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Cyclohexane-1,2-dicarboxylic acid diisononyl ester and metabolite effects on rat epididymal stromal vascular fraction differentiation of adipose tissue

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ABSTRACT

Plastics are generally mixed with additives like plasticizers to enhance their flexibility, pliability, and elasticity proprieties. Plasticizers are easily released into the environment and are absorbed mainly through ingestion, dermal contact, and inhalation. One of the main classes of plasticizers, phthalates, has been associated with endocrine and reproductive diseases. In 2002, 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) was introduced in the market for use in plastic materials and articles intended to come into contact with food, and it received final approval from the European Food Safety Authority in 2006. At present, there is limited knowledge about the safety and potential metabolic and endocrine-disrupting properties of DINCH and its metabolites. The purpose of this study was to evaluate the biological effects of DINCH and its active metabolites, cyclohexane-1,2-dicarboxylic acid (CHDA) and cyclohexane-1,2-dicarboxylic acid mono isononyl ester (MINCH), on rat primary stromal vascular fraction (SVF) of adipose tissue. DINCH and its metabolite, CHDA, were not able to directly affect SVF differentiation. However, exposure of SVF to 50 μM and 100 μM concentrations of MINCH affected the expression of *Cebpa* and *Fabp4*, thus inducing SVF preadipocytes to accumulate lipids and fully differentiate into mature adipocytes. The effect of MINCH was blocked by the specific peroxisome proliferator-activated receptor (PPAR)-α antagonist, GW6471. Taken together, these results suggest that MINCH is a potent PPAR-α agonist and a metabolic disruptor, capable of inducing SVF preadipocyte differentiation, that may interfere with the endocrine system in mammals.

1. Introduction

The discovery of plastic dates back to 1600 B.C., when ancient Mesoamerican people were processing rubber to create balls, human figurines, and other objects (Hosler et al., 1999). The production of plastics continued throughout the 19th century with the introduction of many natural and synthetic polymers in the market, such as bakelite and polyvinyl chloride (PVC) (Andrady and Neal, 2009; Halden, 2010). However, only in the 1940s did the mass production of plastics explode, and it has continued to expand to the present day with an estimated 288 million tons being produced for the year 2012 (Halden, 2010; PlasticsEurope, 2013).

Plastics are generally mixed with additives (*e.g.*, inorganic fillers, plasticizers, and fire retardants) to enhance their performance (Andrady and Neal, 2009). Among these, plasticizers are added to increase PVC flexibility, pliability, and elasticity (Andrady and Neal, 2009; Halden, 2010). Since plasticizers are only dispersed in the polymer, and not actually a part of it (German Federal Environment Agency, 2011; Halden, 2010), they are easily released into the environment and are absorbed through ingestion (Frederiksen et al., 2007; Halden, 2010), dermal contact (Frederiksen et al., 2007), and inhalation (Wilson et al., 2007).

Phthalates are among the most well-known plasticizers, and they have been extensively studied in recent years for their endocrine-disrupting proprieties. Phthalates, are defined as anti-androgenic compounds (German Federal Environment Agency, 2011; Halden, 2010) due to their well-studied effects on the male reproductive system, where they have been shown to induce cryptorchidism, changes in testicular testosterone and Leydig cell

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homeostasis, and a phenotype described as testicular dysgenesis syndrome (Halden, 2010; Yoon et al., 2014). Moreover, phthalates have been implicated in different types of cancers (breast, liver, pancreas, and testicular Leydig cancers) (Yoon et al., 2014), premature breast development in women (Colon et al., 2000), a reduction of litter size, and an increased risk of mid-pregnancy abortions in murine models (Halden, 2010; Yoon et al., 2014). Phthalates are still widely used, and their production in recent years has reached five million tons (German Federal Environment Agency, 2011). In 2005 the use of di-2-ethylhexyl phthalate (DEHP), dibutyl phthalate, and benzyl butyl phthalate was banned from all children's products (European Parliament and European Council, 2005). Despite the availability of more stable alternatives like diisononyl phthalate and diisodecyl phthalate, it has not yet been possible to completely substitute DEHP in medical devices due to their poor flexibility and viscosity proprieties (Nagorka et al., 2011).

The aforementioned evidence of endocrine disruption by DEHP led to the search for new and safer compounds. In 2002, 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) was introduced as an alternative to common plasticizers for plastic materials. But only in 2006 DINCH received final approval for articles that were intended to contact food in Europe, Switzerland, Australia, Japan, Korea, Taiwan, and China (Bhat et al., 2014; European Food Safety Authority, 2006). DINCH has viscosity and flexibility proprieties that are similar to DEHP (Crespo et al., 2007). A report from the European Food Safety Authority (EFSA) showed that DINCH is not genotoxic and it has sex-specific renal and thyroid toxicity at high doses in rats (300–1000 mg/kg body weight/day). No evidence of developmental or reproductive toxicity was observed prenatally in two-generation toxicity studies in Wistar rats and rabbits at doses of up to 1000 mg/kg body weight/day (European Food Safety Authority, 2006).

Studies from our group and other researchers showed that DEHP's bioactive metabolite, mono-2-ethylhexyl phthalate (MEHP), is able to affect the differentiation of preadipocyte cell lines, as well as primary human preadipocytes (Campioli et al., 2011; Ellero-Simatos et al., 2011; Feige et al., 2007). Due to the similar structure of DEHP and DINCH, the purpose of this study was to evaluate the effect of DINCH and two of its major metabolites (cyclohexane-1,2-dicarboxylic acid [CHDA] and cyclohexane-1,2-dicarboxylic acid mono isononyl ester [MINCH]) (European Food Safety Authority, 2006; Koch et al., 2013; Silva et al., 2012, 2013) on the differentiation of rat primary stromal vascular fraction (SVF). To our knowledge, this is the first study investigating a potential metabolic disrupting effect by DINCH.

2. Methods

2.1. Chemicals

1,2-Cyclohexanedicarboxylic anhydride, mono-(2-ethylhexyl) phthalate (MEHP) and CHDA were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Isononyl alcohol was purchased from C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). DINCH was a gift from Hanno Erythropel, Richard Leask, and Milan Maric (McGill University, Montreal, QC, Canada). The peroxisome proliferator-activated receptor α (PPARα) antagonist, *N*-((2*S*)-2-(((1*Z*)-1-Methyl-3-oxo-3-(4-(trifluoromethyl)phenyl)prop-1-enyl) amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy) phenyl)propyl)propanamide (GW6471) was purchased from Tocris (R&D Systems Inc., Minneapolis, MN, USA) and the selective antagonist of PPARγ, 2-chloro-5-nitro-N-4-pyridinyl-benzamide (T0070907), was from Cayman Chemical Company (Ann Arbor, Michigan, USA). The structures of DINCH, DEHP, MINCH, CHDA, and MEHP are shown in Fig. 1A.

2.2. Cyclohexane-1,2-dicarboxylic acid mono isononyl ester (MINCH) synthesis

Isononyl alcohol (1.1 eq.) in dioxane was added to a stirring solution of 1,2-cyclohexanedicarboxylic anhydride (1.0 eq.) in dioxane. The flask was sealed and heated at 100 °C for 3 days. Solvents were evaporated, dried under high vacuum, and the residue was analyzed by nuclear magnetic resonance (NMR) $^{-1}$H. MINCH was purified from the residual isononyl alcohol (10%) by flash chromatography with a 10–15% ethyl acetate/hexanes gradient. Fractions containing the pure compound were pooled, evaporated and dried under high vacuum. MINCH synthesis is depicted in Fig. 1B.

2.3. Stromal vascular fraction (SVF) isolation

Epididymal adipose tissue was obtained from 60-day-old male Sprague–Dawley rats (Charles River Laboratories, Saint-Constant, QC, Canada). Animals were handled according to protocols approved by the McGill University Animal Care and Use Committee. SVF was obtained from the collected adipose tissue following digestion with type II collagenase (Sigma-Aldrich Canada Ltd.) (Campioli et al., 2014; Li et al., 2014). Purified SVF cells in culture medium (10% fetal bovine serum, 1% penicillin–streptomycin solution, and 1% amphotericin B in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12; all reagents purchased from Life Technologies Inc., Burlington, ON, Canada) were filtered through a 40 μm nylon strainer, and they were centrifuged at 350*g* for 5 min at 4 °C. The pellet was resuspended in the appropriate volume of medium and the cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

To induce preadipocyte differentiation, cells were incubated 3 days after the extraction (day 0) in culture medium implemented with 500 μM of 3-isobutyl-1-methylxanthine (IBMX), 1 μM of dexamethasone, and 1.6 μM of insulin (all purchased from Sigma-Aldrich Canada Ltd.). At day 2, the medium was replaced with culture medium containing only 1.6 μM of insulin for 2 days. At days 4 and 7, the medium was replaced only with the culture medium without any differentiating compound.

2.4. Immunoblot analysis

Chemicals were added at days 0 and 2, in addition to the differentiation cocktail described above. At day 4 of differentiation, cell pellets were homogenized in 1X cold radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology Inc., Danvers, MA, USA) implemented with protease inhibitor cocktail (Sigma-Aldrich Canada Ltd.). Protein extracts were obtained by centrifugation of the homogenate at 14,000*g* for 10 min at 4 °C after two cycles of incubation in ice/vortex, and the protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were electrophoretically separated on a NuPAGE® Novex 4–12% Bis-Tris gradient gel (Life Technologies Inc.; Thermo Fisher Scientific, Waltham, MA, USA), then transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories) and blocked for 1 h at room temperature in blocking buffer solution (10% skim milk in TBST solution prepared with 15 mM Trizma hydrochloride, 140 mM NaCl, and 1% Tween-20, from Sigma-Aldrich Canada Ltd.). Membranes were then incubated overnight at 4 °C in a 5% BSA (Roche Applied Science, Laval, QC, Canada)–TBST solution with the following antibodies: rabbit immunoglobulin (Ig)G anti-PPAR-α polyclonal antibody (1:1000 dilution; Abcam Inc, Toronto, ON, Canada), and rabbit IgG anti-PPAR-γ polyclonal antibody (1:1000 dilution; Cell Signaling Technology Inc.). Finally, membranes were washed