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Exposure to DEHP decreased four fatty acid levels in plasma of prepartum mice

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ABSTRACT

Maternal exposure to di(2-ethylhexyl) phthalate (DEHP) decreased the plasma triglyceride in prepartum mice. To identify the fatty acid (FA) species involved and to understand the underlying mechanisms, pregnant Sv/129 wild-type (m*PPARα*), peroxisome proliferator-activated receptor α-null (*Pparα*-null) and humanized PPARα (h*PPARα*) mice were treated with diets containing 0%, 0.01%, 0.05% or 0.1% DEHP. Dams were dissected on gestational day 18 together with fetuses, and on postnatal day 2 together with newborns. n-3/n-6 polyunsaturated, saturated, and monounsaturated FAs in maternal plasma and in liver of wild-type offspring, and representative enzymes for FA desaturation and elongation in maternal liver, were measured. The plasma levels of linoleic acid, α-linolenic acid, palmitic acid and oleic acid were higher in the pregnant control m*PPARa* mice than in *Ppara*-null and h*PPARa* mice. DEHP exposure significantly decreased the levels of these four FAs only in pregnant m*PPARα* mice. Plasma levels of many FAs were higher in pregnant mice than in postpartum ones in a genotype-independent manner, while it was lower in the livers of fetuses than pups. DEHP exposure slightly increased hepatic arachidonic acid, α-linolenic acid, palmitoleic acid and oleic acid in fetuses, but not in pups. However, DEHP exposure did not clearly influence FA desaturase 1 and 2 nor elongase 2 and 5 expressions in the liver of all maternal mice. Taken together, the levels of plasma four FAs with shorter carbon chains were higher in pregnant m*PPARα* mice than in other genotypes, and DEHP exposure decreased these specific FA concentrations only in m*PPARα* mice, similarly to triglyceride levels.

1. Introduction

Plasticizers are used to improve the plasticity and elasticity of the polyvinylchloride (PVC) materials. Phthalate ester is the most commonly used plasticizer in the world, the output of which was about 320,000 tons in 2005 in Japan. Although its output is decreasing year by year and stood at about 200,000 tons in 2011, phthalate ester still accounts for about 75% of all plasticizer production in Japan (Japan Plasticizer Industry Association, 2013; Ministry of Economy, Trade and Industry, 2012). Among all the phthalate esters in Japan, di(2-ethylhexyl) phthalate (DEHP) accounts for more than 60% of PVC products, such as construction materials, wire covering, and vinyl sheets used for agriculture and daily-life products like food wrappers (Japan Plasticizer Industry Association, 2010). DEHP easily elutes from those materials because it does not chemically bind to PVC. As a result, humans are mainly exposed to DEHP through food (Koch et al., 2006).

DEHP exerts adverse effects on liver, kidney, and reproductive organs, and in some organs causes carcinogenicity (Lamb et al., 1987; Rusyn et al., 2006; Christiansen et al., 2009). Hayashi et al. (2011) reported that maternal exposure to DEHP decreased the number of alive fetuses and pups, while it increased those of resorption in wild-type (mouse peroxisome proliferator-activated receptor α: m*PPARα*) and humanized PPARα (h*PPARα*) mice. Interestingly, these adverse effects were not observed in *Pparα*-null mice. A decrease in the concentration of maternal plasma triglyceride (TG) was observed only in m*PPARα* mice, and this was suspected to be a pathogenesis for the adverse effects of DEHP. Therefore, DEHP exposure may also influence the concentration of several fatty acid (FA) components which constitute a major part of TG. Indeed, FAs are essential for early human development (Hornstra, 2001), and exposure to DEHP and/or mono (2-ethylhexyl) phthalate (MEHP) changed serum lipid profiling

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including TG and n-3/n-6 in families of rats (Oishi, 1984) and in a rat placental cell line (Xu et al., 2006), which may have resulted in abnormal fetal development.

DEHP absorbed in the body is metabolized into MEHP by lipase (Gunnarsson et al., 2008), a ligand for PPARα and γ (Maloney and Waxman, 1999). After PPARα is activated, β- or ω-oxidizing enzymes of the FAs are up-regulated, and the metabolism is accelerated. FAs that humans and other animals cannot synthesize de novo and must be ingested from food for optimum development and health, are called essential fatty acids (EFA) (Hornstra, 2001). Linoleic acid (LA, a n-6 FA, carbon chain length (C) 18) and α-linolenic acid (ALA, a n-3 FA, C18) are two EFAs and serve as precursors for the synthesis of the respective n-6 and n-3 longer-chain, polyunsaturated fatty acids (LC-PUFA) via sequential desaturation, elongation and partial degradation steps. Arachidonic acid (AA, C20) is the most important member of n-6 LC-PUFA, and eicosapentaenoic acid (EPA, C20) and docosahexaenoic acid (DHA, C22) are the most important members of n-3 LC-PUFA (Hornstra, 2001; Guillou et al., 2010). In the conversion of linoleic acid and α-linolenic acid to their respective LC-PUFA groups, fatty acid desaturase (Fads) 2 catalyzes the initial and rate-limiting desaturation of linoleic acid and α-linolenic acid. Fads1 promotes desaturation to yield arachidonic acid and EPA, and elongation of very-long-chain FAs by elongase (Elovl) 2 and 5 are more selective for PUFA elongation (Guillou et al., 2010). In contrast to PUFA, saturated FAs such as palmitic acid (C16) and stearic acid (C18), and monounsaturated FAs such as palmitoleic acid (C16) and oleic acid (C18), can be synthesized de novo and have undesirable effects on health, although they are essential barrier components of the plasma membrane (Guillou et al., 2010).

Considering the above, in the present study, we investigated the effects of DEHP exposure on the levels of EFA and n-3/n-6 LC-PUFA, as well as saturated and monounsaturated FAs in the plasma of pregnant and postpartum mice, and on the livers of their offspring. We then discussed the relationship between the decrease in TG and individual FA components induced by DEHP exposure.

2. Materials and methods

2.1. Laboratory animal and diet

Three genotyped male and female mice, i.e. (1) m*PPARα*, (2) *Pparα*-null (Lee et al., 1995) and (3) h*PPARα*,$^{Tet\text{-}Off}$ express human PPARα only in the liver of *Pparα*-null mice (Cheung et al., 2004) with a Sv/129 genetic background, were obtained from the US National Cancer Institute, and housed in a constant environment (23–25 °C room temperature, 57–60% humidity, and 12 h light/dark cycles). They were fed a solid diet (CLEA Rodent Diet CE-2; Japan Clea Co., Tokyo, Japan) and tap water ad libitum. The mice were then bred. At the age of 12 weeks, the genotyped mice were divided into 4 groups, and given diets containing 0, 0.01, 0.05, and 0.1% DEHP, respectively, ad libitum. Their dietary intakes were as follows: 10–12, 55–64 and 119–145 mg per kg body weight per day, respectively, throughout the observed periods.

Four weeks after the commencement of DEHP exposure, the genotyped mice with the same exposure level were mated. The day on which the plug was identified was designated as gestational day (GD) 0. All the pregnant mice were divided into 2 groups: one was dissected on GD18 together with fetuses and the other on postnatal day (PND) 2 together with newborn pups. Under these conditions, DEHP exposures at dosages of 0.05% and/or 0.1% decreased the number of live fetuses and pups, and increased resorption in m*PPARα* and h*PPARα*. In addition, the plasma TG level in pregnant mice was decreased at a 0.1% dose only in m*PPARα* mice (Hayashi et al., 2011). The blood and livers were collected. Blood was processed with heparin and the removed livers were snap-frozen in liquid nitrogen. These samples were stored at −80 °C until use.

All the experiments were conducted according to the Guidelines for Animal Experiments of the Nagoya University Animal Center.

2.2. Measurement of maternal plasma FAs concentration

FA levels in maternal plasma of mice were measured according to the method of Masood et al. (2005): 25 μl of plasma was mixed with 1.2 ml of methanol, 75 μl of acetyl chloride, and 75 μl of 10 μg/100 μl tricosanoic acid ethyl ester/methanol (internal standard) in screw-capped glass test tubes. Air in the test tubes was then replaced by nitrogen and heated at 100 °C for 60 min in a heating block. After the tubes were allowed to cool to room temperature, n-hexane (500 μl) was added and shaken for 30 s by vortex. Then the tubes were centrifuged at 3000 rpm for 2 min, and the upper organic layer was collected and moved into another vial followed by replacing the air of the vial with nitrogen. After the n-hexane extraction was repeated once more, the concentration of FA methyl ester contained in the n-hexane layer was measured by gas chromatography–mass spectrometry (GC–MS). Nine FA components targeted for measurement included palmitic acid and stearic acid of saturated FAs, palmitoleic acid and oleic acid of monounsaturated FAs, linoleic acid and arachidonic acid of the n-6 family, and α-linolenic acid, EPA and DHA of the n-3 family.

Details of the conditions for the GC–MS (Agilent Technologies, GC: 6890N, MS: 5975, Injector: 7683B Series, auto sampler 7683 series) were as follows: capillary column, DB-FFAP (15 m × 0.10 mm × 0.10 μm, Agilent Technologies); carrier gas, helium with velocity of 0.2 ml/min with a constant head pressure of 279.6 kPa. Initial column and injection port temperature, 150 °C with 280 °C in interface temperature. The temperature program was as follows: initial temperature of 150 °C for 0.25 min, raised to 200 °C in the next 1 min, held at 200 °C for 4.5 min, raised to 225 °C in the next 2 min, held at 225 °C for 8.5 min, raised to 245 °C in the next 0.25 min, and then held at 245 °C for 1.5 min. Analysis mode, EI; positive ion, 70 eV; SIM parameters: tricosanoic acid (internal standard), palmitic acid, palmitoleic acid, stearic acid, oleic acid 74 *m/z*, linoleic acid 67 *m/z*, α-linolenic acid, arachidonic acid, EPA, DHA 79 *m/z*. Split ratio was 200:1. Under these conditions, the detection limits were 2.4 μg/ml for palmitic acid, 1.3 μg/ml for stearic acid, 0.69 μg/ml for palmitoleic acid, 3.6 μg/ml for oleic acid, and 2.0 μg/ml for EFA and n-3/n-6 PUFA.

2.3. Measurement of liver FAs in offspring

Because we could not collect enough blood samples from offspring for the measurement of FA, liver concentrations in fetuses and pups were measured. Livers from fetuses and newborn pups were homogenized with 50 mM K_2HPO4/KH_2PO4 buffer with a ratio of 1:3. The lipids were extracted from the homogenate according to the method of Folch et al. (1957). The chloroform layer obtained was evaporated and dried. Then the sediment was resolved by 1.2 ml of methanol, and the 9 FA components were measured by the same method mentioned above.

2.4. Western blot analysis

Fads 1, 2 and Elovl 2, 5 are localized in the microsomes; therefore, maternal liver microsomal fraction was prepared. Samples containing the same quantity of protein were subjected to SDS-PAGE as described elsewhere (Aoyama et al., 1998; Jia et al., 2012; Nakajima et al., 2000; Nakamura et al., 2009). Briefly, the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the membranes were incubated with the following antibodies: Fads 1 (V-21), Fads 2 (M-50), and Elovl2 (S-16) (Santa Cruz Biotechnology, Santa Cruz, CA), and Elovl5 (ab81326) (Abcam, Cambridge, UK). Actin (H-196) (Santa Cruz Biotechnology, Santa Cruz, CA) antibody was performed for loading control. 1-step™ NBT/BCIP (Pierce Biotechnology, Rockford, IL, USA) was used for the detection of specific proteins. Each band was quantified by densitometry using the Lane and Spot Analyzer version 5.0 (ATTO Corporation, Tokyo, Japan).

2.5. Real-time quantitative PCR

Total RNA was extracted from whole livers using RNeasy Mini Kit (Qiagen, Tokyo, Japan). Quantitative real-time PCR analysis was performed using 1× SYBR Green PCR master Mix (Applied Biosystems) as described elsewhere (Ito et al., 2007; Ramdhan et al., 2009; Nakamura et al., 2009). We normalized all of the mRNA levels to glyceraldehyde-3-phosphate dehydrogenase. The sequences for the forward and reverse primers are shown in Supplemental Table 1.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2013.04.010.

2.6. Statistical analysis

All the data are presented as mean ± standard deviation (SD). Statistical analyses were performed by a Tukey–Kramer HSD test while comparing the genotype effects. Dunnett's test was performed to compare the DEHP exposure in each genotyped mouse, and *t*-test was used to compare the control groups of fetuses and pups, and pregnant and postpartum dams. Data were converted into logarithm form when it was not a normal distribution. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Maternal plasma FA concentrations

3.1.1. n-6 family PUFA

Comparing each genotype of pregnant control mice, the levels of linoleic acid were significantly higher in m*PPARα* mice than in