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Triphenyl phosphate enhances adipogenic differentiation, glucose uptake and lipolysis *via* endocrine and noradrenergic mechanisms

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ABSTRACT

The use of triphenyl phosphate (TPhP) as a flame retardant or plasticizer has increased during the last decade, resulting in widespread human exposure without commensurate toxicity assessment. The main objectives of this study were to assess the *in vitro* effect of TPhP and its metabolite diphenyl phosphate (DPhP) on the adipogenic differentiation of 3T3-L1 cells, as well as glucose uptake and lipolysis in differentiated 3T3-L1 adipocytes. TPhP increased pre-adipocyte proliferation and subsequent adipogenic differentiation of 3T3-L1 cells, coinciding with increased transcription in the CEBP and PPARG pathway. Treatment of mature adipocytes with TPhP increased the basal- and insulin stimulated- uptake of the glucose analog 2-[N (-7-nitrobenz-2-oxa1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG). This effect was ablated by inhibition of PI3K, a member of the insulin signaling pathway. DPhP had no significant effect on cell proliferation and, compared to TPhP, a weaker effect on adipogenic differentiation and on 2-NBDG uptake. Both TPhP and DPhT significantly enhanced the isoproterenol-induced lipolysis, most likely by increasing the expression of lipolytic genes during and after differentiation. This study suggests that TPhP increases adipogenic differentiation, glucose uptake, and lipolysis in 3T3-L1 cells through endocrine and noradrenergic mechanisms.

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1. Introduction

Endocrine disrupting chemicals (EDCs) interfere with hormonal action and cause non-monotonic impairments of hormonal functions in the regulation of development, maintenance of homeostasis and physiological processes, including adipogenesis and energy balance (Gore et al. 2015; Zoeller et al. 2012). Further, perinatal exposure to EDCs and other environmental pollutants has been proposed to be a contributing factor to overweight and obesity under the so-called "environmental obesogen" hypothesis (Grun and Blumberg 2006; La Merrill and Birnbaum 2011).

Obesity has been increasing in all countries, with prevalence doubling during the past three decades to reach a global prevalence of 11% of adult men and 15% of adult women in 2014 (Ogden et al. 2014; WHO 2014). If these current trends continue, by 2025, global obesity prevalence will reach 18% in men and surpass 21% in women (NCD-RisC 2016). Developed countries such as United States are stressing the dramatic levels of such trends, where 35% of the adult population was already obese in 2012 (Ogden et al. 2014). During the last decades, studies with twins have demonstrated that the genetic heritability of the variation in obesity-related phenotypes, such as body mass index, is only between 40 and 60% (Qi and Cho 2008; Silventoinen et al. 2016). This substantiates the intuitive notion that genetics alone cannot account for the rapid change in the prevalence of obesity. Environmental factors and their interaction with the genome must then have a substantial role in the change in obesity prevalence. EDCs may well be a component of that environmental contribution to obesity.

Given the relatively recent rise in obesity, the most plausible candidate obesogens are those EDCs that have also risen in use over the same time period. In this regard, phosphate flame retardants are plausible candidate obesogens. The phosphate flame retardants have been

Abbreviations: Acetyl-coenzyme A carboxylase alpha, (Acaca); Adipose tissue triglyceride lipase, (ATGL); (BM1), basal media 1; (BM2), basal media 2; (Cebpa), CCAAT/enhancer binding protein alpha; (Cebpb), CCAAT/enhancer binding protein beta; (Cebpd), CCAAT/enhancer binding protein delta; (DMSO), dimethyl sulfoxide; (DPhP), diphenyl phosphate; (DMEM), Dulbecco's modified Eagle Medium; (EDCs), endocrine disrupting chemicals; (Fasn), fatty acid synthase; (FBS), fetal bovine serum; (GLUT4), glucose transporter type 4; (IBMX), 3-isobutyl-1-methylxanthine; (Lpl), lipoprotein lipase; (Mgll), monoglyceride lipase; (MTT), 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; (NEFAs), non-esterified fatty acids; (NCS), newborn calf serum; (PAGE), polyacrylamide gel electrophoresis; (PBDE), polybrominated diphenyl ether; (PBS), phosphate buffered saline; (PI3K), phosphoinositide 3-kinase; (Pnpla2), patatin-like phospholipase domain containing 2; (Pparg), peroxisome proliferator-activated receptor gamma; (Rxra), retinoid X receptor alpha; (TPhP), triphenyl phosphate; (T2DM), type 2 diabetes mellitus; (2-NBDG)., 2-[N (-7-nitrobenz-2-oxa1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose.

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extensively used, especially in US, as additives to polymers and resins to reduce the flammability of furniture, electronics and construction materials during the last several decades including their recent replacement of the polybrominated diphenyl ether (PBDE) flame retardants (Dodson et al. 2012; van der Veen and de Boer 2012). For example, triphenyl phosphate (TPhP) is a phosphate ester used as a flame retardant in both consumer and industrial products or as a plasticizer (Mendelsohn et al. 2016). Exposure to TPhP is widespread among the general population (Cooper et al. 2011; Dodson et al. 2012; Dodson et al. 2014) and results in a rapid (36 h) hepatic hydrolysis into the main metabolite diphenyl phosphate (DPhP) which is mainly excreted in urine (Cooper et al. 2011; Su et al. 2014).

One exploratory study of developmental Firemaster 550 exposure found this mixture of compounds, which includes 17% TPhP, caused an increased body mass, fasting glucose and glucose intolerance in adult rat offspring (Patisaul et al. 2013). Recently, rats developmentally exposed to TPhP at the same dose and for the same developmental window as was used in the Firemaster 550 study (Patisaul et al. 2013) had increased adiposity and an earlier onset of type 2 diabetes independent of adiposity (Green et al. 2016). This suggested that TPhP could be a primary metabolic toxicant in the Firemaster 550 mixture. In corroboration with these *in vivo* observations, *in vitro* exposure to Firemaster 550 and TPhP initiated differentiation of adipocytes, mediated by the activation of the master regulator peroxisome proliferator-activated receptor γ (PPARγ) (Belcher et al. 2014; Pillai et al. 2014). In follow-up to this emerging body of evidence, the main objectives of this study were to assess the possible endocrine and/or noradrenergic effects of TPhP and its metabolite DPhP on adipogenic differentiation, glucose uptake and lipolysis. This study exploits the 3T3-L1 *in vitro* model because the 3T3-L1 preadipocytes are the only cell line capable of complete differentiation into mature white adipocytes, representing a stable and reproducible model to study obesogen compounds (Green and Kehinde 1975; Pereira-Fernandes et al. 2014).

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was purchased from Gemini Bio Products (Sacramento, California, USA). Newborn calf serum (NCS), rosiglitazone, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide (DMSO), methylene blue dye, DPhP, and insulin from bovine pancreas were purchased from Sigma Chemical (St. Louis, Missouri, USA). TPhP was acquired from Accustandard (New Haven, Connecticut, USA). Phosphate buffered Saline (PBS) was purchased from Boston Biochem (Cambridge, Massachusetts, USA). High glucose (4.5 g/L), low glucose (1 g/L), and plain (no glucose) Dulbecco's modified Eagle Medium (DMEM) and penicillin/streptomycin were purchased from Life Technologies (Grand Island, New York, USA). We used the inhibitor of phosphoinositide 3-kinase (PI3K), LY294002, also from Life Technologies. The fluorescent glucose analog 2-[N (-7-nitrobenz-2-oxa1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) was purchased from Cayman Chemicals (Ann Arbor, Missouri, USA). The 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) cell proliferation assay was purchased from the American Type Culture Collection (Manassas, Virginia, USA). The AdipoSIGHT Lipolysis Assay Kit was purchased from ZEN-BIO Inc., Chapel Hill, NC, USA. The Mem-PER™Plus Membrane Protein Extraction Kit was purchased from Thermo Fisher Scientific (Rockford, Illinois, USA) and the BCA protein assay kit was purchased from Millipore (Milford, Massachusetts, USA). The chemicals were dissolved in DMSO with the exception of 2-NBDG, which was dissolved in ethanol.

2.2. Cell culture and differentiation

3T3-L1 pre-adipocytes (American Type Culture Collection) were maintained in T75 flasks with basal media 1 (BM1), containing high

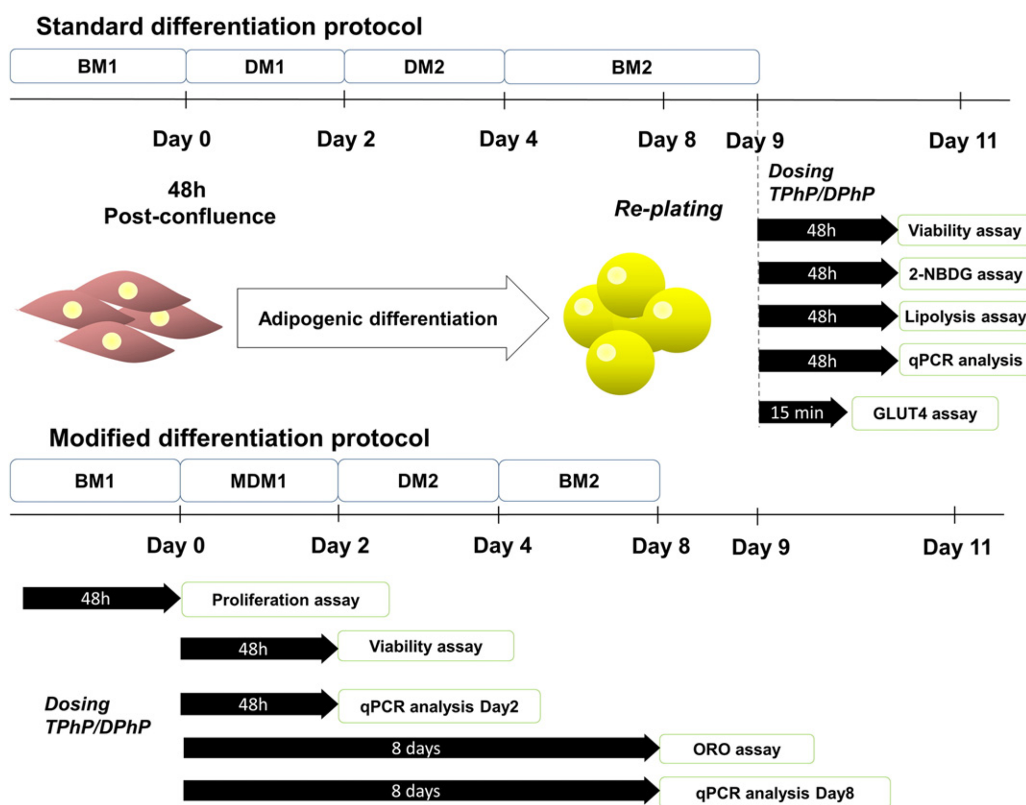

Fig. 1. Study design, differentiation and dosing protocol. BM1, basal media 1; BM2, basal media 2; DM1, differentiation media 1; MDM1, modified differentiation media 1; DM2, differentiation media 2; ORO assay, oil red O assay.

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