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Plasticizers used in food-contact materials affect adipogenesis in 3T3-L1 cells

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ABSTRACT

Recent studies suggest that exposure to some plasticizers, such as Bisphenol A (BPA), play a role in endocrine/metabolic dispruption and can affect lipid accumulation in adipocytes. Here, we investigated the adipogenic activity and nuclear receptor interactions of four plasticizers approved for the manufacturing of food-contact materials (FCMs) and currently considered safer alternatives. Differentiating 3T3-L1 mouse preadipocytes were exposed to scalar concentrations (0.01–25 μM) of DiNP (Di-iso-nonyl-phthalate), DiDP (Di-iso-decyl-phthalate), DEGDB (Diethylene glycol dibenzoate), or TMCP (Tri-m-cresyl phosphate). Rosiglitazone, a well-known pro-adipogenic peroxisome proliferator activated receptor gamma (PPARγ) agonist, and the plasticizer BPA were included as reference compounds. All concentrations of plasticizers were able to enhance lipid accumulation, with TMCP being the most effective one. Accordingly, when comparing *in silico* the ligand binding efficiencies to the nuclear receptors PPARγ and retinoid-X-receptor-alpha (RXRα), TMPC displayed the highest affinity to both receptors. Differently from BPA, the four plasticizers were most effective in enhancing lipid accumulation when added in the mid-late phase of differentiation, thus suggesting the involvement of different intracellular signalling pathways. In line with this, TMCP, DiDP, DiNP and DEGDB were able to activate PPARγ in transient transfection assays, while previous studies demonstrated that BPA acts mainly through other nuclear receptors. qRT-PCR studies showed that all plasticizers were able to increase the expression of CCAAT/enhancer binding protein β (*Cebpβ)* in the early steps of adipogenesis, and the adipogenesis master gene *Pparγ2* in the middle phase, with very similar efficacy to that of Rosiglitazone. In addition, TMCP was able to modulate the expression of both Fatty Acid Binding Protein 4/Adipocyte Protein 2 (*Fabp4/Ap2)* and Lipoprotein Lipase (*Lpl)* transcripts in the late phase of adipogenesis. DEGDB increased the expression of *Lpl* only, while the phthalate DiDP did not change the expression of either late-phase marker genes *Fabp4* and *Lpl.* Taken together, our results suggest that exposure to low, environmentally relevant doses of the plasticizers DiNP, DiDP, DEGDB and TMCP increase lipid accumulation in 3T3-L1 adipocytes, an effect likely mediated through activation of PPARγ and interference at different levels with the transcriptional cascade driving adipogenesis.

1. Introduction

Obesity is the fastest growing health problem in Europe and worldwide. In the European Union, overweight affects between 36% and 67.5% of adults, while obesity affect between 10% and 28% of adults (last update 2014) [1]. In addition to genetic factors, life style factors such as excessive caloric intake, high fat diets, and low physical activity contribute to obesity. However, there is also increasing evidence that environmental pollutants including endocrine-disrupting chemicals (EDCs) may contribute to the development of obesity and metabolic disorders. A subset of EDCs have been named “obesogens” or “metabolic disruptors” [2–5], because of their ability to promote adiposity by altering fat cell development and increasing energy storage of fat tissue, and because of their implication in metabolic syndrome and obesity [6].

The EU regulation (1907/2006 and subsequent updates) regarding

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the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has identified so far 181 substances of very high concern (SVHC) for the environment and human health (last update January 2018). Several SVHC are plasticizers, a class of diverse additives used in plastics production, that are poorly bound or not bound to the polymers. These features facilitate their migration from food-contact materials (FCMs) and several household plastic items, thus coming in contact with humans through food consumption, skin absorption and inhalation [7]. FCMs, including plastic packaging, are not generally perceived to be a chemical health threat when compared to pesticides, veterinary drugs or heavy metals arising from agricultural practices or environmental contamination. However, within the last decade it has been increasingly reported that certain FCMs can act like EDCs [8]; a good example are plastic additives used in food containers like Bisphenol A (BPA), a substance recently included in the SVHC list and whose impact on the endocrine system has been increasingly reported [2,3].

The EFSA (European Food Safety Authority) regulation 10/2011 has provided a list of plasticizers permitted in EU for FCMs manufacturing, which has become a useful source of alternatives to currently used SVHC. In the present work, we focused our attention on four plasticizers employed in food packaging: Di-iso-nonyl-phtalate (DiNP), Di-iso-decyl-phtalate (DiDP), Diethylen glycol dibenzoate (DEGDB), and Tri-m-cresyl phosphate (TMCP). Notably, DiNP and DiDP are comprised in the EFSA list of permitted compounds and are indeed among the most used in the plastic market (33% United States; 63% European Union) as substitutes of di(2-ethylhexyl) phthalate (DEHP), a substance classified as SVHC [9,10]. DEGDB is another emerging plasticizer designed to substitute phthalates, since it is considered more eco-friendly due to its biodegradation pathways [11]. Tri-cresyl phosphates, such as tri-m-cresyl phosphate (TMCP), are mainly used as substitutes of the plasticizers polybrominated diphenyl ethers (e.g. BDE-47) [12]. Along with the increased usage of these SVHC substitutes as alternative plasticizers, new biomonitoring data are becoming available associating the exposure to these chemicals with adverse effects in living beings. Notably, DiNP and DiDP have both been associated with increased insulin resistance in adolescent cohorts [13] and in general with several different adverse effects after peri- and post-natal exposure [14]. Interestingly, *in silico* approaches demonstrated that DiNP and DiDP can act as ligands of human peroxisome proliferator activated receptor γ (PPARγ) and retinoid-X-receptor-α (RXRα), possibly triggering a cascade of intracellular events [15]. DiDP is also a confirmed modulator of PPAR:RXR-dependent gene expression pathways in fish hepatocytes [16]. Similarly, TMCP was found to affect lipid/cholesterol metabolism through a functional interplay between PPARs and liver X receptor (LXR) in a fish in vitro system [17]. Also, in fish DEGDB was demonstrated to have high affinities for PPARα, RXRα and LXR, showing the ability to modulate PPARα transcriptional pathways [18].

The 3T3-L1 preadipocyte cell line has proved to be a useful tool to study in vitro mechanisms by which obesogens can affect lipid accumulation and adipocyte differentiation. In 3T3-L1 cells, these two processes are regulated by a strict transcriptional activity in which PPARγ is the master regulator [19]. During adipocyte differentiation, three different time windows can be distinguished, each one characterized by the upregulation/activation of a different set of transcription factors: an early phase of induction, characterized by the upregulation of *Cebp* (CCAAT/enhancer binding protein) β and δ and the activation of *Cebp*β and *Rxrs*; a middle phase, with RXRα and PPARγ2 as obligate heterodimers; a late phase, where adipocyte specific genes such as *Fabp4/Ap2* (Fatty Acid Binding Protein 4/Adipocyte Protein 2), *Lpl* (Lipoprotein Lipase), *AdipoQ* (adiponectin) and leptin are upregulated [20–22]. Several studies have shown how environmental chemicals can perturb this intracellular cascade by targeting transcription factors and consequently enhance or decrease adipogenesis [5,6,22–24]. For example, certain EDCs may target PPARγ by binding to it directly to activate downstream cascades leading to enhanced lipid accumulation or by increasing *Pparγ* expression to favour its activation [24].

In the present work we used 3T3-L1 preadipocytes to investigate the possible adipogenic effects of plasticizers considered safe SVHC substitutes and used in FCMs manufacturing. First, we evaluated possible modifications in lipid accumulation following exposure to scalar concentrations of the plasticizers DiNP, DiDP, DEGDB and TMCP. Since adipogenesis occurs in 3T3-L1 with a defined timeline of transcription factors and receptors activity, we also evaluated the possible different effects of plasticizer exposure alternatively during 3T3-L1 early or mid-late differentiation. We then verified, by *in silico* molecular docking analysis and reporter gene assays, the ability of these molecules to bind and activate the major transcription factor involved in adipogenesis, namely PPARγ. To better understand the intracellular mechanisms underlying the changes in the adipogenic process, we investigated the regulation of the expression of genes belonging to the early, mid and late phase of adipocyte differentiation.

2. Material and methods

2.1. Chemicals/reagents

All the reagents for cell culture (including medium supplements), Oil Red O (CAS Number 1320-06-5), Rosiglitazone (BRL49653; CAS Number 122320-73-4, purity ≥ 98%), DiNP (di-iso-nonyl-phtalate; CAS Number 28553-12-0, purity ≥ 99%), DiDP (di-iso-decyl-phtalate; CAS Number 26761-40-0, purity ≥ 99%), DEGDB (diethylene glycol dibenzoate; CAS Number 120-55-8, purity 90%), TMCP (tri-m-cresyl phosphate; CAS Number 563-04-2) and BPA (Bisphenol A; CAS Number 80-05-7, purity ≥ 99%) were obtained from Sigma Aldrich (USA).

2.2. 3T3-L1 culture and adipocyte differentiation experiments

3T3-L1 preadipocytes (ATCC® CL-173™; ATCC, USA) were cultured in Dulbecco's modified Eagle's medium high-glucose (DMEM) supplemented with 10% calf serum, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin. 2×10^4 cells/well were seeded in 24-well plates. Two days after reaching confluence (day 0), cells were exposed to the differentiation medium (MDI; DMEM containing 10% fetal bovine serum, 1 µg/mL insulin, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine). Two days later (day 2), MDI medium was replaced with maintenance medium (MM; DMEM 10% FBS, 1 µg/mL insulin). Fresh medium was provided every two days. Experiments were ended after 10 days from the beginning of the differentiation (day 10).

Cells were exposed to the following plasticizers: DiNP, DiDP, DEGDB, TMCP or BPA at concentrations ranging from 0.01 to 25 µM, that were excluded to be toxic by visual analysis. 100 nM Rosiglitazone was used as a positive control. All the chemicals were dissolved in 100% DMSO as vehicle, and cells were exposed to a final concentration of 0.1% DMSO. Cells were treated with chemicals alternatively from day 0 to day 10 (whole differentiation period treatment), from day 0 to day 2 (early phase treatment), or from day 2 to day 10 (middle-late phase treatment). Control cells were kept in MDI plus 0.1% DMSO from day 0 to day 2 and in MM plus 0.1% DMSO from day 2 to day 10.

Three independent replicates were set in each experiment; experiments were repeated three times at different passage numbers (p8-p11).

2.4. Quantification of adipocyte lipid accumulation

Lipid accumulation in 3T3-L1 adipocytes was determined by quantitative Oil Red O (ORO) staining at day 10. Oil Red O was dissolved in isopropanol overnight at a concentration of 0.35%, followed by 0.2 µm filtration, dilution in water to a final concentration of 0.2%, and refiltration. Adipocytes were washed twice with PBS, then they were fixed in 10% paraformaldehyde for 10 min at room temperature. Cells were washed with ddH_2O, allowed to dry, and stained with ORO