Developmental exposure to di(2-ethylhexyl) phthalate impairs endocrine pancreas and leads to long-term adverse effects on glucose homeostasis in the rat

Yi Lin,* Jie Wei,* Yuanyuan Li, Jun Chen, Zhao Zhou, Liqiong Song, Zhengzheng Wei, Ziquan Lv, Xi Chen, Wei Xia, and Shunqing Xu

Ministry of Education, Key Laboratory of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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Lin Y, Wei J, Li Y, Chen J, Zhou Z, Song L, Wei Z, Lv Z, Chen X, Xia W, Xu S. Developmental exposure to di(2-ethylhexyl) phthalate impairs endocrine pancreas and leads to long-term adverse effects on glucose homeostasis in the rat. Am J Physiol Endocrinol Metab 301: E527–E538, 2011. First published June 14, 2011; doi:10.1152/ajpendo.00233.2011.—Di(2-ethylhexyl) phthalate (DEHP), a typical endocrine-disrupting chemical (EDC), is widely used as plasticizer. DEHP exposure in humans is virtually ubiquitous, and those undergoing certain medical procedures can be especially high. In this study, we investigated whether developmental DEHP exposure disrupted glucose homeostasis in the rat and whether this was associated with the early impairment in endocrine pancreas. Pregnant Wistar rats were administered DEHP (1.25 and 6.25 mg·kg⁻¹·day⁻¹) or corn oil throughout gestation and lactation by oral gavage. Body weight, glucose and insulin tolerance, and β-cell morphology and function were examined in offspring during the growth. In this study, developmental DEHP exposure led to abnormal β-cell ultrastructure, reduced β-cell mass, and pancreatic insulin content as well as alterations in the expression of genes involved in pancreas development and β-cell function in offspring at weaning. At adulthood, female DEHP-exposed offspring exhibited elevated blood glucose, reduced serum insulin, impaired glucose tolerance, and insulin secretion. Male DEHP-exposed offspring had increased serum insulin, although there were no significant differences in blood glucose at fasting and during glucose tolerance test. In addition, both male and female DEHP-exposed offspring had significantly lower birth weight and maintained relatively lower body weight up to 27 wk of age. These results suggest that developmental exposure to DEHP gives rise to β-cell dysfunction and the whole body glucometabolic abnormalities in the rat. DEHP exposure in critical periods of development can be a potential risk factor, at least in part, for developing diabetes.

β-cells; insulin secretion

TYPE 2 DIABETES HAS BEEN RISING AT EPIDEMIC RATES OVER THE LAST SEVERAL DECADES IN THE US AND EUROPE AND MORE RECENTLY IN DEVELOPING COUNTRIES. ACCEPTED RISK FACTORS SUCH AS DIET, LIFESTYLE, AND GENETICS CANNOT FULLY EXPLAIN THIS PHENOMENON, AND THERE IS INCREASING EVIDENCE SUGGESTING THAT THE INCREASED PRESENCE OF ENVIRONMENTAL POLLUTANTS PLAYS AN IMPORTANT PART IN THE ELDED INCIDENCE OF TYPE 2 DIABETES (19). ENDOCRINE-DISRUPTING CHEMICALS (EDCs), TO WHICH MANY PEOPLE ARE EXPOSED IN DAILY LIFE, HAVE BEEN RECEIVING CONSIDERABLE ATTENTION RECENTLY BECAUSE OF THEIR CONTRIBUTIONS TO THE ENERGY IMBALANCE AND METABOLIC DISORDERS (7). IT HAS BEEN REPORTED THAT EXPOSURE TO EDCs SUCH AS DIOXINS (39, 42), BISPHENOL A (21), PHTHALATES (37), OR P,P’-DICHLOORODIPHENYLTRICHLOROETHANE (27, 40) IS HIGHLY ASSOCIATED WITH THE INCREASED RISK OF DEVELOPING INSULIN RESISTANCE OR DIABETES, BECAUSE THE SUBPOPULATIONS THAT HAVE THE HIGHEST RATES OF THESE CHRONIC DISEASES ARE ALSO THOSE THAT HAVE GREATEST EXPOSURE TO EDCs.

Di(2-ethylhexyl) phthalate (DEHP), one of the most widespread plasticizers to impart flexibility of polyvinyl chloride (PVC), is commonly used in the manufacture of many daily products, including vinyl flooring, wall covering, food containers, cosmetics, and toys. General human populations are widely exposed to DEHP in everyday life (7), which is supported by a finding that more than 75% of the US population has had measurable levels of DEHP and other phthalate metabolites detected in the urine (31, 33). Most importantly, DEHP is a major plasticizer for medical products such as containers for blood, dialysis, and nutrients, tubings, and catheters, etc. Thus patients undergoing medical treatment, especially for those requiring extensive infusions, can be exposed to high levels of DEHP (5, 17). To cite just one example, premature neonates undergoing intensive medical procedures can be exposed to 10–20 mg·kg⁻¹·day⁻¹ DEHP (20), exceeding the reference dose of the US Environmental Protection Agency (20 μg·kg⁻¹·day⁻¹) (12) by a factor of 1,000.

Although the majority of research in DEHP focuses on the reproductive development or sex differentiation, mounting evidence has pointed to the impact of DEHP exposure on nutritional and metabolic states. DEHP has been reported to induce glucose intolerance and alterations in hepatic glycogen content in rats (24). Indeed, exposure to DEHP at 7.50 mg/kg body wt on alternate days for 14 days also results in a reduction in serum insulin and an increase in blood glucose in female rats (16). In addition to hormones, abnormal glucose intermediate metabolite contents in liver and skeletal muscle were found to be highly associated with impaired glucose tolerance in male Wistar rats that were fed a diet containing 2% (wt/wt) DEHP for 21 days (23). In humans, a recent epidemiological study related DEHP exposure to the increased risk of metabolic disorders, reporting that adult males who have higher body mass index and higher rates of abdominal obesity and insulin resistance are those that have greater exposure to DEHP or other phthalates (37).

It is generally accepted that individuals are more sensitive to chemical exposure during vital developmental periods; however, few studies have examined the effects of developmental DEHP exposure. Therefore, the aim of the present study was to

* Both of these authors contributed equally to this work.

Address for reprint requests and other correspondence: S. Xu, Ministry of Education, Key Laboratory of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China (e-mail: xuscience@hotmail.com).

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investigate whether developmental DEHP exposure disrupts the whole body glucose homeostasis in the rat and, if so, whether the dysglycemia observed in this animal model is mediated by the impairment of endocrine pancreas at an earlier age.

RESEARCH DESIGN AND METHODS

Animals and treatments. The care of animals and all experiments were conducted according to the guidelines for the care and use of animals established by Tongji Medical College, Huazhong University of Science and Technology, and were approved by the Ethics Committee of Tongji Medical College.

Wistar rats were purchased from the Hubei Research Center of Laboratory Animals, China, and housed under special pathogen-free conditions at the Animal Laboratory of Wuhan University. Animals were maintained under controlled temperature, humidity, and a 12:12-h light-dark cycle, with ad libitum access to food and water. Glass water bottles and polypropylene cages were used to avoid potential contamination from sources other than gavage administration. Nulliparous rats (250–300 g) were mated, and the presence of sperm in the vaginal smear was taken at gestation day 0 (GD 0). A total of 34 pregnant rats were housed individually and monitored daily. Among them, 10 received corn oil (CAS no. 8001-30-7; Sigma), 12 received 1.25 mg·kg\(^{-1}\)·day\(^{-1}\) DEHP, and 12 received 6.25 mg·kg\(^{-1}\)·day\(^{-1}\) DEHP (purity 99%, CAS no. 117-81-7; Sigma) by oral gavage beginning on GD 0 until postnatal day 21. DEHP was dissolved in corn oil, and dosage was adjusted daily for maternal body weight changes (2.0 ml/kg body wt).

After spontaneous delivery, litter size, sex ratio, and birth weight of offspring were recorded. Pups from litters containing less than five males or five females and from litters containing 16 or greater were excluded from the study. Remaining pups were equalized to five male and five female pups per dam and kept with their mothers to reduced litter size-related variability in postnatal nutrition. Pups (n = 10 pups/litter) and dams (n = 8 dams/group) were weighed on postnatal days 1, 5, 10, 15, and 21. Pups were sexed and weaned on postnatal day 21. Two male and two female pups from each of the litters were chosen at random and housed up to week 27. Offspring were fed normal diets that contained 12.05% fat, 24.93% protein, and 63.02% carbohydrates, with energy of 3.45 kcal/g. Offspring continued to be weighed from weaning to week 27. Measurements of postweaning body weight included all of the rats in eight litters per treatment group. Remaining data included five male and five female offspring per treatment group, and only one offspring per sex per litter was selected for testing.

Oral glucose tolerance test and insulin tolerance test. At 3, 15, and 26 wk of age, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed in six male and six female offspring from different litters (only 1 offspring was selected per sex per litter). For OGTT, offspring were fasted overnight for 16 h. Blood glucose and serum insulin were determined before and 15, 30, 60, and 120 min after glucose (purity ≥99.5%; CAS no. 50-99-7; Sigma) administration (2.0 g/kg body wt glucose by oral gavage). Blood glucose was determined using the hand-held commercial glucose meter (ACCU-CHEK Active; Roche). Serum insulin was determined by commercially available radioimmunoassay kits (Linco Research, Millipore, Billerica, MA). Three days after the OGTT, the same cohort of animals was fasted for 6 h and intraperitoneally injected with biosynthetic human insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) at 0.75 U/kg body wt. Blood glucose levels were measured at the indicated intervals, as described above.

Pancreatic insulin content determination. At weeks 3 and 27, pancreases from three male and three female offspring (only 1 offspring was selected per sex per litter) were dissected out, weighed, and homogenated. The homogenate was extracted overnight with acid ethanol at 4°C. Insulin content in the supernatant was determined by radioimmunoassay described above.

Transmission electron microscopy. At weeks 3 and 27, pieces of pancreatic tail from three male and three female offspring (only 1 offspring was selected per sex per litter) were collected and fixed in 2.5% glutaraldehyde. Sections were prepared as described (4) and examined with FEI Tecnai 12G\(^2\) transmission electron microscope (FEI, Eindhoven, The Netherlands). Representative photographs of β-cells were analyzed by Image Pro Plus Version 6.0 software (Media Cybernetics, Silver Spring, MD). Secretory granules in β-cells were manually counted, and quantifications were performed on 24 sections from 10 islets/rat. The average area and density of mitochondria were determined by manually circling a minimum of 100 mitochondria within the β-cells per rat.

Immunohistochemistry and morphometry. At weeks 3 and 27, the whole pancreases from three male and female offspring (only 1 offspring was selected per sex per litter) were dissected out and weighed. The tails of the pancreases were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were prepared and immunostained with anti-insulin (Millipore) and anti-glucagon (Abcam, Cambridge, UK) antibodies. β-Cells were defined as insulin-positive cell aggregates. β-Cell mass was calculated by described methods (14). Average β-cell area was measured manually in ≥100 insulin-positive cell aggregates chosen randomly on different sections. Detection was performed in 10 sections (5 μm) separated by 200 μm/rat.

White adipose tissues from gonadal fat pads were collected, weighed, and then fixed in 4% paraformaldehyde and embedded in paraffin at weeks 3 and 27. Sections (5 μm) were stained with hematoxylin-eosin. Area was determined in five serial sections and 250 adipocytes/rat. Analysis included five male and five female offspring per treatment group, and only one offspring per sex per litter was selected for analysis.

Glucose-stimulated insulin secretion. Islets from three male and three female 27-wk-old offspring (only 1 offspring was selected per sex per litter) were isolated by intraductal collagenase (Sigma-Aldrich) digestion and Ficoll step density gradient separation (6). Twenty islets of similar size were cultured for 48 h in RPMI-1640. Thereafter, islets were incubated in medium containing 3.0, 5.8, or 16.7 mmol/l glucose for 1 h at 37°C. Insulin secreted in the media was assayed by radioimmunoassay, and each offspring was tested in six replicate for each glucose concentration.

RNA extraction and mRNA quantification. Total RNA was extracted from pancreases at weaning using Trizol reagent (Invitrogen, Carlsbad, CA). Expression of cDNA was quantified by real-time PCR using an Applied Biosystems model 7900HT Fast Real-Time PCR System. Relative gene expression was normalized to 36B4 and calculated by the 2\(^{-ΔΔCt}\) method. Each offspring was tested in triplicate. Analysis included five male and five female offspring per treatment group, and only one offspring per sex per litter was selected for analysis. Primers sequences are provided in Table 1.

Statistical analysis. All results are expressed as means ± SE. Body weight, blood glucose, and serum insulin of dams, litter size, sex ratio, and birth weight of offspring were identified by one-way analysis of variance, followed by least significant difference and Dunnett’s T3 test using SPSS 13.0 (SPSS, Chicago, IL). The remaining data were analyzed using the appropriate ANCOVA model, with litter size as the covariate, followed by Bonferroni test. Differences between the two groups were analyzed using independent two-tailed t-test. P values <0.05 were considered significant.

RESULTS

Maternal physiology, details of delivery, and postnatal growth of pups. To test whether developmental DEHP exposure predisposed rats to metabolic abnormalities, we studied three groups of rats, including offspring born to dams exposed
to corn oil (control), 1.25 mg·kg⁻¹·day⁻¹ DEHP (DEHP-1.25), and 6.25 mg·kg⁻¹·day⁻¹ DEHP (DEHP-6.25).

Birth weight of DEHP-1.25 and DEHP-6.25 rats was significantly lower than that of controls. Differences in weight among these treatment groups persisted throughout the preweaning period (Fig. 1, A and B). Litter size was comparable among controls (13.5 ± 0.80, n = 10 dams), the DEHP-1.25 group (13.4 ± 0.63, n = 12 dams), and the

### Table 1. Primer sequences for quantitative real-time PCR

<table>
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<tr>
<th>Gene Name</th>
<th>Size, bp</th>
<th>5' Oligonucleotide</th>
<th>3' Oligonucleotide</th>
<th>GenBank</th>
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<td>Bip</td>
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<td>36B4</td>
<td>141</td>
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<td>TGGAGCAGAAGTGCGGTAAC</td>
<td>NM_022402.1</td>
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Pdx-1, pancreatic and duodenal homebox-1. Ucp2, uncoupling protein-2.
DEHP-6.25 group (14.1 ± 0.61, n = 12 dams). No difference was observed in the proportion of females per litter among control (47.69 ± 3.91%, n = 10 dams), DEHP-1.25 group (56.60 ± 3.61%, n = 12 dams), or DEHP-6.25 group (52.08 ± 3.83, n = 12 dams).

There was no significant difference in body weight of dams among the DEHP-1.25, DEHP-6.25, or control groups throughout gestation or lactation (data not shown). At weaning, fasting blood glucose was not significantly different among control (3.82 ± 0.14 mmol/l, n = 8), DEHP-1.25 (3.86 ± 0.14 mmol/l, n = 8), or DEHP-6.25 dams (3.70 ± 0.15 mmol/l, n = 8). Fasting serum insulin was also comparable among control (0.43 ± 0.01 ng/ml, n = 8), DEHP-1.25 (0.44 ± 0.01 ng/ml, n = 8), and DEHP-6.25 dams (0.42 ± 0.01 ng/ml, n = 8).

Body weight and food intake in offspring after weaning. Body weight of DEHP-1.25 rats was lower than that of controls after weaning, but differences did not reach statistical significance starting at week 9 for females and week 7 for males (Fig. 1, C and D). In the DEHP-6.25 group, body weights of both sexes were significantly lower than controls throughout the experimental periods (Fig. 1, C and D). Cumulative food intake was comparable between the DEHP-1.25 and control groups but was reduced in DEHP-6.25 group (Fig. 1E). When the data were expressed relative to body weight, there was no significant difference among all groups (Fig. 1F).

Whole body glucose homeostasis in offspring. At weaning, fasting blood glucose and serum insulin were lower in DEHP-1.25 and DEHP-6.25 rats compared with controls of either sex (Fig. 2, A–D). After administration of glucose, DEHP-1.25 and DEHP-6.25 rats of both sexes exhibited lower blood glucose levels and glucose area under the curve (AUC) compared with controls (Fig. 2, A and C). Insulin secretion in response to glucose loading and insulin AUC were also significantly reduced in DEHP-1.25 and DEHP-6.25 rats of both sexes (Fig. 2, B and D).

By week 15, comparable fasting blood glucose was observed among groups of either sex (Fig. 2, E and G). Fasting serum insulin was elevated in female DEHP-1.25 and DEHP-6.25 rats, whereas it was comparable among males (Fig. 2, F and H). When challenged with OGTT, there was no difference among females in blood glucose levels and glucose AUC (Fig. 2E). Although prolonged elevation of insulin at 30, 60, and 120 min and higher insulin AUC were observed in female DEHP-1.25 and DEHP-6.25 rats, insulin levels were decreased at 15 min after glucose loading. In males, DEHP-1.25 and DEHP-6.25 groups maintained an enhanced glucose tolerance (Fig. 2G). Insulin AUC was comparable among groups, but 15-min insulin response to glucose declined in male DEHP-1.25 and DEHP-6.25 rats (Fig. 2H).

At week 27, fasting blood glucose was elevated, but serum insulin was decreased in female DPHP-1.25 and DEHP-6.25 rats (Fig. 2, I and J). In males, fasting serum insulin levels were higher in DEHP-1.25 and DEHP-6.25 groups than in controls (Fig. 2K), whereas no difference was observed in blood glucose (Fig. 2J). When offspring were challenged with OGTT again, significantly elevated glucose levels throughout the test and remarkably increased glucose AUC were observed in female DEHP-1.25 and DEHP-6.25 rats (Fig. 2I). Insulin secretion pattern during OGTT was reserved, as shown by significantly reduced insulin levels at each time point and lower insulin AUC (Fig. 2J). Male DEHP-1.25 and DEHP-6.25 rats did not alter glucose clearance (Fig. 2K) but had greater insulin AUC, implying that greater insulin was required to clear the glucose loading at this time period. The first-phase insulin level remained lower in male DEHP-1.25 and DEHP-6.25 rats (Fig. 2L).

Fasting serum glucagon levels did not differ among DEHP-1.25, DEHP-6.25, and control groups (both sexes) up to week 27 (data not shown).

Insulin sensitivity in offspring. Relative to controls, the glucose-lowering effects were much more pronounced in DEHP-1.25 and DEHP-6.25 rats of both sexes at week 3 (Fig. 3, A and B). Drops in blood glucose levels after insulin administration were comparable among groups at weeks 15 (data not shown) and 27 (Fig. 3, C and D). Adipocyte size and body fat percentage, reciprocally related to insulin sensitivity, were decreased in DEHP-1.25 and DEHP-6.25 rats at week 3, and no difference was observed in these two parameters among groups at week 27 (Fig. 3, E–J).

Ultrastructure of β-cells in offspring. At week 3, hypertrophic rough endoplasmic reticulum and swollen mitochondria with minimal cristae were observed in female DEHP-1.25 and DEHP-6.25 rats (Fig. 4, B and C) compared with controls (Fig. 4A). Quantitative observation showed that both area and density of mitochondria were increased significantly in female DEHP-1.25 and DEHP-6.25 rats (Fig. 4, D and E). In male DEHP-1.25 and DEHP-6.25 rats, mitochondrial swelling was visible but maintained distinct cristae structure (Fig. 5, B and C). Area of mitochondria was increased, although the density was not altered (Fig. 5, D and E). DEHP-1.25 and DEHP-6.25 of either sex had reduced proportion of filled granule and increased proportion of immature granules at this time point (Figs. 4F and 5F). At week 27, derangements in β-cells were much more prominent in DEHP-1.25 and DEHP-6.25 rats (Figs. 4, H and I, and 5, H and I). Remarkably swollen mitochondria, with essentially complete loss of defined structure within the membrane, were observed in β-cells of female DEHP-1.25 and DEHP-6.25 rats (Fig. 4, H and I). Area and density of mitochondria remained higher than controls (Fig. 4, J and K). For males, higher area of mitochondria was observed in the DEHP-1.25 and DEHP-6.25 groups (Fig. 5J). Mild increase in density of mitochondria was observed in male DEHP-6.25 rats, but it was not altered in male DEHP-1.25 rats (Fig. 5K). In addition to the reduced proportion of filled granules and the increased proportion of immature granules observed at weaning, empty granules were remarkably increased in β-cells of DEHP-1.25 and DEHP-6.25 rats of either sex (Figs. 4L and 5L).

β-Cell morphometry and function in offspring. At week 3, there was no difference in pancreas weight (Fig. 6B) and β-cell area (Fig. 6, A and E) among groups of either sex, but lower β-cell mass and pancreatic insulin content were observed in DEHP-1.25 and DEHP-6.25 rats (Fig. 6, C and D). Up to week 27, despite pancreas weight being increased in female DEHP-1.25 and DEHP-6.25 rats (Fig. 6G), β-cells began to fail, as shown by decreased β-cell area and mass and pancreatic insulin content (Fig. 6, H–J). Moreover, many β-cells of female DEHP-1.25 and DEHP-6.25 rats showed very weak or mildly disrupted immunostaining for insulin (Fig. 6F). In males, a compensatory increase in the area of β-cells was observed in the DEHP-1.25 and DEHP-6.25 groups (Fig. 6, F and J). β-Cell mass tended to be higher in two male
DEHP-exposed groups, although the increase was not significant (Fig. 6H). Pancreatic weight and insulin content were comparable among males (Fig. 6, G and I).

We next evaluated glucose-stimulated insulin secretion ex vivo at week 27. Islets from female DEHP-1.25 and DEHP-6.25 rats secreted lower insulin in both 5.8 and 16.7 mM glucose concentrations compared with controls (Fig. 7A). In male DEHP-1.25 and DEHP-6.25 rats, insulin secretion was increased in 3.0 and 5.8 mM glucose but did not differ in 16.7 mM glucose (Fig. 7B).

Expression of mRNA essential to the development of pancreas and β-cell function in offspring. Relative to controls, significant reductions in the level of Pdx-1 (pancreatic and duodenal homeobox-1) were observed in DEHP-1.25 and DEHP-6.25 rats of both sexes at weaning (Fig. 8). In accordance with the downregulated Pdx-1, the level of insulin was decreased. No difference was observed in the glucagon among groups (Fig. 8). Expression of Atf4, Atf6, and Bip, genes involved in endoplasmic reticulum stress, was increased significantly in DEHP-1.25 and DEHP-6.25 rats of both sexes.
Similarly, female DEHP-1.25 and DEHP-6.25 rats had increased levels of \( \text{Ucp2} \) (uncoupling protein 2; Fig. 8A). \( \text{Ucp2} \) mRNA levels tended to be higher in male DEHP-1.25 and DEHP-6.25 offspring, although this did not reach statistical significance (Fig. 8B).

**DISCUSSION**

The prevalence of diabetes mellitus represents a growing public health concern and has been associated with exposure to environmental pollutants such as environmental estrogens, organophosphorus compounds, persistent organic pollutants, etc. (19). However, litter data on the metabolic effects of developmental DEHP exposure are presently unavailable. In this study, we suggested that developmental exposure to DEHP impaired endocrine pancreas in rats at an early age and thereby disrupted the \( \beta \)-cell function and the whole body glucose homeostasis.

Maternal doses of 1.25 and 6.25 mg·kg\(^{-1}\)·day\(^{-1}\) were chosen in this study because they straddled the NOAEL, 5 mg·kg\(^{-1}\)·day\(^{-1}\) for developmental toxicity, as suggested by the European Union Risk Assessment Report (28), and we found that offspring displayed significant metabolic reprogramming effects at both doses. Despite the fact that it has been established that typical human exposure to DEHP ranges from 3 to 30 mg·kg\(^{-1}\)·day\(^{-1}\) (11), specific medical conditions exist in which DEHP exposure is significantly higher and reaches several orders of magnitude above the exposure levels estimated for the general population (5, 20, 22). For instance, individuals requiring long-term hemodialysis are subjected to exposure to the cumulative level of DEHP, reaching up to 2.2 mg·kg\(^{-1}\)·day\(^{-1}\). Blood transfusions to trauma patients also result in the acute higher DEHP exposure in adults, up to 10 mg·kg\(^{-1}\)·day\(^{-1}\) (30). In addi-
tion, premature neonates have much higher DEHP exposure in relation to body weight, reaching 10–20 mg·kg\(^{-1}\)·day\(^{-1}\) (20, 22), because they usually have lighter body weight and require a combination of several medical interventions. Consequently, doses chosen in this study are within the range of possible exposure levels and can extend to the human population.

It is well known that chemical exposure in critical periods of development is associated with chronic diseases (1, 36), so we hypothesize that developmental DEHP exposure would predispose rats to metabolic disorders. Indeed, female DEHP-exposed offspring were hyperglycemic and hypoinsulinemic and developed progressive, severe glucose intolerance by week 27. But it was interesting that adipocyte size and body fat percentage, parameters reciprocally related to insulin sensitivity, were comparable among groups. In performing an ITT, we further confirmed that the decrease in glucose utilization after developmental DEHP exposure is not due to alterations in peripheral insulin sensitivity. Developmental exposure to DEHP impairs glucose homeostasis in rats even in the absence of insulin resistance.

If not insulin resistance, what is the origin of glucose intolerance in female DEHP-exposed offspring? We hypothesize that DEHP can act particularly in the endocrine pancreas of offspring in early life. As anticipated, exposure to DEHP led to reduction in pancreatic insulin content, loss in \(\beta\)-cell mass, and abnormal \(\beta\)-cell ultrastructure at weaning. Moreover, first-phase insulin response was decreased in DEHP-exposed offspring, indicating that \(\beta\)-cells had not been able to produce and/or secrete normal amounts of insulin early in life. Enhanced glucose tolerance observed at this time point is due to DEHP-exposed offspring being more insulin sensitive than

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**Fig. 4.** Effects of developmental DEHP exposure on \(\beta\)-cell ultrastructure in female offspring at weeks 3 (A–F) and 27 (G–L). A–C and G–I: representative electron microscopy of \(\beta\)-cells in control (A and G), DEHP-1.25 (B and H), and DEHP-6.25 (C and I) rats. N, nucleus; Gr, secret granulation. Gr is identified as filled (dense core), immature (light gray core), and empty (no core). Immature granules are indicated by black arrows. Rough endoplasmic reticulums are indicated by black arrowheads. Mitochondria are indicated by black boxes. Photographs were taken at \(\times9,700\) magnification. D and J: average mitochondrial area [ANCOVA: \(P\) (litter size) > 0.05]. E and K: mitochondrial optical density [ANCOVA: \(P\) (litter size) > 0.05]. Higher optical density values (a measurement of brightness) was an indication of mitochondrial swelling. F and L: manual quantifications of the percentage of different granules (filled, immature, and empty) in \(\beta\)-cells [ANCOVA: \(P\) (litter size) > 0.05]. Values are given as means \(\pm\) SE of 3 female offspring from 3 litters (only 1 offspring/litter was selected). \(\dagger\) \(P < 0.05\) for DEHP-1.25 vs. control; \(\dagger\dagger\) \(P < 0.01\) for DEHP-1.25 vs. control; * \(P < 0.05\) for DEHP-6.25 vs. control; ** \(P < 0.01\) for DEHP-6.25 vs. control.
controls. By the age of 15 wk, elevated serum insulin levels in female DEHP-exposed offspring suggested that β-cells tended to secrete enough insulin to compensate for the defects in insulin action. However, because the production of new β-cells in adulthood is low (41), deficiencies occurring in early life comprise β-cell mass expansion, resulting in β-cells failing with age, as shown by decreased pancreatic insulin content, reduced β-cell mass and area, and evident degranulation of β-cells in female DEHP-exposed offspring at week 27. Diminished ability to secrete insulin in response to glucose stimulus in vivo and ex vivo was also observed following developmental DEHP exposure. Most importantly, impaired first-phase insulin secretion displayed early was observed throughout the lives of both male and female DEHP-exposed offspring, which is similar to the human predestined to develop diabetes.

In this study, we indicated that expression of key genes involved in endocrine pancreas development and β-cell function was altered in DEHP-exposed rats at weaning. Pdx-1, a gene regulating the early development of endocrine pancreas and the formation of islets (15, 35), was significantly downregulated in DEHP-exposed offspring. Because Pdx-1 plays an important role in islet formation and gene transcription (3), suppression of Pdx-1 substantially impairs insulin expression and reduces insulin output and then gives rise to reduced β-cell mass and decreased pancreatic insulin content in both male and female DEHP-exposed offspring. Reductions in Pdx-1 also have been reported to impair mitochondrial function, resulting in blunted insulin secretion (3). This is indeed what happened in DEHP-exposed rats in this study. Mitochondrial ultrastructure damages, characterized by increased area and density, were accompanied by a defect in glucose-stimulated insulin release.
secretion. \textit{Ucp2}, involved in the decreased metabolic efficiency of mitochondrial ATP production \cite{8,9}, was significant up-regulated in female offspring following DEHP exposure. Since \textit{H9252}-cells require large amounts of ATP to allow glucose-stimulated insulin secretion to take place, we suggest that abnormal expression of \textit{Ucp2} partly results in \textit{H9252}-cell dysfunction in female offspring later in life. Ultrastructural analysis also showed that rough endoplasmic reticulum was hypertrophied in \textit{H9252}-cells of DEHP-exposed offspring of both sexes. Levels of three endoplasmic reticulum (ER) stress gene markers serving to mitigate stress \cite{13,32}, \textit{Atf4}, \textit{Bip}, and \textit{Atf6}, were increased in DEHP-exposed offspring. Because rough ER plays an essential role in the processing and assembly of insulin \cite{25}, and ER stress responses contribute to \textit{β}-cell survival and autoimmunity in the pathogenesis of diabetes \cite{18,26}, DEHP-induced damage in rough ER would be another mechanism underlying \textit{β}-cell dysfunction in rats, which needs to be elucidated in our further study.

In the present study, developmental DEHP exposure results in reductions in \textit{β}-cell area and mass and pancreatic insulin content in both male and female rats at weaning, but sex-related differences in glucose homeostasis and \textit{β}-cell function are examined in DEHP-exposed rats along with age. At week 27, decreased \textit{β}-cell area, \textit{β}-cell mass, and pancreatic insulin content were exhibited in DEHP-exposed female offspring. Moreover, elevated blood glucose and glucose intolerance were also observed in the presence of reduced serum insulin. In contrast, male DEHP-exposed offspring had significantly increased fasting circulating insulin and \textit{β}-cell area at this time point, and they recovered \textit{β}-cell mass and pancreatic insulin content to control levels. These results suggest that female DEHP-exposed offspring develop severe glucose intolerance at adulthood due to their being relatively more insulin deficient, whereas male DEHP-exposed offspring develop compensatory hyperinsulinemia to maintain glucose homeostasis with age. Adverse effects of developmental exposure to DEHP on glucose homeostasis are much more severe in female rats than males, because females develop glucose intolerance more rapidly. The cause for sex differences in glucose homeostasis and \textit{β}-cell function along with age following developmental DEHP exposure remains to be determined in further research.

In this study, the body weight of both male and female pups perinatally exposed to 1.25 and 6.25 mg·kg$^{-1}$·day$^{-1}$ DEHP was decreased at birth; moreover, effects of DEHP on body weight persisted at the postnatal stage and throughout life. These observations are consistent with previous results demonstrating that perinatal exposure to DEHP disturbed body growth in different rodent species. In a recent study, daily
exposure to 100 mg·kg\(^{-1}\)·day\(^{-1}\) DEHP in Sprague-Dawley rats starting from GD 12 by oral gavage results in lower birth weight of offspring. Moreover, this study reports that reduced body weight is observed in offspring at weaning, when exposure to DEHP continued during lactation at a much lower dose (10 mg·kg\(^{-1}\)·day\(^{-1}\)) (10). In our study, body weight was decreased significantly at birth in Wistar rat offspring when DEHP exposure starting from GD 0 was 1.25 and 6.25 mg·kg\(^{-1}\)·day\(^{-1}\). Therefore, we suggested that exposure to DEHP throughout the period of gestation would lead to more serious adverse impacts on birth weight in offspring even when the exposure level is as low as 1.25 mg·kg\(^{-1}\)·day\(^{-1}\). Our study also observed that the effects of perinatal DEHP exposure on body weight persisted beyond the end of exposure, in line with results reporting that Institute of Cancer Research mice offspring exposed to 1 mg·kg\(^{-1}\)·day\(^{-1}\) DEHP during gestation and lactation present lower body weight at 2, 4, and 6 wk of age (38). Notably, our study suggested that DEHP exposure does not affect the litter size and the maternal body weight, so the reduction of body weight cannot be explained solely by the general DEHP toxicity to the mother. Further researches are needed to achieve a comprehensive understanding of the potential mechanisms leading to lower body weight in rats developmentally exposed to DEHP. Because experimental and epidemiological studies suggest that lower birth weight is a risk factor for type 2 diabetes later in life (2, 29, 34), growth retardation induced by developmental DEHP exposure could be regarded as another potential contributor to the further deterioration of glucose homeostasis in offspring.

Taken together, our study indicates that developmental exposure to DEHP impairs endocrine pancreas early in life and leads to overt glucose intolerance in adult female rats. Doses chosen in this study are relevant to certain risk groups of high DEHP exposure. As a result, the risk of DEHP exposure in pregnant and neonate must be considered, particularly when they undergo intensive medical procedures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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