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Research Paper

Evidence for estrogeno-mimetic effects of a mixture of low-dose pollutants in a model of ovariectomized mice

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ABSTRACT

We recently hypothesized that a mixture of low-dosed dioxin, polychlorobiphenyl, phthalate and bisphenol may induce estrogeno-mimetic activities in a model of lifelong-exposed female mice. Herein, we evaluated the impact of this mixture in estrogen deficiency conditions. Based on the protective effects of estrogens against metabolic disorders, we reasoned that exposure to pollutants should attenuate the deleterious metabolic effects induced by ovariectomy. In line with the hypothesis, exposure to pollutants was found to reduce the impact of ovariectomy on glucose intolerance and insulin resistance, to enhance the expression levels of the hepatic estrogen receptor α and to attenuate the ovariectomy-induced enhancement of the chemokine MCP-1/CCL2 considered as an indicator of estrogen signalling. Because of the very low doses of pollutants used in mixture, these findings may have strong implications in terms of understanding the potential role of environmental contaminants in the development of metabolic diseases, specifically in females during menopausal transition.

1. Introduction

Convincing evidences have demonstrated that environmental pollutants contribute to the aetiology of obesity and associated metabolic disorders including diabetes and cardiovascular diseases (WHO, 2016; Casals-Casas and Desvergne, 2011; Gore et al., 2015; Lee et al., 2014). These multifactorial diseases have risen dramatically these last few decades and they constitute a true challenge for Public Health in terms of quality of life and life expectancy but also because of the heavy economic cost for the society (Trasande et al., 2016). WHO, 2016 reported a doubling of the worldwide prevalence of obesity between 1980 and 2014 so that 39% of adults were overweight in 2014 and the global prevalence of diabetes has also risen during this period of time to affect 8.5% of adults.

It has been suggested that mechanisms by which chemicals interfere with energy homeostasis could be related to their hormono-mimetic properties. An endocrine disrupting chemical (EDC) is an exogenous substance or mixture of chemicals that interfere with any aspect of hormone action (Zoeller et al., 2012). About a thousand of chemicals could display ED activities and a subset was identified as metabolic disruptors (Heindel et al., 2017; Nadal et al., 2017; Casals-Casas and Desvergne, 2011; Janesick and Blumberg, 2016). Indeed, it is well documented that energy balance is a highly controlled process depending on a multitude of hormonal inputs and signalling molecules interconnecting central and peripheral organs to meet energy demands and preserve homeostasis (Mauvais-Jarvis et al., 2013). Identified chemicals concerned dioxins which are by-products issued from industrial processes or polychlorobiphenyls (PCBs) produced for their fire-resistant characteristics. Although dioxin production is limited and PCBs forbidden, their persistent properties made them still present in the environment. Other EDCs may have short half-life (e.g., phthalates, bisphenols). However because they are massively produced by industry

Abbreviation: EDC, endocrine disrupting chemical; PCB, polychlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BPA, bisphenol A; ER, estrogen receptor; EST, estrogen sulfotransferase; HFHS, high-fat high-sucrose; DEHP, Di-[2-ethylhexyl]-phthalate; GTT, glucose tolerance test; TDI, tolerable daily intake; OVX, ovariectomy; FFA, free fatty acids; AT, adipose tissue; MCP-1, monocyte chemoattractant protein-1; Ccl5, Chemokine (C-C motif) ligand 5; IL6, IL1: interleukin 6 interleukin 1; Gusb, b-glucuronidase; HPRT, hypoxanthine ribosyl transferase; TBP, TATA-Box Binding Protein; PPAR, peroxisome proliferator-activated receptor; SREBP1, sterol response element binding protein 1; CPT1, carnitine palmitoyl transferase

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for everyday plastic objects or medical equipment among others, everyone is continuously exposed as reflected by the constant detectable levels of several of them in blood, urine or hair (Casals-Casas and Desvergne, 2011; Lee et al., 2014; Appenzeller et al., 2017; Vandenberg et al., 2012). Importantly, these chemicals have been identified as bearing estrogeno-mimetic, anti-estrogenic or anti-androgenic properties which explain that EDCs were first identified as altering the reproductive function and fertility (Vandenberg et al., 2012; Zoeller et al., 2012). The challenge of EDCs resides in that they may act at low and environmental doses in a manner possibly depending on the tissue considered, the sex of the individual and the timing of exposure, all in a context of multi-exposure (Vandenberg et al., 2012; Zoeller et al., 2012; Casals-2011Casas and Desvergne, 2011; Gore et al., 2015; Le Magueresse-Battistoni et al., 2017; Heindel et al., 2017; Barouki et al., 2012).

To enhance our understanding on the metabolic impact of EDCs in mixture, we have developed in the laboratory a model of lifelong exposure to a mixture made of 4 well-spread chemicals including a dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]), PCB153, diethylhexylphthalate (DEHP) and bisphenol A (BPA) at doses in the range of the Tolerable Daily Intake (TDI), thus without expected adverse health effects (Dorne, 2010). Further, these pollutants could be found in a Western-type of diet and are archetypal examples of EDCs suspected to be involved in the aetiology of metabolic disturbances (Lee et al., 2014; Heindel et al., 2017; Le Magueresse-Battistoni et al., 2017; Le Magueresse-Battistoni et al., 2015; Nadal et al., 2017). Importantly, using this model we reported an aggravation of glucose intolerance and hepatic insulin resistance in adult female mice but not in the sibling males. Specifically, we found reduced expression levels of the estrogen receptor α (ERα) and induction of the estrogen sulfotransferase (EST) that inactivates estrogens, all consistent with reduced hepatic estrogen signalling (Naville et al., 2013). Conversely, in immature female mice characterized by low circulating levels of estrogens, lifelong exposure to the pollutant mixture induced improvement of glucose tolerance, enhanced lean mass, and reduced inflammation of the adipose tissue consistent with enhanced estrogen signalling (Naville et al., 2015). We therefore put forward the hypothesis that the mixture of pollutants could exert estrogeno-mimetic effects. Indeed, it is well established that estrogens protect females against metabolic disorders and diabetes for concentrations which stay within physiological range values (Mauvais-Jarvis, 2015), and that the metabolic protective effect of estrogens requires intact hepatic estrogen signalling through its receptor α (ERα) (Zhu et al., 2013). To give further insight to the hypothesis, we have evaluated in the present study the impact of the mixture of pollutants in conditions of estrogen deficiency. We reasoned that if the hypothesis still holds true, then the mixture of pollutants should attenuate the deleterious metabolic effects induced by ovariectomy.

2. Materials and methods

2.1. Animals, diets and experimental design

All procedures were performed with the approval of the Regional Committee of Ethics for Animal Experiments and the French Ministry for Higher Education and Research. After one week acclimatization, five week-old C57Bl6 female mice (Envigo, Gannat, France) were fed a high fat-high sucrose diet (HFHS) (from Envigo) containing (HFp) or not (HF0) a mixture of pollutants. The diet was given 5 weeks before mating with standard-chow adult C57Bl6 males and continued during gestation and lactation. At weaning, the offspring (F1 mice) received the same diet than their dams. By 5 weeks, half of the F1 females were ovariectomized (OVX) and the other half was sham-operated, generating 4 groups of 7–9 animals per group: HF0-OVX and HF0-sham not exposed to the pollutant mixture and HFp-OVX and HFp-sham exposed to the pollutant mixture. Surgeries were performed under anaesthesia using a mixture of ketamine (100 µg/g) and xylazine (10 µg/g) injected intraperitoneally. Ovaries were removed (OVX) or not (sham-operated) after two small flank incisions. The analgesic meloxicam was added to the water during the first days after surgery.

With this protocol, HFp dams underwent normal gestation. All parameters (litter size, sex-ratio and pup weight) were in the normal range, consistently with the previous data of the laboratory (Naville et al., 2013). Body weight and food intake were recorded weekly throughout the protocol until mice were 12-weeks of age. Validation of the surgery was assessed at the time of sacrifice with the observation of uterine atrophy in the OVX females. We also quantified 17ß estradiol levels and found them below the detection threshold of the assay (Interchim, Cayman EIA kit, Montluçon, France) both in HF0- and HFp-OVX mice.

The pollutant mixture was made of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, CAS n° 1746-01-6), Polychlorinated biphenyl (PCB) 153 (CAS n°35065-27-1), Bisphenol A (BPA, CAS n°80-05-7) and Di-[2-ethylhexyl]-phthalate (DEHP, CAS n°117-81-7) (all from Sigma-Aldrich, Saint-Quentin Fallavier, France). Each pollutant was used at a dose close to its tolerable daily intake (TDI) reference dose of either the pollutant itself (DEHP, BPA) or representative congeners (TCDD, PCB153) (EUROPEAN, 2005; EFSA Panel on Food Additives 2015, WHO, 2003; Van Leeuwen et al., 2000). Pollutants dissolved in dimethylsulfoxide (DMSO) were diluted in corn oil to facilitate uniform homogenization in the food. Pollutant-free diet contained volumes of DMSO and corn oil identical to the pollutant-containing food. The composition of the diets is given in the supplemental data (Supplementary Table 1). Thus, it resulted in a daily exposure to 2 pg/kg bw/d of TCDD, 80 ng/kg bw/d of PCB153, 50 µg/ kg bw/d of DEHP and 5 µg/ kg bw/d of BPA, as previously described (3). (Naville et al., 2013; Naville et al., 2015). An additional pellet of pollutant-free HFSD diet was provided to animals to ensure they were fed ad libitum.

2.2. Metabolic tests

After 16 h of fasting, mice were injected intraperitoneally with glucose 1 mg/g of body weight for the glucose tolerance test (GTT). Blood glucose was measured using OneTouchUltra glucometer (Lifescan, Issy-Les-Moulineaux, France). At times 0 and 15 min of the GTT, about 20 µl of blood was collected from tail using heparinized glass capillary tubes. Recovered plasma was used for the measurement of insulin by ELISA (Mouse Ultrasensitive ELISA, Eurobio, Courtaboeuf, France).

2.3. Blood and tissue collection

Mice were fasted six hours and blood was collected by retro-orbital sampling. Mice were euthanized by cervical dislocation and liver, gastronecmius muscle, subcutaneous and visceral (periovarian + parametrial) adipose tissue were quickly dissected and snap-frozen in nitrogen liquid. We measured blood concentration of glucose (glucometer), plasma levels of insulin (Mouse Ultrasensitive ELISA, Eurobio), free fatty acids (FFA) (Sigma-Aldrich), triglycerides (Biolabo, Maizy, France), and leptin (Crystal Chem, Zaandam, Netherlands). Triglycerides were also measured in the liver after extraction of lipids from frozen liver samples using a lipid Extraction kit chloroform-free (Clinisciences, Nanterre, France).

2.4. Real time PCR analyses

Total RNA extracted from frozen liver and adipose tissue samples was reverse-transcribed and analyzed by real-time PCR as described (Naville et al., 2011) using a set of specific primers (Supplementary Table 2). Data were normalized relatively to house-keeping genes which were Gusb (encoding b-glucuronidase) for liver, HPRT (encoding hypoxanthine ribosyl transferase) for subcutaneous adipose tissue and TBP (encoding TATA-Box Binding Protein) for visceral adipose tissue.