Bisphenol A Promotes Adiposity and Inflammation in a Nonmonotonic Dose-response Way in 5-week-old Male and Female C57BL/6J Mice Fed a Low-calorie Diet

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A growing body of epidemiological research show that Bisphenol A (BPA) is positively correlated with obesity and metabolic disorders. However, the mechanisms of BPA on adiposity remain largely unknown. In this study, we found that 5-week-old male and female C57BL/6J mice exposed to four dosages of BPA (5, 50, 500, and 5000 μg/kg/d) by oral intake for 30 days showed significantly increased body weight and fat mass in a nonmonotonic dose-dependent manner when fed a chow diet. The effect occurred even at the lowest concentration (5μg/kg/d), lower than the tolerable daily intake of 50 μg/kg/day for BPA. However, no significant difference in body weight and fat mass was observed in either male or female mice fed a high-fat diet, suggesting that BPA may interact with diet in promoting obesity risk. In vitro study showed that BPA treatment drives the differentiation of white adipocyte progenitors from the stromal vascular fraction, partially through glucocorticoid receptor. BPA exposure increased circulating inflammatory factors and the local inflammation in white adipose tissues in both genders fed a chow diet, but not under high-fat diet. We further found that BPA concentration was associated with increased circulating inflammatory factors, including leptin and TNFα, in lean female subjects (body mass index < 23.0 kg/m^2) but not in lean male subjects or in both sexes of overweight/obese subjects (body mass index > 25.0 kg/m^2). In conclusion, we demonstrated the nonmonotonic dose effects of BPA on adiposity and chronic inflammation in 5-week-old mice, which is related to caloric uptake. **(*Endocrinology* 157: 2333–2345, 2016)**

Obesity has become a global epidemic, contributing to the increased prevalence of hypertension, cardiovascular disease, type 2 diabetes, and certain cancers (1). A large number of epidemiological studies demonstrated that exposure to endocrine -disrupting chemicals (EDCs) was associated with obesity and its related metabolic disorders (2–4). EDC exposure in the European Union contributes substantially to obesity and diabetes with a conservative estimated cost of €18 billion per year (5). Recently, the Endocrine Society's Second Scientific Statement on EDCs systematically reviewed the advances in EDC research for seven topical areas in the past five years and highlighted three features of action for EDCs, including nonmonotonic dose-responses, low-dose effects, and developmental vulnerability (6). Our previous epidemiological studies also provided critical evidence for these

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Abbreviations: BMI, body mass index; BPA, Bisphenol A; CD, chow diet; EDC, endocrine-disrupting chemical; eWAT, epididymal white adipose tissue; GR, glucocorticoid receptor; HFD, high-fat diet; Hs-CRP, high-sensitivity C-reactive protein; IPGTT, intraperitoneal glucose tolerance test; iWAT, inguinal white adipose tissue; NHANES, National Health and Nutrition Examination Survey; SVF, stromal vascular fraction; TDI, tolerable daily intake.

findings, including the nonmonotonic dose association with diabetes and obesity for the important EDC, Bisphenol A (BPA) (7, 8). However, these cross-sectional studies with diet as an important confounding risk factor could provide the association but not the causal relationship between BPA and the development of metabolic disorders. In particular, we previously observed that the highest quartile of urinary BPA concentration was associated with high prevalence of insulin resistance in lean subjects with a body mass index (BMI) less than 24.0 kg/m^2; however, this association was not observed in overweight/obese subjects with a BMI greater than 24.0 kg/m^2 (8), which suggests different obese status may affect BPA's pathogenic function. To date, these three features of BPA's action on obesity were not comprehensively investigated in one integrated epidemiological and/or animal study. In addition, the direct effects of BPA exposure on adiposity remained unclear.

BPA is a widely used industrial chemical that commonly appears in various products for daily use including water pipes, electronic equipment, and toys (2). Data from the National Health and Nutrition Examination Survey (NHANES) also suggested that exposure to BPA elevates the risk for human obesity, diabetes, and heart diseases (9). Interestingly, others and our human studies showed BPA exposure was associated with metabolic diseases in a "nonmonotonic dose-response" way, indicating that lower levels of exposure also increased the risk (7, 8). The U.S. Environmental Protection Agency and the European Food Safety Agency have established a tolerable daily intake (TDI) of 50 μg/kg/day for BPA. Recent animal studies revealed that exposure to BPA at doses below the TDI also alters biological functions and metabolic processes in adult mice by interfering with endocrine signaling pathways, such as reproductive functions, brain functions, lipid synthesis, and liver triglyceride accumulation, as well as glucose homeostasis and pancreatic β-cell function in adult male mice (10–13). Animal studies also showed that maternal exposure to BPA during the fetal and/or lactation period resulted in an increase in the body weight of male or female adult offspring (14–16). There are also studies illustrating the sex-specific effects of maternal exposure to BPA on the body weight of offspring (17). Furthermore, some studies have suggested that BPA could induce adipocyte differentiation through a nonclassical estrogen receptor pathway or promote adipogenesis in the 3T3-L1 cell line through the glucocorticoid receptor (GR) pathway (18, 19). However, there are few comprehensive investigations on the direct effects of BPA exposure on obesity especially in young male and female animal models; and the effect of BPA on adipogenesis by using primary progenitors of adipocytes has been less well studied.

In this study, we treated male and female 5-week-old C57BL/6J mice with four dosages of BPA (5, 50, 500, and 5000 μg/kg/d) by oral intake for 30 days and found that both male and female BPA-treated mice showed significantly increased body weight and fat mass in a nonmonotonic dose-dependent manner when fed a chow diet (CD). Given that the inflammation reaction is the core pathogenic step in obesity-induced metabolic disorders (20), we further evaluated the effects of BPA exposure at different doses on the circulating and adipose-local inflammatory factors in mice under low- and high-calorie diet respectively, and also detected the association of urinary BPA levels with circulating inflammatory factors in lean and overweight/obese human subjects. Interestingly, we observed that BPA exposure promoted an inflammation reaction in a nonmonotonic dose-response manner in both male and female mice fed a low-calorie diet but not a high-fat diet (HFD). BPA was associated with increased circulating inflammatory factors particularly in the lean female subjects but not in the lean male subjects or in both sexes of overweight/obese subjects. We therefore provided new evidence for the effects of BPA exposure on adiposity especially in 5-week old male and female mice, and more interestingly, the interactive effects of BPA with diet on inflammation further supported our previous large-scale cross-sectional epidemiological studies.

Materials and Methods

Animals

Five-week-old male and female C57BL/6J mice (SLAC) were randomly divided into five groups without difference in body weight (n = 9–12 per group) and were fed BPA-contaminated CD or HFD for 30 days (housing at 22 ± 2°C, 12-h light/dark cycle). Procedures were shown in Supplemental Figure 1. Basic composition (percentage of protein, carbohydrate, and fat) of the CD and HFD used in this study were shown in Supplemental Table 1. BPA (4, 40-dihydroxy-2, 2-diphenyl -propane, Sigma-Aldrich) was incorporated into CD (10% kcal in fat) and HFD (45% kcal in fat) (Medicience Ltd) at 0 (controls), 50, 500, 5 000 or 50 000 μg/kg. Assuming a consumption of 10% of the body weight per day (10), this corresponds to an exposure of 0 (controls), 5, 50 (TDI), 500, or 5000 μg/kg/d. Fat mass was assessed by magnetic resonance image–based body composition analysis (Echo MRI, Echo Medical Systems). All procedures were approved by the Animal Care Committee of Shanghai Jiao-Tong University School of Medicine.

Blood and organ sampling

For evaluating the systemic inflammatory reaction, plasma was collected following anesthesia through retro-orbital bleeding into heparin-treated tubes, centrifuged for 10 minutes at

2000× *g*, and kept at −80°C until usage. Leptin and resistin levels were measured using a commercial ELISA kit (Millipore) following the manufacturer's guidelines. The inguinal white adipose tissues (iWAT) and epididymal white adipose tissues (eWAT) were removed, weighed, and dissected. Portions of the samples were frozen in liquid nitrogen, with a small part fixed in 4% neutral buffered formalin, and embedded in paraffin. Sections of 5-μm thickness were stained with hematoxylin and eosin according to standard protocols. All the representative images were repeated in at least three mice. Quantification of adipocyte diameter was performed in iWAT from male mice in the CD group. Data were collected from hematoxylin and eosin–stained sections from three individual mice in each group and three fields per mouse, using ImageJ software (National Institutes of Health).

Glucose tolerance tests

For intraperitoneal glucose tolerance test (IPGTT), the mice were fasted for 16 hours and received an injection of D-glucose (2 g/kg body weight) intraperitoneally. We measured mouse blood glucose levels with whole blood from the tail vein using a glucose meter (LifeScan).

Stromal vascular fractions isolation, white adipocyte differentiation and Oil Red O staining

Stromal vascular fractions (SVFs) were isolated as follows. In brief, adipose tissue from adult male C57BL/6J mice was minced and digested with 2 mg/mL collagenase type II (Sigma-Aldrich) in HEPES-buffered solution (pH 7.4) for 30 minutes at 37°C. The cell suspension was placed on ice for 20 minutes to allow mature fat cells and lipid droplets to float. Cell suspensions were then filtered through a 40-μm strainer (BD Biosciences) and centrifuged, washed, and plated onto 10-cm dishes. Cells were cultured in DMEM/F12 (GIBCO) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), and 10 ng/mL murine basic fibroblast growth factor (R&D Systems). SVFs were then plated onto 48-well plates to reach confluence. White adipocyte differentiation of confluent cells was carried out in growth medium supplemented with 5 μg/mL insulin, 0.5mM isobutylmethylxanthine (Sigma-Aldrich), and 1μM dexamethasone (Sigma-Aldrich) for 48 hours, and further in growth medium supplemented with 5 μg/mL insulin for 6 days. From day −2 to day 8, the indicated concentrations of BPA and/or GR antagonist RU486 (Sigma-Aldrich) were included in the differentiation medium, which was replenished every 2 days. For Oil Red O staining, fully differentiated SVFs were fixed with 4% neutral buffered formalin for 20 minutes, followed by Oil Red O (Sigma-Aldrich) incubation for 30 minutes.

Protein preparation and Western blot analysis

Protein preparation and Western blot were performed as follows. Proteins were extracted using RIPA (Beyotime) reagent according to the manufacturer's protocol. The protein concentration was determined by the BCA protein assay (Pierce Biotechnology, Inc). Total protein was denatured by boiling, separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane (Millipore). After blocking in 10% BSA, the membranes were incubated overnight at 4°C with primary antibodies directed against C/EBP-α, PPAR-γ and AP2, or incubated at room temperature for 1 hour with the horseradish peroxidase-linked GAPDH antibody. Antibodies used for Western blotting were provided in Table 1. The secondary antibody used was antirabbit IgG (LI-COR) and the resulting bands were visualized using the Imagine Quant LAS 4000 imaging system (GE Healthcare).

RNA isolation and real-time PCR

Total RNA was extracted from cells or tissues using Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. The Reverse Transcription System was used to transcribe 1 μg RNA to cDNA (Promega). Real-time PCR was carried out on the Lighter cycler 480 system (Roche) using SYBR Green Supermix (Takara) or QuantiNova Probe PCR Kit (QIAGEN). Primers used in this study are provided in Supplemental Table 2. Data were normalized to 36B4 and analyzed using the $2^{-\Delta\Delta CT}$ method.

Human study design and participants

Subjects were recruited from Songnan Community, Baoshan District, Shanghai, China, as reported previously (7). Morning urine samples were collected to test urinary BPA concentration. Total (free and conjugated) urinary BPA concentrations were measured by a sensitive and selective liquid chromatography–tandem mass spectrometry as described previously (7). The procedure used to enroll subjects from the community population were shown in a schematic flow diagram (Supplemental Figure 2). Subjects were preliminarily divided into two groups: lean group defined as BMI less than 23.0 kg/m^2 and overweight/obese group defined as BMI greater than 25.0 kg/m^2. Both groups of participants were categorized into four subgroups according to urinary BPA concentrations. We randomly selected participants

Table 1. Antibody Table

Peptide/Protein Target	Name of Antibody	Manufacturer, Catalog Number	Species Raised In	Dilution Used
C/EBP-α	CCAAT/enhancer binding protein (C/EBP), alpha	Proteintech, 18311-1-AP	Rabbit	IB (1:1000)
PPAR-γ	Peroxisome proliferator-activated receptor γ	Cell Signaling Technology, 2435	Rabbit	IB (1:1000)
AP2	Fatty acid binding protein	Cell Signaling Technology, 3544	Rabbit	IB (1:1000)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Kangcheng, KC5G5	Mouse	IB (1:10 000)

from the four subgroups in a ratio of 1:1:1:1. This survey included 228 people subdivided into 114 people in each group. Overall, the mean age was 61.9 ± 9.3 years in the group with BMI less than 23.0 kg/m^2 and 38.6% were men. The median urinary BPA level was 1.12 ng/mL (interquartile range, 0.63–1.86 ng/mL) (Supplemental Figure 3). In the participants with BMI of greater than 25.0 kg/m^2, the mean age was 63.4 ± 9.5 years and 40.3% were men. The median urinary BPA level was 1.16 ng/mL (interquartile range, 0.67–1.88 ng/mL) (Supplemental Figure 4). All procedures used in this study were in accordance with institutional guidelines. The Committee on Human Research at Rui-Jin Hospital, Shanghai Jiao-Tong University School of Medicine, approved the study protocol, and all study participants provided written informed consent.

Data collection

Sociodemographic characteristics and a medical history were conducted by trained personnel as reported previously (7). All the participants underwent a 75-g oral glucose tolerance test, and the plasma glucose were obtained at 0 and 2 hours during the test. Plasma alanine aminotransferase, γ-glutamyltransferase, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, and high-sensitivity C-reactive protein (hs-CRP) were tested as reported previously (7). The basic clinical data were shown in (Supplemental Tables 3 and 4). Leptin and TNFα were detected in those subjects with the Human Adipokine Panel 2 (Millipore).

Statistical analysis

SAS version 8.1 (SAS Institute) was used for all statistical analyses. Unless otherwise stated, data are presented as the mean ± SEM. Data were tested for normal distribution and logarithmically transformed for statistical analyses when required. Comparisons between two groups were analyzed by Student *t* test. For multiple-group comparisons, ANOVA followed by the post least significant differences test was used. Differences were considered to be significant at two-tailed $P < .05$.

Results

BPA exposure promoted adiposity in a nonmonotonic dose-response way in CD mice

We first compared body weight gain using 5-week old mice fed a CD with four dosages of BPA (5, 50, 500, and 5000 μg/kg/d) and found that both male and female mice treated with BPA exhibited increased body weight when compared with mice fed with a diet free of BPA (Figure 1A). Furthermore, mice fed with BPA displayed a significant increase in body fat content, even at the lowest dosage of 5 μg/kg/day, below the safety dosage of the TDI (50 μg/kg/d). Following exposure to BPA, the body fat content, iWAT and eWAT content in male and female mice were all increased in a nonmonotonic dose-response manner (Figure 1, B–D), similar to body weight. However, we

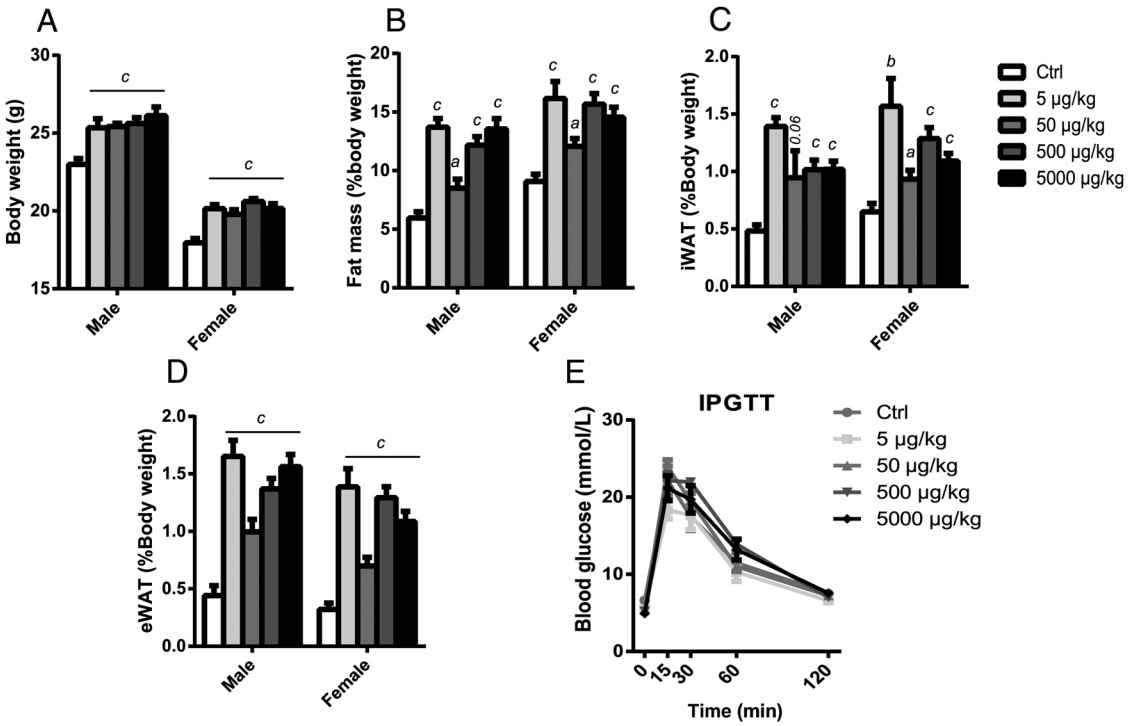

Figure 1. BPA increases fat accumulation of male and female mice fed a chow diet. Body weight (A), fat mass (B), iWAT (C), eWAT (D) relative to body weight and (E) IPGTT of 5-week-old C57BL/6J mice exposed orally for 30 d to different BPA dosages (0, 5, 50, 500, and 5000 μg/kg/d), n = 9–12/group. IPGTT was performed on male mice. Significant results compared with control mice are denoted as follows: a, $P < .05$; b, $P < .01$, c, $P < .001$. All data are presented as the mean ± SEM.

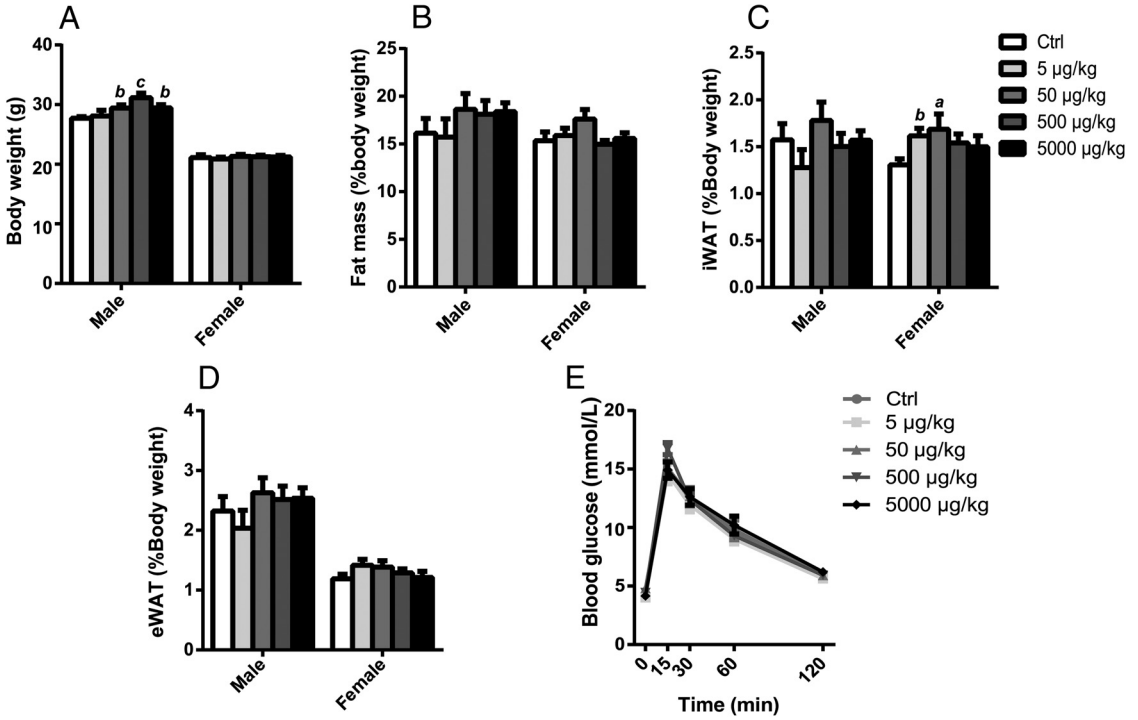

Figure 2. Effects of BPA exposure on adiposity in male and female mice under HFD. Body weight (A), fat mass (B), iWAT (C), eWAT (D) relative to body weight and (E) IPGTT of 5-week-old C57BL/6J mice exposed orally for 30 d to different BPA dosages (0, 5, 50, 500, and 5000 μg/kg/d), n = 9–12/group. IPGTT was performed on male mice. Significant results compared with control mice are noted as follows: a, $P < .05$; b, $P < .01$; c, $P < .001$. All data are presented as the mean ± SEM.

did not observe changed glucose tolerance in BPA-treated male mice (Figure 1E). To assess the potential interaction of BPA with diet, we also performed the same BPA treatment in mice fed a HFD. Interestingly, male mice treated with BPA only showed a mild increase of body weight at the dosage above TDI, whereas no changes were observed in most doses of male mice or in all doses of female mice (Figure 2A). Consistently, no significant change was observed in fat mass and fat content in both sexes (Figure 2, B–D), and glucose tolerance was either unaltered in male mice (Figure 2E). Besides, treatment with BPA in male and female adult mice beginning at 8 weeks old did not show significant changes in body weight (Supplemental Figure 3). These findings demonstrate that BPA exposure significantly promoted body weight increase and adiposity especially in 5-week old mice fed a low-calorie diet, which was mitigated by a HFD.

BPA exposure increased the expression of genes related to adipogenesis and lipogenesis in vivo

Adipogenesis and lipogenesis are tightly linked with fat accumulation in adipose tissues. Thus, we next evaluated the effects of different BPA dosages on the expression of the related genes in the male mice fed a CD. As shown in Figure 3A, BPA exposure significantly increased the expression of the master genes of adipogenesis, such as *C/ebp-α*, *Ppar-γ*, and *Ap2* in the iWAT of male mice fed a CD (Figure 3A). However, no significant effects were observed on the expression of thermogenesis genes, such as *Ucp1* and *Pgc-1α* (Supplemental Figure 4). In addition, lipogenic genes such as *Fas*, *Srebp-1c*, and *Scd-1* were also significantly increased by BPA exposure (Figure 3B). Consistently, histological morphology analysis suggested that the adipocyte size of iWAT was enlarged after BPA treatment, which suggests that the adipose tissue has increased lipid storage in response to BPA exposure under CD condition (Figure 3C). Moreover, by counting the diameter of the inguinal adipocytes in the CD group, we discovered that the percentage of small adipocytes was reduced, whereas the percentage of large cells was increased in mice exposed to different BPA levels, which also follows a nonmonotonic dose-response curve (Figure 3D). Taken together, these findings strongly suggested that BPA exposure enhanced expression of genes related to adipogenesis, fat synthesis, and storage, leading to increased adiposity.

BPA exposure induced the differentiation of adipose progenitors partially by GR activation in vitro

To further evaluate the BPA's effects on adipocyte differentiation, we isolated primary SVF from iWAT,

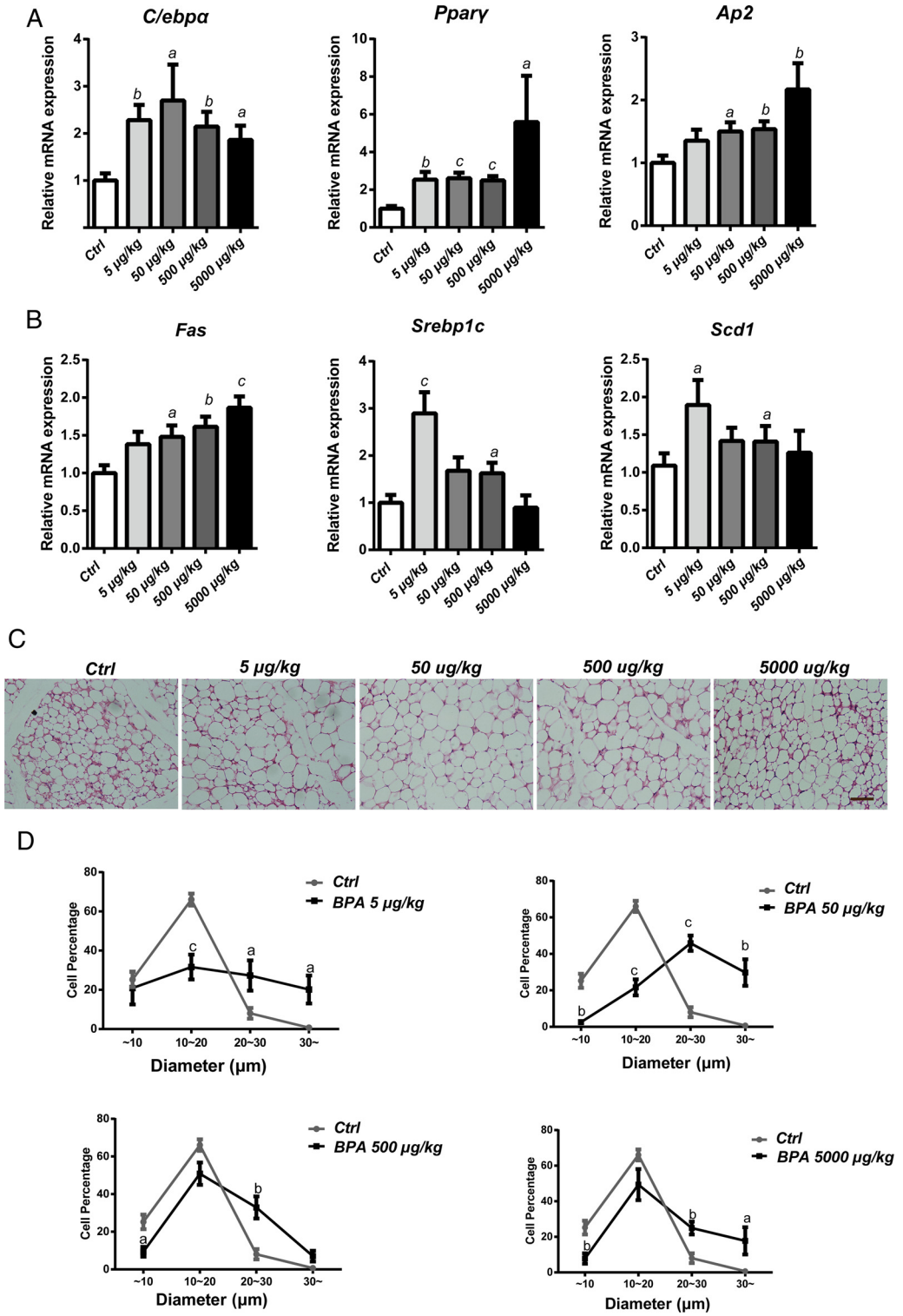

Figure 3. BPA exposure induces expression of genes related to adipogenesis and lipogenesis in vivo. A, mRNA levels of the adipogenesis master genes, *C/ebp-α*, *Ppar-γ*, and *Ap2*, were significantly increased in the iWAT of male mice exposed to different dosages of BPA in the CD group. B, mRNA levels of the lipogenic genes, *Fas*, *Srebp-1c*, and *Scd1*, were significantly increased in the iWAT of male mice exposed to BPA in the CD group. C, Representative hematoxylin and eosin staining of the iWAT in male mice with or without BPA exposure in the CD group. Scar bar, 50 μm. D, Quantification of the adipocyte diameter of iWAT from male mice in the CD group. Significant results compared with control mice are indicated by a, $P < .05$; b, $P < .01$; c, $P < .001$. All data are presented as the mean ± SEM.

which contains a high percentage of adipocyte progenitors (21). Next, the SVF was induced into white adipocytes in vitro and treated with different dosages of BPA or vehicle during differentiation period. When compared with vehicle, the fully differentiated adipocytes in the 50μM BPA-treated group showed a sub-

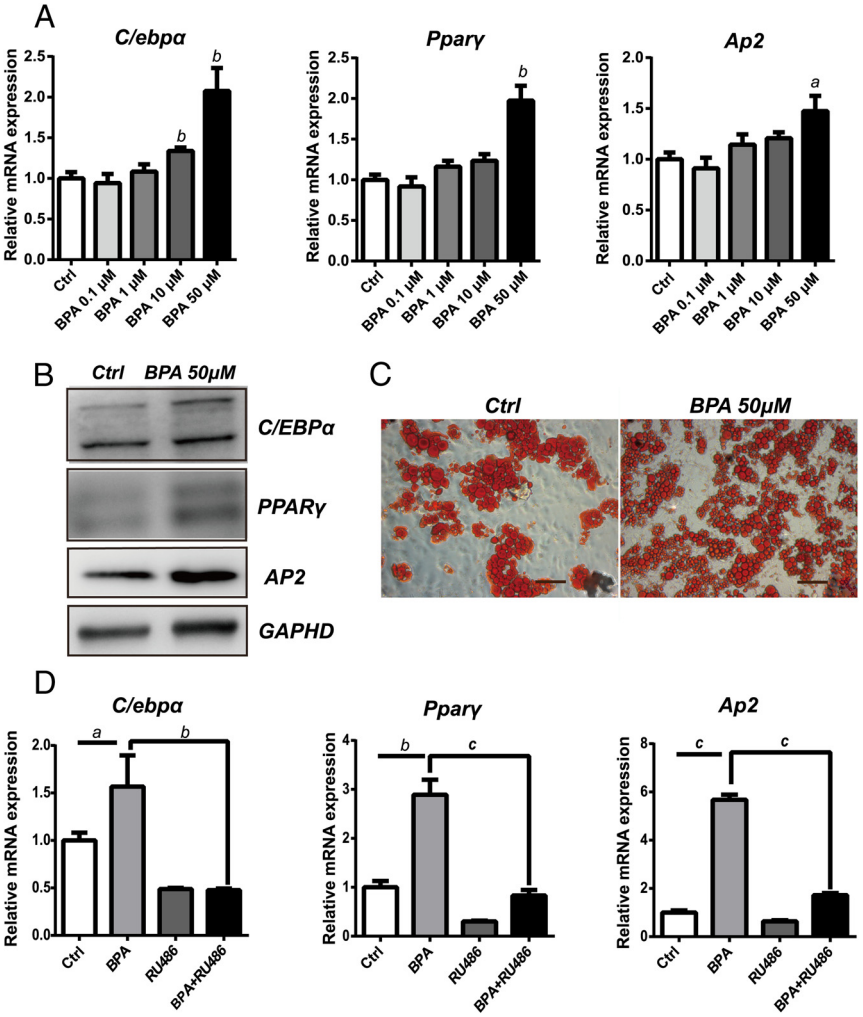

Figure 4. BPA enhances the differentiation of SVF toward white adipocytes. A, mRNA levels of *C/ebp-α*, *Ppar-γ*, and *Ap2* were significantly increased in BPA-treated SVF from iWAT. B, Western blot of lysates from adipocytes differentiated from SVFs at day 8 after induction with or without 50μM BPA treatment (n = 3). C, Primary SVF treated with 50μM BPA showed increased Oil Red O lipid staining. Scar bar, 100 μm. D, RU486, the GR-specific antagonist, blunts BPA-induced differentiation of primary SVF. BPA, 50μM; RU486, 1μM. a, $P < .05$; b, $P < .01$; c, $P < .001$ compared with vehicle. All data are presented as the mean ± SEM.

stantial elevation in *C/ebpα*, *Pparγ*, and *Ap2* gene expression (Figure 4, A and B) as well as an increase in the Oil Red O staining (Figure 4C), indicating that BPA induced adipocyte differentiation by activating the transcriptional cascade of adipogenic genes. It was reported that BPA significantly stimulates GR-mediated luciferase expression in the 3T3-L1 cell line (19). We further used GR antagonist RU486 to evaluate whether the effect of BPA on adipocyte differentiation from primary SVF was through GR signaling. Interestingly, GR inactivation significantly inhibited the effects of BPA on white adipocyte differentiation (Figure 4D), suggesting that BPA induces adipogenesis partially through GR signaling.

BPA treatment increased systemic and local inflammation in the CD mice but not in the HFD-induced obese mice

Excess fat accumulation is usually accompanied by chronic inflammation in mammals. Therefore, we examined the circulating inflammatory factors and found that plasma leptin and resistin levels were markedly increased in both male and female mice exposed to BPA under CD condition (Figure 5A). Meanwhile, macrophage-related genes such as *F4/80*, *Cd11c*, and *Mcp-1* (Figure 5B), and other inflammatory genes, such as *Il-6*, *Tnfα*, *Il-1β*, *Ifnγ*, and *iNos2* (Figure 5C), were all significantly increased in iWAT of male mice in response to BPA treatment at the highest dosage. However, circulating inflammatory factors such as leptin and resistin levels were not increased in both sexes exposed to BPA under the HFD condition (Figure 5D).

The association of BPA levels with circulating inflammation factors in lean female subjects but not in lean male subjects or in both sexes with overweight/obesity

We determined the effects of BPA exposure on promoting adiposity and inflammation in both male and female mice fed a low-calorie diet but not a high-calorie diet. Thus, we further assessed the association of BPA exposure with the inflammation reaction in human. Our results revealed that BPA was associated with inflammation in lean subjects but not in overweight/obese subjects (Figure 6, A–D). When stratified by sex, we noticed that plasma leptin and TNFα levels were significantly higher in the female subjects with the highest quartile of BPA concentration than those with the lowest quartile in the lean group with a BMI less than 23.0 kg/m^2 (Figure 6, E and F). However, this association disappeared in female subjects in the overweight/obese group (Figure 6, G and H) and male subjects in both lean and obese group (Figure 6, I–L). These results overall suggested that the association of BPA concentration with chronic inflammation reaction in human was at-

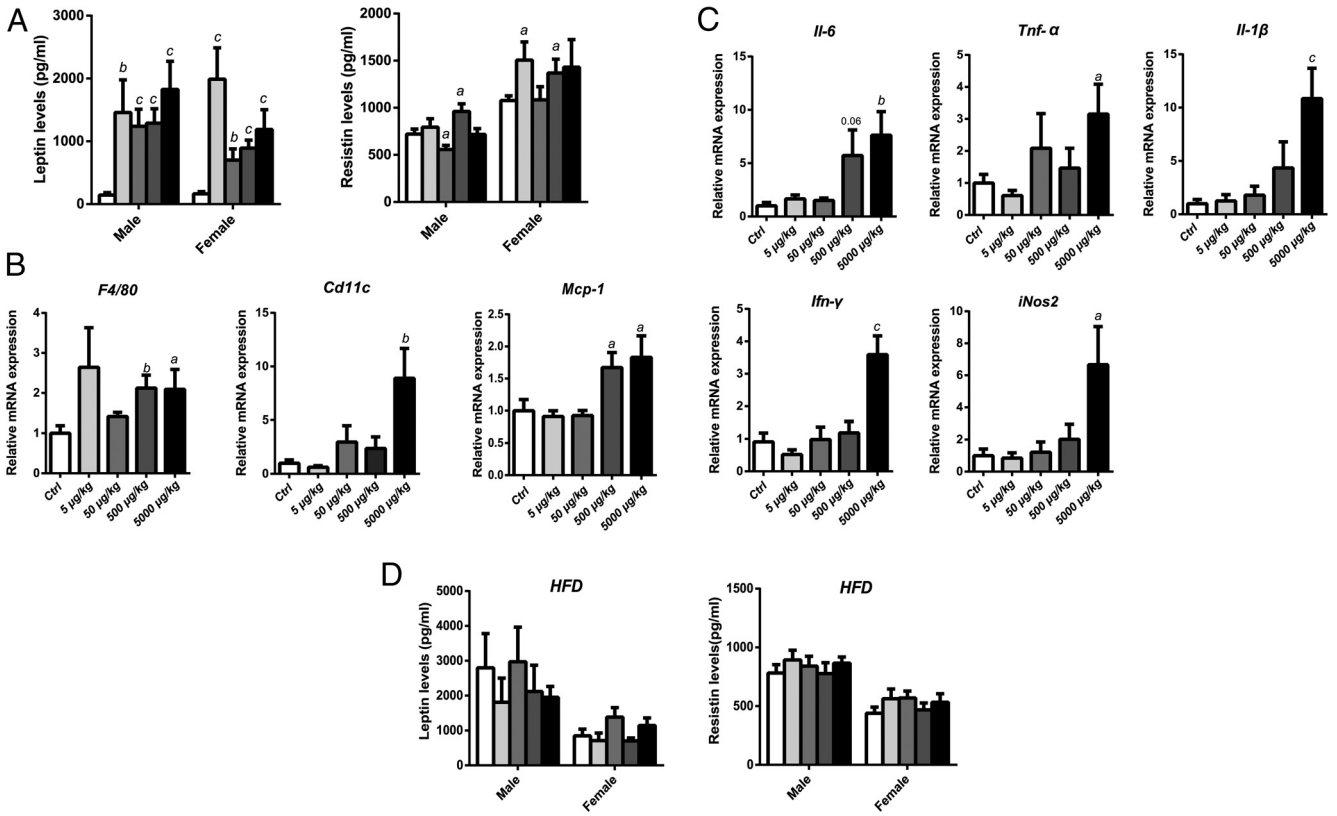

Figure 5. BPA increases circulating and localized inflammation reactions. A, Increased plasma leptin and resistin levels in the mice fed a CD exposed to different dosages of BPA. B, Macrophage-related genes *F4/80*, *Cd11c*, and *Mcp-1* were increased in male mice fed a CD after BPA exposure. C, Exposure to BPA promotes expression of inflammatory genes such as *Il-6*, *Tnfα*, *Il-1β*, *Ifn-γ*, and *iNos2* in WAT in the high-dosages group in male mice fed a CD. D, Plasma leptin and resistin levels in mice fed a HFD. n = 9–12/group. A, $P < .05$; b, $P < .01$; c $P < .001$ compared with control mice. All data are presented as the mean ± SEM.

tenuated by overweight or obesity and this association might also be affected by sex.

Discussion

BPA is an extensively used chemical compound that enters into the diet primarily from food and beverage containers (22, 23). Detectable levels of BPA were found in 92.6% of urinary samples from 2003–2004 NHANES (24). Many lines of evidence from epidemiological and animal studies suggest that BPA disturbs the endocrine systems of mammals. Therefore, it is important to illustrate BPA's effects on the epidemic metabolic diseases, such as obesity and type 2 diabetes. Our previous large-scale cross-sectional epidemiological studies demonstrated BPA's nonmonotonic dose-response with diabetes and obesity (7, 8), although several confounding risk factors, including diet in particular, made it difficult to infer the association between BPA and metabolic disorders. In contrast, obesity has become a global epidemic. Interventions promoting weight maintenance are urgently needed for people in their adolescence because this is the time when many people adopt unhealthy lifestyles and may be more sensitive to environment factors (25). To illustrate the effects of BPA on adiposity, we studied 5-week-old mice fed a low- or high-calorie diet treated orally with four dosages of BPA (ranging from 5 to 5000 μg/kg/d) for 30 days. We found that BPA increased body weight and fat mass in a nonmonotonic dose-response manner in both male and female mice fed a low- but not a high-calorie diet. Further molecular data suggested that BPA promoted obesity by increasing adipogenesis of adipose progenitors and lipogenesis in white adipocytes partially through GR activation. Finally, higher BPA exposure increased systemic and adipose inflammation in male and female mice fed a CD but not a HFD, and high urinary BPA concentrations were associated with high circulating levels of leptin and TNFα in lean female subjects but not in lean male subjects or in both sexes of overweight/obese subjects.

We found that BPA increased the body weight and fat mass in a nonmonotonic dose-response manner in 5-week old male and female mice fed a low-calorie diet but not in

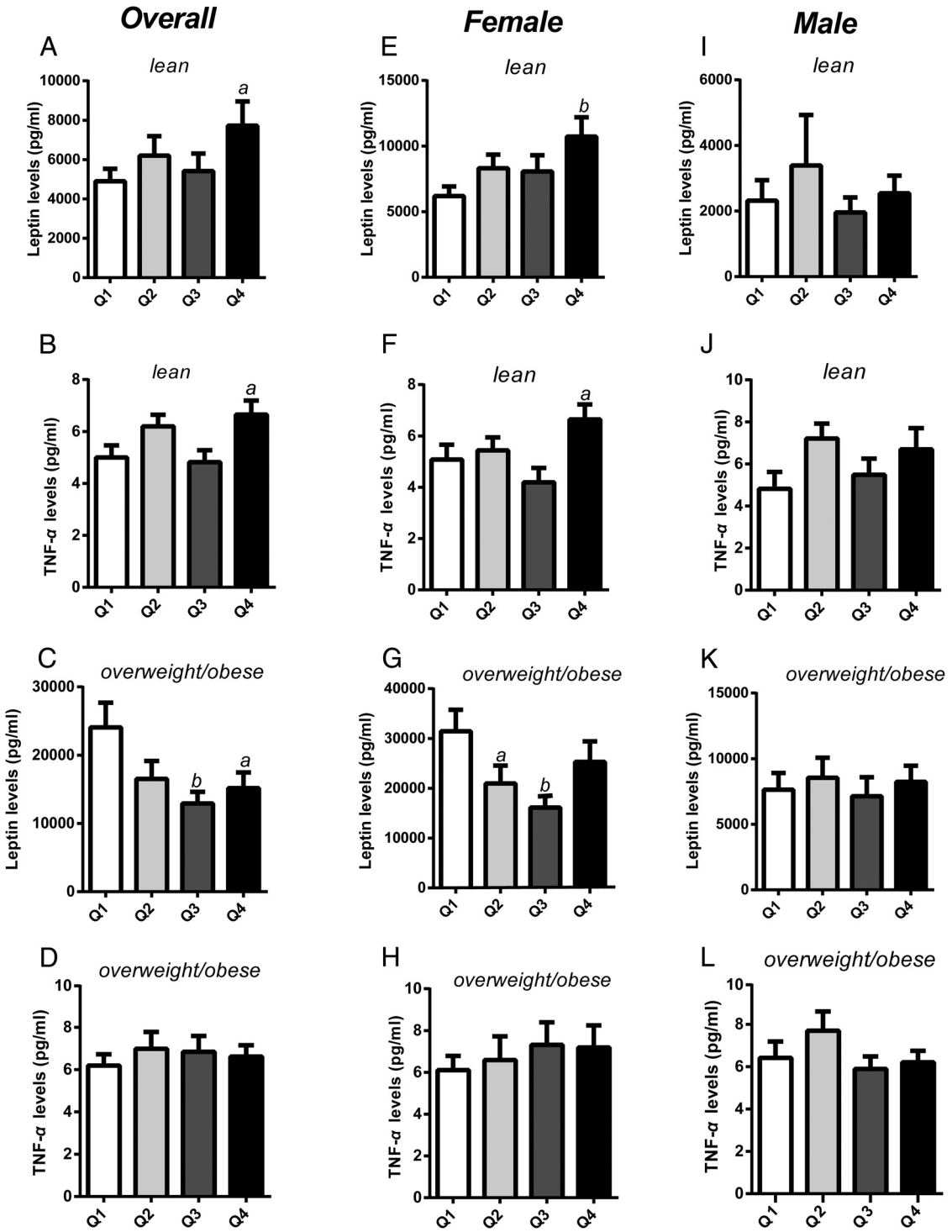

Figure 6. The association of BPA levels with circulating inflammation factors in lean female subjects but not in lean male subjects or in both genders with overweight/obesity. A–D, A nonmonotonic dose-response manner of plasma leptin and TNFα levels increases with urinary BPA quartiles in lean subjects but no increase in overweight/obese subjects. E and F, Plasma leptin and TNFα levels were significantly higher in the female subjects with the highest quartile of BPA concentration than those with the lowest quartile in the lean group. No such association was observed in female subjects in the overweight/obese group (G and H) and male subjects in both lean and overweight/obese group (I–L). a, $P < .05$; b, $P < .01$ compared with Q1 group. All data are presented as the mean ± SEM.

those fed a high-calorie diet. BPA's promoting effects on adiposity may be dependent on the exposure in the early age of mice given that similar effects were not observed in male and female adult mice exposed to BPA beginning at age 8 weeks. Based on our data (Supplemental Figure 3) and previous studies in adult mice showing that treatment

with BPA for 4, 8, or even 28 days under CD condition did not increase body weight (10, 12, 13). In addition to mouse age when exposing BPA, other factors such as exposure duration, exposure mode, doses and genetic background of mice used may also affect the effects of BPA on adiposity. In those mice subjected to short-term treatment with BPA (4 days or 8 d) at 100 μg/kg/d, Swiss albino OF1 male mice were used, and BPA was injected subcutaneously (12, 13). Marmugi et al (10) reveal that oral intake of BPA at the same doses to our study for 28 days induces gene expression related to lipid synthesis and trigger triglyceride accumulation in liver of adult mice, but has no significant effects on body weight, although the duration time, exposure mode, and doses used are similar as in our study. We noticed that male CD1 mice were used for this study, whereas C56BL/6J mice were used in our study, suggesting the genetic background might also affect the role of BPA on adiposity. Previous evidence has also demonstrated that C56BL/6J mice were susceptible to HFD-induced obesity compared with other strains (26). These findings together suggested that BPA's effects on adiposity may differ in response to mouse ages of BPA exposure, diet, and genetic background of mice used. In contrast, lean female adult humans with higher BPA exposure also showed higher plasma levels of inflammation factors. This might be due to long-term and early-age exposure in adult human compared with a relative short-term exposure in those 8-week-old mouse experiments. Of note, previous studies revealed a sex difference regarding BPA's effects on offspring of mothers exposed to BPA (16, 17, 27). Somm et al (17) reported that gestational exposure to BPA increased body weight only in female offspring at weaning time. Furthermore, other studies also support that BPA exposure led to consistent increase in body weight in female offspring (16, 27). We found that both male and female mice showed increased body weight and fat mass in response to BPA exposure beginning at age 5 weeks and lasting 30 days when fed a chow diet (CD). These findings together suggested that sex difference may not be a dominant factor for the effects of BPA exposure on body weight especially for the adolescent mice, and BPA-by-sex interaction may be influenced by mouse age when exposed to BPA.

Consistent with the risk effects of BPA on human obesity by epidemiological studies (8, 28, 29), we showed that BPA exposure at all dosages increased body weight and adiposity of adolescent mice, which provided additional important evidence to previous mouse studies addressing the risk of maternal exposure to BPA on the adiposity of offspring (17, 30). Other studies demonstrate that exposure to environmentally relevant levels of BPA at doses below the TDI (50 μg/kg/d) could alter various biological functions (11). It is reported that BPA at 5 μg/kg/day results in accumulation of triglycerides in the liver (10). Consistently, our results showed that BPA at 5 μg/kg/day, the lowest BPA dosage, could increase body weight in adolescent mice. Because of the effect at the lowest dosage in our study, BPA increased body weight and fat mass in a nonmonotonic dose-response manner following the main principles of most EDCs' action in metabolic and other disorders. The mechanisms of EDCs functions in the body seem to be complicated because EDCs do not interact with hormone receptors with the same specificity and affinity as endogenous hormones (6).

Animal studies have suggested that exposure to BPA may impair glucose homeostasis and contribute to the development of type 2 diabetes in adult mice (31, 32). It is reported that sc injection of BPA decreases blood glucose parallel to an increased plasma insulin within 30 minutes (13), whereas BPA treatment for 4 or 8 days at 100 μg/kg/day induces glucose and insulin intolerance in adult mice (12, 13). However, we did not find significant changes in glucose tolerance in 5-week old mice exposed to BPA under CD or HFD for 30 days. Plasma insulin levels were either unaltered, except in female mice fed a CD at two doses (Supplemental Figure 5). Marmugi et al (10), also reported that BPA exposure for 28 days had no significant effect on plasma glucose in CD1 adult mice, but they observed hyperinsulinemia as well. Overall, these findings suggested that acute exposure (within 30 min) and chronic exposure to BPA may cause different results on blood glucose, as acute exposure to BPA lead to a rapid increase in insulin secretion in β-cells lowering blood glucose, whereas chronic exposure (4, 8, 28 d) may result in a complementary reaction in the body and hyperinsulinemia. Besides, the unaltered plasma insulin levels in 5-week old C56BL/6J mice exposed to BPA, as revealed in our study, suggest that the age or genetic background of the mice may influence the effects of BPA on glucose homeostasis. The exact mechanisms remained to be determined in future study.

We further detected higher expression levels of adipogenic genes and lipogenic enzymes in iWAT of BPA-exposed male mice fed a CD, indicating elevated adipogenesis and lipid biosynthesis in response to BPA exposure. In vitro studies on SVF suggested a direct role of BPA in the differentiation of adipose progenitors toward white adipocytes. It was previously reported that BPA acts as a potent estrogen via nonclassical estrogen triggered pathways or GR-mediated effects (19, 33). Our study supported the finding that BPA promoted adipocyte differentiation partially through GR activation in primary progenitors. Therefore, our current results reinforce the

effect of BPA on adipogenesis and provide a new mechanism of BPA's induction of obesity.

Notably, our study revealed that BPA had no significant effect on body weight and fat mass in mice fed a HFD, consistent with our previous observation regarding the effects of BPA on insulin resistance in lean vs overweight/obese subjects (8). It is generally thought that a HFD could enlarge the difference between treatment groups; however, there are also some exceptions. For instance, one study demonstrated that BMP4 (Bone Morphogenetic Protein 4) transgenic mice gained more weight than control mice when fed with CD and the difference in body weight was less pronounced in transgenic and control mice on the HFD, indicating the HFD could reverse the BMP4's obesogenic effects (34). Garcia-Arevalo's study revealed that the male offspring of pregnant mice exposed to BPA accumulated more fat than controls fed a CD but not a HFD, further indicating a HFD might reverse the risk of metabolic disorders caused by the exposure to BPA at the beginning stages of life (15). The mechanisms still need to be examined in future studies.

Chronic inflammation constitutes an important link between obesity and its metabolic consequences (20, 35, 36). For instance, large numbers of adipose tissue macrophages accumulated following excess fat storage (37, 38), which are linked to insulin resistance (38, 39). Several studies have investigated the association of BPA with inflammation (40, 41). One study showed that BPA exposure was associated with CRP in postmenopausal women (40). Our mouse study showed that circulating levels of inflammation factors such as leptin and TNFα were significantly increased in both sexes fed a CD in response to BPA exposure. We further detected increased expression of *F4/80*, *Cd11c*, and *Mcp-1* in white adipose tissue from BPA-treated male mice fed a CD, indicating microphage filtration in WAT. Inflammation factors including *Ifn-*γ, *Il-6*, *Il-1*β, *Tnf*α, and *iNos2* were also increased in the WAT of BPA exposed mice. These data suggest a critical role for BPA in chronic inflammation. Noticeably, these effects were not seen in mice fed a HFD. Moreover, we also found that high urinary BPA concentrations were associated with high circulating levels of leptin and TNFα, particularly in lean female subjects with a BMI less than 23.0 kg/m^2 but not in subjects with a BMI greater than 25.0 kg/m^2. Participants with a BMI of 23.0–25.0 kg/m^2 were not included in our human study, which could have provided additional information regarding the differential effects of BPA on inflammation among subjects with varied obesity status. These results suggest that the effects of BPA on adiposity or chronic inflammation may be modulated by diet status or obese status, and the lean subjects especially females seemed to be more sensitive to BPA exposure for the development of chronic inflammation.

We want to mention that urinary BPA concentrations were associated with circulating inflammation only in lean females but not in males, which was consistent with one epidemiological study showing that urinary BPA levels were significantly associated with overweight in elderly women but not elderly men (42), similar to the age range of our subjects. However, Trasande et al (28) revealed a significant association between urinary BPA concentration and obesity in children and adolescents of both sexes. And another study on children age 6–18 years showed that the positive association between increasing levels of urinary BPA and obesity more predominantly presented in boys than girls (43). These findings again suggested that BPA's effects on obesity and chronic inflammation may depend on the age and sex; and BPA-by-sex interaction may be influenced by the age of the study population. The stratification by sex and age should be taken into account in future analysis of BPA's effects on obesity and inflammation. The limitation of the study is that adolescent male and female subjects were not included in our human study, which could provide additional information regarding the sex difference of BPA's effects on obesity, especially in early-age population.

In summary, our study demonstrated the effects of a wide range of BPA dosages (including low, tolerance safety, and high dosages) on adiposity and inflammation in a nonmonotonic dose-response manner in 5-week-old male and female mice. The interaction of BPA with diet was first revealed by the evidence of the diverse effects of BPA on mice fed the CD or HFD. In addition, our study also revealed that lean subjects, especially females, seemed to be more sensitive to BPA exposure for the development of chronic inflammation. A more selective substitution of BPA would be helpful to provide for proper industrial use without the development of negative metabolic effects as the lower dosage of BPA under TDI also exerted obesogenic effects. In addition, our study suggested that BPA may interact with diet composition and/or BMI to affect adiposity or inflammation, which is important for further population stratification analysis according to different caloric uptake or BMI in epidemiological studies.

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