Effect of Di-(2-ethylhexyl) phthalate on the hypothalamus-pituitary-thyroid axis in adolescent rat

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Abstract. Di-(2-ethylhexyl) phthalate (DEHP) is extensively used in many personal care and consumer products, which has resulted in widespread human exposure. Limited studies have suggested that exposure to DEHP may affect thyroid function, but little is known about the effect and mechanisms of DEHP exposure on the hypothalamic-pituitary-thyroid axis (HPTA). The present study was conducted to elucidate the potential mechanisms in which DEHP disrupts the function of the HPTA. Wistar rats were administered DEHP by gavage at 0, 5, 50, and 500 mg/kg/day for 28 days and then sacrificed within 24 h following the last dose. Hormones of HPTA was quantified with radioimmunoassay and enzyme-linked immunosorbent assay, protein levels of thyrotropin-releasing hormone receptor (TRHR) and thyroid-stimulating hormone receptor (TSHR) were analyzed by Western blot and immunohistochemistry, expression levels of TRHR and TSHR mRNA were measured by quantitative real-time PCR. Rats treated with DEHP resulted in increased bodyweight, on the HPTA, down-regulated the protein levels of TRH in the hypothalamus, up-regulated the protein and mRNA levels of TRHR in the pituitary, down-regulated mRNA expression of TSHR in the thyroid, while the difference of TSH in various dose groups was not statistically significant and T3, T4, FT3, FT4 levels in serum were decreased compared with control. DEHP could interfere with the balance of HPTA of adolescent rats, and increase the body weight, down-regulate the homeostasis of thyroid related hormones and receptors expression levels.

Key words: Phthalates, Di-(2-ethylhexyl) phthalate, Endocrine disruptors, Hypothalamic-pituitary-thyroid axis, Thyroid
especially for the nervous system, reproductive system and endocrine system. More importantly, they are also essential for energy homeostasis and many crucial metabolic pathways. Interestingly, reports show that minor changes in THs may also adversely direct impact on human health, including chronic fatigue syndrome, hypomnesia, goiter, and osteoporosis [15-17]. Limited studies suggest that exposure to DEHP may be associated with altered thyroid function. Some studies have observed an inverse association between MEHP urinary concentrations and free T4 and T3 serum levels in men and similar negative correlations have been found in pregnant women [18, 19]. In animal studies, rats and fishes fed with diets contaminated with DEHP were found to have thyroid alterations and lower plasma T4 concentrations [20, 21]. But the detailed mechanisms that are involved in this process are unclear.

The levels of THs are regulated by thyrotropin-releasing hormone (TRH) and thyroid-stimulating hormone (TSH). Thyroid related hormones are regulated by a feedback mechanism through the hypothalamic-pituitary-thyroid axis (HPTA) to keep internal environment of vivo invariably. Factors, what can influence the balance of the HPTA, may cause abnormal thyroid related hormone levels. As an endocrine disruptor, whether DEHP affects thyroid function by influencing the balance of HPTA is still unknown. In the present study, we explored the effects of DEHP on HPTA in adolescent rats by detecting and analyzing the changes of HPTA related protein and mRNA levels after DEHP exposure. It is important for reviewing the DEHP toxicity comprehensively and systematically and supplying scientific thoughtfulness for the human endocrine system.

Materials and Methods

Animals and treatments

21-d-old healthy Wistar rats were acquired from the Experimental Animal Center of Jilin University, Changchun, China, and acclimatized for one week before exposure. Eighty rats (40 males and 40 females) were randomly divided into four groups (n = 20, 10 males 10 females each group), different sex rats in separate cages. All rats were housed individually at 23 ± 1°C, in humidity set at 55 ± 5% in a 12 h cycle lighting control system room, and ensure uninterrupted food and water supply. Observed and recorded changes of rats twice daily. Animals were daily conducted by intragastric administration for 28 days with corn oil dissolved DEHP (Sinopharm Chemical Reagent, Shanghai, China, purity >99%) at 0 (control), 5, 50, and 500 mg/kg body weight, intragastric dose is 5 mL/kg.

24 hours after the last dose, rats were hucossed with chloral hydrate (10%) anesthesia. Blood was then collected and centrifuged at 1,500 rpm for 10 min to separate the serum. The separated serum was stored at −20°C for measurement. The detached hypothalamus, pituitary and thyroid were washed with physiological saline and weighed after removal from the rats. Then we rapidly dissected them into two parts: one was frozen immediately in liquid nitrogen and then kept at −80°C; another was fixed with 10% formalin for immunohistochemistry. All procedures on animals were in agreement with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of People’s Republic of China.

Radioimmunoassay for T4, T3, FT4, FT3 and TSH

Thyroid hormones T4, T3, FT4, and FT3, as well as TSH in serum were measured by radioimmunoassay kits (3V Biological Technology, Shandong, China). The tracer of radioimmunoassay kits is 125I. Experimental operation was carried out in strict accordance with the instructions. There were no apparent cross reactions observed while conducting the experiment. All samples were done in duplicate.

ELISA for TRH

The level of thyrotropin-releasing hormone was measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems China Co. Ltd., Shanghai, China) in hypothalamus of rat. The total protein was extracted from the hypothalamus under asepsis condition. Micro-porous plate coated with TRH-antibody was prepared by adding 10-μL diluted sample and incubated at 37°C for 30 min. After five washes with distilled water, added 50-μL horseradish peroxidase-labeled streptavidin to the sample mixture and incubated at 37°C for 30 min. Washing five times, and then covered chromogenic solution (50-μL A and 50-μL B) from the kit for 10 min at 37°C in darkness. In the end, the 50-μL stop solution is injected into holes, the reaction was stopped. Microplate Reader (BioTek Instruments, Inc., Vermont, USA) was used to measure the absorbance at 450 nm wavelength within 15 min of quenching the reaction.
Immunohistochemistry for protein expression of TRHR and TSHR

After paraffin section, deparaffinization and rehydration, the specimens of pituitary and thyroid were processed by antigen retrieval in 0.1 M citric acid solution in a microwave oven for 5 min. The sections were then incubated with 3% H$_2$O$_2$ in methanol for 10 min at room temperature to quench the endogenous peroxidase activity. The sections were blotted with normal serum blocking solution at 37°C for 30 min, and incubated with goat polyclonal anti-TRHR primary antibody (Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit polyclonal anti-TSHR primary antibody (Santa Cruz Biotechnology) (1:200 by PBS) in a moist chamber overnight at 4°C. After three washes in PBS, the treated specimens were combined with peroxidase-conjugated secondary antibody (Bioss Biotechnology Company, Beijing, China) (1:200 dilution by PBS) for 30 min at 37°C. With above steps complete, the staining of TRHR and TSHR was visualized by rat immunohistochemistry kits (Bioss Biotechnology Company). Histological sections were observed and photographed by the optical microscope (Nikon Instruments Co. Ltd., Tokyo, Japan).

Western blot analysis for protein levels of TRHR and TSHR

The pituitary and thyroid of rats were homogenized with cell lysis buffer (Beyotime Biotech Inc., Beijing, China). Use BCA Protein Assay Kit (Beyotime Biotech Inc.) to measure the concentrations of total tissues protein. Thirty micrograms of proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel and transferred the isolated proteins to a nitrocellulose membrane. Put the membranes into blocking buffer containing 5% nonfat dry milk for 2 h at room temperature and then incubated with primary antibodies (Santa Cruz Biotechnology, dilution: 1:100) and anti-β-actin (Proteintech Inc., Chicago, USA, dilution: 1:2,000) overnight at 4°C. Then, put the membranes into horseradish peroxidase conjugated secondary antibody and incubated for 45 min at 37°C. Used ECL chemiluminescence Kit (Proteintech Inc.) and covered the up side of membranes with the detection solution for 5 min to react. Exposed the protein side of the membranes to X-ray films which were used to scan and analyze. Image-ProPlus 6.0 (Media Cybernetics) was used to calculate grey value.

Quantitative real-time PCR for mRNA levels of TRHR and TSHR

Total RNA was extracted from the pituitary and thyroid with Trizol reagent (TaKaRa Biotechnology, Dalian, China). Quantitative real-time PCR (QPCR) was used to detect gene expression with Real-Time System (Stratagene MX3000p, California, USA). Reverse transcription was carried out with 500 ng total RNA in a 10-μL reaction (TaKaRa Biotechnology). Then we added 10-μL cDNA into 25-μL mixture, which contained primers and SYBR-Green Supermix (SYBR premix Ex Tapi II, TaKaRa Biotechnology). Temperature and cycle times were 45 cycles of 95 °C for 20 s and 60 °C for 20 s. β-actin gene of rat was reference gene. The mRNA levels were quantified via 2$^{-\Delta\Delta CT}$. The primers were listed in Table 1.

Statistical analysis

The experimental results were listed as mean ± standard deviation, SPSS 13.0 statistical software (SPSS Inc., Chicago, Illinois, USA) is used for statistical analysis. According to normality and homogeneity of variance, experimental data were tested for one-way ANOVA or non-parametric test in SPSS. $p < 0.05$ was considered that the difference was statistically significant.

Results

Effect of DEHP on body weight

As shown in Table 2, administered 50 and 500 mg/kg/d DEHP for three weeks, the mean weights of rats were significantly higher than control rats ($p < 0.05$). After four weeks, the mean weight of 5, 50, 500 mg/kg/d DEHP exposed group was significantly higher than controls ($p < 0.05$).

Effect of DEHP on hormone levels

In the hypothalamus, as the Fig. 1 showed that TRH
levels of rats treated with 500 mg/kg/d DEHP were significantly higher than control group and 5 mg/kg/d DEHP group \( (p < 0.05) \). There was no significant difference in serum TSH level among the various groups \( (p > 0.05) \). The serum levels of T3, T4, FT3 and FT4 in the group treated with 500 mg/kg/d DEHP were significantly lower compared with the control group \( (p < 0.05) \).

**Protein expression levels of TRHR and TSHR**

Fig. 2 was the photos of immunohistochemistry sections of TRHR and TSHR staining. In the pituitary, positive staining for TRHR was found in the cytoplasm and membranes of cells (Fig. 2A). In the thyroid, positive staining for TSHR was found in the cytoplasm and membranes of cells (Fig. 2B).

Fig. 3 was the protein bands of western blot and the contrast situation of gray values. In the pituitary, the protein expression of TRHR treated with 500 mg/kg/d group was significantly higher than the control, 50 mg/kg/d and 50 mg/kg/d group \( (p < 0.05) \), Fig. 3A, Fig. 3C). In the thyroid, the protein expression of TSHR treated with 500 mg/kg/d group was significantly lower than the control and 5 mg/kg/d group, 50 mg/kg/d group was significantly lower than control and 5 mg/kg/d group \( (p < 0.05) \), Fig. 3B, Fig. 3D).

**Gene expression levels of TRHR and TSHR**

Fig. 4 was the mRNA expression levels of TRHR and TSHR. In the pituitary, TRHR mRNA expression levels of treated with 500 mg/kg/d group was significantly higher than controls, 50 mg/kg/d group was significantly higher than controls \( (p < 0.05) \), Fig. 4A). Furthermore, in the thyroid, the mRNA expression levels of TSHR treated with 500 mg/kg/d group were significantly lower than control and 5 mg/kg/d group \( (p < 0.05) \), Fig. 4B).

**Discussion**

As a kind of environmental endocrine disruptors (EEDs), DEHP has a huge potential hazard. And more
and more researchers pay attention on DEHP. Studies about the threshold of DEHP had shown that 5 mg/kg/day was the no observed adverse effect level (NOAEL); 500 mg/kg/day DEHP levels can cause obvious toxicity in rats; 600 mg/kg was the threshold for the occurrence of acute toxicity in Wistar rat in the short term; and the LD50 of rats to DEHP was about 30 g/kg/day [22-25]. In order to better observe the toxic effects and mechanisms. Our dosage was selected as follows: 500 mg/kg/day (1/60 LD50), 50 mg/kg/day (1/600 LD50), 5 mg/kg/day (1/6,000 LD50).

Adolescence is the key period to the growth and
development. Compared with adults, adolescents are more sensitive to EEDs. Han-Bin Huang et al. found that the free T4 level was positively associated with urinary mono benzyl phthalate levels among minors, while it was not positive association among adult [26]. So we chose 4 weeks old adolescent rats to explore the effects of DEHP on the hypothalamus-pituitary-thyroid axis.

In the present study, our experimental data show that DEHP exposure resulted in increased body weight, up-regulated the TRH in the hypothalamus, up-regulated the protein and mRNA expression of TRHR in the pituitary, down-regulated the protein and mRNA expression of TSHR in the thyroid, while THs levels in serum were decreased compared with control.

Body weight is the most considerable indicator of developmental changes among adolescents. The adolescent rats exposed to DEHP resulted higher body weight is identical to others results of study. The available data verified that treated with a low dose of endocrine disruptors during adolescent development can interfere with the normal homeostasis of the endocrine system, and may lead to the occurrence of overweight and obesity [27-29]. The function of THs is energy metabolism regulation and the levels of those were decreased with DEHP exposure, this is possible to be a cause for fat accumulation. Because of the specific mechanism is not clear, future studies are necessary to deeply research whether other reasons are responsible for fat metabolism disorders.

DEHP may cause many adverse effects, in addition to reproductive toxicity or embryo toxicity [11-13], a possible regulating mechanism in the HPTA could account for the influence of thyroid. As previously demonstrated during growth of rats, TRH is regulated by the hypothalamic system to release into the pituitary and stimulate TSH production. This hormone in serum has an effect on the thyroid and causes the thyroid to secrete THs. With the levels of T4 and T3 in serum decreasing, they will negatively regulate the biosynthesis and secretion of TRH and TSH [30, 31]. The results of our study showed that DEHP can directly influence the adolescent rat thyroid and contribute to the lack of hormones. In the hypothalamus, the lowered circulating levels of T4 and T3 in the DEHP-treated rats may primarily be the consequence of thyroid toxicity, resulting in the increased TRH expression. These results, DEHP down-regulated the levels of THs and increased TRH expression in rats, are consistent with a previous study [20]. Another report that involved animal studies described that decreased the levels of serum T4 with prolonged DEHP exposure [21]. Similar results were found in epidemiological studies [18, 19]. Moreover, other endocrine disruptors, such as polychlorinated biphenyls, have been shown to decrease of THs expression [32]. Due to low-levels of circulating thyroid hormones negative feedback increased secretion of serum TSH and TRH also promoted TSH secretion, but the difference of TSH in various dose groups was not statistically significant. We try to put forward a hypothesis that, in addition to decreasing THs, DEHP had toxic effects on pituitary and interfere with the normal secretion of TSH. This kind of mechanism of disturbance is similar to estrogen-like effects of DEHP.

TRHR and TSHR are the key elements in HPTA. The regulatory mechanism, in mammals, is that stimulated hypothalamic secretes TRH and works on the pituitary, binding of TRH to TRHR on the surface of pituitary gland cells, to increase synthesis and the release of TSH [32, 33]. The TSH acts on the thyroid by interacting with TSHR to promote the secretion of THs [34, 35]. The results of this study show that DEHP exposure could
raise the levels of both TRH in the hypothalamus and TRHR in the pituitary. Contrasted with untreated control group, mRNA expression levels of TRHR were significantly higher than rats in 50 and 500 mg/kg/d groups, and similar tendency were observed with TRHR protein expression. Interestingly, the levels of TSH were slightly altered, but the TSHR protein expression in the 500 mg/kg/d group was significantly lower than the lower-dose groups. Therefore, it is highly likely that the administration of DEHP to adolescent rats affected the hypothalamus little and changes of TRH and TRHR should be a reflex of negative feedback regulation of THs decline. DEHP thyroid toxicity could lead to reducing levels of THs and TSHR, besides, the stimulate effect of TSH could be inhibited by the decreasing of TSHR and continue to reduce THs.

In conclusion, DEHP could interfere with the balance of HPTA of adolescent rats, down-regulate the homeostasis of thyroid related hormones and receptors expression levels and increase the body weight.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No. 81573184).

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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