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Maternal diethylhexyl phthalate exposure affects adiposity and insulin tolerance in offspring in a PCNA-dependent manner

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ABSTRACT

The ubiquitous plasticizer, diethylhexyl phthalate (DEHP), is a known endocrine disruptor. However, DEHP exposure effects are not well understood. Changes in industrial and agricultural practices have resulted in increased prevalence of DEHP exposure and has coincided with the heightened occurrence of metabolic syndrome and obesity. DEHP and its metabolites are detected in the umbilical cord blood of newborns; however, the prenatal and perinatal effects of DEHP exposure have not been intensively studied. Previously, we discovered that phosphorylation (p) of proliferating cell nuclear antigen (PCNA) at tyrosine 114 (Y114) is required for adipogenesis and diet-induced obesity in mice. Here, we show the unique ability of DEHP to induce p-Y114 in PCNA *in vitro*. We also show that while DEHP promotes adipogenesis of wild type (WT) murine embryonic fibroblasts, mutation of Y114 to phenylalanine (Y114F) in PCNA blocked adipocyte differentiation. Given the induction of p-Y114 in PCNA by DEHP and the relationship to obesity, WT and Y114F PCNA mice were exposed to DEHP during gestation or lactation, followed by high fat diet feeding. Paradoxically, *in utero* exposure of Y114F PCNA females to DEHP led to a significant increase in body mass and was associated with augmented expression of PPARγ, a critical regulator of obesity, compared to WT controls. *In utero* exposure of WT mice to DEHP led to insulin sensitivity while Y114F mutation ablated this phenotype, indicating that PCNA is an important regulator of early DEHP exposure and ensuing metabolic phenotypes.

1. Introduction

For several decades, diethylhexyl phthalate (DEHP) has been used as an industrial plasticizer in the production of polyvinylchloride among other universal products; DEHP is present within plumbing, medical instruments, food products, and more. The release of DEHP into the environment, *via* manufacturing and leaching from DEHP-containing products, is well documented, posing an environmental risk which is compounded by low environmental degradation rate of the chemical (Wams, 1987). As a result, large populations of industrialized nations are exposed to DEHP daily (Snyder et al., 2003; Casals-Casas et al., 2008). There is increasing evidence supporting that endocrine disorders correlate with phthalates (including DEHP) owing to their activity as endocrine disruptors. The ubiquitous nature of DEHP poses complex challenges for expecting mothers, as DEHP and its metabolites (most notably the monoester MEHP) have been detected in the umbilical cord blood of newborns in a multitude of studies (Latini et al., 2003; Jurewicz and Hanke, 2011; Huang et al., 2014; Darbre, 2017; Minatoya et al., 2017); however, the effects of the introduction of DEHP during gestation and shortly after birth are not well understood.

A gap in knowledge exists with regards to both the mechanism of action and extent of influence of phthalates in human disease. Modern calorie-rich diets are tightly linked to the pandemic of metabolic disorders including obesity and diabetes, which may be compounded in the presence of environmental toxins. Indeed, increased prevalence of metabolic diseases has been found to correlate with changes in agricultural and industrial practices which are known to introduce endocrine disruptors into the environment. A subset of endocrine disruptors leads to metabolic deregulation, manifested by increased adipose deposition and disorder of glucose metabolism (Casals-Casas and

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Desvergne, 2011; Heindel et al., 2015). However, much of the current knowledge about *in utero* and postnatal exposure to endocrine disruptors have been focused on development, particularly of reproductive systems (Barakat et al., 2017; Cardoso et al., 2017). The potential impact of interaction between endocrine disruptors and diet on global metabolic programming remains understudied.

Endocrine disruptors are thought to work through binding to nuclear receptors with similar mechanisms as hormones (Snyder et al., 2003; Henley and Korach, 2006; Casals-Casas and Desvergne, 2011). With respect to DEHP, the nuclear receptor known as peroxisome proliferator-activated receptor gamma (PPARγ) has been identified to be a specific molecular target in which MEHP, the monoester metabolite of DEHP, reportedly functions as an agonist in *in vitro* assays (Feige et al., 2007; Casals-Casas et al., 2008). PPARγ's function lies at the crossroads of both metabolic syndrome and obesity; this protein is required for the deposition of adipose tissues and is known to be the molecular target of insulin- sensitizing compounds thiazolidinediones (TZDs), which are used clinically to treat type 2 diabetes (Spiegelman, 1998; Feige et al., 2007).

Previously, our laboratory has shown that phosphorylation (p) of proliferating cell nuclear antigen (PCNA) at tyrosine 114 (Y114) plays a significant role in adipocyte maturation and subsequent clonal expansion in mice (Lo et al., 2013). When placed on a high-fat diet (HFD), mice defective for Y114 phosphorylation on PCNA exhibit a less obese phenotype compared to the wild type mice over a prolonged time course, establishing a role of Y114 phosphorylation (p-Y114) of PCNA to HFD-induced obesity. As the functional consequences of PCNA phosphorylation with respect to metabolic endocrine regulation are not well understood, particularly in the context of exposure to endocrine disruptors, we reasoned that our mouse model was suitable to study the effects of early exposure to DEHP in the context of a calorie-rich diet. In this study, we conducted analyses for the *in vivo* mechanism of DEHP exposure and subsequent obesity and metabolic morbidity. Using *in utero* and postnatal (lactational) DEHP exposure models with dosing congruent to that of human exposure (Kavlock et al., 2002; Hao et al., 2012), we characterized the effects on the offspring of DEHP exposed mothers during this sensitive developmental window with a focus on obesity and metabolic outcome.

2. Materials and methods

2.1. Chemicals and antibodies

Antibodies for analysis included: anti-PCNA (Santa Cruz), anti-PPARγ (Cell Signaling Technologies), anti-actin C4 antibody (Abcam), and anti-Phospho Y114 PCNA generated *via* rabbits immunized with a KLH-conjugated peptide (synthetic sequence CNQEKVSD-pY-EMKLMD; Yenzym) and subsequent isolation of serum and extraction *via* a phosphopeptide affinity matrix and clean-up with an affinity matrix conjugated to unphosphorylated peptides (Loet al, 2013). Diethylhexyl phthalate (DEHP), benzylbutyl phthalate (BBP), diisononyl phthalate (DNP) and Oil Red O were purchased from Sigma.

2.2. In vitro adipogenesis

Mouse embryonic fibroblasts (MEFs) used for *in vitro* studies were previously described (Lo et al., 2013). After plating MEFs and allowing for attachment, media with or without 0.5 μM DEHP was utilized for the totality of the adipogenesis protocol as previously described (Tang et al., 2003). Briefly, confluent MEFs are provided Dulbecco's Modification of Eagle Medium with 10% FBS, 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for two days, then DMEM with 10% Fetal Bovine Serum and 1 μg/ml insulin for two days followed by culturing in DMEM with 10% FBS. Visualization with 0.1% Oil Red O staining occurs 8 days after the initiation of differentiation. Adipogenesis statistics were performed using a Student's *t*-test.

2.3. Mice

Wild type (WT/WT) mice and mice with a point mutation of PCNA at residue 114 which changes tyrosine to phenylalanine ($PCNA^{Y114F/Y114F}$) (Lo et al., 2013) in a FVB background were maintained per approved University of Cincinnati Institutional Animal Care and Use Committee (IACUC) protocols. Mice genotypes were confirmed as previously described (Lo et al., 2013). Exposure in both gestational and lactation exposed mice occurred through oral gavage of the pregnant/lactating mouse of either corn oil (vehicle), 0.05 mg/kg/day DEHP, or 500 mg/kg/day DEHP. Offspring were weaned onto high fat diet (45 kcal% fat, Research Diets, Inc.) at 21 days of age. Body mass monitored weekly until study mice were euthanized at 22 weeks of age. Body weight curve statistics were performed using either a Student's *t*-test of data points at individual time points (Fig. 3**B, C**) or two-way ANOVA analyses of data points at individual time points (Fig. 2**B, C**).

2.4. Tissue examination

Visceral and inguinal fat pads were dissected and weighed at the point of euthanasia. Samples were homogenized in RIPA lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton-X 100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM PMSF, Aprotinin 2 μg/ml, sodium fluoride 5 mM, sodium orthovanadate 1 mM) for analysis *via* immunoblot, or were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological examination of fat pad morphology.

2.5. Glucose tolerance and insulin tolerance testing

Glucose tolerance testing (GTT) was performed using 1 g/kg intraperitoneal glucose injection (Andrikopoulos et al., 2008; Stuart et al., 2015); glucose levels were measured using Accu-Chek Aviva Plus meter and test strips (Roche) from blood collected *via* tail nicks. GTT statistics were performed using two-way ANOVA analyses of data at individual time points. Insulin tolerance testing (ITT) was performed using 0.5 U/kg as per published protocols (Feige et al., 2007; Stuart et al., 2015). GTT and ITT were performed at 12 and 13 weeks of HFD feeding, respectively. ITT statistics were performed using a Fisher's exact test.

3. Results

3.1. DEHP treatment induces phosphorylation of PCNA at Y114 and promotes adipocyte differentiation in vitro

Utilizing mouse embryonic fibroblasts (MEF) derived from wild type (WT) mice cultured *ex vivo*, we sought to examine the effect of phthalates on PCNA protein levels and status of phosphorylation at the Y114 residue. MEFs were exposed to benzylbutyl phthalate (BBP), diisononyl phthalate (DNP), and diethylhexyl phthalate (DEHP) after which whole cell lysates were prepared to be examined p-Y114 by Western blot analysis. As shown in Fig. 1**A**, DEHP strongly induced p-Y114 PCNA whereas BBP and DNP showed no discernable induction over endogenous p-Y114 PCNA levels observed in the vehicle control group. Thus, DEHP was selected to be further studied in a dose course of p-Y114 increasing concentrations of DEHP and cell lysates were examined for p-Y114 PCNA by Western blot analysis (Fig. 1**B**). The result shows that DEHP induces p-Y114 of PCNA in a dose-dependent manner.

DEHP has been shown to induce adipogenesis *in vitro* (Hurst and Waxman, 2003; Bility et al., 2004; Feige et al., 2007; Hao et al., 2012). Previously, we demonstrated that p-Y114 PCNA induction is required for *in vitro* adipogenesis, and therefore sought to understand whether DEHP-induced adipogenesis is through the function of p-Y114 PCNA during adipocyte differentiation. To test this linkage, we compared WT MEFs to MEFs isolated from mice containing a tyrosine to phenylalanine substitution at residue 114 in PCNA ($PCNA^{Y114F/Y114F}$) in an *in*