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The plasticizer BBP selectively inhibits epigenetic regulator sirtuins

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ABSTRACT

The plasticizer benzyl butyl phthalate (BBP) is a well-known endocrine disruptor. Widespread human exposure to phthalates has raised substantial public concern due to its detrimental health effects. However, molecular mechanisms of the phthalates effect require elucidation. In this study, we analyzed: 1) the binding interaction of several phthalates and persistent organic pollutants with epigenetic regulator sirtuins and 2) the effect of BBP on the sirtuins in HepG2 cells. AutoDock molecular docking analysis showed that BBP binds to Sirt1 and Sirt3 proteins similarly to the native ligands with shortest binding free energies (ΔG_b) of −7.35 and −8.3 kcal/mol, respectively; and inhibition constants (K_i) of 4.07 μM and 0.82 μM, respectively. Furthermore, BBP was superimposed onto the co-crystallized ligands within the least root-mean-square deviation (RMSD) of 0.96 Å and 1.55 Å for Sirt1 and Sirt3, respectively, and bound into the sites with a sufficient number of hydrogen bonds, implying the best fit compared to other sirtuins. In HepG2 cells, BBP significantly down-regulated Sirt1 and Sirt3 ($p < 0.05$) gene expression at a concentration as low as 10 nM; other sirtuins remained unaffected. Consistent with decreased gene expression, Sirt1 and Sirt3 protein levels were significantly decreased at 48 h ($p < 0.05$). In addition, mitochondrial biogenesis regulators PGC-1α, NRF-1, and NRF-2, were decreased ($p < 0.05$). SiRNA studies showed that BBP did not regulate PGC-1α *via* sirtuin and BBP requires sirtuin's presence to regulate NRF-1 or NRF-2. BBP significantly increased ROS production ($p < 0.05$) and ROS may be chiefly regulated by NRF-1 and NRF-2 in HepG2 cells under Sirt1 and Sirt3 silenced condition. This is the first report to demonstrate that BBP selectively disrupts specific sirtuins in HepG2 cells. In conclusion, our study suggests that BBP can impair two vital epigenetic regulators and mitochondrial biogenesis regulators in liver cells.

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1. Introduction

Over the last two decades, there has been a dramatic and rapid deterioration in population metabolic health primarily due to the emergence of an obesity and diabetes epidemic (Smyth and Heron, 2006). With an alarming annual obesity-related health cost of approximately $245 billion in the United States, this cost will rise dramatically as diabetes increases from approximately 387 million individuals worldwide in 2013 to a staggering 592 million people by 2035 (ADA, 2013; IDF, 2013). The major causes are most often attributed to genetics and behavioral factors including diet and inactivity (Aguilera et al., 2013; de Beaufort, 2014; Kebede and Attie, 2014; Leech et al., 2014). However, environmental chemicals, such as plasticizer phthalates, are understudied risk factors compared to diet and lifestyle in the development of diabetes and obesity (Fleisch et al., 2012; Goodman et al., 2014; Grun and Blumberg, 2009a, 2009b; Hatch et al., 2008; Kuo et al., 2013; Martinelli et al., 2006; Neel and Sargis, 2011). In addition, epigenetic mechanisms where the environment plays a prominent role remain poorly understood (Dolinoy and Jirtle, 2008; Guerrero-Bosagna and Skinner, 2014; Ho et al., 2012; Janesick et al., 2014; Norman et al., 2013; Rozek et al., 2014).

The interaction between genes and the environment has emerged as a new frontier for the discovery of how networks of modified genes contribute to several major pathologies. One emerging group of epigenetic regulators are the sirtuins, NAD^+ dependent protein deacetylases, that act as cellular sensors to detect energy availability and modulate metabolic processes (Kendrick et al., 2011). Among the seven sirtuin types, Sirt1 and Sirt3 have been the most extensively investigated. Sirt4, 5, and 7 have also been shown to have functional importance in physiological regulation (Flick and Luscher, 2012; Jing and Lin,

Abbreviations: BBP, benzyl butyl phthalate; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NRF-1, nuclear respiratory factor 1; NRF-2, nuclear respiratory factor 2.

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2015; Laurent et al., 2013; Nishida et al., 2015; Shin et al., 2013). Previous studies including our study demonstrated that mice fed a high fat diet (HFD) showed decreased Sirt3 expression and activity and developed clinical features that closely mimic the human metabolic syndrome, including obesity, T2D, insulin resistance, lipid abnormalities, and steatohepatitis (Hirschey et al., 2011; Kendrick et al., 2011). In humans, Sirt3 expression was associated with an extended lifespan (Albani et al., 2014). Sirt1 is also an extensively studied sirtuin for its role in longevity (Bordone and Guarente, 2005) and whole body energy metabolism (Boutant and Canto, 2014; Fernandez-Marcos and Auwerx, 2011), maintaining pancreatic beta cell integrity and function (Luu et al., 2013), reducing myocardial hypertrophy (Planavila et al., 2011), and providing neuroprotection (Jiang et al., 2012). However, the effects of endocrine disruptors on these epigenetic regulators are largely unknown.

Phthalates, a group of endocrine disruptors, have attracted public attention due to their possible adverse environmental and human health effects (Bajkin et al., 2014; Gallinger and Nguyen, 2013; Li and Ko, 2012; Martinez-Arguelles et al., 2013; Meeker and Ferguson, 2014; North and Halden, 2013). The recently proposed obesogen hypothesis (Biemann et al., 2012) suggests that man-made chemical compounds that disrupt normal development and balance of lipid metabolism can lead to increased risks of obesity, Type 2 diabetes (T2D), and cardiovascular disease (CVD). Epidemiological studies reveal increased phthalate metabolites in urine correlated with abdominal obesity and insulin resistance in adolescents and adult males (Goodman et al., 2014). Infants and toddlers are the most vulnerable because: 1) they exhibit more hand-to-mouth activity and 2) they consume the most food as a percent of their body weight (Braun et al., 2013; Fischer et al., 2013; Sathyanarayana, 2008). One of the known plasticizers, benzyl butyl phthalate (BBP), is primarily used to enhance plasticity of industrial polymers and is primarily found in papers and paperboards used as packaging materials for aqueous, fatty, and dry foods. The ubiquitous use of the plasticizers in multiple products results in extensive exposure to humans, and has become an unavoidable part of modern life. Most studies have focused on specific targeted processes in cells or laboratory animals such as cell death, sex differentiation, reproductive defects, and oxidative stress (Lyche et al., 2009; McKee et al., 2004). Reports have shown that BBP can enter the cells, bio-accumulate, and exert mitogenic effects, with the highest toxicities observed in embryonic development (Harris et al., 1997; Sabbieti et al., 2009; Saillenfait et al., 2003). Interestingly, in the 3T3-L1 cell line, butylbenzyl phthalate (BBP) was shown to induce adipogenesis (Pereira-Fernandes et al., 2013). A recent report showed that di-(2-ethylhexyl)phthalate (DEHP) inhibits Sirt1 activity and mitochondrial function in the testis (Li et al., 2014). Another study showed that mono-(2-ethylhexyl)phthalate (MEHP) impacts lipolysis, glucose uptake/glycolysis, and mitochondrial respiration/biogenesis implying that MEHP accumulation disturbs energy metabolism of fat cells (Chiang et al., 2014). However, the effects of BBP have yet to be thoroughly investigated during the development of metabolic diseases. Considering the widespread exposure to phthalates in the population, we hypothesize that phthalates impair the function of one important metabolic regulator group of sirtuins, which can lead to the development of metabolic syndrome. Accordingly, the present study was designed to investigate the ligand binding efficiency of five phthalates (benzyl butyl phthalate (BBP), di-*n*-butyl phthalate (DBP), di-2-ethylhexyl phthalate (DEHP), monobutyl phthalate (MBP), mono-(2-ethylhexyl)phthalate (MEHP)), and two hydrophilic perfluoroalkyl substances (PFAS or known as POPS) [perfluorooctanoic acid (PFOA), and perfluorooctanesulfonic acid (PFOS)] to the ligand binding domains of Sirt1, Sirt2, Sirt3, Sirt5, and Sirt6 in human, using the molecular docking approach. These five phthalates are most commonly found in our surroundings. In addition, a few of them (DEHP, BBP) had been selectively prohibited by European commission or the EPA in selective materials (*e.g.*, baby toys, plastic medical device). Recently EPA investigated the health effects of PFOA and PFOS (POPS or PFAS) for purposes of public comment (scientific views) and peer review (https://peerreview.versar.com/epa/pfoa/). PFOA and PFOS are listed on the third contaminant Candidate List and both chemicals are currently being monitored under the third Unregulated Contaminant Monitoring Rule (http://www2.epa.gov/ccl/contaminant-candidate-list-3-ccl-3). Interestingly, phthalates and POPS have recently been labeled as obesogen in several publications. More studies are needed to establish the effect of plasticizers on metabolic regulators.

The outcomes of this study predict the existence of BBP-sirtuin specific interactions. *In silico* predictions were then complemented by *in vitro* experiments to show possible dose-relationship effects of BBP on sirtuins pathway in HepG2 cells.

2. Materials and methods

2.1. Reagents and antibodies

BBP was purchased from Sigma (St. Louis, MO). Goat polyclonal antibodies to Actin and mouse monoclonal antibodies to Sirt3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the rabbit monoclonal antibodies to Sirt1 were obtained from Cell Signaling Technology (Danvers, MA). Thiazolyl Blue Tetrazolium Bromide powder was used for MTT assay (Sigma).

2.2. Molecular docking method

In silico molecular docking studies were performed using Autodock 4.2 (Morris et al., 2009). Lamarckian genetic algorithmic function of Autodock was used to scan the active site for low energy bindingmodels and orientations. The target macromolecular proteins, human NAD-dependent deacetylase Sirt1 (pdb code: 4I5I) (Zhao et al., 2013), Sirt2 (pdb code: 4L3O) (Yamagata et al., 2014), Sirt3 (pdb code: 4BVH) (Gertz et al., 2013), Sirt5 (pdb code: 4HDA) (Gertz et al., 2012), Sirt6 (pdb code: 3PKJ) (Pan et al., 2011) were retrieved from the 3D structures database in the Protein Data Bank (RCSB PDB) (http://www.rcsb.org/pdb/home/home.do) (Table 2). The corresponding co-crystallized native ligands: 4I5, MES, OCZ, STL402, A2N respectively, were extracted from theirprotein using Accelyrs Discovery Studio v3.5 client software [Accelrysinc, San Diego, CA (2005)]. The key amino acids were identified through the pictorial database of 3D structures in the Protein Data Bank (http://www.ebi.ac.uk/pdbsum/). The results of 10randomly seeded runs were analyzed for each of the docked endocrine disruptors. The clusters were ranked from the average lowest energy obtained for members of the cluster to the highest energy. The analysis was then carried out for the top three docking clusters. Each of the clusters that exhibited significant negative interaction energy was examined by Accelyrs Discovery Studio visualizer. Initially, the co-crystallized ligands (4I5, MES, OCZ, STL402, and A2N) (Table 3) were docked into their corresponding binding sites, and their molecular docking results and orientation were used for reference comparison. The binding affinities of the endocrine disruptors were evaluated according to the binding free energies (ΔG_b, kcal/mol), inhibition constants (K_i), hydrogen bonding, and RMSD values in comparison to the native co-crystallized ligand. RMSD was measured by evaluating distance between two centroids. The endocrine disruptor which exhibits the highest binding affinities has the lowest binding free energies, and the highest numbers of hydrogen bonds within the target sirtuins. In addition, the lowest RMSD values are considered as the best fitted ligands.