Perinatal exposure to diethyl-hexyl-phthalate induces obesity in mice

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
   3.1. Cell culture and treatment
   3.2. Oil red O staining
   3.3. Animal care and DEHP exposure
   3.4. Quantitative real-time PCR analysis
   3.5. Measurement of obesity parameters
   3.6. Data analysis
4. Results
   4.1. DEHP has no effect on adipocyte differentiation in 3T3-L1 cells
   4.2. DEHP induces adipogenic regulators and markers in vivo
   4.3. Perinatal exposure to DEHP leads to obesity of offspring in mice model
5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

The environmental obesogen hypothesis proposes that exposure to endocrine disruptors during developmental “window” contributes to adipogenesis and the development of obesity. Implication of environmental endocrine disruptor such as diethyl-hexyl-phthalate (DEHP) on adipose tissue development has been poorly investigated. Here, we evaluated the effects of DEHP on adipocyte differentiation in vitro and in vivo, and explored potential mechanism involved in its action. DEHP had no effect on adipocyte differentiation in the murine 3T3-L1 cell model, whereas DEHP induced the expression of transcriptional factors peroxisome proliferator-activated receptor (PPAR) gamma, CCAAT/enhancer-binding protein (C/EBP) alpha and sterol regulatory element binding factor 1 (Srebf1) as well as downstream target genes required for adipogenesis in vivo. Furthermore, perinatal exposure to DEHP had an impact on filial adipogenesis. Body weight, adipose tissue deposition, serum lipids and glucose levels were significantly elevated in offspring at postnatal day (PND) 60. Therefore, these results suggested that perinatal exposure to DEHP might be expected to increase the incidence of obesity in offspring and could act as a potential chemical stressor for obesity and obesity-related disorders.

2. INTRODUCTION

The prevalence of obesity is increasing in developed countries, and that in the developing world is rapidly catching up. Obesity can therefore be seen as a global pandemic. The consequences of this are the significant morbidity and premature mortality associated with the serious medical diseases, including diabetes, hyperlipidemia, hypertension, cardiovascular disease, osteoarthritis and many forms of cancer (1-3). Obesity is a problem for all ages, but it is of particular concern for children since the number of children and adolescents who are overweight, or at risk for being overweight, has risen in parallel with that reported in adults (4). While the unbalance between caloric intake and expenditure is thought to be the most common cause of obesity, the reason for its surge might not be only related to excessive intake of food and an increasingly sedentary lifestyle (5). In the modern world, a large and ever increasing number of synthetic chemical products permeate the diet and environment and human exposure to these is unavoidable. Epidemiology studies indicate that the serum/urine concentrations of some of these chemicals have been found to be associated with the onset and incidence rate of obesity and diabetes (6-15). This has led to the hypothesis that a subset of environmental
DEHP promoted adipogenesis

pollutants that interfere with the body’s homeostatic controls might add one more risk factor for obesity and could be considered as “environmental obesogens” (5). The “obesogens” inappropriately regulate lipid metabolism and adipogenesis promoting obesity (16-19).

Phthalates are widespread environmental micro-pollutants used in a variety of products, including cosmetics, shampoos, soap, lubricants, pesticides, and paints; they are also used as a softener of polyvinyl chloride (PVC). Human exposure to phthalates mainly occurs through food, because of the use of PVC in wrapping materials and food processing (20). DEHP is one of the most widely used and studied phthalates, so its biological effects are of major concern but so far elusive. In the past, research on endocrine disruption caused by DEHP has mainly focused on reproductive defects (21-23). Previous studies have shown that monoethyl-hexyl-phthalate (MEHP), one major metabolite of DEHP, is a selective PPAR gamma modulator and induces adipocyte differentiation through PPAR gamma in vitro (24). However, it is unclear whether low doses of in utero DEHP exposure will disrupt adipose tissue homeostasis and promote development of obesity and obesity-associated cardiovascular disease.

In the present study, we demonstrated that DEHP had no effect on adipocyte differentiation in the murine 3T3-L1 cell model, whereas DEHP induced the expression of transcriptional factors PPAR gamma, C/EBP alpha and Srebf1 as well as downstream target genes required for adipogenesis in vivo. To further determine whether perinatal exposure to DEHP could have an impact on filial adipogenesis, we defined body weight, adipose tissue deposition, serum lipids and glucose levels in offspring at PND 60. Our results suggested that perinatal exposure to DEHP might be expected to increase the incidence of obesity in offspring and could act as a potential chemical stressor for obesity and obesity-related disorders.

3. MATERIALS AND METHODS

3.1. Cell culture and treatment

3T3-L1 mouse embryo fibroblasts, purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin. Cells were maintained as subconfluent cultures at 37°C in a humidified 5% CO2 atmosphere with media changes every 2-3 days. For differentiation assays, cells were seeded at 6×10^4 cells per well into polylysine-coated 6-well cell culture plates in 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA)/DMEM, after which cultures were grown for 2 days and then treated with different concentrations of DEHP (Sigma-Aldrich, St. Louis, MO, USA) or troglitazone (Sigma-Aldrich, St. Louis, MO, USA) with 10 µg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA) for 8 days. An 8-day treatment with 10µg/ml insulin only served as a control. Media and drug treatments were renewed every 2 days. After 8 days, cells were stained with Oil Red O for lipid droplet accumulation as described below.

3.2. Oil red O staining

Cells were washed with sterile phosphate-buffered saline (PBS), fixed with 10% formaldehyde for 15 min at room temperature (25), washed with distilled water, and then stained with filtered Oil Red O solution (4 g/liter, 60% isopropanol) for 15 min. Excess stain was removed by washing three times with distilled water.

3.3. Animal care and DEHP exposure

C57BL/6J mice were bred in the animal facility of National Research Institute for Family Planning (mouse protocol SYXK 2009-0033) and housed in a room with a 12-hr light/dark cycle (lights on at 7:30 a.m. and off at 7:30 p.m.) with access to food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of NRIFP. Six-week-old male mice received an intraperitoneal (ip) injection of DEHP (0.5 mg/kg body weight (b.w.)), troglitazone (0.5mg/kg b.w.) or vehicle (olive oil) for 24 hours. Animals were sacrificed by decapitation. Liver and epididymal adipose tissue were collected for total RNA extraction as described above. Pregnant mice were dosed by gavage with DEHP (0.05, 0.25 or 0.5 mg/kg b.w.) or vehicle (olive oil) from day 12 of gestation every 24 h until day 7 of lactation (PND 7 of offspring). Pups were weaned at 3 wk of age (PND 21) and maintained on standard rodent chow.

3.4. Quantitative real-time PCR analysis

Total RNA from C57BL/6j mouse tissues was isolated using RNeasy mini kit (QIAGEN, Germantown, MD, USA) and reversed transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercul es, CA, USA) according to the manufacturer’s instructions. Triplicate cDNA samples (15 ng/reaction) were analyzed by quantitative real-time PCR on an ABI prism 7500 thermal cycler (Applied Biosystems, Foster City, CA, USA) using FastStart universal SYBR Green master (Roche, Mannheim, Germany). Fold changes in expression levels were calculated after normalization to 18s rRNA. Gene-specific primers are shown in Table 1.

3.5. Measurement of obesity parameters

After 8-week-old pups were weighed, animals were sacrificed by asphyxia. Liver, epididymal/parametrial and perirenal fat pads were harvested and weighed. Blood was collected by cardiac puncture after an overnight fasting. Blood glucose, serum total cholesterol and triglycerides (TG) were measured by colorimetric kit assays (Leadman, Beijing, China) and analyzed using a fully-automatic biochemistry analyzer (Hitachi, Tokyo, Japan).

3.6. Data analysis

cDNA samples analyzed by quantitative real-time PCR were performed in triplicate and were repeated three times. All data were obtained from at least three independent experiments. Data are represented as means ± S.E.M., and n indicates the number of experiments. Multiple group comparisons were performed using one-way analysis of variance (ANOVA). For studies examining...
DEHP promoted adipogenesis

4. RESULTS

4.1. DEHP has no effect on adipocyte differentiation in 3T3-L1 cells

In the murine 3T3-L1 preadipocyte cell model, adipogenic signals induce late differentiation markers PPAR gamma and C/EBP alpha, which drive terminal adipocyte differentiation and lipid accumulation (26-29). We therefore examined the effects of DEHP on the differentiation of 3T3-L1 preadipocyte and compared its effect to PPAR gamma agonist troglitazone. An 8-day treatment of 3T3-L1 preadipocyte with the PPAR gamma agonist troglitazone in the presence of insulin strongly induced adipogenesis in a dose-dependent manner, as evidenced by Oil Red O staining, whereas insulin alone had only mild effect (Figure 1). Likewise, DEHP in the presence of insulin had the similar effect on 3T3-L1 preadipocyte differentiation with insulin alone (Figure 1), suggesting DEHP was inefficient in inducing adipocyte differentiation in vitro.

4.2. DEHP induces adipogenic regulators and markers 
in vivo

When ingested, DEHP is converted by intestinal lipases to its monoester equivalent MEHP, which is then preferentially absorbed and functions in vivo (30). Therefore, we asked whether DEHP could perturb key modulators of adipogenesis and lipid homeostasis, and further promote adipogenesis in vivo. To characterize the adipogenic actions of DEHP, we performed gene expression analysis on critical regulators by quantitative reverse transcriptase-PCR. Liver and epididymal adipose tissue of 6-wk-old male mice dosed for 24 h with DEHP (0.5 mg/kg b.w.), troglitazone (0.5 mg/kg b.w.), or vehicle (olive oil) were dissected for total RNA extraction and target genes analysis.

A pronounced expression increase induced by DEHP was observed for adipogenic markers such as PPAR gamma, aP2, LPL (adipocyte-specific fatty acid binding protein), LPL (lipoprotein lipase) and FAS (fatty acid synthetase), whereas troglitazone significantly induced expression of PPAR gamma as well as the downstream target genes such as aP2 and FAS in adipose tissue (Figure 2A).

Liver is a key organ involved in metabolism, as it controls synthesis of many nutrients including lipids and carbohydrates. The results showed that troglitazone significantly promoted PPAR gamma transcription and induced the expression of PPAR gamma target genes such as aP2, LPL and FAS in liver. In addition, troglitazone was also able to clearly up-regulate the expression level of Sreb1 transcript, another critical transcriptional mediator during adipogenesis, which regulates transcription of many lipid genes and participates in the generation of endogenous PPAR gamma ligands (27, 31). Similarly, DEHP significantly increased the expression of adipogenic transcriptional factors PPAR gamma, C/EBP alpha and Sreb1 in liver. In addition, hepatic expression of PPAR gamma target genes such as aP2, FAS and LPL was up-regulated by 2-fold, 6-fold and 1.5-fold, respectively, thus confirming that the newly formed PPAR gamma was functionally active (Figure 2B). Therefore, the coordinate increased expression of adipogenic transcriptional regulators as well as the downstream target genes suggests that DEHP stimulates fatty acid uptake, triglyceride synthesis and lipid accumulation.

Taken together, these results indicate that DEHP exposure enhances adipogenesis in vivo through an increase of transcription of critical genes implicated in adipocyte metabolism.

4.3. Perinatal exposure to DEHP leads to obesity of offspring in mice model

Based on its activation responses in vivo, we reasoned that perinatal exposure to DEHP, which is a sensitive period of adipose tissue development, would disrupt normal endocrine controls and adipogenic signals. Therefore, pregnant mice were dosed by gavage with DEHP (0.05, 0.25 or 0.5 mg/kg b.w.) or vehicle (olive oil) from day 12 of gestation until day 7 of lactation (PND 7 of offspring). Eight-week-old pups were weighed. The results showed that DEHP-treated offspring exhibited obese phenotype at the dose of 0.25 mg/kg b.w., whereas the other two doses of DEHP (0.05 or 0.5 mg/kg b.w.) had no significant effect on body weight gain in offspring despite the mildly obese tendency of female offspring at the dose of 0.05 mg/kg b.w. (data not shown).

In DEHP-exposed female offspring, the mean body weight (16.91±0.61 g) was greater than that of controls (15.86±0.16 g) (Figure 3A). To determine whether the increased body weight reflects body composition changes, we weighed liver and two distinct fat pads (parametrial and perirenal). Liver weight had no significant increase in DEHP-treated group compared with vehicle controls. In contrast, both parametrial (0.20±0.02 g) and perirenal fat mass (0.10±0.01 g) significantly increased compared with
DEHP promoted adipogenesis

Figure 1. Effect of DEHP on 3T3-L1 preadipocyte differentiation. Confluent 3T3-L1 cells were treated for 8 days in the presence of DMSO, 10 µg/ml insulin alone or insulin plus ligands at the indicated concentrations. Differentiated adipocytes were evidenced by Oil Red O staining. Scale bar represents 200 µm. Ins, insulin; Trog, Troglitazone.

controls (parametrial: 0.15±0.01 g; perirenal: 0.05±0.01 g) (Figure 3B). To assess whether these alteration in body weight and fat pads weight were related to changes in circulating levels of nutrients, we measured the levels of serum total cholesterol, TG and blood glucose. Consistent with the increased fat mass, serum total cholesterol, TG and blood glucose levels were increased about 11%, 49% and 45%, respectively, compared with the controls (Figure 3C).

Likewise, in male offspring, perinatal exposure to DEHP significantly increased the body weight gain (22.46±0.86 g) compared with controls (19.62±0.31 g) (Figure 4A). Moreover, the increase in body weight in male offspring was related to an increase in liver and adipose
DEHP promoted adipogenesis

Figure 2. In vivo induction effect of DEHP on adipogenic modulators. Six-week-old C57BL/6J male mice were dosed with vehicle (olive oil), DEHP (0.5 mg/kg b.w.) or troglitazone (0.5 mg/kg b.w.) by ip injection. Animals were sacrificed after 24 hours and cDNA was prepared from epididymal adipose tissue or liver for quantitative real-time PCR analysis. Expression levels were standardized to levels of 18s rRNA mRNA by calculating cycle thresholds (Ct). The data shown are representative of three independent experiments. Values are mean ± S.E.M. (n=3). *P<0.05, **P<0.01, ***P<0.001. Trog, Troglitazone.

depot weight. We tested that liver and epididymal fat pads weight in DEHP-treated group increased by 16% and 48%, respectively, compared with the control group, while perirenal fat pads weight did not differ significantly from the control group (Figure 4B). To determine whether the difference in body weight and body composition had an impact on lipid and glucose homeostasis, we detected serum levels of total cholesterol, TG and blood glucose in both DEHP-exposed and control males. Serum levels of total cholesterol, TG and blood glucose in DEHP-treated group after 16 hr of fasting were increased about 13%, 26% and 30%, respectively, compared with the control group (Figure 4C).

Overall, these results suggest that perinatal exposure to DEHP increases the susceptibility to obesity in offspring, and the susceptibility is highly specific for the exposure dose used.

5. DISCUSSION

In the study, we demonstrated that DEHP induced the expression of transcriptional factors PPAR gamma, C/EBP alpha and Srebfl as well as downstream target genes required for adipogenesis in vivo. Furthermore, perinatal exposure to DEHP increased body weight, liver weight and fat pads weight, and improved serum total cholesterol, triglyceride and glucose levels in offspring. Thus, our results suggested that perinatal exposure to DEHP might be expected to increase the incidence of obesity in offspring and could act as a potential chemical stressor for obesity and obesity-related disorders.
DEHP promoted adipogenesis

Feige et al. (24) showed that MEHP induced adipogenesis by modulating PPAR gamma activity in cellular models, whereas our studies exhibited the DEHP had no effect on inducing adipocyte differentiation in 3T3-L1 cell model. We reason that it may be related to DEHP parent compound which is inactive in vitro, because when ingested through contaminated food and medical devices, DEHP is converted by intestinal lipases to its monoester equivalent MEHP, which is then preferentially absorbed and functions in vivo (30). We therefore detected the action of in vivo exposure to DEHP on adipogenesis and lipogenesis. During adipogenesis, a cascade of transcriptional factors is involved, among which PPAR gamma is considered the crucial determinant of adipocyte fate (27). Moreover, PPAR gamma alone or in cooperation with C/EBP alpha induces the transcription of many adipocyte-specific genes involved in adipogenesis (32). Also important is Srebf1, which regulates transcription of many lipid genes and participates in the generation of endogenous PPAR gamma ligands (27, 31). The analysis of gene expression profiles demonstrated that in vivo exposure to DEHP was efficient in activating critical transcriptional regulators and markers. Especially in liver tissue, DEHP induced the coordinated expression of transcriptional factors PPAR gamma, C/EBP alpha and Srebf1 as well as adipocyte-specific genes aP2, FAS and LPL, suggesting that DEHP could promote obesity once it was converted to MEHP metabolite in vivo.

Previous studies mainly focused on the effects on reproductive toxicity at high dose of phthalates exposure (21-23). Although much proof has demonstrated that low dose of endocrine disrupting chemicals (EDCs) during development can interfere with normal function of endocrine system, consequently inducing overweight and obesity later in life (16, 33-37), it is not fully understood whether low doses of in utero DEHP exposure promotes the onset of obesity and metabolic syndrome in offspring. In the study, pregnant mice were dosed by gavage with DEHP (0.05, 0.25 or 0.5 mg/kg b.w.) or vehicle (olive oil) during the critical stages of differentiation (developmental “window”). Our results demonstrated for the first time that the body weight of 8-week-old offspring including females and males was significantly increased at the dose of 0.25 mg/kg b.w., but the lower (0.05 mg/kg b.w.) or higher (0.5 mg/kg b.w.) dose of DEHP had no effect on body weight gain. We infer that there may be non-traditional dose response curves such as an inverted U-shaped curve in body weight increase in offspring. We also observed that in female offspring, the increase of body weight was associated with an increase of parametrial and perirenal fat pads. It is currently unknown whether the increased adiposity in vivo results from an increase in adipocyte precursor cell number, enhanced adipocyte differentiation from the same number of precursors, an increase in adipocyte size without an increase in number, or some combination of these. Liver weight in female offspring was not significantly different between the two groups, we thus infer that a statistical significance continues to be further studied at older ages. Likewise, in male offspring, the differences of perirenal fat pads weight need to be further studied for the long-term consequences. In addition to increased body weight and body composition, serum total cholesterol, triglycerides and glucose levels were improved in offspring. Therefore, the accumulation of fat mass could...
DEHP promoted adipogenesis

**Figure 4.** Perinatal exposure to DEHP induces obese phenotype in male offspring. Pregnant mice were dosed by gavage with vehicle (olive oil) or DEHP (0.25 mg/kg b.w.) from day 12 of gestation until PND 7. (A) Body weight of 8-week-old male offspring. Body weight of DEHP-treated pups was higher than that of controls. (B) Weight of liver, epididymal and perirenal fat pads in male pups. Liver and epididymal fat pads weight in DEHP-treated pups were significantly higher than those in controls. Perirenal fat mass between the two groups had no significant difference. (C) Serum levels of total cholesterol, TG and blood glucose in DEHP-treated male pups were significantly higher than those in controls. Values are mean ±S.E.M. (n=16 vehicle-treated male pups from five litters; n=11 DEHP-treated male pups from three litters). *p<0.05, **p<0.01, ***p<0.001. TG, triglycerides.

In conclusion, the present study demonstrates that in vivo exposure to DEHP efficiently activates transcriptional factors PPAR gamma, C/EBP alpha and Srebf1 as well as downstream target genes required for adipogenesis. Consequently, direct exposure to DEHP through placenta and milk increases body weight, adipose storage, serum lipid and glucose levels in offspring. Increased adipose storage could be a major public health concern in relation to the epidemic obesity and obesity-related disorders. Nevertheless, more precise molecular mechanisms involved in the adipogenesis stimulated by DEHP are needed to be further explored, and the long-term consequences of DEHP exposure on body weight homeostasis are needed to be further understood.

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5. REFERENCES


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DEHP promoted adipogenesis


DEHP promoted adipogenesis


**Abbreviations:** aP2: adipocyte-specific fatty acid binding protein; C/EBP alpha: CCAAT/enhancer-binding protein alpha; DEHP: diethyl-hexyl-phthalate; EDCs: endocrine disrupting chemicals; FAS: fatty acid synthetase; ip: intraperitoneal; LPL: lipoprotein lipase; MEHP: monoethyl-hexyl-phthalate; PND: postnatal day; PPAR: peroxisome proliferator-activated receptor; PVC: polyvinyl chloride; Srebf1: sterol regulatory element binding factor 1; TG: triglycerides.

**Key Words:** Diethyl-hexyl-phthalate, Perinatal exposure, Peroxisome proliferator-activated receptor gamma, Adipogenesis, Obesity

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