ToxSci Advance Access published January 21, 2014

Contribute to Toxicological Sciences

Metabolomic analysis reveals metabolic changes caused by bisphenol A in rats

Minjian Chen *, †, Kun Zhou *, †, Xiaojiao Chen *, †, Shanlei Qiao *, †, Yanhui Hu *, †, Bo Xu *, †, Bin Xu *, †, Xiumei Han *, †, Rong Tang *, †, Zhilei Mao *, †, Congcong Dong *, †, Di Wu *, †, Yubang Wang ‡, Shoulin Wang *, †, Zuomin Zhou *, Yankai Xia *, †, 1, Xinru Wang *, †, 1

* State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China

† Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing 211166, China

‡ Safety Assessment and Research Center for Drug, Pesticide, and Veterinary Drug of Jiangsu Province, School of Public Health, Nanjing Medical University, Nanjing 210029, China

[1] To whom correspondence should be addressed:

Dr. Yankai Xia and Dr. Xinru Wang

Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China

Phone: +86-25-86868427; Fax: +86-25-86868427

E-mail: yankaixia@njmu.edu.cn and xrwang@njmu.edu.cn

Running Title: Metabolic changes caused by bisphenol A

Abbreviations

ALT alanine transaminase

BPA bisphenol A

CE capillary electrophoresis

CHOL cholesterol

CSA D-camphor-10-sulfonic acid

ESI electrospray ionization

FDR false discovery rate

LC-MS liquid chromatography-mass spectrometry

LOAEL accepted lowest observed adverse effect level

MAT methionine adenosyltransferase

MetSul methionine sulphone

NMR nuclear magnetic resonance

QC quality control

RSDs relative standard deviations

SAMe *S*-adenosylmethionine

TOFMS time-of-flight mass spectrometry

Abstract

Bisphenol A (BPA) is a widely used material known to cause adverse effects in humans and other mammals. To date, little is known about the global metabolomic alterations caused by BPA using urinalysis. Sprague-Dawley rats were orally administrated BPA at the levels of 0, 0.5 μg/kg/d and 50 mg/kg/d covering a low dose and a reference dose for 8 weeks. We conducted a capillary electrophoresis in tandem with electrospray ionization time-of-flight mass spectrometry based nontargeted metabolomic analysis using rat urine. To verify the metabolic alteration at both low and high doses, RT-PCR and western blotting were further conducted to analyze hepatic expression of methionine adenosyltransferase Iα (*Mat1a*) and methionine adenosyltransferase IIα (*Mat2a*). Hepatic *S*-adenosylmethionine (SAMe) was also analyzed. A total of 199 metabolites were profiled. Statistical analysis and pathway mapping indicated that the most significant metabolic perturbations induced by BPA were the increased biotin and riboflavin excretion, increased synthesis of methylated products, elevated purine nucleotide catabolism, and increased flux through the choline metabolism pathway. We found significantly higher mRNA and protein levels of *Mat1a* and *Mat2a*, and significantly higher SAMe levels in rat liver at both low and high doses. These two genes encode critical isoenzymes which catalyze the formation of SAMe, the principal biological methyl donor involved in the choline metabolism. In conclusion, an elevated choline metabolism is underlying the mechanism of highly methylated environment and related metabolic alterations

caused by BPA. The data of BPA-elevated accepted biomarkers of injury indicate that BPA induces DNA methylation damage and broad protein degradation, and the increased deleterious metabolites in choline pathway may also be involved in the toxicity of BPA.

Key words: bisphenol A；metabolomics；choline；methionine adenosyltransferase；methylation；toxicity

INTRODUCTION

Bisphenol A (BPA) is a high-volume industrial chemical used to make polycarbonate plastic and epoxy resins, which are used in baby bottles, lunch boxes as food and beverage packaging materials as well as dental sealants (Vandenberg *et al.*, 2010). Widespread and continuous human exposure to BPA is believed to be via inhalational, dermal and oral contact through foods and beverages as well as air, drinking water, dust, soil, and personal care products (Vandenberg, *et al.*, 2010). As one of the world's highest production volume compounds, measurement of BPA in the urine of population representative samples indicates that the majority (over 90%) of the general population is exposed to BPA (Calafat *et al.*, 2008).

BPA health risk has aroused public concern recently. BPA is one of the endocrine disrupting compounds (Vandenberg, *et al.*, 2010). Chronic health hazard assessments conducted by U.S. EPA indicate that the critical effect caused by BPA exposure is reduced mean body weight (U.S. Environmental Protection Agency 1993). Epidemiological studies indicate that exposure to BPA is related to various diseases including cardiovascular disease (Lang *et al.*, 2008), reproductive abnormalities (Mendiola *et al.*, 2010), liver impairment (Lang, *et al.*, 2008) and cancer (Duan *et al.*, 2012). There are also reports on related adverse effects of BPA in experimental animals and cell cultures (Chitra *et al.*, 2003; Hassan *et al.*, 2012; Pant *et al.*, 2011; Soto and Sonnenschein, 2010).

Metabolomics is a newly emerging technology which holds promise for the discovery of mechanisms linking disease processes and toxic effects of chemicals (Abaffy *et al.*, 2010; Parman *et al.*, 2011; Sreekumar *et al.*, 2009; Stewart and Bolt, 2011; Wang *et al.*, 2011). Deciphering the molecular network which is disturbed by BPA exposure may lead to critical insight into BPA-induced toxic effects. Monitoring metabolites in urine fluid, which is the most commonly used biofluid in metabolomics (Zhang *et al.*, 2012), has become an important way to study metabolomic disturbance caused by chemical exposure (Parman, *et al.*, 2011). Although BPA-induced metabolic changes are important issues on the research agenda, little is known about the global metabolomic alterations induced by BPA using urinalysis. To date, there are few metabolomic studies regarding BPA-induced metabolic changes (Cabaton *et al.*, 2013; Chen *et al.*, 2012; Zeng *et al.*, 2013). Capillary electrophoresis (CE) in tandem with electrospray ionization (ESI) time-of-flight mass spectrometry (TOFMS) has been proven to be a favorable method for polar metabolite analysis in urine (Ramautar *et al.*, 2011). Also, liver is the major organ that affects metabolism in the whole body in mammals. Examining activation/inhibition of hepatic expression of genes encoding critical enzymes in relation to changed metabolites found in a metabolomic study is suitable for expanding our understandings of metabolic alterations (Parman, *et al.*, 2011).

Here, we used a CE in tandem with ESI-TOFMS based nontargeted metabolomic technique, which has been proven to be able to provide a large-scale metabolite profiling covering primary metabolism pathways (Matsumoto *et al.*, 2012; Ooga *et*

al., 2011), to study metabolomic alterations caused by BPA using urinalysis of a model of rat exposed to BPA. This unbiased metabolomic profiling allowed us to conduct a hypothesis-free analysis of metabolomic perturbations of endogenous compounds caused by BPA exposure. Furthermore, a follow-up study was performed using rat liver to better understand the BPA-induced metabolic alteration especially in the low-dose group. The expression of genes encoding critical enzymes involved in the major metabolic alterations was analyzed in this study to validate the metabolomic observations and to understand BPA-induced metabolic changes at both low and high doses from the metabolic enzyme perspective based on the examination of molecular events other than metabolite analysis. Finally, targeted analysis of metabolites in the liver was conducted to further support the metabolic alteration identified by metabolomic profiling of urine and analysis of metabolic enzyme gene expression in the liver.

MATERIALS AND METHODS

Animals and treatments. Sprague-Dawley rats were purchased from Slaccas (Slaccas Laboratory Animal, Shanghai, China). Eighteen male Sprague-Dawley rats (180-200 g; 6-8 weeks) were housed under controlled temperature (22±2°C) and humidity (40-60%) with a 12 h light/dark cycle, and the rats were randomized into vehicle control group, and low and high-dose group (n=6 rats/group). Animals had free access to food and water. The animals were acclimated to the laboratory for 1 week prior to the start of the experiments. Then the rats in vehicle control, low-dose

and high-dose groups were given daily gavage administration of 0.5 ml of corn oil containing 0, 0.5 μg/kg/d, 50 mg/kg/d BPA for 8 weeks, respectively. BPA (purity ≥ 99%) and corn oil were purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO, USA), and BPA dosing solutions for rats were prepared in corn oil. Fresh solution of BPA in corn oil was made each week. The chemical structure of BPA has been shown in Fig. 1. The dose of 0.5 μg/kg/d was given according to the reported BPA exposure level in humans (UK Food Standards Agency 2001); a reference dose of 50 mg/kg/d was also used, which is the currently accepted lowest observed adverse effect level (LOAEL) (U.S. Environmental Protection Agency 1993). Using this gavage method, none of the rats died, and no injuries in the stomach, esophagus or trachea were found. Body weight of all rats was recorded weekly. After 1 week acclimation, at 55 days after dosing, 24-h urine was collected using a metabolic cage that was placed under an ice bath so as to avoid the degradation of metabolites and urine was immediately stored at -80°C (Patterson *et al.*, 2009). Food and water consumption in the 24-h period at 55 days after dosing was recorded. At 56 days after dosing, rats were maintained under fasting condition for 12 h after which blood samples were collected. Then the livers were dissected and weighed in order to calculate the organ/body weight ratios for each animal. Several liver sections from each rat were placed into cryo-vials. After snap frozen in liquid nitrogen, they were preserved at −80°C. This study was carried out strictly in accordance with the international standards on animal welfare and the guidelines of the Institute for Laboratory Animal Research of Nanjing Medical University.

Metabolomic profiling. The metabolomic measurement and data processing were performed with a well validated metabolomic method as previously described (Matsumoto*, et al.*, 2012; Ooga*, et al.*, 2011). Briefly, 20 μL of rat urine was mixed with 80 μL of 125 μM each of methionine sulphone (MetSul) and D-camphor-10-sulfonic acid (CSA). MetSul and CSA were used as internal standards for cationic and anionic metabolites, respectively. The mixtures were then filtrated with a 5-kDa cut-off filter (Millipore, Ultra-free MC PLHCC Human Metabolome Technologies) to remove proteins and macromolecules prior to CE-TOFMS analysis. CE-TOFMS was carried out using an Agilent CE (capillary: fused silica capillary i.d. 50 μm × 80 cm)-TOFMS system (Agilent Technologies, Inc.). Run buffers (Solution ID H3301-1001 for the cation mode and H3302-1021 for the anion mode) were used (Human Metabolome Technologies Inc., Tsuruoka, Japan), and rinse buffers (Solution ID H3301-1001 for the cation mode and H3302-1022 for the anion mode) were used (Human Metabolome Technologies Inc., Tsuruoka, Japan). Sample was injected with a pressure injection of 50 mbar for 10 seconds for cation mode, and was injected with a pressure injection of 50 mbar for 25 seconds for anion mode. The CE voltage was set at positive, 27 kV for cation mode and positive, 30 kV for anion mode. Compound was ionized in ESI positive or ESI negative mode. MS capillary voltage was 4000 V at cation mode and 3500 V at anion mode. MS scan range was set to 50-1000 *m/z*. Sheath liquid (H3301-1020, Human Metabolome Technologies Inc., Tsuruoka, Japan) was used. All samples were analyzed in a randomized fashion to avoid complications related to the injection order. The alignment of detected peaks was performed

according to migration time on CE and *m*/*z* value determined by TOFMS. Annotation tables of compounds profiled were produced from CE-TOFMS measurement of standard compounds and were aligned with the datasets according to similar *m/z* value and migration time. Quality control (QC) sample was prepared by mixing equal volumes (100 μl) from each original sample according to a previous metabolomic study (Gika *et al.*, 2008), and was analyzed with every 6 urine samples. We found that the relative standard deviations % (RSDs) of relative level in most compounds profiled (>70%) were at the ≤30% level among QC samples, indicating this metabolomic analysis could provide reproducible and reliable data (Gika, *et al.*, 2008).

Real-time PCR analysis. Total RNA was homogenized and extracted from snap-frozen liver fragments by use of Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Reverse transcription was performed using PrimeScript RT Reagent Kit (Takara, Dalian, China) in accordance with the manufacturer's recommendations. All real-time PCR reactions were carried out on ABI7900 HT Fast Real-Time System (Applied Bio systems, Foster City, CA, USA) using SYBR Green PCR Master Mix reagent kits (Takara, Dalian, China) according to the manufacturer's instructions for quantification of gene expression. Rat-specific primers and reaction conditions for the genes of interest are listed in Table 1. All of the PCRs were performed in triplicate, and the specificity of the PCR products was confirmed using melting curve analyses. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression (Ferreira ID, *et al.*, 2006). The housekeeping gene *Gapdh* was

used as an internal control. The levels of the methionine adenosyltransferase Iα (*Mat1a*) and methionine adenosyltransferase IIα (*Mat2a*) gene were normalized relative to the expression levels of the gene *Gapdh*.

Western blot analysis. Livers were homogenized in RIPA buffer, and 80 μg of protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membranes were incubated in blocking buffer for 2 h at room temperature, followed by overnight incubation at 4°C with rabbit polyclonal antibodies for MAT1A (Proteintech Group Inc, Chicago, IL, USA, 1:2000 dilution) and MAT2A (Beijing Biosynthesis Biotechnology CO., Ltd, Beijing, China, 1:500 dilution), and mouse polyclonal antibodies for GAPDH (Beyotime, China, 1:1000 dilution). The membranes were washed with TBST three times (10 mins each) and incubated for 1 h at room temperature with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Beyotime, China, 1:2000 dilution) or goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Beyotime, China, 1:1000 dilution). Detection was performed using the ECL Western blot detection kit (Millipore) according to the manufacturer's instructions. Densitometry was performed using Image J software (National Institutes of Health). All values were normalized to GAPDH content and are expressed as fold increase over the control group.

ELISA analysis. Liver tissue was homogenized in 9 volumes of cold phosphate-buffered saline (PBS; 0.01M, pH=7.4) with a homogenizer on ice. To further break the cells, the homogenate was sonicated with an ultrasonic cell disruptor.

The homogenate was then centrifuged at 5000 g for 5 minutes to get the supernatant. The levels of *S*-adenosylmethionine (SAMe) in the liver homogenate supernatants were assayed using commercially available ELISA kit (Cat. No. E-EL-0043, Elabscience Biotechnoogy, Wuhan, China) according to the manufacturer's protocol. The data are presented as fold change compared with the mean value of the control group according to the previous report (Shyh-Chang N, *et al.*, 2013).

Serum alanine transaminase and cholesterol analysis. Fresh blood was transferred to non-anticoagulant-treated tubes and kept standing for 3 h at 4ºC. After 15 min centrifugation at 3000 rpm, the serum was separated from the blood sample. Serum (150 µl) was drawn into a new sample tube, and serum biochemical parameters including serum alanine transaminase (ALT) and cholesterol (CHOL) were analyzed using an automatic biochemical analyzer (Hitachi, Tokyo, Japan).

Statistical analysis. Statistical analysis of the data was performed using Stata statistical package (Version 9.2, Stata Corp, LP), SPSS version 15.0 (SPSS, Inc., Chicago, IL) and ''R'' (http://cran.r-project.org/), which is a freely available open source software package. Statistical comparisons between vehicle control group and low-dose and high-dose BPA treatment groups were performed by ANOVA following by Dunnet's test. *Mat1a* and *Mat2a* mRNA levels were log transformed before further parametric analysis was done. According to previous metabolomics-based reports (Matsumoto*, et al.*, 2012; Parman*, et al.*, 2011), in order to compare compound levels between experimental groups, Satterthwaite t tests were performed. Moreover, in order to improve statistical robustness, multiple comparisons were made with the false

discovery rate (FDR) method, and each FDR was estimated using *q* values. A *p*-value of < 0.05 was considered statistically significant.

RESULTS

Body weight is decreased after BPA exposure. As shown in Fig. 2, when compared with vehicle control group, the body weight of rats was significantly decreased in the high-dose group ($p<0.05$). There was no significant difference in body weight between the low-dose group and the control group ($p=0.66$). Liver weight was also significantly decreased in the high-dose group ($p<0.05$), but liver organ coefficients were not significantly altered after BPA exposure (Supplementary Table 1). Serum ALT and CHOL levels were not significantly different between control and treatment groups (Supplementary Table 1). Food and water consumption was similar in BPA treated and control groups (Supplementary Table 1).

Metabolomic profiles. By using the CE-TOFMS platform, 199 metabolites were profiled in rat urine (120 metabolites from cation mode and 79 metabolites from anion mode, respectively). The prevalence of metabolite alterations in rat urine after daily administration of BPA has been summarized in Table 2. There were 11 metabolites with altered levels after exposure to 0.5 μg/kg/d of BPA ($p<0.05$), while there were 32 metabolites with altered levels after exposure to 50 mg/kg/d of BPA ($p<0.05$). To test the statistical robustness, multiple comparisons for this data set were made with the FDR method, and each FDR was estimated using *q* values. When a cut-off value of $q<0.2$ for the FDR was applied according to previous

metabolomic studies (Fiehn *et al.*, 2010; Putluri *et al.*, 2011; Wei *et al.*, 2013), we found that 50 out of 51 (98%) statistically significant ($p<0.05$) or suggestive differences ($0.05\leq p<0.10$) between high-dose group and vehicle control group had q values less than the cut-point value 0.2 after the correction for multiple testing with FDR, suggesting that alterations observed between high-dose group and vehicle control group are statistically robust. However, when the strict FDR correction was applied, metabolite alteration between low-dose group and vehicle control group does not appear to be statistically robust (differential metabolites ($p<0.05$) with FDR q value>0.2), suggesting that additional information other than urine metabolite profiling is needed to support and verify metabolic changes in the low-dose group. Also, a previous metabolomics-based report suggested that differential metabolites with high FDR q value might be still significant if additional information was supportive (Sreekumar*, et al.*, 2009). Thus, our focus was directed towards understanding the metabolites which showed robust alterations ($p<0.05$ and FDR q value<0.2, vehicle control group v high-dose group), and the whole metabolomic data were put into biological network to improve the reliability of data interpretation. Also, according to biological and statistical significance, we considered differences between the low-dose and vehicle control group noteworthy when the relative levels of these metabolites were different between high-dose group and vehicle control group. Furthermore, we verified key metabolic events caused by exposure to low-dose of BPA with additional independent experiments by the targeted analysis of critical metabolic enzyme gene expression and key metabolite in rat liver.

Individual known metabolites and the fold change with *p* value and FDR *q*-value are presented in Supplementary Table 2. After classifying the chemicals and mapping the metabolites into general biochemical pathways as illustrated in the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/), it became clear that metabolomic disturbances caused by BPA exposure involve biotin and riboflavin excretion, metabolism of methylated products, purine nucleotide catabolism as well as choline metabolism.

BPA alters urinary biotin and riboflavin excretion. Increases of urinary biotin and riboflavin were among the most dramatic changes induced by BPA treatment at doses of the 0.5 μg/kg/d and the 50 mg/kg/d (Fig. 3). Riboflavin and biotin output was elevated significantly at the 50 mg/kg/d dose ($p<0.05$ and FDR q value<0.2). Additionally, given the biological significance, the statistically significant differences of output of biotin ($p<0.05$) between low-dose and control group were noteworthy. Also, alterations of output of riboflavin ($p<0.10$) between low-dose and control group should also be taken into consideration.

Methylated products are increased after BPA exposure. As shown in Fig. 4 and Supplementary Table 2, notable increases in a wide range of methylated products were observed in rat urine after BPA treatment. The relative levels of N-methylalanine, N-methylglutamic acid and 3-methylhistidine were positively correlated with BPA doses by Spearman's correlation test in the three groups (r= 0.9258, p= 0.0080, for N-methylalanine; r= 0.8000, p= 0.0001, for N-methylglutamic acid; r= 0.6164, p= 0.0064, for 3-methylhistidine), indicating a

dose-related alteration of these methylated products. Significant increases of methylguanidine were also found at low dose ($p<0.05$). Increases of methylated purines, including 3-methylguanine ($p<0.05$ and FDR q value<0.2) and 3-methyladenine ($p<0.10$ and FDR q value<0.2), were also observed in the high-dose group when compared with vehicle control group.

BPA elevates purine degradation products. Uric acid is the breakdown product of catabolism of nucleic acid. Uric acid is further oxidized to allantoin which is also excreted in urine in rodents (Yang *et al.*, 2006). In this study, significant changes in the purine nucleotide catabolism pathway by BPA treatment were observed (Fig. 5). When compared with vehicle control group, uric acid level was elevated significantly following BPA dosing at 50 mg/kg/d ($p<0.05$ and FDR q value<0.2), while allantoin was also increased at 50 mg/kg/d ($p<0.10$ and FDR q value<0.2). In addition, in the upper part of purine nucleotide catabolism pathway, the average relative levels of adenine and guanine in the high-dose group were also higher than vehicle control group.

Choline metabolism is increased after BPA treatment. As shown in Fig. 6, choline is partly derived from the degradation of membrane phosphatidylcholine via glycerophosphocholine in rats (Zeisel, 1985). The catabolism of choline occurs through a series of demethylation steps to form betaine, N,N-dimethylglycine and sarcosine (Park, *et al.*, 1999; Sreekumar, *et al.*, 2009; Wang, *et al.*, 2011). The reaction of betaine converted into N,N-dimethylglycine donors methyl group for the formation of SAMe (Park, *et al.*, 1999), which is the precursor of numerous

methylated biochemical compounds including 3-methylhistidine in rats (Munro and Young, 1978; Yang *et al.*, 2004). In this study, increases of glycerophosphocholine (p<0.05 and FDR q value<0.2), betaine (p<0.05 and FDR q value<0.2), N,N-dimethylglycine (p<0.10 and FDR q value<0.2), trimethylamine N-oxide (p<0.05 and FDR q value<0.2) (a product that requires gut flora to be produced and is also derived from the choline) (Wang, *et al.*, 2011) were observed when compared with vehicle control group. Also, the average relative levels of choline and sarcosine in the high-dose group were higher than vehicle control group.

BPA activates hepatic *Mat1a* and *Mat2a* gene expression and increases hepatic SAMe. SAMe is the principal biological methyl donor, and is mainly synthesized and consumed in the liver where it serves as a methyl donor in numerous metabolic reactions (Yang *et al.*, 2004). As a highly methylated environment was observed after BPA exposure in metabolomic analysis of urine, the expression of the 2 genes (*Mat1a, Mat2a*) encoding MAT, a critical enzyme that catalyzes the formation of SAMe in liver, was further analyzed in this study (Ko *et al.*, 2008). The mRNA levels of hepatic *Mat1a* were both significantly increased at low and high doses of BPA (p<0.05). When compared with vehicle control group, the mRNA levels of *Mat2a* were elevated significantly at 50 mg/kg/d (p<0.05), and significant increases were also found in the low-dose group (p<0.05) (Fig. 7A). The protein levels of both MAT1A and MAT2A in rat liver were significantly increased at both low and high doses of BPA (p<0.05) (Fig. 7B). Furthermore, we found that hepatic SAMe was significantly increased at both low and high doses (p<0.05) (Fig. 7C).

DISCUSSION

In this study, by conducting a hypothesis-free metabolomic profiling of rat urine after BPA exposure, we identified various BPA-induced metabolomic perturbations and further found expression levels of genes encoding MAT and SAMe involved in BPA-disrupted metabolism were altered in the liver. Additionally, in this rat model, we found that the body weight of rats was decreased in the 50 mg/kg/day BPA dose group (Fig. 2), and no significant change in food and water consumption was observed (Supplementary Table 1). These results are consistent with the document of Integrated Risk Information System of U.S. EPA describing that rats orally exposed to BPA at LOAEL showed evident decreased body weight with no alteration in food consumption (U.S. Environmental Protection Agency 1993).

In this study, we identified increased excretion of riboflavin and biotin at doses of 0.5 μg/kg/d and the 50 mg/kg/d, indicating that the metabolism of these vitamins is very sensitive to BPA exposure and may be used as potential biomarkers for BPA exposure (Fig. 3). Previous report indicated that elevated urinary riboflavin excretion is a biomarker of tissue flavoproteins catabolism (Windmueller *et al.*, 1964). Similarly, given the fact that biotin occurs mainly bound to proteins or polypeptides and is abundant in liver, kidney and pancreas (Duval *et al.*, 1994), it is plausible that the elevation of biotin output is also related to protein degradation. Interestingly, a notable finding of BPA-induced metabolic perturbation was that increased methylated amino acids were excreted into urine following oral BPA exposures. It is

reported that amino acid methylation is irreversible, and methylation of proteins may increase protein degradation rates (Teerlink, 2005). Therefore, elevated amino acid methylation caused by BPA exposure may result in increased protein degradation which induces the observed elevated riboflavin and biotin excretion. Indeed, urinary excretion of 3-methylhistidine, which is formed by the methylation of peptide-bound histidine by SAMe, was also found to be highly indicative of protein catabolism (Munro and Young, 1978). Furthermore, methylguanidine, which is also derived from protein catabolism (Marzocco *et al.*, 2004), was significantly increased at low dose, which is well consistent with the increased excretion of riboflavin and biotin at low dose. These data suggest the effect of BPA on methylation environment even at low dose. Since protein degradation is related to body weight loss (Khan *et al.*, 2003), BPA-induced protein degradation was also supported by the observed body weight loss.

Notably, BPA-induced highly methylated environment also included increased methylation of purines. 3-methyladenine and 3-methylguanine are potentially lethal methylation products (Branch *et al.*, 1995). Alkylation of DNA may lead to possible miscoding, and is the critical molecular event in the induction of tumors (Margison *et al.*, 1976). Urinary 3-methyladenine has been used as a biomarker for genetic damage by alkylation (Braybrooke *et al.*, 2000). The finding of elevated methylated purines output is consistent with those previous reports in which exposure of rats to BPA increased urinary hydroxymethyl-2'-deoxyuridine and 8-hydroxy-2'-deoxyguanosine which are highly related to DNA methylation damage

(Cho *et al.*, 2009; Woo *et al.*, 2009). DNA methylation damage observed in this study also agrees with previous reports that BPA could cause genotoxic effects and cancer (Duan, *et al.*, 2012; Soto and Sonnenschein, 2010).

As reported previously, the increments in methylated purine output are related to elevated uric acid excretion (George and Chandrakasan, 2000). In order to validate the increased methylated purine output and further explore the changes in nucleotide metabolism after BPA exposure, we examined the metabolomic data with the focus on the metabolites in purine nucleotide catabolism pathway in which uric acid is the product (George and Chandrakasan, 2000; Yang, *et al.*, 2006). We found uric acid and its metabolite, allantoin, were increased after BPA exposure (Yang, *et al.*, 2006) (Fig. 5), which is supported by a related study on effects of BPA on the metabolome at low doses (Zeng *et al.*, 2013). Hyperactivation of purine degradation pathway resulted from BPA exposure was also supported by the increased excretion of average relative levels of uric acid precursors, including adenine and guanine. These data indicate an overall rise in purine compound degradation induced by BPA exposure. It is also noteworthy that the conversion of hypoxanthine to xanthine followed by xanthine to uric acid which is involved in purine catabolism pathway increases the oxidative stress burden of superoxide radical *in vivo* (Li *et al.*, 2011). Oxidative stress caused by BPA exposure has been widely reported in rats at 50 mg/kg/d and low dose level similar to exposure level in humans (Chitra, *et al.*, 2003; Hassan, *et al.*, 2012). Asides from DNA methylation damage, oxidative stress is also an accepted cause of DNA impairment and it has been found to be involved in

BPA-induced hepatotoxicity and reproductive toxicity (Hassan, *et al.*, 2012; Wu *et al.*, 2013). Oxidative stress was also observed in testes in a metabolomic study regarding BPA reproductive toxicity (Chen, *et al.*, 2012).

The fact that the methylated products were simultaneously increased by BPA suggested that they shared a common linkage. It is worth noting that these compounds share choline as a precursor (Park, *et al.*, 1999; Lyles and McDougall, 1989). Choline metabolism also is found to be closely interrelated to methyl group metabolism (Wang, *et al.*, 2011). Therefore, the indication of the widely elevated methylated product output by BPA is centered on the hyper-activation of choline metabolism pathway. To validate this hypothesis, we focused on the metabolomic data regarding choline and related metabolites (Fig. 6). We found that glycerophosphocholine (the precursor of choline) (Zeisel, 1985), betaine as well as trimethylamine N-oxide (two products of choline metabolism) were significantly increased after BPA exposure (Wang, *et al.*, 2011), while the increase of N,N-dimethylglycine (another product of choline metabolism) was also suggestive (Park, *et al.*, 1999). Besides, we also found that the average relative levels of choline and sarcosine (a precursor of methylamine) (Horner and Mackenzie, 1950) were increased in BPA-exposed group. These results indicate that the metabolic disturbance caused by BPA exposure is characterized by the dramatically increased flux through the choline metabolism pathway, which provides the precursors of a wide range of methylated products. Increased cholines in whole body and serum after low-dose BPA exposure were also found in a related study (Cabaton, *et al.*,

2013).

Liver is the major organ that affects metabolism in the whole body. SAMe is the principal biological methyl donor (e.g. methyl donor for 3-methylhistidine) (Munro and Young, 1978), and is mainly produced and consumed in the liver (Yang *et al.*, 2004). MAT which catalyzes the formation of SAMe is a critical methyl-donor related enzyme involved in choline metabolism pathway. *Mat1a* is expressed mostly in the liver while *Mat2a* is widely distributed. In mammals, these two genes encode two homologous MAT catalytic subunits (Ko, *et al.*, 2008). As highly methylated environment is the focus of the various BPA-induced metabolic alterations, we further examined expression of *Mat1a* and *Mat2a* and SAMe in rat liver. The fact that *Mat1a* and *Mat2a* mRNA and protein levels and SAMe levels were significantly increased at both low and high doses supports our metabolomic findings in urine (Fig. 7), and indicates BPA-induced metabolic disturbance regarding methylation at low dose. Collectively, the obvious perturbations in choline metabolism pathway not only add to the evidence that methylated products were broadly increased after BPA exposure, but also provide us an insight into the underlying mechanism of the highly methylated environment caused by BPA treatment.

Choline metabolism is considered to be essential in mammals (Wang, *et al.*, 2011). However, recent metabolomic studies have revealed that elevated metabolites in choline metabolism are extremely harmful to health (Abaffy, *et al.*, 2010; Sreekumar, *et al.*, 2009; Wang, *et al.*, 2011). Highly methylated environment which was related to elevated choline metabolism was found in melanoma (Abaffy, *et al.*, 2010).

Moreover, trimethylamine N-oxide and sarcosine have been proven to be the key factors in the pathogenesis of cardiovascular disease and prostate cancer, respectively (Sreekumar, *et al.*, 2009; Wang, *et al.*, 2011). Therefore, the changes in choline metabolism may not only cause DNA methylation damage and protein degradation, but also may play an important role in escalating BPA-induced toxicological consequences including cardiovascular disease and prostate cancer through deleterious metabolites (Lang, *et al.*, 2008; Pant, *et al.*, 2011; Soto and Sonnenschein, 2010).

In this study, by the integrated analyses of various metabolites such as riboflavin, biotin and methylguanidine in urine and expression of *Mat1a* and *Mat2a* as well as SAMe in the liver, we revealed consistent metabolic alterations caused by BPA even at dose as low as 0.5 μg/kg/d. Because of the long-term exposure and possible accumulation of BPA in humans (Chitra, *et al.*, 2003), the metabolic changes observed in our rat model with time-limited exposure should arouse attention and may be relevant to clinical conditions of public health. These biomarkers for impairment and/or disease identified in this study are well consistent with diseases in relation to BPA exposure in humans (Braybrooke, *et al.*, 2000; Sreekumar, *et al.*, 2009; Wang, *et al.*, 2011), and may provide novel mechanistic insights into BPA-induced diseases in humans (Lang, *et al.*, 2008; Mendiola, *et al.*, 2010; Soto and Sonnenschein, 2010). Therefore, the biomarkers identified in this study need further investigation in humans.

In conclusion, our study explored BPA-induced metabolic changes using

urinalysis with nontargeted metabolomic profiling and following targeted analysis of metabolic enzyme gene expression as well as key metabolite in the liver. Consistent results indicate that notable changes of choline metabolism is an underlying mechanism of highly methylated environment and related metabolic changes caused by BPA exposure. Our study identifies BPA-elevated accepted biomarkers of injury in relation to BPA toxic effect, and reveals that BPA induces DNA methylation damage and broad protein degradation, and the increased deleterious metabolites in choline pathway may also be involved in the toxicity of BPA. Our study represents a significant mechanistic advance in understanding the potential toxicological effects of BPA, and underscores the integration of metabolomics and analysis of metabolic enzyme gene expression as a promising tool in providing mechanistic understanding of chemical toxicity.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

This study was supported by National 973 Program (2012CBA01306); National Science Fund for Outstanding Young Scholars (81322039); National Natural Science Foundation (31371524); Distinguished Young Scholars of Jiangsu Province (BK20130041); Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

ACKNOWLEDGEMENTS

We thank Yuka Matsuyama for CE-TOFMS analyses, and Hajime Sato and

Takamasa Ishikawa for kind guidance with the data analysis. We are also grateful to Takushi Ooga for fruitful discussions in the process of data analysis. We thank Xuan Wang, Zhan Zhang and Jun Gu for rat BPA exposure model establishment and sample collection. We also thank Ankur Sharan for language editing of this manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abaffy, T., Duncan, R., Riemer, D. D., Tietje, O., Elgart, G., Milikowski, C. and DeFazio, R. A. (2010). Differential volatile signatures from skin, naevi and melanoma: a novel approach to detect a pathological process. *PloS one* **5,** e13813.

Branch, P., Hampson, R. and Karran, P. (1995). DNA mismatch binding defects, DNA damage tolerance, and mutator phenotypes in human colorectal carcinoma cell lines. *Cancer Res* **55,** 2304-2309.

Braybrooke, J. P., Houlbrook, S., Crawley, J. E., Propper, D. J., O'Byrne, K. J., Stratford, I. J., Harris, A. L., Shuker, D. E. and Talbot, D. C. (2000). Evaluation of the alkaline comet assay and urinary 3-methyladenine excretion for monitoring DNA damage in melanoma patients treated with dacarbazine and tamoxifen. *Cancer Chemother Pharmacol* **45,** 111-119.

Cabaton, N. J., Canlet, C., Wadia, P. R., Tremblay-Franco, M., Gautier, R., Molina, J., Sonnenschein, C., Cravedi, J. P., Rubin, B. S., Soto, A. M. and Zalko, D. (2013). Effects of Low Doses of Bisphenol A on the Metabolome of Perinatally Exposed CD-1

Mice. *Environ Health Perspect* **121,** 586-593.

Calafat, A. M., Ye, X., Wong, L. Y., Reidy, J. A. and Needham, L. L. (2008). Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. *Environ Health Perspect* **116,** 39-44.

Chen, M., Xu, B., Ji, W., Qiao, S., Hu, N., Hu, Y., Wu, W., Qiu, L., Zhang, R., Wang, Y., Wang, S., Zhou, Z., Xia, Y. and Wang, X. (2012). Bisphenol A alters n-6 fatty acid composition and decreases antioxidant enzyme levels in rat testes: a LC-QTOF-based metabolomics study. *PLoS One* **7,** e44754.

Chitra, K. C., Latchoumycandane, C. and Mathur, P. P. (2003). Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. *Toxicology* **185,** 119-127.

Cho, S. H., Choi, M. H., Kwon, O. S., Lee, W. Y. and Chung, B. C. (2009). Metabolic significance of bisphenol A-induced oxidative stress in rat urine measured by liquid chromatography-mass spectrometry. *J Appl Toxicol* **29,** 110-117.

Duan, B., Hu, X., Zhao, H., Qin, J. and Luo, J. (2012). The relationship between urinary bisphenol A levels and meningioma in Chinese adults. *Int J Clin Oncol* **18,** 492-497.

Duval, M., Job, C., Alban, C., Douce, R. and Job, D. (1994). Developmental patterns of free and protein-bound biotin during maturation and germination of seeds of Pisum sativum: characterization of a novel seed-specific biotinylated protein. *Biochem J* **299,** 141-150.

Ferreira I.D., Rosário V.E., and Cravo P.V.(2006). Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in Plasmodium falciparum.*Malar J* **5,**1.

Fiehn, O., Garvey, W. T., Newman, J. W., Lok, K. H., Hoppel, C. L. and Adams, S. H.

(2010). Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. *PloS one* **5,** e15234.

George, J. and Chandrakasan, G. (2000). Biochemical abnormalities during the progression of hepatic fibrosis induced by dimethylnitrosamine. *Clin Biochem* **33,** 563-570.

Gika, H. G., Macpherson, E., Theodoridis, G. A. and Wilson, I. D. (2008). Evaluation of the repeatability of ultra-performance liquid chromatography-TOF-MS for global metabolic profiling of human urine samples. *J Chromatogr B Analyt Technol Biomed Life Sci* **871,** 299-305.

Hassan, Z. K., Elobeid, M. A., Virk, P., Omer, S. A., Elamin, M., Daghestani, M. H. and Alolayan, E. M. (2012). Bisphenol A Induces Hepatotoxicity through Oxidative Stress in Rat Model. *Oxid Med Cell Longev* **2012,** 194829.

Horner, W. H. and Mackenzie, C. G. (1950). The biological formation of sarcosine. *J Biol Chem* **187,** 15-22.

Khan, Z. H., Simpson, E. J., Cole, A. T., Holt, M., MacDonald, I., Pye, D., Austin, A. and Freeman, J. G. (2003). Oesophageal cancer and cachexia: the effect of short-term treatment with thalidomide on weight loss and lean body mass. *Aliment Pharmacol Ther* **17,** 677-682.

Klubes, P. and Jondorf, W. R. (1971). Dimethylnitrosamine formation from sodium nitrite and dimethylamine by bacterial flora of rat intestine. *Res Commun Chem Pathol Pharmacol* **2,** 24-34.

Ko, K., Yang, H., Noureddin, M., Iglesia-Ara, A., Xia, M., Wagner, C., Luka, Z., Mato, J. M. and Lu, S. C. (2008). Changes in S-adenosylmethionine and GSH homeostasis during

endotoxemia in mice. *Lab Invest* **88,** 1121-1129.

Lang, I. A., Galloway, T. S., Scarlett, A., Henley, W. E., Depledge, M., Wallace, R. B. and Melzer, D. (2008). Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. *JAMA* **300,** 1303-1310.

Latypova, E., Yang, S., Wang, Y. S., Wang, T., Chavkin, T. A., Hackett, M., Schafer, H. and Kalyuzhnaya, M. G. (2010). Genetics of the glutamate-mediated methylamine utilization pathway in the facultative methylotrophic beta-proteobacterium Methyloversatilis universalis FAM5. *Mol Microbiol* **75,** 426-439.

Li, H., Yan, Z., Zhu, J., Yang, J. and He, J. (2011). Neuroprotective effects of resveratrol on ischemic injury mediated by improving brain energy metabolism and alleviating oxidative stress in rats. *Neuropharmacology* **60,** 252-258.

Lyles, G. A. and McDougall, S. A. (1989). The enhanced daily excretion of urinary methylamine in rats treated with semicarbazide or hydralazine may be related to the inhibition of semicarbazide-sensitive amine oxidase activities. *J Pharm Pharmacol* **41,** 97-100.

Margison, G. P., Margison, J. M. and Montesano, R. (1976). Methylated purines in the deoxyribonucleic acid of various Syrian-golden-hamster tissues after administration of a hepatocarcinogenic dose of dimethylnitrosamine. *Biochem J* **157,** 627-634.

Marzocco, S., Di Paola, R., Ribecco, M.T., Sorrentino, R., Domenico, B., Genesio, M., Pinto, A., Autore, G. and Cuzzocrea, S. (2004) Effect of methylguanidine in a model of septic shock induced by LPS. *Free Radic Res* **38,** 1143-1153

Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., Kurihara, S., Sawaki, E., Koga, Y. and Benno,

Y. (2012). Impact of intestinal microbiota on intestinal luminal metabolome. *Sci Rep* **2,** 233.

Mendiola, J., Jorgensen, N., Andersson, A. M., Calafat, A. M., Ye, X., Redmon, J. B., Drobnis, E. Z., Wang, C., Sparks, A., Thurston, S. W., Liu, F. and Swan, S. H. (2010). Are environmental levels of bisphenol a associated with reproductive function in fertile men? *Environ Health Perspect* **118,** 1286-1291.

Munro, H. N. and Young, V. R. (1978). Urinary excretion of N gamma-methylihistidine (3-methylihistidine): a tool to study metabolic responses in relation to nutrient and hormonal status in health and disease of man. *Am J Clin Nutr* **31,** 1608-1614.

Ooga, T., Sato, H., Nagashima, A., Sasaki, K., Tomita, M., Soga, T. and Ohashi, Y. (2011). Metabolomic anatomy of an animal model revealing homeostatic imbalances in dyslipidaemia. *Mol Biosyst* **7,** 1217-1223.

Pant, J., Ranjan, P. and Deshpande, S. B. (2011). Bisphenol A decreases atrial contractility involving NO-dependent G-cyclase signaling pathway. *J Appl Toxicol* **31,** 698-702.

Park, E.I. and Garrow, T.A. (1999). Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. *J Biol Chem* **274,** 7816-7824.

Parman, T., Bunin, D. I., Ng, H. H., McDunn, J. E., Wulff, J. E., Wang, A., Swezey, R., Rasay, L., Fairchild, D. G., Kapetanovic, I. M. and Green, C. E. (2011). Toxicogenomics and metabolomics of pentamethylchromanol (PMCol)-induced hepatotoxicity. *Toxicol Sci* **124,** 487-501.

Patterson, A. D., Slanar, O., Krausz, K. W., Li, F., Hofer, C. C., Perlik, F., Gonzalez, F. J. and

Idle, J. R. (2009). Human urinary metabolomic profile of PPARalpha induced fatty acid beta-oxidation. *J Proteome Res* **8,** 4293-4300.

Putluri, N., Shojaie, A., Vasu, V. T., Vareed, S. K., Nalluri, S., Putluri, V., Thangjam, G. S., Panzitt, K., Tallman, C. T., Butler, C., Sana, T. R., Fischer, S. M., Sica, G., Brat, D. J., Shi, H., Palapattu, G. S., Lotan, Y., Weizer, A. Z., Terris, M. K., Shariat, S. F., Michailidis, G. and Sreekumar, A. (2011). Metabolomic profiling reveals potential markers and bioprocesses altered in bladder cancer progression. *Cancer Res* **71,** 7376-7386.

Ramautar, R., Mayboroda, O. A., Somsen, G. W. and de Jong, G. J. (2011). CE-MS for metabolomics: Developments and applications in the period 2008-2010. *Electrophoresis* **32,** 52-65.

Shyh-Chang, N., Locasale, J.W., Lyssiotis, C.A., Zheng, Y., Teo, R.Y., Ratanasirintrawoot, S., Zhang, J., Onder, T., Unternaehrer, J.J., Zhu, H., Asara, J.M., Daley, G.Q. and Cantley, L.C. (2013) Influence of threonine metabolism on S-adenosylmethionine and histone methylation. *Science* **339,** 222-226.

Soto, A. M. and Sonnenschein, C. (2010). Environmental causes of cancer: endocrine disruptors as carcinogens. *Nat Rev Endocrinol* **6,** 363-370.

Sreekumar, A., Poisson, L. M., Rajendiran, T. M., Khan, A. P., Cao, Q., Yu, J., Laxman, B., Mehra, R., Lonigro, R. J., Li, Y., Nyati, M. K., Ahsan, A., Kalyana-Sundaram, S., Han, B., Cao, X., Byun, J., Omenn, G. S., Ghosh, D., Pennathur, S., Alexander, D. C., Berger, A., Shuster, J. R., Wei, J. T., Varambally, S., Beecher, C. and Chinnaiyan, A. M. (2009). Metabolomic profiles delineate potential role for sarcosine in prostate cancer

progression. *Nature* **457,** 910-914.

Stewart, J. D. and Bolt, H. M. (2011). Metabolomics: biomarkers of disease and drug toxicity. *Arch Toxicol* **85,** 3-4.

UK Food Standards Agency (2001) Survey of Bisphenols in Canned Foods (Number 13/01). Avaiable at: http://www.food.gov.uk/science/research/surveillance/fsis2001/bisphenols

U.S. Environmental Protection Agency (1993) Bisphenol A (CASRN 80-05-7), Integrated Risk Information System (IRIS). Avaiable at: http://www.epa.gov/iris/subst/0356.htm

Teerlink, T. (2005). ADMA metabolism and clearance. *Vasc Med* **10 Suppl 1**, S73-81.

Vandenberg, L. N., Chahoud, I., Heindel, J. J., Padmanabhan, V., Paumgartten, F. J. and Schoenfelder, G. (2010). Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environ Health Perspect* **118,** 1055-1070.

Wang, Z., Klipfell, E., Bennett, B. J., Koeth, R., Levison, B. S., Dugar, B., Feldstein, A. E., Britt, E. B., Fu, X., Chung, Y. M., Wu, Y., Schauer, P., Smith, J. D., Allayee, H., Tang, W. H., DiDonato, J. A., Lusis, A. J. and Hazen, S. L. (2011). Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* **472,** 57-63.

Wei, X., Shi, X., Zhong, W., Zhao, Y., Tang, Y., Sun, W., Yin, X., Bogdanov, B., Kim, S., McClain, C., Zhou, Z. and Zhang, X. (2013). Chronic alcohol exposure disturbs lipid homeostasis at the adipose tissue-liver axis in mice: analysis of triacylglycerols using high-resolution mass spectrometry in combination with in vivo metabolite deuterium labeling. *PLoS One* **8,** e55382.

Windmueller, H. G., Anderson, A. A. and Mickelsen, O. (1964). Elevated riboflavin levels in

urine of fasting human subjects. *Am J Clin Nutr* **15,** 73-76.

Woo, H. M., Kim, K. M., Choi, M. H., Jung, B. H., Lee, J., Kong, G., Nam, S. J., Kim, S., Bai, S. W. and Chung, B. C. (2009). Mass spectrometry based metabolomic approaches in urinary biomarker study of women's cancers. *Clin Chim Acta* **400,** 63-69.

Wu, H. J., Liu, C., Duan, W. X., Xu, S. C., He, M. D., Chen, C. H., Wang, Y., Zhou, Z., Yu, Z. P., Zhang, L. and Chen, Y. (2013). Melatonin ameliorates bisphenol A-induced DNA damage in the germ cells of adult male rats. *Mutat Res* **752,** 57-67.

Yang, H., Sadda, M.R., Li, M., Zeng, Y., Chen, L., Bae, W., Ou, X., Runnegar, M.T., Mato, J.M. and Lu, S.C. (2004) S-adenosylmethionine and its metabolite induce apoptosis in HepG2 cells: Role of protein phosphatase 1 and Bcl-x(S). *Hepatology* **40**, 221-231.

Yang, Y. D., Zhang, J. Z., Sun, C., Yu, H. M., Li, Q. and Hong, M. (2006). Heroin affects purine nucleotides catabolism in rats in vivo. *Life Sci* **78,** 1413-1418.

Zeisel, S. H. (1985). Formation of unesterified choline by rat brain. *Biochim Biophys Acta* **835,** 331-343.

Zeng, J., Kuang, H., Hu, C., Shi, X., Yan, M., Xu, L., Wang, L., Xu, C. and Xu, G. (2013) Effect of bisphenol A on rat metabolic profiling studied by using capillary electrophoresis time-of-flight mass spectrometry. *Environ Sci Technol* **47,** 7457-7465.

Zhang, A., Sun, H., Wang, P., Han, Y. and Wang, X. (2012). Recent and potential developments of biofluid analyses in metabolomics. *J Proteomics* **75,** 1079-1088.

Figure Legends:

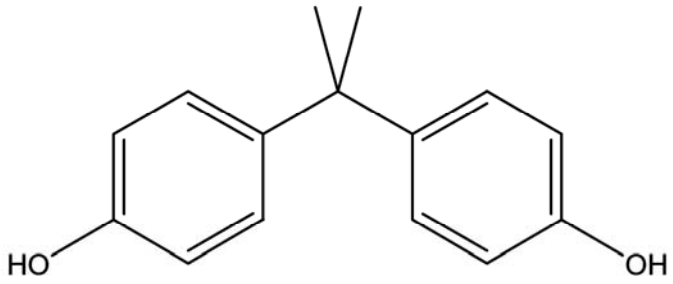

Fig. 1 The chemical structure of BPA.

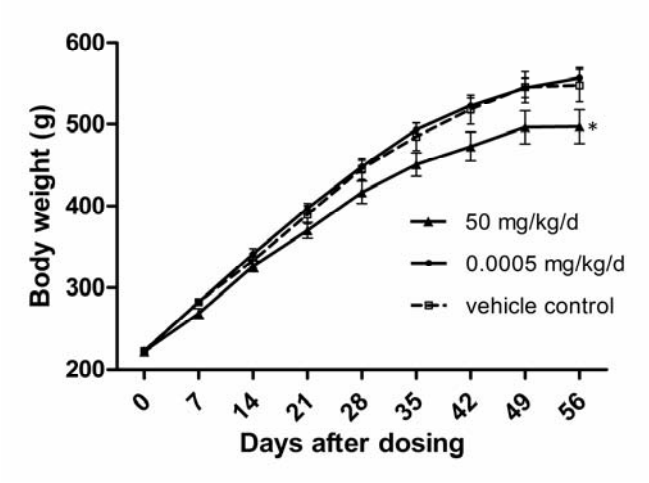

Fig. 2 Effect of oral BPA exposure on body weight of rats. Rats were either untreated (vehicle control, n = 6 rats) or treated with 0.5 µg/kg/d (n = 6 rats), or 50 mg/kg/d (n = 6 rats) BPA. Values are mean ± SEM. * attached to high-dose group represents significant difference comparing with vehicle control group (p<0.05).

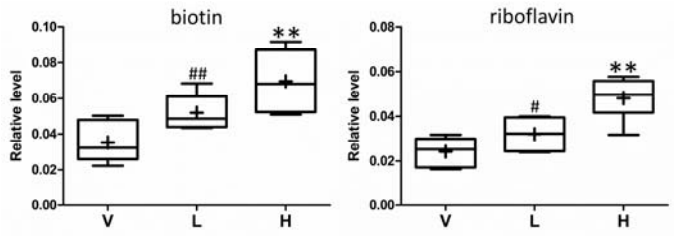

Fig. 3 The metabolomic data for biotin and riboflavin in rat urine are presented as interquartile range box plots. The name of each metabolite is indicated on the top of the plot. The top and bottom of each box represents the 75th and 25th percentile, respectively. Also shown are the median (—) separating the inner quartiles, the mean (+), ‘‘whiskers’’ from the maximum to the minimum. The y-axis indicates the relative level of the indicated metabolite.

V = vehicle control group, L= low-dose group (0.5 μg/kg/day), H = high-dose group (50 mg/kg/day). The *p* values and FDR *q*-values for all comparisons are referenced in Supplementary Table 2; **, $p<0.05$ and FDR q-value<0.2; ##, $p<0.05$ and FDR q-value>0.2; #, $0.05\leq p<0.1$ and FDR q-value>0.2.

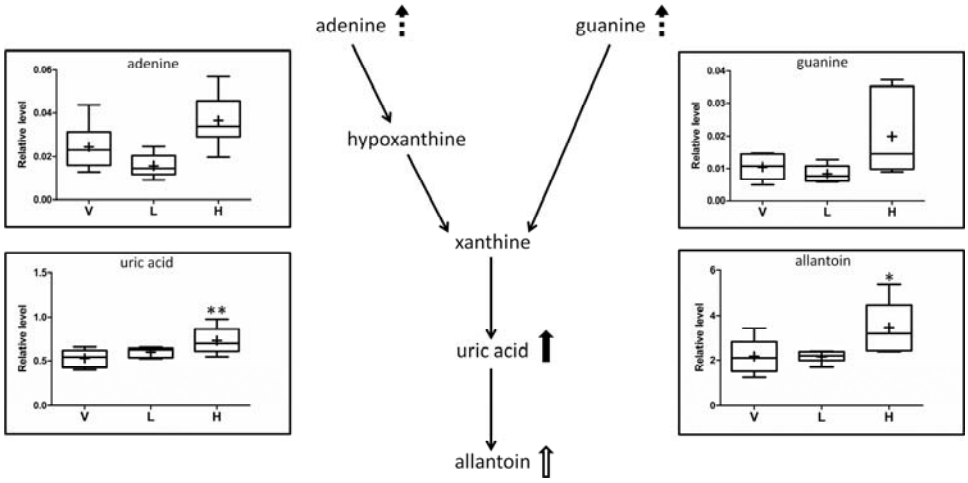

Fig. 4 The metabolomic data for methylated products in rat urine are presented as interquartile range box plots. The explanation for the box plots can be found in the legend of Fig. 3. The *p* and FDR-*q* values for all comparisons are referenced in Supplementary Table 2; **, $p<0.05$ and FDR q-value<0.2; *, $0.05\leq p<0.1$ and FDR q-value<0.2.

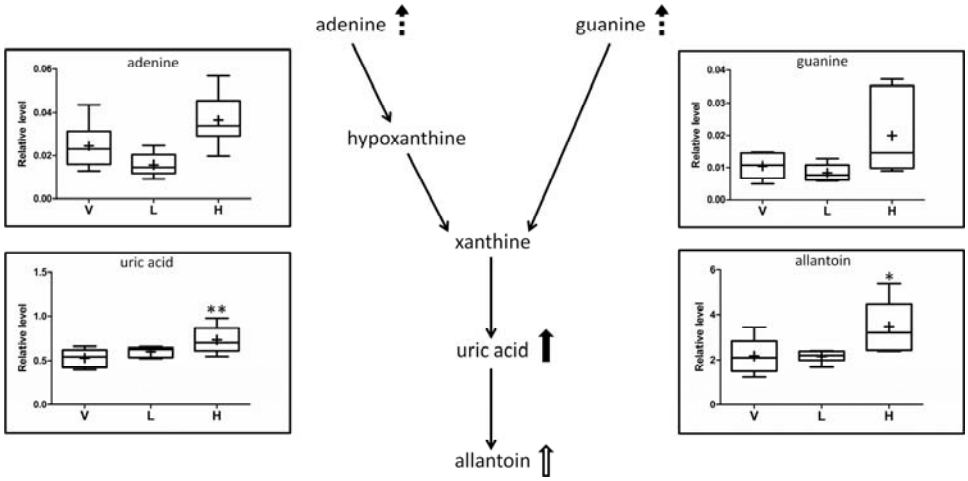

Fig. 5 Purine nucleotide catabolism pathway and the interquartile range box plots of related metabolites in rat urine. The explanation for the box plots can be found in the legend of Fig.

3. The *p* and FDR-*q* values for all comparisons are referenced in Supplementary Table 2; **, $p<0.05$ and FDR q-value<0.2; *, $0.05\leq p<0.1$ and FDR q-value<0.2. Bold up arrow in the pathway indicates statistically significant increases ($p<0.05$ and FDR q-value<0.2); hollow arrow indicates statistically suggestive changes ($0.05\leq p<0.10$ and FDR q-value<0.2); dotted arrow indicates a non-statistically significant trend with a fold change (high-dose versus vehicle-control) greater than 1.2.

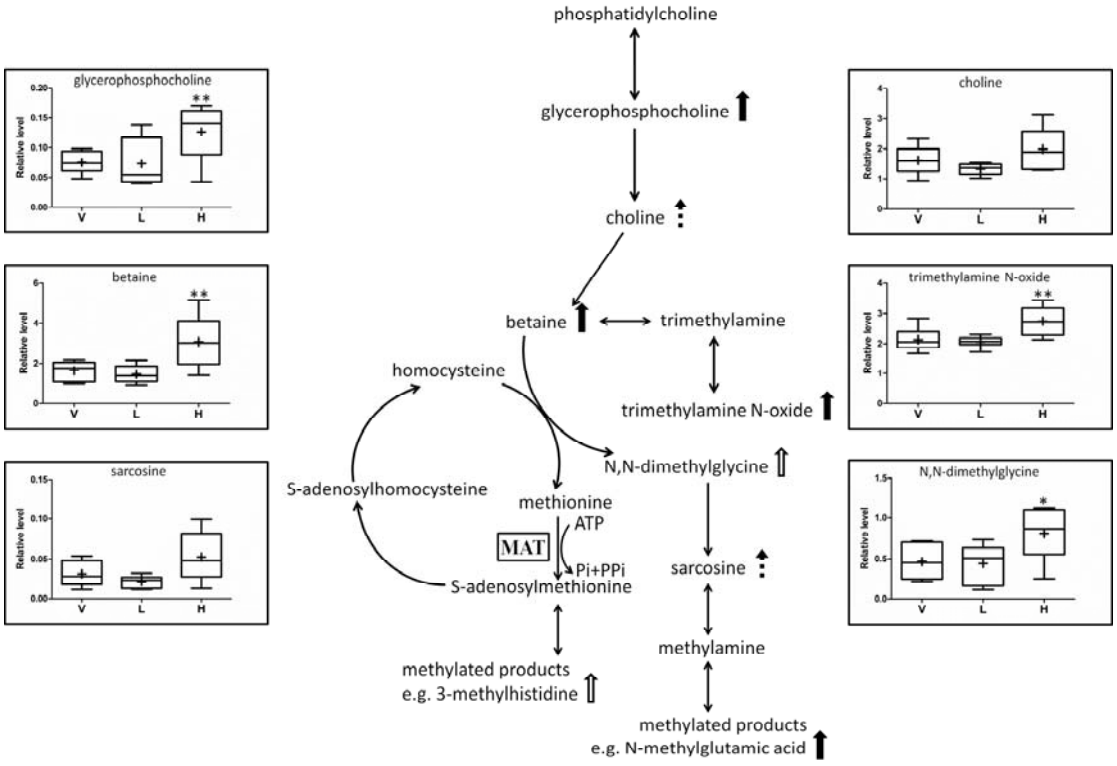

Fig. 6 Choline metabolism pathway and the interquartile range box plots of related metabolites in rat urine. MAT, methionine adenosyltransferase. The explanation for the box plots can be found in the legend of Fig. 3. The *p* and FDR *q*-values for all comparisons are referenced in Supplementary Table 2; **, $p<0.05$ and FDR q-value<0.2; *, $0.05\leq p<0.1$ and FDR q-value<0.2. Bold up arrow in the pathway indicates statistically significant increases ($p<0.05$ and FDR q-value<0.2); hollow arrow indicates statistically suggestive changes ($0.05\leq p<0.10$ and FDR q-value<0.2); dotted arrow indicates a non-statistically significant

trend with a fold change (high-dose versus vehicle-control) greater than 1.2.

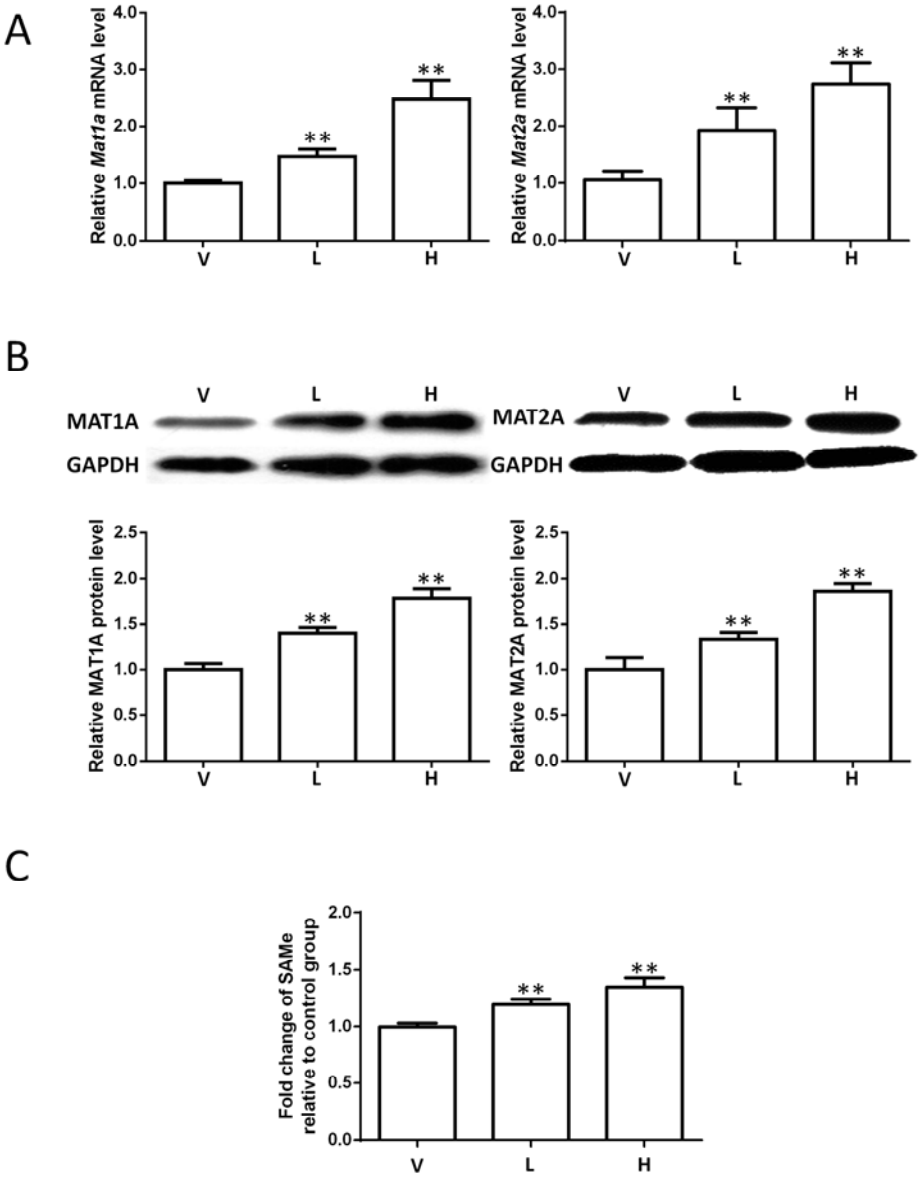

Fig. 7 Effects of BPA on expression of *Mat1a* and *Mat2a* genes and SAMe in the liver. **A** Effects of BPA on mRNA levels of *Mat1a* and *Mat2a* genes in rat liver. Each bar represents the mean ± SEM, n = 6 for each group. The data shown are representative of the results of three independent RT-PCR analysis. Each value was normalized to the mRNA expression of *Gapdh*, and summary of results was expressed as % of control mRNA levels. V = vehicle control group, L= low-dose group (0.5 μg/kg/day), H = high-dose group (50 mg/kg/day).

Significance level: **, $p<0.05$ compared with controls. **B** Effects of BPA on the expression of hepatic proteins of MAT1A and MAT2A. Representative western blots show expression of MAT1A (left) and MAT2A (right) and corresponding GAPDH. The lower trace of each panel shows the bar graph summarizing the immunoblot data. Densitometric results are expressed as mean ± SEM (n = 6 for each group). Each value was normalized to GAPDH, and the relative expression levels were generated compared with the control value. Significance level: **, $p<0.05$ compared with controls. **C** Effects of BPA on hepatic SAMe levels. Results are expressed as mean ± SEM (n = 6 for each group), and % of control SAMe levels. Significance level: **, $p<0.05$ compared with controls.

Table 1 Primers for Real-time PCR

Target gene	GenBank accession no.	Product length (bp)	Primer sequences	Tm (◦C)
Mat1a	NM_012860.2	155	Sense: 5'-AGGAGCAGACACCTTGCATC-3' Anti-sense: 5'-CCTGACCCCATGGAAAGACC-3'	60
Mat2a	NM_134351.1	125	Sense: 5'-GAGTCGCCTTCTCTCATCGC-3' Anti-sense: 5'-ATCTTATCTGGATGACCTTCCCC-3'	60
Gapdh	NM_017008.4	252	Sense: 5'-ACAGCAACAGGGTGGTGGAC-3' Anti-sense: 5'-TTTGAGGGTGCAGCGAACTT-3'	60

Table 2 Summary of Named Metabolite Changes by BPA Administration

Changes	0.5 µg/kg/day versus vehicle	50 mg/kg/day versus vehicle
Increased metabolites with $p<0.05$	4	23[a]
Increased metabolites with $0.05\leq p<0.10$	2	17(16[a])
Decreased metabolites with $p<0.05$	7	9[a]
Decreased metabolites with $0.05\leq p<0.10$	4	2[a]

[a] FDR q value<0.2.