NMR-based metabonomic analysis of the hepatotoxicity induced by combined exposure to PCBs and TCDD in rats

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ABSTRACT

A metabonomic approach using ¹H NMR spectroscopy was adopted to investigate the metabonomic pattern of rat urine after oral administration of environmental endocrine disruptors (EDs) polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alone or in combination and to explore the possible hepatotoxic mechanisms of combined exposure to PCBs and TCDD. ¹H NMR spectra of urines collected 24 h before and after exposure were analyzed via pattern recognition by using principal component analysis (PCA). Serum biochemistry and liver histopathology indicated significant hepatotoxicity in the rats of the combined group. The PCA scores plots of urinary ¹H NMR data showed that all the treatment groups could be easily distinguished from the control group, so could the PCBs or TCDD group and the combined group. The loadings plots of the PCA revealed remarkable increases in the levels of lactate, glucose, taurine, creatine, and 2-hydroxy-isovaleric acid and reductions in the levels of 2-oxoglutarate, citrate, succinate, hippurate, and trimethylamine-*N*-oxide in rat urine after exposure. These changes were more striking in the combined group. The changed metabolites may be considered possible biomarker for the hepatotoxicity. The present study demonstrates that combined exposure to PCBs and TCDD induced significant hepatotoxicity in rats, and mitochondrial dysfunction and fatty acid metabolism perturbations might contribute to the hepatotoxicity. There was good conformity between changes in the urine metabonomic pattern and those in serum biochemistry and liver histopathology. These results showed that the NMR-based metabonomic approach may provide a promising technique for the evaluation of the combined toxicity of EDs.

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Introduction

Endocrine disruptors (EDs), as widely dispersed environmental and food contaminants that pose a great risk to public health, have been one of the widespread concerns in health safety, and of especial concern is their toxicities of combined exposure. Polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are typical EDs and persistent organic pollutants (POPs). They are currently one of the hot spots in the field of environmental toxicology because of their extensive disposition and coexistence in the environment, persistence, bioaccumulation, migration, and high toxicity (Walker et al., 2005; Nagayama et al., 2007; Aragon et al., 2008; Ma, 2009). Most researches on the toxicity and human health hazards of these EDs have focused on toxicological studies on

individual compounds so that conventional techniques for toxicity evaluation remain the mainstream while the possible interactions of combined exposure to EDs have rarely been investigated systematically (Wade et al., 2002; Desaulniers et al., 2005). Considering the fact that humans are ubiquitously simultaneously exposed daily to complex mixtures of environmental contaminants, the assessment of the mixture effects of multiple pollutants using a new approach is of more theoretical and actual significance.

Metabonomics, the global analysis of metabolites, is defined as the “quantitative measurement of time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” (Nicholson et al., 1999). More practically, metabonomics is achieved by maximum data captured from biofluid or tissue analysis through the use of techniques such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry, followed by pattern recognition statistics (Tiziani et al., 2009). This technique represents a potentially powerful method for determining the systemic response to toxicity or disease. NMR spectroscopy is one

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of the major techniques used in metabonomic studies. NMR spectra possess a wealth of metabolic information, containing signals from thousands of individual metabolites which are observed simultaneously and which can partially overlap. Because of the information density of NMR spectra, data are routinely analyzed by a combination of data reduction and pattern recognition methods using multivariate statistical analysis, such as principal components analysis (PCA) (Holmes and Antti, 2002). This strategy results in the identification of groups with similar metabolic patterns. In addition, individual metabolites discriminating between these experimental groups can be identified. The PCA is a standard technique for pattern recognition and multivariate data analysis, particularly suited to the analysis of a set of data where each individual measurement contains a multitude of data—as is the case with a series of NMR spectra, containing signals from hundreds of individual metabolites. PCA compares all measurements with each other and explains the variation inherent in the dataset in terms of artificial components called principal components (PCs). The first principal component (PC1) is responsible for the largest amount of variation in the data; the second PC is orthogonal to PC1 and explains the largest amount of unexplained variation, and so forth with higher PCs. In effect, the first two PCs explain the vast majority of variation present in the data.

Over the past 10 years, increasing researches have demonstrated that metabonomic data are useful for assessment of toxic mechanisms, prediction of toxicity, and identification of clinically useful biomarkers (Jordan and Cheng, 2007; Bain et al., 2009). The most common metabolites identified by NMR analyses, especially in urine, are typically those associated with major endogenous metabolic pathways such as the tricarboxylic acid (TCA) cycle, and include intermediates (Nicholson et al., 2002). However, there is clear evidence that the pattern of changes noted, even with these commonly occurring metabolites, is altered in a toxicity- and tissue-specific manner, a feature that is extremely powerful with respect to the predictive utility of metabonomic data. At the same time, there is the potential to identify unique and/or specific endogenous metabolites that are associated with a specific type of change, enabling the identification of biomarkers of toxic changes. It was shown that urine NMR analysis had at least a 4- to 16-fold higher sensitivity than histopathology and clinical chemistry in detecting adaptive and/or toxic changes caused by some compounds (Schoonen et al., 2007). The high sensitivity of NMR urinalysis and the available knowledge from other studies which apply urine NMR spectroscopy to determine the effects of hepatotoxic compounds (Kleno et al., 2004; Azmi et al., 2005; Bollard et al., 2005) led us to the belief that the NMR approach to hepatotoxicity is feasible (DellaVecchia et al., 2007). Metabolomics has been successfully applied in the research fields of pharmacology, toxicology (Nicholson et al., 2002; Keun, 2006), and medical screening (Odunsi et al., 2005; Serkova et al., 2007; Lewis et al., 2008). However, very few studies concerning the metabonomics of TCDD and PCBs have been reported.

In our recent study, it was proved that combined exposure to TCDD and PCBs induced hepatotoxicity in rats. However, the hepatotoxic mechanism, especially the changes in metabolic profiling, remains unknown. In the present study, ^1H NMR-based metabonomic approach was used to reveal the possible hepatotoxic mechanisms and the possible biomarkers of combined exposure to TCDD and PCBs. Moreover, the feasibility of studying the combined toxicities of EDs metabonomically was also evaluated.

Materials and methods

Reagents and chemicals. TCDD was purchased from Cerilliant Corporation (Round Rock, TX; Lot no. ER011005-01) and the purity was 99%. Aroclor 1254 was obtained from AccuStandard, Inc (New Haven, CT; Lot no. 124-191-B). 2,2',3,3'-Deuterio(trimethylsilyl)propionic acid (TSP) was purchased from Merck Corporation. All other chemicals were of analytical grade and commercially available.

Animals and housing. Male Sprague-Dawley (SD) rats of 6–8 weeks of age (180–200 g) were purchased from Beijing Vital River Laboratory Animal Co. (Beijing, China) and bred in the Laboratory Animal Center of Academy of Military Medical Sciences (Beijing, China). The laboratory conditions were maintained under a 12 h light/dark cycle at $22 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity. The animals were provided with standard commercial pelleted feed and water *ad libitum*.

Experimental design. All animal procedures were approved by the Institutional Animal Care and Use Committee of Academy of Military Medical Sciences and were performed in strict accordance with current animal care guidelines. Animals were allowed to acclimate for 1 week before the start of the study. After acclimatization, the animals were assigned randomly into four groups of five each, including a control group and three treatment groups. Rats in the treatment groups received $10\text{ }\mu\text{g/kg}$ of TCDD, 10 mg/kg of PCBs (Aroclor 1254) or the combination of TCDD ($10\text{ }\mu\text{g/kg}$) and Aroclor 1254 (10 mg/kg), respectively. Control rats were administered with equivalent volumes of olive oil only. All animals were treated by intragastric administration (2.5 ml/kg) daily for 6 consecutive days. TCDD and Aroclor 1254 were initially dissolved in DMSO and further diluted with olive oil.

Rats were individually housed in metabolism cages, body weights were recorded daily, and the urine samples in 24 hours were collected before and after exposure. The urine samples were kept at -80°C pending ^1H NMR spectroscopic analyses. On the next morning of the last administration, the rats were subject to anesthesia, and the blood was collected for serum biochemical analysis. The livers were immediately isolated and weighed, and the left lobe of each liver was stored in formalin for histopathological evaluation.

Serum biochemical analysis. Biochemical analyses of the serum obtained from the collected blood were performed by an automated analyzer (Hitachi, Japan) for the following parameters: activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), and the levels of cholesterol (CHOL), triglyceride (TG) and total bilirubin (TBIL). Data were presented as mean $\pm$ standard deviations (SD). Statistical analyses were performed using the two-way analysis of variance (2-way ANOVA) followed by the post-hoc Dunnett *t* test. A *p* value less than 0.05 was regarded as statistically significant.

Histopathological examination. Formalin-fixed livers were processed according to the routine procedure, and $4\text{ }\mu\text{m}$ of thickness was sectioned. The sections were stained with haematoxylin and eosin (H&E), and the slides were examined by an expert in histopathologic evaluation. Histopathologic alterations were evaluated with a grading scale: NC, no obvious changes; +, minimal alterations; ++, intermediate alterations; and +++, extensive alterations.

^1H NMR spectroscopy. The preparation of the urine for NMR analysis was based on a protocol previously described (Beckwith-Hall et al., 1998). Urine samples were prepared by mixing $400\text{ }\mu\text{l}$ of urine with $200\text{ }\mu\text{l}$ of 0.2 M sodium phosphate buffer (pH 7.4) and kept for 10 min. Then the samples were centrifuged at $13,000 \times g$ for 10 min to remove any precipitates. Five hundred-microliter aliquots of the resulting supernatant were transferred into 5-mm NMR tubes, to which $50\text{ }\mu\text{l}$ of a solution of TSP in deuterium oxide (D_2O) was added (final concentration = 1 mM). The D_2O and TSP provided a field frequency lock for the NMR spectrometer and the chemical shift reference ($\delta 0.0$), respectively. ^1H NMR spectra of all the urine samples were obtained at 599.69 MHz ^1H frequency by using a Varian INOVA 600 NMR spectrometer. One-dimensional spectrum was acquired by using a standard NOESY pulse sequence with water suppression during the mixing time of 0.15 s and the relaxation delay of 2 s . Sixty-four free induction decays (FIDs) were collected into 64 K data points, and the

number of scans collected for each urine sample was 256. The data were zero-filled by a factor, and the FIDs were multiplied by an exponential function equivalent to a line broadening of 0.3 Hz Fourier transformation (FT).

¹H NMR data analysis. All NMR spectra were phased and baseline corrected and then data-reduced to 225 integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.52 to δ 9.48 using the VNMR 6.1C software package (Varian, Inc). The region of δ 4.6 to δ 6.2 in the urine spectra was excluded from the recognition analysis to remove the uncertainty of residual water signal and urea. Normalization to the total sum of the spectrum was carried out on the data before pattern recognition analyses, and the values of all variables were mean-centered and pareto-scaled (Webb-Robertson et al., 2005; Craig et al., 2006) before PCA using SIMCA-P 10.0 software package (Umetrics AB, Umeå, Sweden). Pareto scaling gives each variable a variance numerically equal to its standard deviation. Data were visualized by using the first two PCs scores and loadings plots. Each point on the scores plot represents an individual sample, and each point on the loadings plot represents a single NMR spectral region that was altered as a result of PCBs or TCDD dosing. The scores and loadings plots are complementary. Mean data were calculated for each integrated regions for each time point. Thus, biochemical components responsible for the differences between samples detected in the scores plot can be extracted from the corresponding loadings.

Results

Body weight and relative liver weight

There was no significant difference in body weights of rats in the PCBs group when compared with the control group. However, body weights in the TCDD and the combined groups were significantly reduced from the 5th and 4th days, respectively, as compared with the control group, and the maximal reduction was found in the combined group (Table 1).

The relative liver weight (liver weight / body weight $\times$ 100%) in all the treatment groups showed significant increase when compared with the control group ($p < 0.05$), and the maximal increase was found in the combined group ($p < 0.05$). There was significant difference between the PCBs or TCDD group and the combined group ($p < 0.05$; Table 1).

Serum biochemistry

Biochemical changes of serum are presented in Table 2. Combined exposure to PCBs and TCDD induced significant changes in serum biochemistry. Serum TG, CHOL, TBIL levels, and AST activity were increased by 85.7%, 163.8%, 104.5%, and 112.6% as compared to the control value, respectively ($p < 0.05$), indicating the occurrence of liver injuries. In addition, there were statistical differences in several biochemical parameters between the PCBs or TCDD group and the combined group ($p < 0.05$).

Table 1

Effects of exposure to PCBs and TCDD alone or in combination on body weights and relative liver weights of SD rats. Results are displayed as the mean $\pm$ SD of five animals per group. Two-way ANOVA was used followed by the post-hoc Dunnett *t* test.

Treatment	<i>n</i>	Body weight (g)	Liver weight (g)	Relative liver weight (%)
Control	5	227.9 $\pm$ 14.1	7.0 $\pm$ 0.4	3.1 $\pm$ 0.2
PCBs	5	221.3 $\pm$ 10.5 ^a	11.3 $\pm$ 0.4 ^b	5.1 $\pm$ 0.2 ^{a,b}
TCDD	5	203.0 $\pm$ 3.9 ^b	9.3 $\pm$ 1.1 ^{a,b}	4.6 $\pm$ 0.5 ^{a,b}
TCDD + PCBs	5	181.7 $\pm$ 15.1 ^b	12.2 $\pm$ 1.2 ^b	6.7 $\pm$ 0.5 ^b

^a $p < 0.05$ versus combined group.

^b $p < 0.05$ versus control group.

Liver histopathology

Liver histopathologic examination was performed to further evaluate the hepatotoxicity of PCBs and TCDD. Control livers showed no signs of obvious abnormality (Fig. 1A). In contrast, there was slight hepatocellular swelling and a few vacuolations in the livers of PCBs-treated rats (Fig. 1B). Mild hepatocellular swelling and vacuolation were noted in the TCDD group (Fig. 1C). Combined exposure to PCBs and TCDD induced marked swelling, mild to moderate hepatocellular vacuolation, necrosis and inflammatory infiltration in the livers (Fig. 1D). Generally, the histological changes of the liver in the combined group were more remarkable than those in the PCBs or TCDD group (Table 3).

¹H NMR spectra of rat urine

Typical ¹H NMR spectra obtained from urine samples of rats exposed to PCBs and TCDD after 1, 3, and 6 days were shown in Fig. 2. The spectra were dominated by a number of metabolites, including citrate, trimethylamine-*N*-oxide (TMAO), 2-oxoglutarate, creatine, creatinine, lactate, succinate, taurine, dimethylamine (DMA), hippurate, 2-hydroxy-isovalerate, 2-oxoisovalerate and glucose. ¹H NMR spectra revealed differences in overall composition between the urines from control animals and PCBs and TCDD-treated animals. Most changes in the ¹H NMR spectra are related to endogenous metabolites involved in carbohydrate metabolism such as the TCA cycle intermediates, e.g., citrate, 2-oxoglutarate, succinate, and the glycolysis end product lactate (Fig. 2C).

PCA of ¹H NMR data

The PCA was performed to facilitate a general overview of metabolite patterns and to identify potential biomarkers. The PCA scores plots of the urinary ¹H NMR data are shown in Fig. 3A. Each data point corresponds to one NMR spectrum and represents one urine sample, and the position in the plot was determined by the difference of that spectrum with respect to the average of the respective PC. The distance between points in the scores plot is an indication of the similarity between samples. The scores plots revealed that all the treatment groups could be easily distinguished from the control group both along PC1 and PC2, and the distinction was the most striking for the combined group. Furthermore, the PCBs or TCDD group was also separated from the combined group. The corresponding loadings plots were shown in Fig. 3A. Each data point represents one bucket (with the chemical shift indicated explicitly). The plot identifies which spectral regions are responsible for the differences between the spectra observed in the scores plots. This means that it is possible to identify from the loadings plots which spectral regions and thus which chemical compounds are responsible for any grouping of data points observed in the scores plots. Further analysis of loading factors of PCA and ¹H NMR spectrum itself can show biomarkers corresponding to toxicity. Compared to the control group, in the PCBs group, the major biochemical changes identified in urine from corresponding loadings plot (Fig. 3B) and ¹H NMR spectra were the increase in the levels of 2-hydroxy-isovaleric acid (δ 3.82, δ 3.86), lactate (δ 4.14), and glucose (δ 3.62, δ 3.66, δ 3.74) and the decrease in the levels of citrate (δ 2.54, δ 2.58, δ 2.66, δ 2.7), creatine (δ 3.02), 2-oxoglutarate (δ 2.46), and TMAO (δ 3.26) ($p < 0.05$). Fig. 3C shows that elevation in the levels of lactate (δ 4.14), 2-hydroxy-isovaleric acid (δ 3.82, δ 3.86), and glucose (δ 3.74) was observed, while reduction in the levels of citrate (δ 2.54, δ 2.58, δ 2.66, δ 2.7) ($p < 0.05$) was found in the TCDD group. The predominant changes identified in the PCA of the urinary ¹H NMR of the combined group (Fig. 3D) included remarkable increase in the levels of lactate (δ 4.14), taurine (δ 3.42), creatine (δ 3.9), 2-hydroxy-isovaleric acid (δ 3.82, δ 3.86), and glucose (δ 3.62, δ 3.66, δ 3.7, δ 3.74), accompanied by reduction in the levels of 2-oxoglutarate (δ 2.46), citrate (δ 2.54, δ 2.58,

Table 2

Effect of exposure to PCBs and TCDD alone or in combination on serum biochemistry of SD rats. Data are presented as the mean $\pm$ SD of $n = 5$ animals per groups. Two-way ANOVA was used followed by the post-hoc Dunnett t test.

Treatment	<i>n</i>	ALT (U/L)	AST (U/L)	ALP (U/L)	TG (nmol/L)	CHOL (mmol/L)	TBIL (μ mol/L)
Control	5	36.2 $\pm$ 5.3	178.8 $\pm$ 23.6	257.4 $\pm$ 39.4	0.35 $\pm$ 0.06	0.94 $\pm$ 0.11	0.22 $\pm$ 0.01
PCBs	5	56.6 $\pm$ 12.1	205.8 $\pm$ 51.3 ^a	222.0 $\pm$ 22.0 ^a	0.47 $\pm$ 0.18	1.60 $\pm$ 0.37 ^{a,b}	0.34 $\pm$ 0.14
TCDD	5	35.0 $\pm$ 4.2	234.4 $\pm$ 20.6	197.2 $\pm$ 32.7 ^{a,b}	0.33 $\pm$ 0.04 ^a	1.28 $\pm$ 0.19 ^a	0.52 $\pm$ 0.22 ^b
TCDD + PCBs	5	53.4 $\pm$ 33.3	380.2 $\pm$ 59.1 ^b	284.4 $\pm$ 63.7	0.65 $\pm$ 0.36 ^b	2.48 $\pm$ 0.81 ^b	0.45 $\pm$ 0.07 ^b

Abbreviations: ALT alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TG, triglyceride; CHOL, cholesterol; TBIL, total bilirubin.

^a $p < 0.05$ versus combined group.

^b $p < 0.05$ versus control group.

$\delta 2.66$, $\delta 2.7$), hippurate ($\delta 3.98$, $\delta 7.82$), and succinate ($\delta 2.42$) ($p < 0.05$) (Table 4).

Discussion

Several studies have demonstrated that exposure to PCBs or TCDD induced hepatotoxicity (Boverhof et al., 2006; Korenaga et al., 2007). In the present research, traditional toxicity evaluations showed that all treatments elicited hepatotoxicity, as characterized by the increased liver weight, elevated serum TG and CHOL levels and AST activity, and liver histopathologic alterations. Moreover, the combined exposure induced more severe hepatotoxicity than exposure to PCBs or TCDD alone. Hepatocellular vacuolation, necrosis, and inflammatory cell infiltration were observed in the histopathologic examination of the combined group.

To explore the hepatotoxic mechanism of PCBs and TCDD, the high sensitivity of NMR urinalysis technique was used. It can inform on the state and severity of target organ dysfunction because the biochemical compositions of a biofluid will be modified if cell function varies (Lindon et al., 2004). Recent studies have shown that NMR-based metabonomic approach can be used as a rapid analytical tool for the

study of the effects of hepatotoxic compounds (Kleno et al., 2004; Azmi et al., 2005; Bollard et al., 2005). In this investigation, ^1H NMR-based metabonomic technique was utilized to study the alterations of urine metabolite profiles in rats after combined exposure to TCDD and PCBs, and the data were analyzed by using PCA. The PCA showed that the samples were primarily distributed in four regions of the scoring oval (95% confidence intervals). The urine metabonomic patterns of each treatment group could obviously be distinguished from that of control group, so could the PCBs or TCDD group and the combined group, suggesting that all the groups had distinct metabonomic spectra. The results suggest that this metabonomic technique can be used to characterize the metabolic consequence of combined exposure to PCBs and TCDD and to elucidate the toxic effects. Conventional toxicity evaluation techniques showed that all treatments elicited different degrees of hepatotoxicity, and the combined hepatotoxicity was more severe than that of individual exposure. These results indicated that the results of urine metabonomic pattern changes were in accordance with those of conventional toxicity evaluations.

The PCA of urinary ^1H NMR data showed that the levels of 2-oxoglutarate, citrate, succinate, creatine, taurine, lactate, TMAO,

Fig. 1. Liver histopathology of rats following exposure to PCBs and TCDD alone or in combination. (A) Vehicle control rat liver. (B) PCBs-treated liver, arrows indicate hepatocellular swelling. (C) TCDD-treated liver, arrows indicate mild hepatocellular vacuolation and hepatocellular swelling. (D) Combination-treated liver, arrows indicate mild to moderate hepatocellular vacuolation and inflammatory cell infiltration. H&E original stain, 20 $\times$ objective.

Table 3

Histopathologic alterations of rats following exposure to PCBs and TCDD alone or in combination. NC, no obvious changes; +, minimal alterations; ++, intermediate alterations, and +++, extensive alterations.

Treatment	Hepatocellular swelling	Inflammatory cell infiltration	Hepatocellular vacuolation
Control	NC	NC	NC
PCBs	+	NC	+
TCDD	++	+	++
TCDD + PCBs	+++	++	+++

hippurate, 2-hydroxy-isovaleric acid, and glucose in rat urine were altered by exposure to PCBs and TCDD, and most of these changes were more evident in the combined group. These changed metabolites may be considered possible biomarkers for the combined hepatotoxicity. Furthermore, latent toxic mechanisms were hinted by analyzing the formation and the transformation of these metabolites. A prominent finding was the depletion of the TCA cycle intermediates which were mainly located in liver mitochondria, including citrate, succinate, and 2-oxoglutarate, in urine samples from the combined group. These organic acids may be more universal markers for cellular liver pathology (Ishihara et al., 2006). The decreased levels of citrate, 2-oxoglutarate, and succinate in urine following PCBs and TCDD treatment could be a result of decreased fatty acid catabolism, since these metabolites are intermediates of the TCA cycle which occurs largely in liver mitochondria. When hepatic insufficiency occurs, glycolysis and gluconeogenesis are often accelerated. Enhanced gluconeogenesis and glycolysis prevent acetyl-CoA entering the TCA cycle, reducing the levels of citrate, 2-oxoglutarate, and succinate (Yokoyama et al., 2005). Similar changes were observed in ANIT, carbon tetrachloride, and hydrazine-induced hepatotoxicity (Wu et al., 2005). The reduced organic acid levels suggested that oxidative phosphorylation was inhibited, glycolysis was increased (Siculella et al., 2002; Connor et al., 2004), and that exposure to PCBs and TCDD affected the mitochondrial energy metabolism in liver, although the effect of other factors could not be excluded. The level of glucose in the urine was increased significantly in all treatment groups, which suggested that the rates of gluconeogenesis and glycolysis increased because the lipid metabolism in these animals failed. Clinical chemistry and liver histopathology examination also showed clear changes for the liver tissues, the increases of serum TG,

CHOL levels, and the appearance of vacuolation in the liver could indicate changes in fatty acid metabolism. Meanwhile, the lactate level in the urine was increased too, which offers a support to the increased rate of glycolysis. In addition, the increased levels of glucose and lactate in the urine from treated animals are consistent with mitochondrial impairment; this proposed inability may result from a lack of acetyl-CoA in the mitochondria as a result of a reduction in the rate of oxidative phosphorylation and fatty acid β -oxidation (McGarry and Foster, 1980). These results provided evidence for the assumption that the cells switched to gluconeogenesis and glycolysis to compensate for the loss of ATP coming from fatty acid β -oxidation (Marty-Teyssset et al., 1996). Our clinical chemistry data and liver histopathology examination could support it also. These indicated that mitochondrial dysfunction and abnormality of fatty acid metabolism probably were the most important mechanisms for the hepatotoxicity of PCBs and TCDD. The work of Mortishire-Smith et al. (2004) also provided evidence of altered fatty acid metabolism as a mechanism of hepatotoxicity. Wang et al. (2006) studied the hepatotoxicity of Z24 on Wistar rats by using metabolomic analysis of urinary ^1H NMR spectra, the results discovered that Z24 inhibited mitochondrial function through altering the energy and lipid metabolism in rats. In addition, a decrease in the formation of hippurate is another common marker for cellular liver pathology (Connor et al., 2004). Decreased oxidative phosphorylation will also lead to a decreased formation of hippurate in rat liver mitochondria. The hippurate formation depends on the metabolism of benzoate (Yavuz et al., 2005); the whole process depends on a good supply of ATP via the oxidative phosphorylation (Sprague et al., 2004). Therefore, the reduced production of ATP may lead to a reduced formation of hippurate in the combined group as observed in the present study. In addition, the levels of taurine and creatine in the urine were increased significantly in the combined group, and the combined elevations in the levels of taurine and creatine had been found previously as a biomarker for liver damage (Waterfield et al., 1993; Beckwith-Hall et al., 1998).

In summary, the present investigation has demonstrated that combined exposure to PCBs and TCDD induced significant hepatotoxicity in rats, and mitochondrial dysfunction and fatty acid metabolism perturbation might contribute to the hepatotoxicity. The metabolomic data obtained in this study provide important mechanistic clues about the effects of PCBs and TCDD on liver metabolism and energy utilization which may be important for the understanding of the pathogenesis of liver injury. We propose that the changed metabolites

Fig. 2. Typical ^1H NMR spectra of urine samples of rats exposed to PCBs and TCDD alone or in combination after 1 (A), 3 (B), and 6 days (C). N, control group; P, PCBs group; T, TCDD group; C, combined group.

Fig. 3. Scores plots and corresponding loadings plots of PCA of urinary ^1H NMR spectra from four groups (A), the PCBs and control groups (B), the TCDD and control groups (C), and the combined and control groups (D). In scores plot of PCA, each data point represents one rat urine sample, and the distance between points in the scores plot is an indication of the similarity between samples. In loadings plot for the corresponding scores plot, each data point represents one bucket (with the chemical shift indicated explicitly). The plot identifies which spectral regions (and thus which chemical compounds) are responsible for the differences between the spectra observed in the scores plot. X axis is PC1, Y axis is PC2. $\bullet$ N, control group; $\blacklozenge$ P, PCBs group; $\blacktriangle$ T, TCDD group; $\blacksquare$ C, combined group.

could be considered as potential noninvasive biomarkers for PCBs and TCDD hepatotoxicity, while further studies will be necessary to verify these metabolites as biomarkers of PCBs and TCDD hepatotoxicity. Furthermore, there was good conformity between changes in the urine metabonomic pattern and those in serum biochemistry and liver histopathology. The results of the present study imply that the NMR-based metabonomic approach represents a promising and sensitive technique for the combined toxicity of EDs.

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Table 4

Effect of PCBs and TCDD on the ^1H NMR spectra relative integral area of metabolites. Data are presented as the mean $\pm$ SD of five animals per group. Two-way ANOVA was used followed by the post-hoc Dunnett *t* test.

Treatment	2-Hydroxy-isovalerate	Lactate	Glucose	Taurine	Creatine	Citrate	2-Oxoglutarate	Succinate	Hippurate	TMAO
Control	191.7 $\pm$ 10.9	85.7 $\pm$ 4.6	231.6 $\pm$ 28.1	82.2 $\pm$ 13.4	384.1 $\pm$ 83.2	290.2 $\pm$ 75.5	314.4 $\pm$ 51.9	210.8 $\pm$ 13.9	216.0 $\pm$ 36.7	258.7 $\pm$ 31.76
PCBs	368.9 $\pm$ 58.0 ^a	211.4 $\pm$ 50.6 ^a	500.2 $\pm$ 94.4 ^a	79.8 $\pm$ 11.7	231.6 $\pm$ 48.3 ^a	154.9 $\pm$ 46.8 ^a	210.1 $\pm$ 76.2 ^a	196.5 $\pm$ 53.8	191.9 $\pm$ 27.7	212.3 $\pm$ 17.6 ^a
TCDD	276.0 $\pm$ 24.6 ^a	159.9 $\pm$ 23.9 ^a	431.3 $\pm$ 83.7 ^a	113.8 $\pm$ 37.4	430.9 $\pm$ 56.5	122.7 $\pm$ 5.5 ^a	359.7 $\pm$ 44.7	201.4 $\pm$ 14.7	177.9 $\pm$ 5.5	235.6 $\pm$ 65.4
TCDD + PCBs	711.3 $\pm$ 98.1 ^a	513.0 $\pm$ 79.7 ^a	805.2 $\pm$ 35.7 ^a	146.2 $\pm$ 23.5 ^a	506.1 $\pm$ 57.7 ^a	49.3 $\pm$ 9.3 ^a	121.3 $\pm$ 51.8 ^a	87.1 $\pm$ 12.5 ^a	119.8 $\pm$ 22.9 ^a	220.7 $\pm$ 53.7

^a *p* < 0.05 versus control group.

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