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Gestational bisphenol A exposure impairs hepatic lipid metabolism by altering mTOR/CRTC2/SREBP1 in male rat offspring

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Abstract

Lipid metabolism is an important biochemical process in the body. Recent studies have found that environmental endocrine disruptors play an important role in the regulation of lipid metabolism. Bisphenol A (BPA), a common environmental endocrine disruptor, has adverse effects on lipid metabolism, but the mechanism is still unclear. This study aimed to investigate the effects of gestational BPA exposure on hepatic lipid metabolism and its possible mechanism in male offspring. The pregnant Sprague-Dawley rats were exposed to BPA (0, 0.05, 0.5, 5 mg/kg/day) from day 5 to day 19 of gestation to investigate the levels of triglyceride (TG) and total cholesterol (TC), and the expression of liver lipid metabolism-related genes in male offspring rats. The results showed that compared with the control group, the TG and TC levels in serum and liver in BPA-exposed groups was increased. And the expressions of liver fatty acid oxidation related genes, such as peroxisome proliferators-activated receptor α (PPARα) and carnitine palmitoyl transferase 1α (CPT1α), were down-regulated. However, the expressions of fatty acid synthesis related genes, such as sterol regulatory element binding proteins 1 (SREBP-1), acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD-1), were up-regulated. The increased protein levels of mTOR and p-CRTC2 suggested that CREB-regulated transcription co-activator 2 (CRTC2) might be an important mediator in the mTOR/SREBP-1 pathway. In conclusion, these results demonstrated that mTOR/CRTC2/SREBP-1 could be affected by gestational BPA exposure, which may involve in the lipid metabolic disorders in later life.

Keywords

Gestational BPA exposure, male offspring, lipid metabolism, mTOR, CRTC2, SREBP-1

Introduction

Environmental endocrine disruptors (EEDs) pollution has become an important issue in the field of public health. In particular, the application of phenolic environmental estrogens (PEEs) has attracted extensive attention in recent years. As a typical PEEs, bisphenol A (BPA) is used to manufacture polycarbonate plastics and epoxy resins, such as food containers, sports equipment, medical and dental equipment, children's toys, and thermal paper receipts.[1–3] Demand for BPA is growing worldwide, with global consumption of BPA expected to rise to 10.6 million metric tons this year from 7.7 million metric tons in 2015.[4] As the polymer degrades, BPA can be released from plastic products into the external environment. Currently, it has been detected in food, water, air and soil, and humans can be exposed to BPA through ingestion, inhalation and skin contact.[5] Studies have shown that the urine of more than

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90% of the population in western countries contains BPA, and the level of BPA in workers engaged in BPA production or use is much higher than that in the general population, even reaching 492,000 ng/m^3.[6–8] Due to the mimic activity of estrogen, anti-androgen and thyroid hormone, BPA may cause adverse effects on multiple internal systems of human, such as reproductive, nervous, immune and metabolic systems.[9,10] What's more, BPA can also cross the placental barrier and affect the growth and development of fetus.[11] As the expression of BPA metabolizing enzymes in fetal liver needs to be delayed until after birth, fetuses and newborns may be at a higher risk of exposure to BPA than older children and adults.[12] The adverse effects of BPA exposure early in life are now a major health concern.

BPA has been shown to act as an obesogen and to disrupt lipid metabolism. The existing epidemiological studies have shown that BPA is positively correlated with obesity, which is more prominent in females than in males, and 1 ng/mL increase in BPA increased the risk of obesity by 11%.[13,14] Moreover, in animal experiments, BPA can cause liver fat accumulation in both acute and chronic exposure.[15] It has even been shown that BPA may accelerate fat formation at non-observed adverse effect levels (NOAEL).[16] Results of rodent studies have shown that BPA is capable of disturbing the carbohydrate and lipid metabolism after chronic exposure, which are associated with increased inflammation, increased levels of gene and protein catalyzing lipogenesis, and decreased levels of those catalyzing lipolysis, accompanied by dyslipidemia, insulin resistance and hepatic fat accumulation.[17,18] Studies also have shown that prenatal BPA exposure can impair liver lipid metabolism in female rat offspring.[19] And after prenatal BPA exposure, alterations in lipid profiles are more prominent in the offspring than those in the rat dams, suggesting that exposure to BPA in utero is more serious than adult exposure.[20] Lipid metabolic disorders may lead to obesity, type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), atherosclerosis and other diseases.[21] However, the mechanism of BPA exposure during pregnancy on lipid metabolism of male offspring is rarely studied.

The sterol regulatory element binding proteins (SREBPs), including SREBP-1 and SREBP-2, are a family of transcription factors that regulate lipid biosynthesis. SREBP-1 has been confirmed to mainly regulate fatty acid synthesis, and SREBP-2 is involved in cholesterol synthesis.[22] Studies have shown that BPA can interfere with the binding of SREBP-1 to their SREs through DNA methylation in the 5′ side region of carnitine palmitoyl transferase 1 (CPT1), thus affecting fatty acid β-oxidation.[23] It was also reported that BPA could decrease the DNA methylation level of SREBP-2 in mice, and upregulate the expression of genes related to cholesterol synthesis in liver, which led to liver lipid accumulation and liver steatosis.[24,25]

mTOR (Mammalian target of Rapamycin) is a serine/threonine phosphoinoside kinase belonging to the PI3K related kinase family. mTOR signaling pathway has been implicated in regulation of lipid synthesis in liver. Studies have found that estrogen combined with ESRs inhibits fat synthesis and promotes fat hydrolysis by inhibiting the PI3K/Akt/mTOR signaling pathway and promoting the expression of AMPK in lipolytic pathway.[26,27] A recent study showed that BPA mainly inhibited the phosphorylation of AMPK, thereby activating the mTOR/SREBP-1c pathway and the expression of downstream adipogenesis related factors.[28] In addition, researches have demonstrated that CREB-regulated transcription coactivator 2 (CRTC2), as a critical mediator of mTOR, may play a key role in mTOR-dependent regulation of SREBP-1.[29,30]

These findings prompt us to investigate whether CRTC2 can mediate the mTOR/SREBP1 pathway, which may be an important pathway for the effects of gestational BPA exposure on lipid metabolism in male offspring.

Materials and methods

Materials

BPA was purchased from Tokyo Chemical Industry (>99%, Japan). Corn oil was purchased from Aladdin (Shanghai, China). Diethyl ether was purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). TG assay kits were obtained from Enzyme-linked Biotechnology Co, Ltd (Shanghai, China). Trizol was purchased from Vazyme Biotech Co, Ltd (Nanjing, China). Primers were obtained from Sangon Biotech (Shanghai) Co, Ltd (Shanghai, China). RIPA lysis buffer, phosphatase inhibitor, 1% phenylmethylsulfonyl fluoride (PMSF) and bicinchonininc acid (BCA) protein assay kit was obtained from Beyotime Biotechnology (Shanghai, China). PAGE Gel Fast Preparation Kit was purchased from Epizyme Biotech (Shanghai, China). Chemistar ECL Western Blotting Substrate was purchased from Tanon Science and Technology Company (Shanghai, China). Goat anti-Rabbit IgG (H&L)-HRP (SA00001-2), Goat anti-Mouse IgG (H&L)-HRP (SA00001-1), CPT1α (15184-1-AP), mTOR (66888-1-Ig), CRTC2 (12497-1-AP) and β-actin (20536-1-AP) were purchased from Proteintech (Wuhan, China). p-mTOR (AP0094) and SCD-1 (A16429) were purchased from ABclonal Technology (Wuhan, China). p-CRTC2 (AF8328), PPARα (AF5301) and SREBP-1 (AF6283) were purchased from Affinity Biosciences Ltd (Jiangsu, China).

Animals and treatments

The experimental animals were 9-week-old Sprague-Dawley rats purchased from Liaoning Changsheng Biotechnology Co, Ltd (Certificate No. SCXK2020-0001, Liaoning of China). The animals were raised in SPF Experimental Animal Center of Shenyang Medical College, and the breeding conditions met the relevant requirements of "Environment and Facilities for Experimental Animals" (GB14925-2010); indoor relative temperature 25 ± 2°C, humidity 55 ± 5%, 12 h/12 h day-night light cycle, free eating and drinking (all drinking bottles were glass bottles). After 1 week of adaptive feeding, female and male rats were randomly mated in a 2:1 ratio. Pregnancy was confirmed by vaginal smear. The date of microscopic sperm observation was gestation day 0 (GD0).[10] 40 pregnant rats were randomly divided into four groups with 10 rats in each group. BPA was dissolved in corn oil. Rats were orally gavaged with corn oil containing 0, 0.05, 0.5, and 5 mg/kg/d BPA respectively, from GD5 to GD19. The day of birth was denoted as postnatal day (PND) 1, and male pups at PND21 and 56 were selected as research objects. At PND21 and 56, the male offspring rats were weighed, anesthetized and then sacrificed. Serum and liver tissues were collected and stored at −80°C for further study. The liver was weighed, and the organ coefficient was calculated. Calculation formula: Organ coefficient (%) = organ wet weight (g)/body weight (g) × 100%.

Lipid content assay

The contents of TG and total cholesterol (TC) in serum and liver were detected by double-antibody one-step sandwich enzyme linked immunosorbent assay ELISA kit. Serum was collected by centrifugation at 3000 r/min for 15 min after hematopexis. Liver tissue was rinsed with 1×PBS, homogenized in 1 mL of 1×PBS, and then centrifuged for 10 min at 3000 r/min. The supernate was assayed immediately. Avoid repeated freeze-thaw cycles. Experimental steps and methods strictly follow the instructions in the kit. Refer to the Assay Layout Sheet to determine the number of wells to be used. 50 μL of standard or sample (10 μL sample +40 μL sample dilution buffer) was added per well. 100 μL of horseradish peroxidase (HRP) labeled detection antibody was added to standard wells and sample wells except blank wells. The reaction wells were covered with a sealing membrane and incubated at 37°C for 60 min. After incubation, the liquid was removed and the wells were washed 5 times. 50 μL of substrate A and B solution (TMB substrate) were added to each well and incubated at 37°C for 15 min 50 μL of stop solution was added to each well. The absorbance (OD) value was measured at 450 nm using a microplate reader, and the sample concentration was calculated.

Real-time PCR

Trizol was used to extract total RNA from male offspring's liver tissue (six in each group), then the cDNA was synthesized using PrimeScript RT kit. The SYBR Premix Ex Taq II kit was used to mix cDNA and primers, and real-time fluorescence quantitative PCR was performed with ABI 7500 fast Real-Time PCR System (California, USA). The housekeeping gene *GAPDH* was used as an internal control. The relative mRNA expression of each gene was calculated by $2^{-\Delta\Delta CT}$ method. Gene-specificity primers were shown in Table 1.

Western blotting

RIPA with phosphatase inhibitors and PMSF was used to lyse liver tissue (six in each group), and then protein concentration was detected by BCA assay kit. Western Blotting was used to detect the samples after boiling and denaturation. The protein samples (50 μg) were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto the nitrocellulose membranes. After that, blocking of the membranes with 5% skimmed milk powder or 5% BSA was done; followed by incubation with primary antibodies for overnight at 4°C. Secondary antibodies were incubated at room temperature for 1 h. The gel imaging system (Tanon-5200 Muti, Tanon, China) was used to take pictures. Image J was used to quantify the gray value of protein bands. The relative protein expression was normalized to β-actin.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by One-way ANOVA followed by LSD or Dunnett's test using SPSS 20.0 software (Illinois, USA). $p < 0.05$ was regarded as statistically significant.

Results

Gestational BPA exposure affected the body weight and liver coefficient of male offspring

From birth to sexual maturity, all offspring rats were in a good state and no abnormal appearance, posture and behavior, and ate normally. At PND21, there was no significant difference in body weight and liver coefficient of male offspring between the BPA exposed groups and the control group ($p > 0.05$). However, at PND56, the body weight of male rats was decreased in the BPA exposed groups, especially in the 0.5 mg/kg group ($p < 0.05$). Moreover, the liver coefficient of male rats was increased significantly in

Table 1. Primer sequences.

Gene	Primer sequence (5′-3′)
mTOR	Forward: CGGTCTGTGGGAAAGTCCTCATTG
	Reverse: CCCTGCTGTGTCTACAAGCTGAAG
CRTC2	Forward: CCCTACCTGACCTCACCAACCTAC
	Reverse: CAGACCTCCACTGATGCCCAAATG
SREBP-1	Forward: CATCGACTACATCCGCTTCTTACAGTC
	Reverse: TTTCAGTGATTTGCTTTTGTGA
ACC1	Forward: AGGAAGATGGTGTCCGCTCTG
	Reverse: GGGGAGATGTGCTGGGTCAT
FAS	Forward: CTCTGGAAGTGCATGCTGTAAGA
	Reverse: GGTAGATGTCATTTGCGAAAGGT
SCD-1	Forward: CCTTAACCCTGAGATCCCGTAGA
	Reverse: AGCCCATAAAAGATTTCTGCAAA
PPARα	Forward: ACGATGCTGTCCTCCTTGATGAAC
	Reverse: ATGATGTCGCAGAATGGCTTCCTC
CPT1α	Forward: GCCCATGTTGTACAGCTTCCA
	Reverse: AGTCTTCTTCCTTCATCAGTGGC
GAPDH	Forward: GCAAGAGAGAGGCCCTCAG
	Reverse: TGTGAGGGAGATGCTCACTG

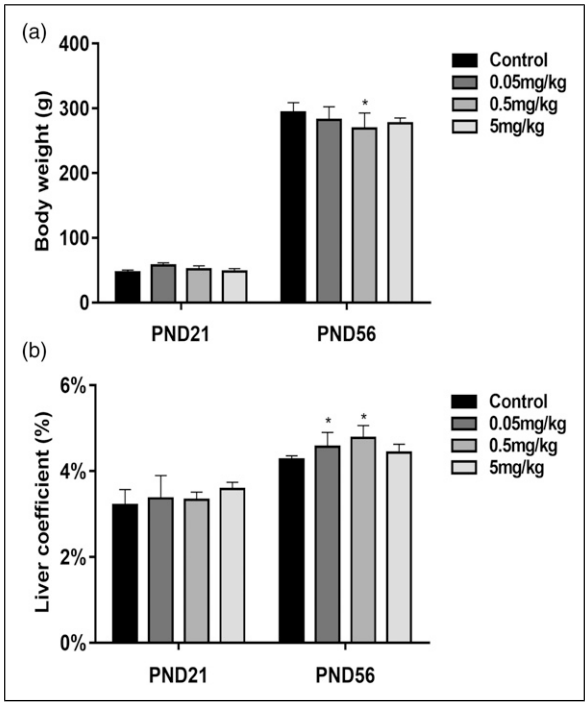

Figure 1. Effects of gestational BPA exposure on body weight and liver coefficient in male offspring. (a) Body weight of male offspring at PND21 and PND56. (b) Liver coefficient of male offspring at PND21 and PND56. Data were shown as means ± SD and analyzed with One-way ANOVA (n = 8). *p < 0.05 vs. Control.

0.05 and 0.5 mg/kg groups compared to the control group (p < 0.05) (Figure 1).

Gestational BPA exposure increased the levels of TG and TC in male offspring

To explore the effects of gestational exposure to BPA on lipid metabolism in male offspring, we examined the TG and TC levels in serum and liver tissue. As shown in Figure 2(a) and (b), at PND21, the TG and TC levels in serum and liver in BPA exposed groups were significantly higher than those in the control group (p < 0.05). And the TG level at PND56 in BPA exposed group was also significantly higher than that in the control group (p < 0.05). However, the TC level at PND56 in BPA exposed group was increased in serum (p < 0.05), but not in liver (p > 0.05) (Figure 2(c) and (d)).

Gestational BPA exposure decreased the expression of liver fatty acid oxidation related genes in male offspring

We hypothesized that BPA may affected TG and TC levels through fatty acid oxidation and fatty acid synthesis. Therefore, we detected the expression levels of two key genes in fatty acid oxidation pathway, PPARα and CPT1α. As shown in Figure 3(a) and (b), at PND21, the mRNA expression of PPARα was significantly decreased in all BPA exposure groups compared with the control group, and the mRNA expression of CPT1α was significantly decreased only in the 5 mg/kg group. Meanwhile, the protein levels of PPARα and CPT1α were significantly decreased in 5 mg/kg group (p < 0.05). At PND56, the mRNA and protein expression of PPARα decreased significantly in 0.5 mg/kg and 5 mg/kg BPA exposure groups, while the protein expression of CPT1α decreased significantly only in 5 mg/kg BPA exposure group (p < 0.05) (Figure 3(c) and (d)).

Gestational BPA exposure increased the expression of liver fatty acid synthesis related genes in male offspring

Furthermore, we detected the expression of liver fatty acid synthesis related factors. As shown in Figure 4(a), at PND21, compared with the control group, the mRNA levels of SREBP-1, ACC1, FAS and SCD-1 were significantly increased in BPA exposure groups (p < 0.05). Consistently, at PND56, the mRNA levels of these factors in BPA exposure groups were also significantly increased (p < 0.05) (Figure 4(c)). The protein levels of SREBP-1 and SCD-1 were detected, which were also significantly increased in

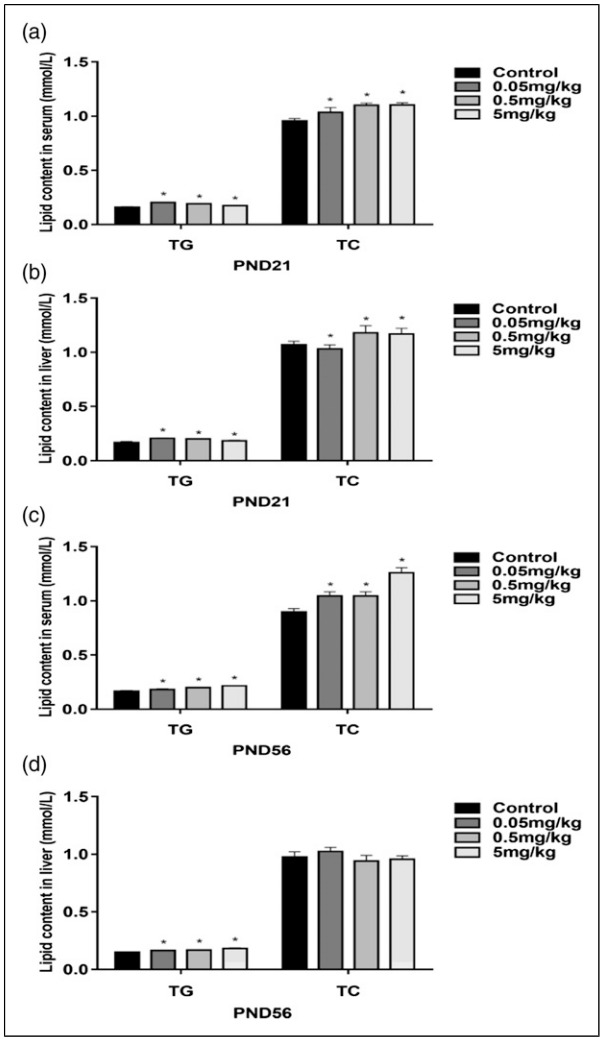

Figure 2. Effects of gestational BPA exposure on TG and TC contents in male offspring. (a) Serum TG and TC contents of male offspring at PND21. (b) Liver TG and TC contents of male offspring at PND21. (c) Serum TG and TC contents of male offspring at PND56. (d) Liver TG and TC contents of male offspring at PND56. Data were shown as means ± SD and analyzed with One-way ANOVA (n = 6). *p < 0.05 vs. Control.

BPA exposed groups at PND21 (Figure 4(b)) and 56 (Figure 4(d)).

Gestational BPA exposure affected the liver fatty acid synthesis by regulating mTOR/CRTC2 pathway in male offspring

Since CRTC2 could be phosphorylated by mTOR, which promoted SREBP1-mediated de novo lipogenesis, we furtherly investigated the effects of gestational BPA exposure on mTOR/CRTC2 pathway. At PND21, the mRNA expression of mTOR in 0.05 mg/kg group was significantly increased than that in the control group ($p < 0.05$), and the mRNA expression of CRTC2 in all BPA exposure groups were significantly increased ($p < 0.05$) (Figure 5(a)). Although the phosphorylation of mTOR did not change significantly, the protein level of mTOR in BPA exposure groups was significantly increased ($p < 0.05$), meanwhile, the phosphorylation of CRTC2 in 0.05 and 0.5 mg/kg groups was significantly increased ($p < 0.05$) (Figure 5(b)). At PND56, compared with the control group, the mRNA expression of mTOR was significantly increased in the 5 mg/kg group ($p < 0.05$), and the mRNA expression of CRTC2 in 0.05 and 0.5 mg/kg groups was significantly increased ($p < 0.05$) (Figure 5(c)). Notably, the protein level of mTOR and the phosphorylation of CRTC2 were also significantly increased in 0.5 and 5 mg/kg groups ($p < 0.05$) (Figure 5(d)), which indicated that gestational BPA exposure might affect the liver fatty acid synthesis in male offspring through the mTOR/CRTC2/SREBP-1 pathway.

Discussion

It is estimated that by 2035, about 592 million people worldwide will be affected by T2DM.[31] It has been reported that obesity (body mass index >30 kg/m^2) is a major independent risk factor for T2DM and an important cause of NAFLD.[32] In the United States alone, 35.7% of adults and about 17% of children are obese.[33] Lipid metabolism disorders have a significant impact on morbidity and mortality of metabolic diseases, which have become major global public health challenges. EEDs capable of disrupting endocrine regulation have been recognized as Environmental Obesogens.[34] The study has shown that gestational urinary BPA concentration is associated with the prevalence of obesity in children and adolescents.[35] Therefore, the present study focused on the effects of BPA exposure during pregnancy, a critical period of organ formation, and 0.05 mg/kg (Tolerable daily intake, TDI) and 5 mg/kg (NOAEL) were selected as the lowest and highest exposure doses respectively. 0.05 mg/kg was also recognized by FDA as the lifetime safe oral dose of BPA.[36,37]

In the present study, the body weight of male rat offspring did not change significantly at PND21. In another study of perinatal BPA exposure, the weight of BPA-exposed male and female pups increased at PND1, however, the weight was increased only in female pups at PND21.[38] These results showed that after weaning, perinatal BPA exposure predisposed to overweight in a sex-dependent manner. The liver organ coefficient of male offspring at PND56 was higher than that in the control group, and was significant in 0.05 and 0.5 mg/kg groups. Organ coefficient, as a common indicator in toxicology experiments, not only can reflect the toxicity of poisons but also observe the possibility of histopathological changes from the side, and help to find toxic target organs. In

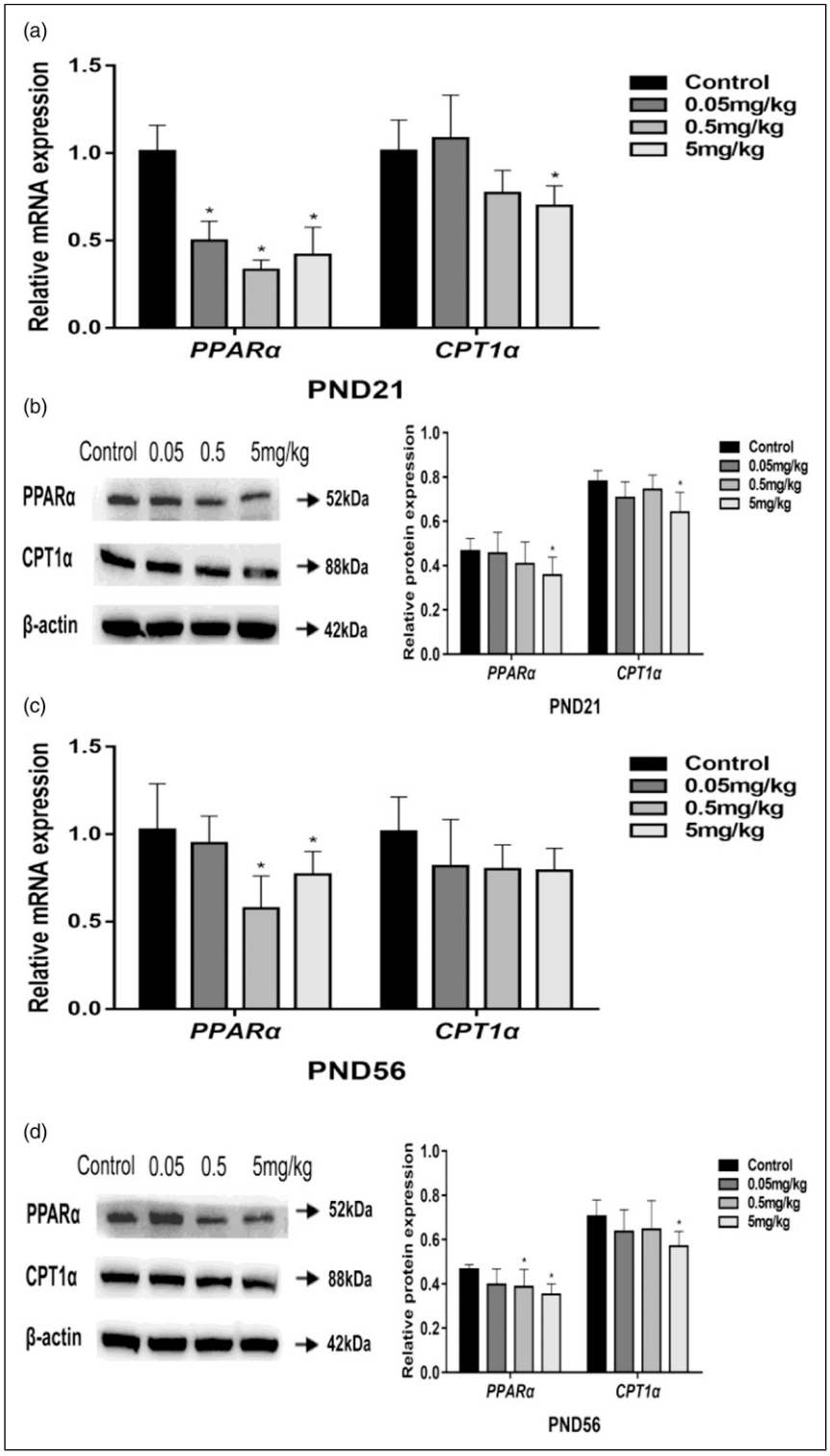

Figure 3. Effects of gestational BPA exposure on expressions of fatty acid oxidation related genes in liver of male offspring. (a) The mRNA levels of PPARα and CPT1α at PND21. (b) Protein levels of PPARα and CPT1α at PND21. (c) The mRNA levels of PPARα and CPT1α at PND56. (d) Protein levels of PPARα and CPT1α at PND56. Western blot bands represent the detection of the protein from three independent tests. The relative intensities were expressed in the bar chart. All data were normalized to β-actin level within the same lane/blot. Data were shown as means ± SD and analyzed with One-way ANOVA ($n = 6$). *$p < 0.05$ vs. Control.

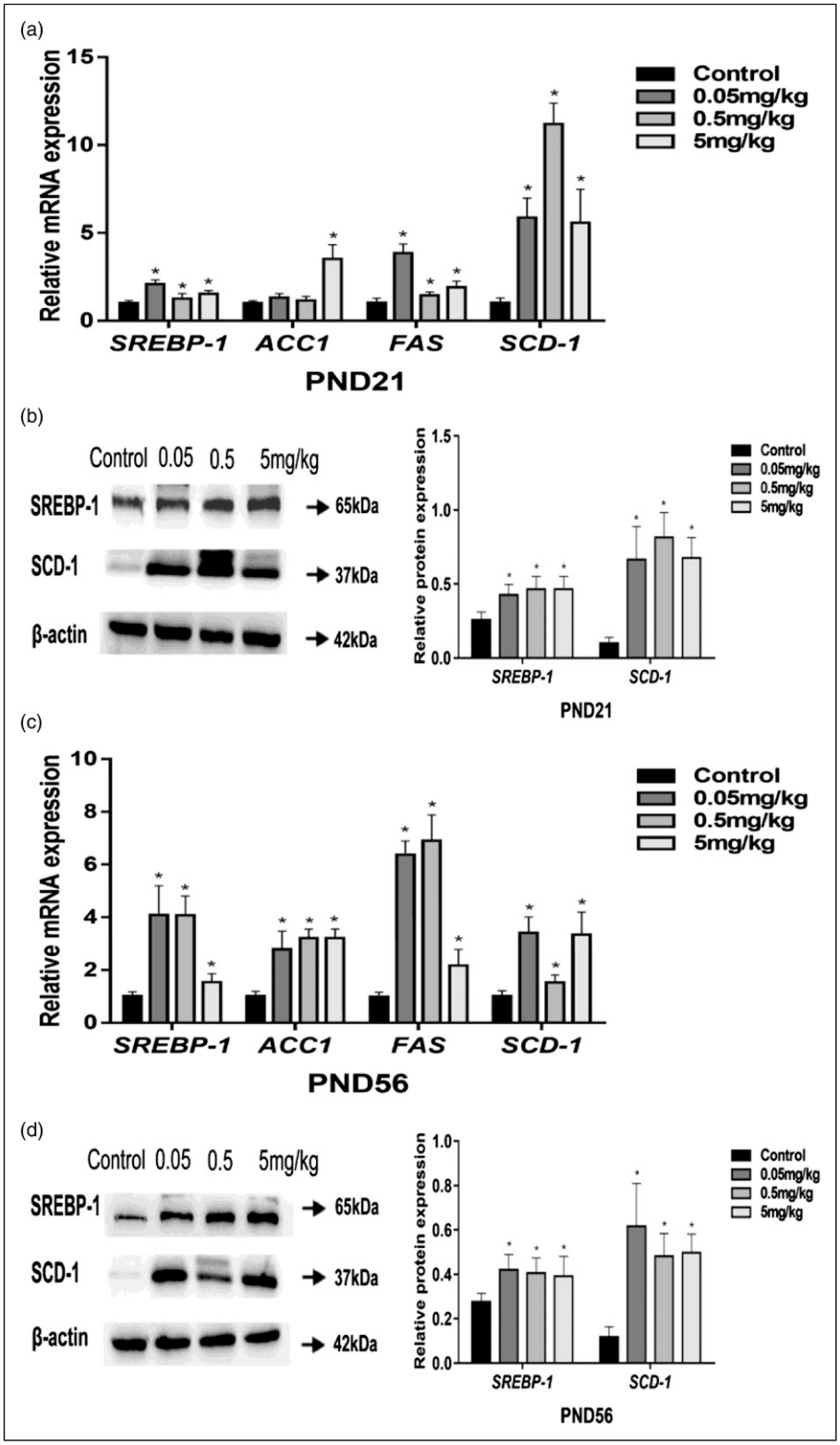

Figure 4. Effects of gestational BPA exposure on the expression of fatty acid synthesis related genes in liver of male offspring. (a) The mRNA levels of SREBP-1, ACC1, FAS and SCD-1 at PND21. (b) Protein levels of SREBP-1 and SCD-1 at PND21. (c) The mRNA levels of SREBP-1, ACC1, FAS and SCD-1 at PND56. (d) Protein levels of SREBP-1 and SCD-1 at PND56. Western blot bands represent the detection of the protein from three independent tests. The relative intensities were expressed in the bar chart. All data were normalized to β-actin level within the same lane/blot. Data were shown as means ± SD and analyzed with One-way ANOVA ($n = 6$). *$p < 0.05$ vs. Control.

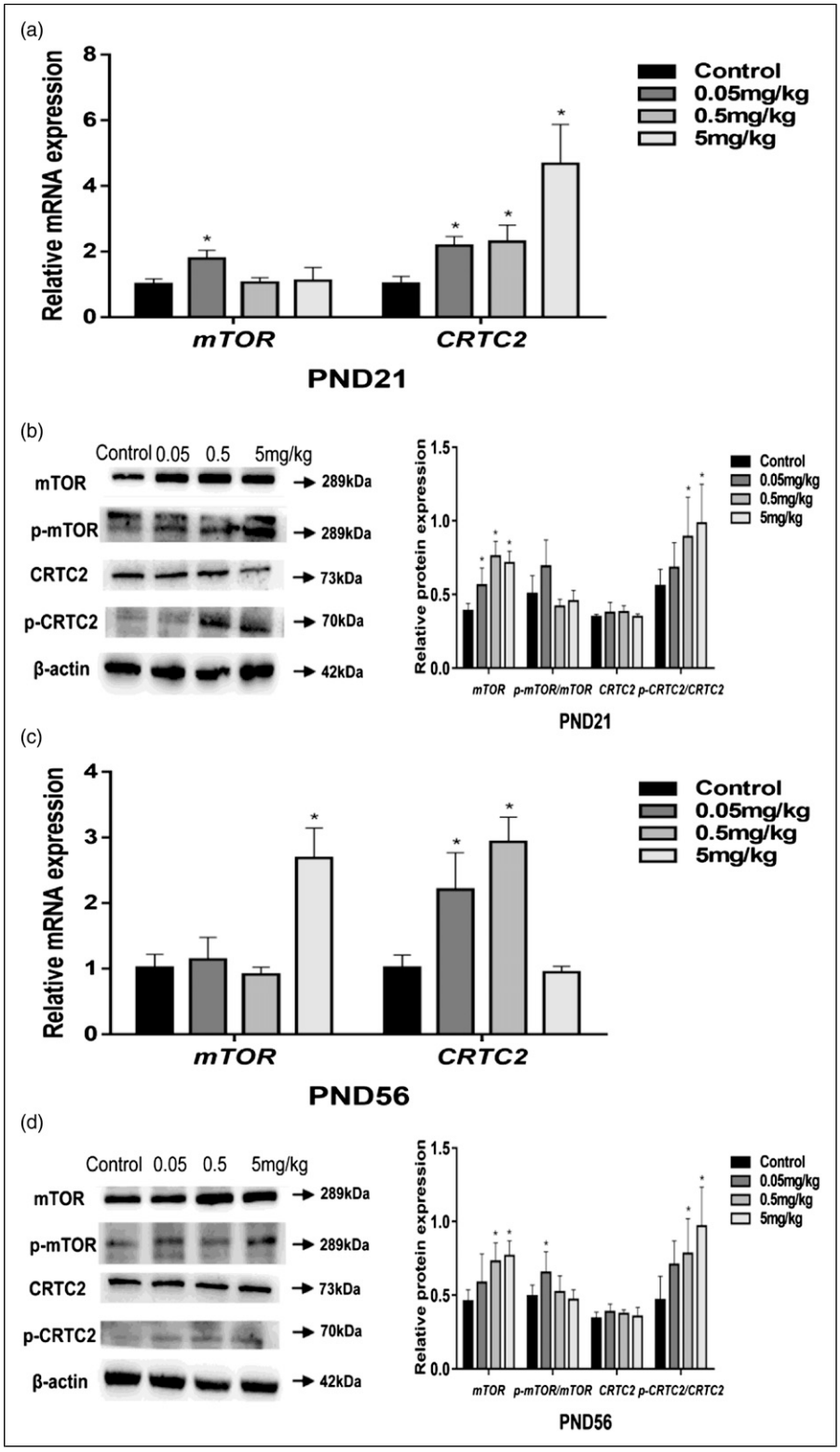

Figure 5. Effects of gestational BPA exposure on mTOR/CRTC2 pathway in male offspring. (a) The mRNA levels of mTOR and CRTC2 at PND21. (b) Protein levels of mTOR, p-mTOR, CRTC2 and p-CRTC2 at PND21. (c) The mRNA levels of mTOR and CRTC2 at PND56. (d) Protein levels of mTOR, p-mTOR, CRTC2 and p-CRTC2 at PND56. Western blot bands represent the detection of the protein from four independent tests. The relative intensities were expressed in the bar chart. All data were normalized to β-actin level within the same lane/blot, except for the p-CRTC2: mature CRTC2 ratio and the p-mTOR: mature mTOR ratio. Data were shown as means ± SD and analyzed with One-way ANOVA ($n = 6$). *$p < 0.05$ vs. Control.

general, their elevation suggests that exposure to BPA may cause damage to offspring's organs. Relevant experiments have shown that BPA exposure can cause mild cellular swelling and steatosis in rat liver tissue,[39] and similar results are obtained in two other studies on vertebrates.[40,41]

TC is the main component of cell membrane, and its serum concentration can be used as an indicator of lipid metabolism. TG is an important form of energy storage and oxidative energy supply in the body, and is often used as a key indicator to determine fatty acid biosynthesis in lipid metabolism. In this study, we measured the contents of TG and TC in serum and liver. The results showed that gestational BPA exposure increased the TG level in male offspring at PND21 and 56, and the increasing trend of TG in serum and liver was basically the same. Our results are consistent with several male rodent studies,[42,43] but not famale.[19,38] Tonini C et al.[19] reported that the effects of maternal BPA exposure on hepatic lipid metabolism are dependent on sex, being observable only in female rat fetuses. The inconsistency of these results may be due to the different dose and duration of exposure. Several population-based epidemiological studies have also reported the association between BPA and lipid metabolism. Urinary BPA levels in adults are also associated with elevated FFA and TG levels in humans.[44,45] A cohort study from Greece reported that prenatal BPA was negatively associated with BMI and adiposity in girls and positively in boys.[46] This result is not consistent with the animal studies on female offspring, and the effect of maternal BPA exposure on lipid metabolism in male offspring should not be ignored. At PND21 and 56, the changes of serum TC and TG were consistent, but at PND56, the liver TC level did not change significantly. This might be because rats at PND56 were in the compensatory stage, and excessive TC synthesized in the liver was transferred into the blood. Anyway, our results demonstrated that intrauterine BPA exposure could lead to lipid accumulation in male progeny, indicating its role of promoting adipogenesis.

Two important pathways involved in the regulation of liver lipid metabolism are fatty acid synthesis and fatty acid oxidation, among which PPAR plays a very important role in fatty acid oxidation. PPARα is highly expressed in liver and promotes fatty acid oxidation by stimulating the transcription of rate-limiting enzyme CPT1.[47] In this study, we found that mRNA and protein expressions of PPARα and CPT1α were decreased in the liver at both stages of 5 mg/kg BPA exposure, suggesting that BPA inhibits liver fatty acid oxidation to increase TG accumulation, which is consistent with previous study.[48]

In addition, to further investigate whether BPA-induced effects were related to the abnormal expression of key genes and proteins in lipid synthesis, SREBP-1 and its regulated genes SCD-1, ACC1, FAS were detected. Results showed that the mRNA expressions of these factors were significantly increased at both stages. SCD-1 is a key control point for lipid synthesis in liver and can catalyze the formation of saturated fatty acids into monounsaturated fatty acids. During fat synthesis, monounsaturated fatty acids are more likely to become substrates of ACAT (ACYL-CoA cholesterol acyltransferase) and DGAT (Diacylglycerol acyltransferase) than saturated fatty acids, which produce cholesterol esters and TG.[49] Therefore, we detected the protein levels of SCD-1 and its key regulatory factor SREBP-1, and found that they were consistent with the results of mRNA. These results suggested that BPA could induce SREBP-1 activation in male offspring, thereby increasing the expression of downstream key genes and leading to TG accumulation in vivo. Similar effects have been reported in previous studies, and overweight in female offspring is also associated with adipocyte hypertrophy and overexpression of lipogenic genes, but males and females exhibited differential susceptibility to the different doses of BPA.[38,50,51] Notably, even very low dose of BPA (1 μg/kg/day) exposure during pregnancy may also lead to dysregulation of lipid metabolism-related regulatory factors in offspring.[51] In addition, Zhang et al.[52] also found that inhibition of SREBP-1 expression could effectively reduce TG accumulation. At present, existing evidence fully shows that the expression of SREBP-1 is positively correlated with the content of TG, which is also an important target of BPA exposure affecting lipid metabolism.

Mammalian mTOR is an important regulator of cell proliferation, differentiation, apoptosis and protein synthesis, and also plays an important role in lipid metabolism. Our results suggested that 0.05 mg/kg BPA exposure increased the p-mTOR/mTOR ratio at PND56, which is consistent with the well-known pattern of mTOR regulating fatty acid synthesis.[53] In 0.5 and 5 mg/kg groups, the total protein level of mTOR increased, while the phosphorylation level did not change significantly. We also found that the phosphorylation level of CRTC2 at PND21 and 56 was significantly increased in 0.5 and 5 mg/kg groups, suggesting that mTOR may phosphorylates CRTC2 and then regulates the expression of SREBP-1. Previous studies on LO2 cells in vitro have also shown that phosphorylation of CRTC2 activates nuclear SREBP-1 activity and subsequent adipogenesis,[54] but the difference is that phosphorylation of mTOR is not well defined. Zhang et al.[55] found that in adipose tissue, mTORC1 played a corresponding regulatory role through phosphorylation of CRTC2. Other studies also reported that CRTC2 knockdown or overexpression did not affect the phosphorylation of mTOR,[56] which strongly supported our research results. However, the effect of BPA on mTOR/CRTC2 pathway has not been reported. Our data demonstrate that gestational BPA exposure may increase the liver fatty acid synthesis by regulating mTOR/CRTC2/SREBP-1 pathway in male offspring. In addition, in this study, the expression

results of mTOR and CRTC2 at mRNA and protein levels are inconsistent, which may be due to the influence of post-transcriptional regulation.

In summary, the present study proved that gestational BPA exposure could affect liver lipid levels by disturbing lipid metabolism, including inhibition of fatty acid oxidation and promotion of fatty acid synthesis. We first propose that the mTOR/CRTC2/SREBP-1 pathway may play an important role in the effects of prenatal BPA exposure on liver fatty acid synthesis in male offspring. In this process, as a downstream mediator of mTOR, CRTC2 may play a potential role in the regulation of SREBP-1 and thus promoting lipid synthesis, which provides a novel insight into the established correlations between early-life BPA exposure and lipid metabolism disorders. Furthermore, these data may partially indicate that prenatal BPA exposure induces long-lasting effects on basal gene expression of lipid metabolism in adult rat liver. This will strengthen the evidence for prevention of exposure to environmental chemicals during pregnancy.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The present study was designed to minimize the animal suffering and use amount according to the National Institutes of Health Guidelines in China, which was also permitted by the Animal Ethics Committee of Shenyang Medical College (Permit number: SYYXY2020092002). Also, the study was performed in compliance with the ARRIVE guidelines.

Data availability

All data generated or analyzed during this study are included in this published article.

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