Reactive Oxygen Species and Nucleotide Metabolism Jointly Contribute to HepG2 Cytotoxicity Induced by Tetrabromobisphenol A

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Abstract. Owing to high lipophilicity and recalcitrance, low volatility and high production, tetrabromobisphenol A (TBBPA) is used as flame retardant worldwide and have been detected in various matrices. Although TBBPA exposure can induce adverse effects on the nervous system, immune system and reproductive system, molecular mechanisms of metabolic toxicity are limited and unclear. Herein, an integrated method including molecular toxicology and mass spectrometry (MS)-based global metabolism was employed to investigate the cytotoxicity of TBBPA on reactive oxygen species (ROS), oxidation/ antioxidation balance and metabolic alterations using HepG2 cell line. Our results elucidated that TBBPA promotes oxidative damage in accordance with the anomalous variations of HepG2 metabolome. TBBPA exposure perturbed the balance statue of oxidation and antioxidation by increasing ROS, 8-oxo-2'-deoxyguanosine (8-oxo-dG) and malondialdehyde (MDA), whereas by decreasing superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity and glutathione level. More importantly, five major metabolites (guanine, guanosine, adenine, adenosine and hypoxanthine) were down-regulated significantly in nucleotide metabolism pathway following TBBPA exposure. We also, showed that metabolism plays a key role in regulation of molecular mechanisms and induction of oxidative damage of bisphenol A (BPA) analogue.

Keywords: Metabolomics, DNA oxidative damage, TBBPA, Metabolic toxicity, HepG2.

1. Introduction

Among the brominated flame retardants (BFRs), tetrabromobisphenol A (TBBPA) has the largest throughput (approximate 30% of all BFRs) and used as electrical components with widespread industrial application and to be release into the environment (WHO/ICPS, 1995). TBBPA is also a highly lipophilic persistent organic pollutant (POP), and has been found in human blood (Jakobsson *et al*., 2002), adipose tissue (Cariou *et al*., 2008), fish (Morris *et al*., 2004), sewage sludges (Chu *et al*., 2005) and indoor environment (Tasaki *et al*., 2004).

As a bisphenol A (BPA) analog, TBBPA has weak estrogenicity than BPA, could consider as an antiandrogenic ligand (Christen *et al*., 2010) to disrupt thyroid hormone (Sun *et al*., 2009). Fukuda *et al*. (2004) found that lower doses of TBBPA (200 mg/kg body weight) caused renal impairment in newborn rats comparing with higher doses of TBBPA (1000 mg/kg) *in vivo*. Moreover, toxicology of TBBPA appeared significant endocrine disruptive effect by competitive binding to transthyretin in the thyroid hormone system (Legler *et al*., 2003). TBBPA could induce various effects, and acts as a neurotoxic agent, a cytotoxic agent and a thyroid hormone agonist (Kitamura *et al*., 2005) *in vitro*. Suh *et al*. (2017) investigated that TBBPA on rat pancreatic β-cells. TBBPA exposure resulted in cell damage by triggering mitochondrial dysfunction and inducing apoptosis. It also could increase the secretion of inflammatory cytokines, generation of nitric oxide, intracellular ROS, and mitochondrial superoxide (Suh *et al*., 2017).

Numerous evidences suggested that environmental pollutant also disordered the metabolites or signaling pathway to increase the risk of diseases or cancer (Zhao *et al*., 2014). Metabolomics can serve as a valuable approach to investigate the global metabolic networks in understanding the toxicity mechanisms of environmental stimulus. By using global metabolic profiling, large numbers of small molecules can be detected qualitatively and quantitatively, and potential biomarkers which linking signaling pathways to toxicity mechanisms are being screened by multivariate statistical analysis. However, metabolic toxicities underlying the characteristics of TBBPA are limited and unclear. Herein, we explored oxidative, antioxidative and regulation of cellular metabolic pathway, and obtained the metabolic biomarkers of oxidative stress to evaluate the TBBPA cytotoxicity comprehensively.

2. Materials and methods

2.1. Cell culture and TBBPA treatment

HepG2 human hepatoblastoma cell (American Type Culture Collection) was maintained in culture medium Gibco RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U/mL penicillin G and 100 U/mL streptomycin with 10% fetal bovine serum (Thermo Fisher Scientific) in a humidified 37 ^{o}C incubator supplied with 5% CO_2. Cells were diluted to proper density from 80-90% confluence using trypsin. Cells were treated with 0.002% DMSO or TBBPA at concentration of 10, 20 or 50 μM for 24 h. Seven replicate experiments were detected for control and TBBPA-treated group.

2.2. Toxicological experiments

Intracellular ROS level was assessed using a fluorometric intracellular ROS kit (Sigma, St Louis, MO, USA). Fluorescence intensity was measured at λ_{ex}520 nm / λ_{em}605 nm with a microplate reader. GSH-Px and SOD activities were assessed using fluorometric kit (Beyotime, Haimen, China). GSH-Px activity was calculated using the formula: $A_{340nm,\ BPs\text{-}treated\ group}$ / min - $A_{340nm,\ control\ group}$ /min / 0.00622. SOD activity was calculated using the formula: $A_{560\ nm,\ Control\ group}$ -$A_{560\ nm,\ BPs\text{-}treated\ group}$ / $A_{560\ nm,\ Control\ group}$ × 100%. MDA level was assessed using thiobarbituric acid reactive substances (TBARS) assay kit (Beyotime). The concentration of intracellular reduced GSH was measured using Intracellular GSH Assay Kit (Abcam, Cambridge, USA). The fluorescence intensity was measured at λ_{ex}490 nm / λ_{em}529 nm with a microplate reader.

Genomic DNA of HepG2 was extracted by a Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacturer's instructions. Genomic DNA was digested in to mononucleosides by 0.01 U of snake venom phosphodiesterase, 2.0 U of DNase I and 4.0 U of CIP at 37 °C for 24 h, and then were filtered by ultrafiltration tubes (3 KDa) and analyzed by Orbitrap Fusion Tribird MS system (Thermo Fisher Scientific). Eight-oxo-dG and dG detection were performed in positive mode, monitoring *m/z* 284.2 →168.2 and 268.1→152.2, respectively. Relative abundance of 8-oxo-dG was calculated using peak area ratio of 8-oxo-dG and dG.

2.3 Metabolite extraction, LC-MS/MS-based global metabonomics and data processing

For extraction and quenching of intracellular metabolites, 800 μL of ice cold 60% methanol was added to culture dish, and immediately transferred to the -80 °C freezer and incubated for 7 min. Cells were scraped and transferred to a fresh 1.5 mL-tube, quenched by liquid nitrogen for 3 times. Cells were centrifuged at 12,000 g for 15 min at -6 ^{o}C. Supernatants were collected and transferred to 1.5 mL-tube and dried in a freeze-dryer.

Analysis of intracellular metabolites was performed using Q Exactive focus orbitrap LC-MS/MS system (Thermo Fisher Scientific) with a 2.1 mm × 100 mm ACQUITY UPLC HSS 1.8 μm T3 column (Waters, Milford, MA, USA). Mobile phage (A) contained water with 0.1% formic acid. Mobile phage (B) contained acetonitrile with 0.1% formic acid. Mobile phase gradient was as follow: 0.0-1.0 min at 2% (B), 1.0-20.0 min from 2% (B) to 100% (B) and kept for 2.0 min, 22.0-22.1 min to 2% (B) and 22.1-25.0 min at 2% (B). Flow rate was 0.3 mL/min, and injected sample amount was 5.0 μL each time.

Data processing were carried out as described in Zhao *et al*. (2017).

3. Results and discussion

3.1 Effect of TBBPA on oxidation damage

To evaluate the cytotoxicity of TBBPA on HepG2, ROS and potential oxidation damage were measured. As shown in Figure 1, we found that ROS (Figure 1A) and MDA levels (Figure 1B) displayed a remarkable increase in a concentration-dependent manner, whereas GSH levels (Figure 1C), SOD activity (Figure 1D) and GSH-Px activity (Figure 1E) showed a remarkable decrease in response to TBBPA treatment. TBBPA concentrations at 10, 20 and 50 μM induced about 2.4-, 2.7- and 5.2-fold increase in ROS, and about 0.2-, 0.5- and 3-fold increase in MDA, respectively ($P < 0.05$). Conversely, at a concentration of 50 μM TBBPA, a 1.2-fold decrease in GSH levels (Figure 1C), a 2.2-fold decrease in SOD activity (Figure 1D) and a 1.0-fold decrease in GSH-Px activity (Figure 1E) were observed. Excess ROS can improve oxidative stress that effects proteins, DNA and membrane lipids (Trine *et al*., 2005). In our study, elevation of ROS and MDA levels and impairment of GSH, SOD and GSH-Px activities suggested that TBBPA-treatment significantly induced oxidative damage of HepG2, and perturbed the balance of oxidation and antioxidation system. Other models also have characteristic of oxidative stress by TBBPA treatment. In human neutrophilic granulocytes, Trine *et al*. (2005) investigated that TBBPA triggered ROS generation by a concentration-dependent manner. In rat model, Szymanska *et al*. (2000) found that TBBPA exposure induced decreased GSH levels and increased MDA levels comparing with control group. In earthworm tissue, Xue *et al*. (2009) demonstrated that TBBPA promoted the generation of hydroxyl radical in accordance with production of oxidative stress.

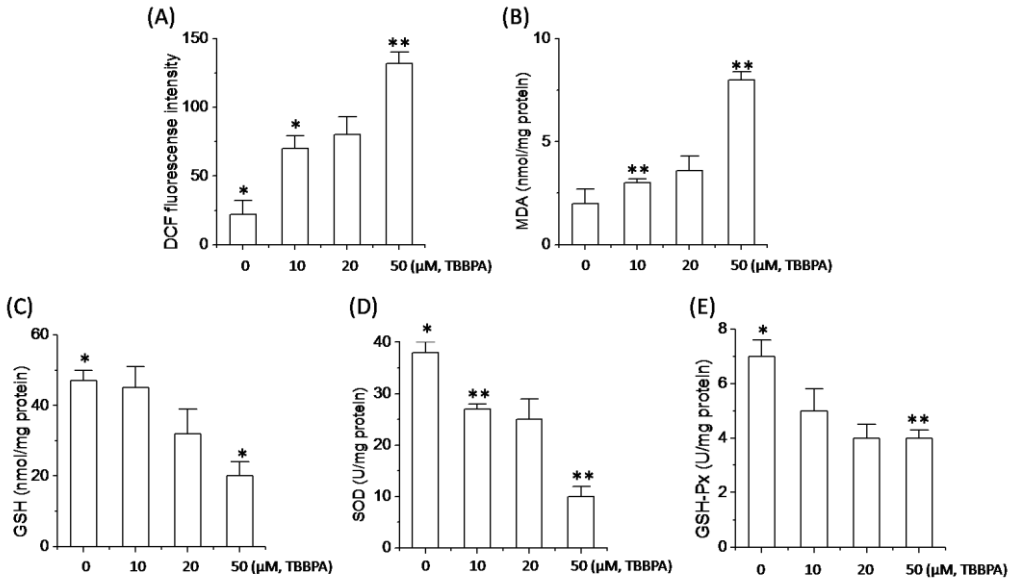

Figure 1. ROS response of TBBPA on HepG2. (A) ROS level; (B) MDA level; (C) GSH level; (D) SOD activity; (E) GSH-Px activity. Asterisk (*) represents statistical significance between treated and control group in seven replicate experiments, * $P < 0.05$, ** $P < 0.01$.

Eight-oxo-2'-deoxyguanosine (8-oxo-dG) is considered as a major product of DNA oxidative damage, and is used as a biomarkers to evaluate oxidative stress (Hattori-Nakakuki *et al*., 1994). To provide intensive insights into the potential association between the oxidative damage of HepG2 and TBBPA treatment, LC-MS/MS method was employed the detection of relative abundance of 8-oxo-dG. Relative abundance of 8-oxo-dG (8-oxo-dG/dG×100%) in HepG2 genomic DNA averaged 122% (P=0.01) in 20 μM of TBBPA-treated group, and 141% (P=0.04) in 50 μM of TBBPA-treated group, while the levels were much lower in 10 μM-treated group, averaging 81% (P = 0.05). Detected significant differences in 8-oxo-dG adduct also provide powerful support that TBBPA exposure could trigger DNA oxidative damage in HepG2 cell line. Above results agree with previous observation that nucleic acids (such as dG and 8-oxo-dG) are more susceptible to pollutant exposure (Prasad *et al*., 2015).

Combining with ROS response of TBBPA on HepG2 (Figure 1), significant increased 8-oxo-dG levels, and imbalance of oxidation and antioxidation system potentially caused DNA oxidative damage during glycolysis or oxidation phosphorylation. Therefore, DNA oxidative damage may be one critical mechanism of toxic effect by TBBPA exposure. In addition, inefficiency of DNA repair mechanisms and deficiency of protective histones also produced DNA damage (Mambo *et al*., 2003).

3.2 Metabolites analysis in HepG2 following TBBPA exposure

Global metabolites profiling reflect the variation of biological system to environmental pollutant exposure. The regulation of oxidative stress signaling pathway is often relate to ROS. In our study, metabolic profiling of HepG2 cell line was analyzed by an orbitrap LC-MS/MS system in positive and negative mode, the concentration of 10, 20 and 50 μM of TBBPA were employed for comparison of cell metabolites. In the PLS-DA score plots, TBBPA and control groups could be separated completely (data not shown) in positive and negative ionization mode, demonstrating that metabolome of TBBPA-treated group was remarkable varied in HepG2 cell line. Metabolites of differential expression were selected according to VIP≥1.5 by SIEVE and SIMCA-P, and further identified according to Biofluid Metabolites Database (https://metlin.scripps.edu/fragment_search.php). The identified potential biomarkers were displayed in Table 1. At the concentration of 10, 20 and 50 μM of TBBPA, five metabolites displayed a significant decrease focusing on the pathway of nucleotide metabolism, including guanine, guanosine, adenine, adenosine and hypoxanthine, whereas xanthine showed significant up-regulation. As shown in Table 1, TBBPA concentration at 50 μM induced 0.31-fold decrease in guanosine which is the most variable of six metabolites. Moreover, TBBPA also induced 2.42-fold increase in xanthine. It suggested that remarkable decreased guanosine and increased xanthine were closely correlated with the generation of 8-oxo-dG.

Table 1. Endogenous differential metabolites induced by TBBPA exposure in HepG2

Compound name, *m/z*	10 μM / Control		20 μM / Control		50 μM / Control	
	P value	Ratio	P value	Ratio	P value	Ratio
adenosine, 268.1613	0.031	0.92	0.102	0.84	0.004	0.85
adenine,136.0467	0.004	0.90	0.004	0.76	0.001	0.72
hypoxanthine, 137.0458	0.192	0.92	0.003	0.67	0.005	0.60
guanine, 152.0567	0.005	0.91	0.002	0.87	0.003	0.70
guanosine, 283.0917	0.032	0.61	0.001	0.45	0.003	0.31
xanthine,153.0407	0.003	1.21	0.028	2.50	0.021	2.42

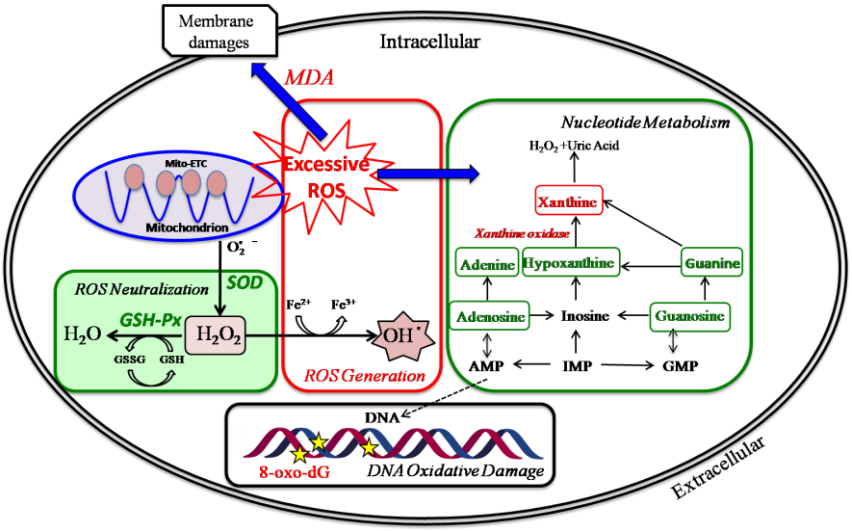

Figure 2. ROS and nucleotide metabolism contribute to HepG2 cytotoxicity induced by TBBPA

3.3 Effect of TBBPA on nucleotide metabolism

We performed metabolomics with a goal to understand the underlying biochemical variations following the TBBPA exposure to discover the biomarkers. The key finding of our study was nucleotide metabolism which was prominent variations associated with TBBPA exposure. Nucleotide plays an essential role in the regulation of DNA replication in human cells. The imbalance of nucleotide level reduces the fidelity of DNA replication and induces mitochondrial depletion and oxidative stress-related DNA damage. In our study, purine showed more perturbations in the nucleotide metabolism relative to pyrimidine. Purine bases can be degraded to uric acid by nucleoside phosphorylases and nucleotidases, while pyrimidine bases can be degraded to H2O and CO2 completely. Guanine can be degraded to hypoxanthine, xanthine, uric acid and H2O2 by xanthine oxidase. The related overactive xanthine oxidase (gene expression fold is 2.1 at mRNA level; data not shown) and xanthine have potential to generate excess uric acid and ROS. Herein, six major metabolites (adenosine, adenine, hypoxanthine, xanthine, guanine and guanosine) and the mRNA levels of xanthine oxidase changed significantly in TBBPA-treated group following an increased ROS level. These results suggested that TBBPA promotes ROS generation in accordance with the variation of nucleotide metabolism in HepG2 cell line (Figure 2). ROS generation, further induced oxidative damage by attacking cell membrane, DNA, RNA and resulted in cell damage ultimately.

4. Conclusions

In this study, we first analyzed ROS effect and 8-oxo-dG adduct generation by TBBPA exposure on HepG2. Then, we examined the variation of global metabolome by LC-MS/MS. Together, our study suggested that oxidative stress and nucleotide metabolism could play an critical role in HepG2 cytotoxicity due to TBBPA exposure. MS-based global metabolomics analysis is an important approach for the molecular mechanism study.

5. Acknowledgments

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