rats (Table 2). As shown in Table 2, the age at VO and first estrous cycle were advanced in DEHP-exposed rats in both experiments 1 and 2. In experiment 1, VO occurred on PND 32.0 in the 0-mg/m³ group and on PNDs 30.3 and 29.7 in the 5- and 25-mg/m³ groups, respectively. In experiment 2, DEHP had nearly the same effect on the first estrous cycle as in experiment 1 (Table 2). The body weight at VO and first estrous cycle were not significantly lower in DEHP-exposed rats compared with controls in experiment 1 and 2, while adjusting for age.

Estrous Cyclicity, Endocrine Status, and Serum Concentrations of FSH, LH, Testosterone, and Estradiol

As shown in Figure 2, irregular estrous cycles were significantly more prevalent \((p < 0.05)\) in the 25-mg/m³ DEHP group (29%) compared with the 5-mg/m³ (12%) and unexposed (14%) groups in experiment 2.

In experiment 1, DEHP exposure did not significantly affect serum concentrations of FSH, LH, and estradiol when rats were

### TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Cyclophilin</td>
<td>CAATGGTGGCAAGTCCATTAC</td>
<td>CATTTGTGTTTGGTCCATT</td>
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<tr>
<td>STAR</td>
<td>ACTGCTAGACCAAGCTGGA</td>
<td>TGATTTCCCTTGACATTTG</td>
</tr>
<tr>
<td>P450xxc</td>
<td>TCCAGAGGACAGGTTCA</td>
<td>CAGGAGGTTCAATCTTTG</td>
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<tr>
<td>3β-HSD</td>
<td>CTAGTCACAGCAGCTTCTTATT</td>
<td>ACTACGCTGCTAGTTGCA</td>
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<tr>
<td>P450-17α</td>
<td>ACTACCGAGAAAGTGCTGTTAT</td>
<td>TGAGTCACAGTTAGCTTTG</td>
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<tr>
<td>17β-HSD</td>
<td>AAAGTCAGGTTCTGCAAGGT</td>
<td>ATGCCACTGCAAGGAGATG</td>
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<tr>
<td>CYP19</td>
<td>TGGAATCCATCAAGCAGCATT</td>
<td>GCGTGTGTAAGTGCTCCAGCAT</td>
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</table>

**Note.** Rat-specific primers were designed using Primer Express Software. Amplicon length = 80–150 base pairs and primer length = 20–24 base pairs.

### TABLE 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0 mg/m³</th>
<th>5 mg/m³</th>
<th>25 mg/m³</th>
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<tbody>
<tr>
<td>Age at VO (days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>32.0 ± 2.0</td>
<td>30.3 ± 1.3*</td>
<td>29.7 ± 1.3*</td>
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<tr>
<td>Experiment 2</td>
<td>31.8 ± 2.6</td>
<td>29.2 ± 2.0*</td>
<td>29.5 ± 2.8*</td>
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<tr>
<td>Body weight (g) at VO</td>
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<td></td>
<td></td>
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<tr>
<td>Experiment 1</td>
<td>87.7 ± 1.3</td>
<td>86.3 ± 1.2</td>
<td>85.2 ± 1.2</td>
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<tr>
<td>Experiment 2</td>
<td>76.7 ± 2.1</td>
<td>76.4 ± 2.0</td>
<td>77.5 ± 2.0</td>
</tr>
<tr>
<td>Age at first estrous (days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>32.7 ± 2.3</td>
<td>30.6 ± 1.3*</td>
<td>29.8 ± 1.2*</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>33.4 ± 2.3</td>
<td>31.0 ± 2.1*</td>
<td>30.6 ± 2.7*</td>
</tr>
<tr>
<td>Body weight (g) at first estrous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>90.8 ± 1.4</td>
<td>88.4 ± 1.1</td>
<td>86.8 ± 1.2</td>
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<tr>
<td>Experiment 2</td>
<td>86.4 ± 2.1</td>
<td>83.3 ± 2.0</td>
<td>84.6 ± 2.0</td>
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</table>

**Note.** Values are expressed as means ± SDs \((n = 10\) per group in experiment 1 and \(n = 12\) per group in experiment 2).

*Significantly different from 0 mg/m³ by Dunn’s multiple comparisons test comparing exposed group to control, \(p < 0.05\).

The data were analyzed by general linear model with adjustment for age.
sacrificed during the diestrous phase, while serum level of testosterone showed a tendency to decrease in DEHP-exposed rats, this decrease does not reach statistical significance (Fig. 3). Endocrine status (diestrous, proestrous, and estrous) for individual rats was examined the day on which the female was sacrificed in experiment 2, and DEHP exposure did not affect endocrine status by $\chi^2$ evaluation (Table 3). In experiment 2, serum from rats exposed to 25 mg/m$^3$ DEHP had significantly higher levels of estradiol (compared with the control) and LH (compared with the 5 mg/m$^3$ group and the control) (Fig. 4). Because of insufficient sample size, we could not statistically evaluate serum hormone levels at different stages of the estrous cycle.

**Serum Cholesterol Levels, and Gene Expression of Estradiol Biosynthetic Enzymes in the Ovary**

Compared with control rats, DEHP-exposed rats had a significantly lower average serum cholesterol level in experiment 1 but had a significantly higher level in experiment 2 (Table 4). We also used RT-PCR to measure mRNA expression from genes encoding all the enzymes involved in estradiol biosynthesis, including steroiogenic acute regulatory protein (StAR), P450 cholesterol side chain–cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxylase/17,20 lyase (P450-17α), 17β-hydroxysteroid dehydrogenase (17β-HSD), and aromatase (CYP19). In experiment 1, the mRNA level of aromatase, a rate-limiting enzyme in the conversion of testosterone to estradiol, was elevated by 145% in the 25 mg/m$^3$ DEHP group relative to the unexposed group (Fig. 5). There were no significant differences in mRNA levels of the other enzymes among groups, and this was the case for all the estradiol biosynthesis enzyme mRNAs in experiment 2 (Fig. 6).

**DISCUSSION**

To the best of our knowledge, our data are the first to demonstrate that non-oral exposure (inhalation exposure) to DEHP may affect the female reproductive system in animal model. We followed the onset of puberty and postpubertal reproductive functions in rats and found that exposure to inhaled DEHP may advance puberty onset and alter postpubertal reproductive functions accompanying the changes in hormone levels and total cholesterol in circulating blood.

**FIG. 2.** Effects of DEHP exposure on estrous cyclicity of female rats from PNDs 49 to 84. Regular cycle is defined as having a 4-day cycle length with 2 days of proestrous and estrous. Asterisk indicates significantly different from 0 mg/m$^3$, $p<0.05$. Hash mark indicates significantly different from 5 mg/m$^3$, $p<0.05$.

**FIG. 3.** Serum levels of FSH, LH, and estradiol in DEHP-exposed and control rats sacrificed during the diestrous stage of experiment 1. Values are expressed as means ± SDs ($n=10$ per group). Differences among groups were analyzed by one-way analysis of variance.

**FIG. 4.** Serum levels of FSH, LH, and estradiol in DEHP-exposed and control rats sacrificed on PND 42 of experiment 2. Values are expressed as means ± SDs ($n=12$ per group). Asterisk indicates significantly different from 0 or 5 mg/m$^3$ by Dunn’s multiple comparisons test, $p<0.05$.  

**TABLE 3**  
Endocrine Status at Necropsy on PND 42 in Experiment 2

<table>
<thead>
<tr>
<th></th>
<th>0 mg/m$^3$</th>
<th>5 mg/m$^3$</th>
<th>25 mg/m$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diestrous</td>
<td>5</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Proestrous/estrous</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Total animals</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

*Note. Data represent the number of observed animals in each stage of the estrous cycle at necropsy, as characterized by vaginal epithelial cytology.*
Although DEHP toxicity to the male reproductive system has been studied (Kavlock et al., 2002), there is a little information on DEHP toxicity to the female reproductive system (Davis et al., 1994a; Hirosawa et al., 2006; Lovekamp-Swan and Davis, 2003). Some studies have clearly shown the susceptibility of young (neonatal/pubertal) rats to testicular insult by DEHP compared to their adult counterparts (Dostal et al., 1988). We thus chose the prepubertal female as our study subject and referred the Female Pubertal Protocol on the influence of prepubertal exposure to endocrine-disrupting chemicals reviewed by Goldman et al. (2000). VO is indicative of puberty in female rats and usually occurs on the day after the first preovulatory surge of gonadotropins (Goldman et al., 2000). We thus used VO and the first estrous cycle as measures of the end of puberty onset, which is followed by important hormonal changes in reproductive organs. When the onset of puberty advanced in experiment 1, we used experiment 2 to verify our observations with regard to puberty development and to investigate the changes in circulating hormone levels upon the onset of puberty. We found that the age at VO and the first estrous cycle were advanced in DEHP-exposed rats in both experiments 1 and 2. Advanced puberty may derive from either gonadal or pituitary disorders (Goldman et al., 2000). When hormone levels were determined, serum estradiol and LH levels were significantly higher in rats exposed to high levels of inhaled DEHP. Females with high LH and estradiol experience early onset of puberty, which may reflect an LH-mediated triggering of final ovarian follicle maturation and an increase in estrogen secretion (Docke and Dornner, 1979). Our results support the above conclusion. It has been suggested that assessing the onset of puberty may be a sensitive marker for interactions between environmental conditions and genetic susceptibility that may influence the pubertal process (Parent et al., 2003). Our results indicate that inhaled DEHP may induce LH secretion by the pituitary and lead to a high estradiol level, which may affect pubertal development. Therefore, short-time DEHP exposure via inhalation to prepubertal animals might affect hypothalamic-pituitary-ovarian axis function and alter the timing of female puberty. Previous studies have demonstrated that some phthalate esters, including DEHP, are weakly estrogenic in vitro but have no estrogenic activity in vivo (Jobling et al., 1995; Zacharewski et al., 1998). Our present results, however, do not support these findings. In utero and lactational exposure to DEHP may delay pubertal onset in female offspring (Grande et al., 2006). Our results may provide a mechanism for the report of premature mammary gland development in young girls associated with high serum DEHP levels (Colon et al., 2000). Puberty has been considered as a period of greater vulnerability to the effects of DEHP (Akingbemi et al., 2004). A recent follow-up study on extracorporeal membrane oxygenation in adolescents exposed to DEHP as neonates (Rais-Bahrami et al., 2004) showed no significant adverse effects of DEHP on physical growth and pubertal maturity in males or females; this conclusion, however, was based on a small sample population and thus must be confirmed by another study.

Regarding adult reproductive functions, the number of irregular estrous cycles was significantly increased in the 25 mg/m³ DEHP group in experiment 1, although there were no significant changes in hormone levels. Davis et al. (1994a) showed that DEHP decreased serum estradiol and prolonged estrous cycles. By comparison, our study shows that serum estradiol level did not change significantly upon DEHP

### TABLE 4

<table>
<thead>
<tr>
<th>DEHP (mg/m³)</th>
<th>Experiment 1 (mM)</th>
<th>Experiment 2 (mM)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>1.65 ± 0.17</td>
<td>1.54 ± 0.23</td>
</tr>
<tr>
<td>5</td>
<td>1.36 ± 0.24*</td>
<td>1.83 ± 0.27*</td>
</tr>
<tr>
<td>25</td>
<td>1.31 ± 0.30*</td>
<td>1.93 ± 0.32*</td>
</tr>
</tbody>
</table>

*Significantly different from 0 mg/m³, p < 0.05.

**Note.** Values are expressed as means ± SDs (n = 10 per group in experiment 1 and n = 12 per group in experiment 2).

**FIG. 5.** Effects of DEHP exposure on gene expression of estradiol biosynthesis enzymes in the ovary in rats sacrificed during the diestrous stage of experiment 1. Gene expression data for the hormone biosynthetic enzymes were logarithmically transformed. Values are expressed as means + SDs (n = 10 per group). Asterisk indicates significantly different from 0 or 5 mg/m³ by Dunn’s multiple comparisons test, p < 0.05.

**FIG. 6.** Effects of DEHP exposure on gene expression of estradiol biosynthesis enzymes in the ovary in rats sacrificed on PND 42 of experiment 2. Gene expression data for the hormone biosynthetic enzymes were logarithmically transformed. Values are expressed as means + SDs (n = 12 per group). Differences among groups were analyzed by one-way analysis of variance.
DEHP effects on reproductive functions

We thank Drs Akihiro Matsuura and Yasuko Matsuura for technical support and assistance with animal care. This work was supported by the Fund for Endocrine Disrupters provided by the Japan Ministry of Environment and Health Science Research Grant from the Japan Ministry of Health, Labor and Welfare.

Acknowledgments

Our study has a few notable limitations. First, according to the Female Pubertal Protocol (Goldman et al., 2000), treatments should be administered daily beginning on PND 22 and continue through PND 42, whereas the treatments in our study were conducted for 6 h/day for 5 contiguous days/week because of the limited availability of the inhalation device. A second limitation of our study is that we considered ovary as the target organ based on well-established research on ovary toxicity (Lovekamp-Swan and Davis, 2003), and we did not collect pituitary samples. However, our data show that inhalation of DEHP may cause the pituitary to induce LH secretion during pubertal development. Third, since we did not use a physiologically based pharmacokinetic model of DEHP exposure via inhalation, we could not obtain data on the distribution of DEHP and its metabolites in target tissues.

In conclusion, our study shows that DEHP exposure via inhalation may advance puberty onset and alter postpubertal reproductive functions. Although the general population has little chance of exposure to DEHP via inhalation at doses used in our study (5 mg/m^3 is recommended for occupational exposure to DEHP during the compounding of DEHP with PVC resins), similar (or other) effects of DEHP may occur at lower levels, and thus, the consequences of DEHP inhalation should be studied in detail. Future work is needed to determine the distribution of DEHP and its metabolites in target tissues using physiologically based pharmacokinetic models via inhalation exposure.

AcKnoWleDgments

We thank Drs Akihiro Matsuura and Yasuko Matsuura for technical support and assistance with animal care. This work was supported by the Fund for Endocrine Disrupters provided by the Japan Ministry of Environment and Health Science Research Grant from the Japan Ministry of Health, Labor and Welfare.
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