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Benzyl butyl phthalate induces epigenetic stress to enhance adipogenesis in mesenchymal stem cells

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ABSTRACT

Endocrine disruptors, phthalates, may have contributed to recent global obesity health crisis. Our study investigated the potential of benzyl butyl phthalate (BBP) to regulate the mesenchymal stem cell epigenome to drive adipogenesis. BBP exposure enhanced lipid accumulation and adipogenesis in a dose-dependent manner compared to control ($P < 0.001$). Adipogenesis markers, PPARγ ($P < 0.001$), C/EBPα ($P < 0.01$), and aP2 ($P < 0.001$) were significantly upregulated by increasing concentrations of BBP when compared to DMSO. BBP enhanced H3K9 acetylation while decreasing H3K9 dimethylation. Fifty μM BBP increased histone acetyltransferases, p300 ($P < 0.05$) and GCN5 ($P < 0.01$) gene expression. Furthermore, histone deacetylases (HDACs), HDAC3 ($P < 0.01$) and HDAC10 ($P < 0.01$, 10 μM BBP; $P < 0.001$, 50 μM BBP) and histone methyltransferases, SETDB1 ($P < 0.01$) and G9a ($P < 0.01$), were significantly downregulated by BBP exposure. BBP acts, in part, through PPARγ, as PPARγ knockdown led to decreased H3K9ac and rescued H3K9me2 during BBP exposure. In conclusion, BBP regulated MSCs towards adipogenesis by tipping the epigenomic balance.

1. Introduction

Obesity is an epidemic in the United States with more than 78 million people considered obese (WHO, 2015). Obesity is associated with substantial health risks including type 2 diabetes, cardiovascular disease, and stroke (Mayans, 2015). Adolescent obesity is on the rise and children are presenting with increasing cases of type 2 diabetes, once thought to be an adult disease (Hannon and Arslanian, 2015).

Endocrine disruptors (EDs) pose a significant health threat because they interfere with hormonal processes and can lead to perturbed development, reproduction, and adult diseases (Yang et al., 2015). EDs have recently been implicated in adipogenesis and obesity, and those EDs that affect adipocytes and can alter adipogenesis are classified as obesogens (Grun and Blumberg, 2009; Yang et al., 2015). Animal studies reported that exposure to several EDs increased adiposity and body weight (Hao et al., 2012; Zuo et al., 2011). However, there is a need to further investigate EDs and obesity. Phthalates, an endocrine disruptor group, are used to increase the flexibility and softness of plastics. Phthalates are also a major source of contamination for humans because of its presence in food (Wormuth et al., 2006). Recent epidemiological studies have linked phthalate exposure to obesity (Goodman et al., 2014; Kim and Park, 2014) though more investigation is warranted.

Studies have shown that EDs can enhance adipogenesis, in part, by interfering with the nuclear hormone receptor family, peroxisome proliferator-activated receptors (PPARs) (Grun and Blumberg, 2009; Janesick and Blumberg, 2011; Yang et al., 2015). The phthalates, bis(2-ethyl-hexyl)phthalate (DEHP) and tributyltin (TBT), have been shown to affect the adipogenic commitment and differentiation in a murine mesenchymal stem cell (MSC) line, C3H10T1/2 (Biemann et al., 2012). Benzyl butyl phthalate (BBP) is most commonly found in vinyl products including flooring, but also paints, adhesives, children's toys, food packaging, synthetic leather, cosmetics, perfumes, and deodorants (Al-Saleh and Elkhatib, 2016; Braun et al., 2013; Kim and Park, 2014; Lin et al., 2011; Wormuth et al., 2006). Although BBP is one of the most common phthalates found in the environment, it is one of the least studied. BBP has been shown to induce adipogenesis in the 3T3-L1 fibroblast cell line, and the obesogenic phenotype has been shown to be linked with PPARγ (Pereira-Fernandes et al., 2013). However, BBP has not been studied in stem cell differentiation. An important aspect to

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consider is the risk of phthalate exposure to pregnant women. Prenatal exposure to phthalates can affect the fetus, not only during gestation but throughout adulthood. These changes can influence the epigenome, a heritable but not genetic trait. Our work investigated, for the first time, the impact of BBP on the adipogenic potential of MSCs via epigenetic regulation.

A well-studied, complex and coordinated transcriptional program drives the adipocyte differentiation process (Otto and Lane, 2005; Rosen and MacDougald, 2006). MSCs are multipotent and can give rise to different lineages of cells including adipocytes, muscle, bone, and cartilage tissue (Otto and Lane, 2005; Park et al., 2014). MSCs receive stimuli from surrounding stromal cells that commit MSCs to the adipocyte lineage. MSCs undergo growth arrest, mitotic clonal expansion and, finally, terminal differentiation into preadipocytes that then differentiate into mature adipocytes. Histones can be epigenetically modified leading to alterations in transcription. Recent evidence showed that histone modifications play an important role in regulating adipogenesis (Okamura et al., 2010; Zhou et al., 2014). Histone acetylation is associated with the activation of transcription (Berger, 2007; Kouzarides, 2007) and is controlled by the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (de Ruijter et al., 2003). In adipogenesis, HATs increase adipogenic target genes such as PPARγ, CEBPα, and CEBPβ to drive adipogenesis (Yoo et al., 2006; Zhang et al., 2012; Zhou et al., 2014). Lysine 9 of histone 3 (H3K9) has been shown to be enhanced at the promoter regions of PPARγ, CEBPα, and aP2 throughout MSC differentiation and in mature adipocytes (Jin et al., 2014; Noer et al., 2009; Steger et al., 2010). Alternatively, HDACs are negative regulators of adipogenesis (Zhou et al., 2014). On the other hand, histone methylation, which can be a transcriptionally active or repressive mark (Berger, 2007), is mediated by histone methyltransferases (HMTs) and histone demethylases (HDMs). These histone methylation regulators have also been shown to regulate adipogenic gene transcription (Okamura et al., 2010; Okuno et al., 2013). Histone methyltransferases (HMTs), together with HATs and HDACs, play an important role in the dynamic regulation of adipogenic transcriptional program. To date, there are no reports studying the effect of phthalates or any EDs on histone modifications during adipogenesis. These studies show, for the first time that BBP enhances the differentiation of the murine MSC line, C3H10T1/2, through alterations in histone acetylation and methylation.

2. Materials and methods

2.1. Cell culture, differentiation, and treatment

The mouse mesenchymal stem cell line C3H10T1/2 (10T1/2) was purchased from the American Type Culture Collection (ATCC). Cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2 in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin antibiotics. For adipogenic induction, cells were seeded in 24-multiwell plates (mRNA extraction) and 100 × 20 mm dishes (protein extraction). Two days post-confluence; culture medium was replaced with differentiation medium [medium containing insulin 10 μg/ml, 3-isobutyl-1-methylxanthine 0.5 mM, and dexamethasone 1 μM (MDI)]. This medium was replaced after 48 h with medium containing 10 μg/ml insulin. BBP (0.1 μM, 10 μM, or 50 μM) or DMSO (not exceeding 0.1%) was added to cells during differentiation at days 2–8.

2.2. Oil Red O staining and quantification

The differentiated mature adipocytes were washed 2 times with phosphate-buffered saline (PBS, pH 7.4). Lipid droplets were stained with Oil Red O (ORO) (0.36% in 60% isopropanol) for 30 min. Wells were washed twice with PBS to remove excess ORO. PBS was aspirated, and images (40× magnification) were acquired on a microscope (NIKON ECLIPSE 80i). ORO dye was extracted from cells using 100% isopropanol and absorbance (Tecan Infinite® M1000 PRO) was measured at 492 nm.

2.3. Cell viability assay

10T1/2 cells were cultured in the presence or absence of increasing concentrations of BBP for 48 h in a 96-well plate (1×10^4 cells per well). At 48 h, 10 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetraolium bromide (MTT, Sigma) (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37 °C. The supernatant was removed and 200 μl of DMSO was added and mixed gently. The absorbance was read in a spectrophotometer (Tecan Infinite® M1000 PRO) at 490 nm.

2.4. Preparation of nuclear fractions and immunoblot analysis

Nuclear fractions and immunoblot analysis was performed as previously described (Choudhury and Shukla, 2008). Briefly, equal amounts of nuclear extracts were loaded on SDS-PAGE gels and western blot was performed against Acetyl-Histone H3 (Lys9) (H3K9ac 1:1000, CST dilution in 5% BSA), Di-Methyl-Histone H3 (Lys9) (H3K9me2 1:1000, CST dilution in 5% BSA), p300 (p300 1:1000, Abcam dilution in 5% BSA), GCN5 (GCNA 1:1000, Abcam dilution in 5% BSA), histone deacetylase 3 (HDAC3 1:1000, CST dilution in 5% BSA), SETDB1 (SETDB1 1:1000, Abcam dilution in 5% BSA), G9a (G9a 1:1000, Abcam dilution in 5% BSA). Target proteins were detected using C-digit Blot scanner (Li-cor). Histone H3 (H3 1:1000, CST dilution in 5% BSA) or α/ß Tubulin (Tubulin 1:1000, CST dilution in 5% BSA) was used to validate equal loading in each lane.

2.5. Quantitative real-time PCR

Total RNA and quantitative real-time PCR was performed as previously described (Zhang et al., 2015). Briefly, total RNA was isolated from 10T1/2 cells using E.Z.N.A. Total RNA Kit I (Omega Bio-Tek) according to manufacturer's protocol. cDNA synthesis was performed with the Applied Biosystem cDNA Synthesis kit according to manufacturer's protocol. Real-time PCR was performed using SYBR select master mix (Applied Biosystem). Relative gene expression was calculated against 18S as a reference gene using the

Table 1 Primer sequences for qRT-PCR.

Gene name	Primer sequence
PPAR gamma	**F**: TCTGGGAGATTCTCCTGTTGA **R**: GGTGGGCCAGAATGGCATCT
C/EBPα	**F**: CAAGAACAGCAACGAGTACCG **R**: GTCACTGGTCAACTCCAGCAC
aP2	**F**: GATGCCTTTGTGGGAACCT **R**: CTGTCGTCTGCGGTGATTT
G9a	**F**: TTCCTTGTCTCCCCTCCCAG **R**: GACGGTGACAGTGACAGAGG
SETDB1	**F**: GCGGAGCCCTGGAGTAAAA **R**: TCAGCGCACCTCAGTCCG
Gcn5	**F**: CTGAAGACCATGACTGAGCGG **R**: TCGGCCACAAAGAGCTTCC
P300	**F**: AACAAAGCCAGCCATCTGGA **R**: GAGGCCACACCAGCATTTTC
HDAC3	**F**: TTCCCTCAAACTCTCACGGC **R**: AGTTGCTGGGGCTCATTACC
HDAC10	**F**: ATGCCATAGCAACCACAACTG **R**: TGAGCCACAGAATTCTCCCATC