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Low-dose pollutant mixture triggers metabolic disturbances in female mice leading to common and specific features as compared to a high-fat diet☆

Emmanuel Labaronne, Claudie Pinteur[1], Nathalie Vega[1], Sandra Pesenti, Benoit Julien, Emmanuelle Meugnier-Fouilloux, Hubert Vidal, Danielle Naville[2], Brigitte Le Magueresse-Battistoni*,[2]

Univ-Lyon, CarMeN laboratory, INSERM U1060, INRA U1397, Université Claude Bernard Lyon1, INSA Lyon, Charles Mérieux Medical School, F-69600 Oullins, France

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Abstract

Environmental pollutants are potential etiologic factors of obesity and diabetes that reach epidemic proportions worldwide. However, it is important to determine if pollutants could exert metabolic defects without directly inducing obesity. The metabolic disturbances triggered in nonobese mice lifelong exposed to a mixture of low-dose pollutants (2,3,7,8-tetrachlorodibenzo-p-dioxine, polychlorinated biphenyl 153, diethylhexyl-phthalate, and bisphenol A) were compared with changes provoked by a high-fat high-sucrose (HFHS) diet not containing the pollutant mixture. Interestingly, females exposed to pollutants exhibited modifications in lipid homeostasis including a significant increase of hepatic triglycerides but also distinct features from those observed in diet-induced obese mice. For example, they did not gain weight nor was glucose tolerance impacted. To get more insight, a transcriptomic analysis was performed in liver for comparison. We observed that in addition to the xenobiotic/drug metabolism pathway, analysis of the hepatic signature illustrated that the steroid/cholesterol, fatty acid/lipid and circadian clock metabolic pathways were targeted in response to pollutants as observed in the diet-induced obese mice. However, the specific sets of dysregulated annotated genes (>1300) did not overlap more than 40% between both challenges with some genes specifically altered only in response to pollutant exposure. Collectively, results show that pollutants and HFHS affect common metabolic pathways, but by different, albeit overlapping, mechanisms. This is highly relevant for understanding the synergistic effects between pollutants and the obesogenic diet reported in the literature.

Keywords: Cholesterol; Circadian clock; Dyslipidemia; Endocrine disruptors; Metabolic disorders; Hepatic transcriptome

1. Introduction

Environmental pollutants are potential etiologic factors of obesity and diabetes, major chronic diseases that reach epidemic proportions worldwide [1,2]. The 2016 World Health Organization (WHO) update [3] indicates a doubling of obese people since 1980 with 1.9 billion overweight adults and a fourfold increase in the number of people with diabetes since 1980, reaching 422 million in 2014. Pollutants are indeed now viewed as metabolic disruptors in addition to their endocrine disruption activities initially evidenced in reproductive organs [4]. Accordingly, a number of chemicals, which exponential manufacturing coincides with obesity trends [5], interfere with lipid metabolism and can promote adipocyte differentiation and fat storage leading to obesity and obesity-related metabolic dysfunctions [6–8]. However, we recently demonstrated that exposure to a mixture of food pollutants could trigger metabolic disturbances in the progeny of obese mice without additional obesogenic effect [9–11], suggesting independent actions of chemicals on metabolic parameters. In this model of lifelong exposure (i.e., from preconception to 12 weeks of age) to a mixture of low-dose pollutants (2,3,7,8-Tetrachlorodibenzo-p-dioxine, TCDD; Bisphenol A, BPA; Diethylhexyl-phthalate, DEHP and Polychlorinated biphenyl, PCB153) in mice fed a high-fat high-sucrose (HFHS) diet, we demonstrated a marked deterioration of glucose tolerance and liver insulin resistance associated with impaired hepatic estrogen signaling in females. Importantly, mice exhibited no extra weight gain in addition to the HFHS diet, and this metabolic phenotype was restricted to the females [9,12]. This suggests that under some

Abbreviations: AUC, area-under-curve; BPA, bisphenol A; CE, cholesteryl ester; DEHP, diethylhexyl phthalate; DMSO, dimethylsulfoxide; FFA, free fatty acid; GTT, glucose tolerance test; HFHS, high- fat high sucrose; IST, insulin sensitivity test; PCB, polychlorinated biphenyl; POP, Persistent organic pollutant; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TDI, Tolerable Daily Intake; TG, triglyceride.

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* Corresponding author at: CarMeN laboratory, INSERM U1060, Faculté de Médecine Lyon-Sud, Chemin du Grand Revoyet, 69600 Oullins, France. Tel.: +33-426235919; fax: +33-426235916.

E-mail address: brigitte.lemagueresse@inserm.fr (B. Le Magueresse-Battistoni).

[1] NV and CP contributed equally to this work.

[2] DN and BLMB share the last authorship.

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circumstances, pollutants could exert metabolic defects without directly inducing obesity.

Consistently, metabolically unhealthy obese patients had higher plasma levels of persistent organic pollutants (POPs) than metabolically healthy obese subjects [13]. In addition, epidemiological studies evidence strong relations between high circulating levels of POPs and insulin resistance, prevalence of metabolic syndrome or diabetes, after adjusting for obesity, in the US general population of the National Health and Nutrition Examination Survey (NHANES) 1999–2002 [2]. The association is even higher among the most obese persons suggesting interactions between POPs and obesity on the risk of Type 2 diabetes [2]. In line with these epidemiological findings, experimental studies demonstrate a worsening of metabolic consequences with a high-fat diet compared with standard diet, in rodent models of exposure to chemicals [7,8,14,15]. Altogether, this important bulk of recent data pinpoints specific actions of chemicals on metabolic parameters in a context of obesity. However, it is important to determine whether effects of pollutants and high-fat diet are mediated by common or distinct mechanisms.

To get more insight in this significant issue, we herein characterized the transcriptomic signature in the liver of mice fed a standard diet containing the mixture of pollutants [9] because the liver is a major site of detoxification and a critical organ for energy homeostasis and insulin sensitivity. The pollutant signature was compared to the list of genes dysregulated in the liver of mice fed an HFHSD but not exposed to pollutants. This study mostly focused on females because of their strong phenotypic response to pollutant exposure previously described in an obesity context [9,12].

2. Materials and methods

2.1. Diets and animals

Mouse studies were performed with the approval of the Regional Committee of Ethics for Animal Experiments. Four week-old C57BL/6 J female mice (Envigo, Gannat, France) were housed in polypropylene cages with a normal light/dark cycle and free access to water (polypropylene bottles) and standard chow (LASQCdiet® Rod16-A, Genobios, Laval, France). After an acclimation period of 1 week, mice were randomly divided into two groups fed a standard diet supplemented (ST*pol* group) or not (ST*veh* group) with a mixture of TCDD (LGC-Promochem, Molsheim, France), BPA, DEHP and PCB153 (all from Sigma-Aldrich, Lyon, France) used at doses in the range of the TDI reference dose of each pollutant [9]. In practice, powdered standard diet (Envigo) was thoroughly mixed in the presence of a solution of gelatin (12 g in 600 ml of water, for 1 kg of powder) to obtain a soft paste. The four DMSO-dissolved pollutants were then added in a small volume of corn oil to facilitate uniform homogenization and make the polluted standard diet. The same amount of DMSO and corn oil was added to the standard diet as vehicle (Supplementary data 1). To ensure that mice were exposed to the similar amounts of polluted food, 1 g of contaminated diet for 17 g of body weight (bw) was added each day (d), completed with the standard diet containing vehicle provided *ad libitum*. Thus, it resulted in a mouse 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 [9]. Female mice were fed these diets for 5 weeks before mating with 8-week-old standard-fed male mice and then during the periods of gestation and lactation. After weaning, the progeny was fed the same diet than their dam until 12 weeks of age. Body weight and food intake were recorded weekly. An additional group of females, following the same experimental design described above, was fed an HFHS diet (Envigo) (Supplementary data 1) without pollutant but containing the same amount of DMSO and corn oil used as vehicle (HFHS*veh*).

2.2. Metabolic tests

Glucose tolerance test (GTT) and insulin sensitivity test (IST) were performed on the female progeny of 11 weeks as previously described [16].

2.3. Tissue collection and biochemical assays

By week 12, the female progeny was euthanized after 6 h of fasting. Blood was collected, liver removed, the duodenum cut out and the intestinal content flushed out. Tissues collected were quickly frozen in liquid nitrogen. Blood glucose concentration was measured (OneTouchUltra glucometer, Lifescan, Issy-les-Moulineaux, France) as well as plasma levels of insulin (Mouse Ultrasensitive ELISA, Eurobio, Courtaboeuf, France). Triglycerides (Biolabo, Maizy, France), free fatty acid, total cholesterol and cholesteryl esters (Sigma-Aldrich) were also assessed in plasma.

Lipids were extracted from frozen liver samples using a lipid extraction kit chloroform free (Clinisciences, Nanterre, France) to measure hepatic triglycerides and total cholesterol.

2.4. Microarray and transcriptomic data analyses

RNA profiling in mice liver was performed using a Mouse GE 4×44K v2 Microarray Kit (Agilent Technologies, Massy, France). Briefly, 100 ng of total RNA isolated from frozen liver samples were labeled using the Low Input Quick Amp Labeling kit (CY3) (Agilent Technologies). Samples from 3 ST*veh*, 5 ST*pol* and 5 HFHS*veh* female mice were used in the study. Microarrays were then hybridized and scanned following the manufacturer's instructions. Data extraction was performed using Agilent Feature Extraction Software 11.5.1.1. Quality analysis and treatment of the data were performed with bioconductor using Agi4×44PreProcess and Limma packages [17]. Background subtraction was performed using the Normexp correction with offset=50. Quantile normalization was then applied. Features flagged in feature extraction as control, as nonuniform outliers, saturating or too weak were excluded. Statistical analysis was performed on 26,082 probes with the Limma package. Datasets are available from the GEO database (GSE86582). Then, for further analyses, probes from the ST*pol* and the HFHS*veh* groups with *p*-value less than 0.05 and with a fold change greater than 1.2 compared to STveh were retrieved. The Database for Annotation, Visualization and Integrated Discovery (*DAVID* v6.7) (https://david.ncifcrf.gov/) was used to identify enrichments in biological functions and pathways in the list of regulated genes.

2.5. Real-time PCR analyses

Total RNA was extracted from frozen liver samples, reverse transcribed and analyzed by real-time quantitative PCR in the presence of specific primer pairs (Supplementary data 2) as previously described [18]. Data were normalized using *Gusb* (encoding β-glucuronidase) and *Hprt* (encoding hypoxanthine guanine phosphoribosyl transferase) as reference genes for liver and duodenum, respectively.

2.6. Statistical analyses

Statistical analyses were performed using one-way ANOVA followed, when appropriate, by *post hoc* testing with Fisher's protected least significant difference test, to assess differences between groups. Mann–Whitney tests were applied when values were not normally distributed. Results are expressed as means ± S.E.M., and differences were considered significant at *P*-value <0.05.

3. Results

3.1. Metabolic alterations in lifelong pollutant-exposed female mice

Upon feeding the standard diet, the mixture of pollutants did not induce any weight gain in the female progeny (Fig.1A) nor changes in food intake (data not shown), as described in a context of obesity [9]. Pollutants did not affect fasting blood glucose or fasting plasma insulin, free fatty acids (FFA) and triglyceride (TG) levels (Fig. 1B–E). In contrast, plasma cholesterol levels showed a tendency toward a decrease (P=.06) while cholesteryl esters (CEs) were significantly decreased (P=.03) (Fig. 1F). There was a significant doubling of the hepatic TG level (Fig. 1G) while total cholesterol concentration was not affected (Fig. 1H). GTT and IST studies (Fig. 1I, J) showed no modifications in glucose tolerance but a tendency toward decreased insulin sensitivity in ST*pol* females (ST*pol* male siblings only exhibiting an enhanced blood glucose level, Supplementary data 3). Compared to females fed the HFHS diet characterized by enhanced body weight, higher blood glucose, increased plasma insulin, cholesterol and CE concentrations, enhanced hepatic TG levels and significant degradation of glucose tolerance (Supplementary data 4) [9], these data indicated that, when provided separately, the mixture of pollutants and the HFHS diet did not induce the same metabolic disturbances allowing the comparison of the metabolic pathways impacted by either challenge. In males, like in females, metabolic disturbances were dependent on the metabolic stressor (Supplementary data 3).

3.2. Transcriptomic signatures of liver in the STpol and HFHSveh groups

Analysis of the microarray data performed in the liver of fasted mice revealed that 2006 probes corresponding to 1473 unique annotated genes show significant changes (*P-value* <0.05, fold change