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The endocrine disruptor diethylstilbestrol induces adipocyte differentiation and promotes obesity in mice

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ABSTRACT

Epidemiology studies indicate that exposure to endocrine disruptors during developmental "window" contributes to adipogenesis and the development of obesity. Implication of endocrine disruptor such as diethylstilbestrol (DES) on adipose tissue development has been poorly investigated. Here we evaluated the effects of DES on adipocyte differentiation *in vitro* and *in vivo*, and explored potential mechanism involved in its action. DES induced 3T3-L1 preadipocyte differentiation in a dose-dependent manner, and activated the expression of estrogen receptor (ER) and peroxisome proliferator-acivated receptor (PPAR) γ as well as its target genes required for adipogenesis *in vitro*. ER mediated the enhancement of DES-induced PPARγ activity. Moreover, DES perturbed key regulators of adipogenesis and lipogenic pathway *in vivo*. *In utero* exposure to low dose of DES significantly increased body weight, liver weight and fat mass in female offspring at postnatal day (PND) 60. In addition, serum triglyceride and glucose levels were also significantly elevated. These results suggest that perinatal exposure to DES may be expected to increase the incidence of obesity in a sex-dependent manner and can act as a potential chemical stressor for obesity and obesity-related disorders.

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 (Collins, 2005; Mokdad et al., 1999, 2003). 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 (Ogden et al., 2002). 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 (Desvergne et al., 2009).

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 (Gladen et al., 2000; Goncharov et al., 2008; Hatch et al., 2008; Lee et al., 2007, 2008; Pelletier et al., 2002; Smink et al., 2008; Stahlhut et al., 2007; Takeuchi et al., 2004; Vasiliu et al., 2006). This has led to the hypothesis that a subset of chemical toxins that interfere with the body's homeostatic controls might add one more risk factor for obesity and could be considered as " obesogens". The "obesogens" inappropriately regulate lipid metabolism and adipogenesis promoting obesity (Baillie-Hamilton, 2002; Grün and Blumberg, 2007; Heindel, 2003; Newbold, 2010; Tremblay and Chaput, 2008).

Diethylstilbestrol (DES), a highly potent orally available synthetic estrogen, was widely prescribed to women for estrogen deficient states as hormone replacement therapy between the 1940s and 1980s. An estimated 2–8 million pregnant women worldwide are at risk of miscarriage. To date, studies of DES exposure have focused largely on the reproductive systems, which established the long-term endocrine disrupting consequences of DES exposure for multiple generations. DES exposed mothers have an increased risk of developing breast cancer, whereas DES daughters display a high incidence of reproductive tract abnormalities, vaginal adenocarcinoma, infertility and autoimmune disorders (Giusti et al., 1995); DES sons also exhibit increased reproductive health risks (Palmer et al., 2009). There are a limited number of

Abbreviations: aP2, adipocyte-specific fatty acid binding protein; C/EBPα, CCAAT/enhancer-binding protein alpha; DES, diethylstilbestrol; EDCs, endocrine disrupting chemicals; ER, estrogen receptor; ERR, estrogen-related receptor; FAS, fatty acid sythetase; ip, intraperitoneal; LPL, lipoprotein lipase; PND, postnatal day; PPARγ, peroxisome proliferator-activated receptor gamma; PVC, polyvinyl chloride; Srebf1, sterol regulatory element binding factor 1; TG, triglycerides.

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studies focusing on obesity and obesity-related disorders caused by developmental exposure to DES. Newbold et al. demonstrated that neonatal exposure to a low dose of DES (0.001 mg/kg/day) on days 1–5 caused an increase in body weight starting at 6 weeks of age in mice (Newbold et al., 2005), whereas a high dose (1 mg/kg/day) administered caused an initial body weight reduction, followed by a period of "catch-up" growth (Newbold et al., 2008). The biological effects of DES on body homeostasis are hence of major concern but so far elusive.

In the present study, we tested the dose-dependent effects of DES on adipocyte differentiation using the murine 3T3-L1 cell model. The confluent cultures of 3T3-L1 fibroblasts treated by DES presented an increase in glycerol-3-phosphate dehydrogenase (GPDH) activity, suggesting that DES by itself can promote 3T3-L1 fibroblasts to differentiate into adipocytes. During differentiation, DES induced the expression of estrogen receptor (ER) and peroxisome proliferator-acivated receptor (PPAR) γ as well as its target genes required for adipogenesis. Furthermore, we observed that DES activated PPARγ and its target genes *in vivo*. To determine whether perinatal exposure to DES could have an impact on filial adipogenesis, we defined adipose tissue deposition, serum lipids and glucose levels in offspring at PND 60. Our results suggested that perinatal exposure to DES might be expected to increase the incidence of obesity in female offspring and could act as a potential chemical stressor for obesity and obesity-related disorders.

Materials and methods

Cell culture and treatment. 3T3-L1 mouse embryo fibroblasts, purchased from Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) were maintained in standard Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% Bovine Calf Serum (BCS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin. Cells were maintained as subconfluent cultures at 37 °C in a humidified 5% CO_2 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 by different concentrations of DES (1 μM or 10 μM; Sigma-Aldrich, St. Louis, MO, USA) with 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA) for 8 days. In addition, 3T3-L1 cells were treated by ER antagonist ICI-182780 (1 μM; Sigma-Aldrich, St. Louis, MO, USA), PPARγ antagonist GW9662 (20 μM; Sigma-Aldrich, St. Louis, MO, USA) or PPARγ activator troglitazone (10 μM; Sigma-Aldrich, St. Louis, MO, USA) with or without DES for 8 days. An 8-day treatment with 10 μg/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.

Oil Red O staining. Cells were washed with sterile phosphate-buffered saline (PBS), fixed with 10% formaldehyde for 15 min at room temperature (Hanlon et al., 2003), washed with distilled water, and then stained with filtered Oil Red O solution (4 g/l, 60% isopropanol) for 15 min. Excess stain was removed by washing three times with distilled water.

GPDH activity. Cells were rinsed with ice-cold PBS, scraped into 0.2 ml extraction buffer (GPDH assay kit; TAKARA Bio Inc., Shiga, Japan), and centrifuged for 10 min at 4 °C. GPDH activity was assayed in the supernatant by monitoring the decrease in absorbance at 340 nm of NADH in the presence of dihydroxyacetone phosphate (Wise and Green, 1979).

Quantitative real-time PCR analysis. Total RNA from 3T3-L1 cells or C57BL/6J mouse tissues was isolated using RNeasy mini kit (QIAGEN, Germantown, MD, USA) and reversed transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, 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 7000 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.

Western blot analysis. Seventy-five microgram proteins were separated by electrophoresis, and the proteins in the gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Amersham) by electrophoretic transfer. The membrane was incubated with rabbit anti-PPARγ and C/EBPα polyclonal antibody (Bioworld technology inc., St. Louis Park, MN, USA), anti-AP2, FAS, SREBP1 and LPL polyclonal antibody (Abcam, Cambridge, MA USA), or mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4 °C. The specific protein–antibody complexes were detected by using horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). Detection by the chemiluminescence reaction was carried using the enhanced luminol-based chemiluminescent (ECL) kit (Millipore, Billerica, MA, USA). The β-actin signal was used as a loading control. The experiment has been repeated at least three times. The bands were analyzed using Quantity One analyzing system (Bio-Rad, Hercules, CA, USA).

Animal care and DES exposure. C57BL/6J mice were bred in the animal facility of NRIFP (National Research Institute for Family Planning) (mouse protocol SYXK 2009-0033) and housed in a room with a 12-h 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 DES [0.5 mg/kg body weight (b.w.)], troglitazone (0.5 mg/kg b.w.) or vehicle (olive oil) for 24 h. 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 DES (0.01, 0.025, 0.05 or 0.1 mg/kg b.w.) or vehicle (olive oil) from day 12 of gestation until day 7 of lactation (PND 7 of offspring). Pups were weaned at 3 weeks of age (PND 21) and maintained on standard rodent chow.

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 were measured by colorimetric kit assays (Leadman, Beijing, China) and analyzed using a fully-automatic biochemistry analyzer (Hitachi, Tokyo, Japan).

Data analysis. For GPDH activity assay and quantitative real-time PCR assay, data were analyzed using one-way analysis of variance (ANOVA), followed by a Tukey's test. For studies examining obesity

Table 1
Primer sequences for real-time PCR.

Gene	Forward	Reverse
18srRNA	CTCTGTTCCGCCTAGTCCTG	AATGAGCCATTCGCAGTTTC
PPARγ	TGGGTGAAACTCTGGGAGATTC	AATTTCTTGTGAAGTGCTCATAGGC
C/EBPα	CCAAGAAGTCGGTGGACAAGA	CGGTCATTGTCACTGGTCAACT
aP2	GAATTCGATGAAATCACCGCA	CTCTTTATTGTGGTCGACTTTCCA
FAS	TCGGGTGTGGTGGGTTTGGTGAAT	ACTTGGGGGCGTGAGATGTGTTGC
Srebf1	GCCCCTGCCCACCTCAAACCT	ACTGGCACGGGCATCCTTCCTC
LPL	GTGTTGCTTGCCATTCTC	TCTCCTGATGACGCTGAT
ERα	GCTCCTAACTTGCTCCTGGAC	AGCAACATGTCAAAGATCTCCA
ERβ	AAAGAAGCATTCAAGGACATAATGACT	GCTTTTACGCCGGTTCTTGT
ERRα	AGATGTCAGTACTGCAGAGCGT	GCTTCATACTCCAGCAGG
ERRβ	ACCACTCTCTGTGACTTGGCAGAT	TCAGGTTGGAGAAACCTGGGATGT
ERRγ	GACTTGACTCGCCACCTCTC	GTGGTACCCAGAAGCGATGT
β-actin	CAGAAGGAGATTACTGCTCTGGCT	GGAGCCACCGATCCACACA