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Characterization of TCHQ-induced genotoxicity and mutagenesis using the pSP189 shuttle vector in mammalian cells

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ABSTRACT

Tetrachlorohydroquinone (TCHQ) is a major toxic metabolite of the widely used wood preservative, pentachlorophenol (PCP), and it has also been implicated in PCP genotoxicity. However, the underlying mechanisms of genotoxicity and mutagenesis induced by TCHQ remain unclear. In this study, we examined the genotoxicity of TCHQ by using comet assays to detect DNA breakage and formation of TCHQ-DNA adducts. Then, we further verified the levels of mutagenesis by using the pSP189 shuttle vector in A549 human lung carcinoma cells. We demonstrated that TCHQ causes significant genotoxicity by inducing DNA breakage and forming DNA adducts. Additionally, DNA sequence analysis of the TCHQ-induced mutations revealed that 85.36% were single base substitutions, 9.76% were single base insertions, and 4.88% were large fragment deletions. More than 80% of the base substitutions occurred at G:C base pairs, and the mutations were G:C to C:G, G:C to T:A or G:C to A:T transversions and transitions. The most common types of mutations in A549 cells were G:C to A:T (37.14%) and A:T to C:G transitions (14.29%) and G:C to C:G (34.29%) and G:C to T:A (11.43%) transversions. We identified hotspots at nucleotides 129, 141, and 155 in the *supF* gene of plasmid pSP189. These mutation hotspots accounted for 63% of all single base substitutions. We conclude that TCHQ induces sequence-specific DNA mutations at high frequencies. Therefore, the safety of using this product would be carefully examined.

1. Introduction

Pentachlorophenol (PCP) is a major industrial and agricultural biocide that has been used extensively as a wood preservative [1]. PCP has become a ubiquitous environmental pollutant due to its worldwide usage, relative stability and improper disposal [1–3]. PCP has been detected in the bodies of people who are not occupationally exposed to PCP, in addition to workers who manufacture PCP. In these individuals, PCP was detected ubiquitously in both bodily fluids (e.g., urine, serum, milk) and in tissues. Additionally, previous studies demonstrated that PCP is metabolized to tetrachlorohydroquinone (TCHQ) by rodents in vivo [2,4] and by liver enzymes from rats and humans in vitro [4,5]. Although PCP has not shown reactivity towards DNA, both PCP and TCHQ were shown to be tumor promoters in previous studies using a mouse skin model in CD-1 mice [3,6].

Tetrachlorohydroquinone (TCHQ) is a major toxic metabolite of PCP and is derived from PCP by oxidization and dechloridation; TCHQ is then further oxidized via tetrachlorosemiquinone radicals (TCSQ•) to form tetrachloro-1,4-benzoquinone (TCBQ) (Fig. 1). In the presence of oxygen, superoxide radicals can be produced by cycles of autoxidation and reduction between TCHQ and its corresponding semiquinone radical under certain physiological conditions [7,8]. Additionally, TCHQ was found to bind DNA to form DNA adducts, which induce single strand breaks in human fibroblasts [7,9] V79 cells [10], Chinese hamster ovary cells [11], and mouse livers [12,13]. TCHQ is also able to induce micronuclei and mutations at the HPRT locus of V79 cells [14] and induce 8-hydroxy-2-deoxyguanosine (8-OH-dG) formation in V79 cells and B6C3F1 mice [12,15]. At low concentrations, TCHQ reduced colony-forming abilities of human fibroblasts and inhibited cell growth in Chinese hamster ovary cells. *p*53 protein accumulation, glutathione depletion and cellular transformation were also observed in mice treated with TCHQ [13]. These data indicate that TCHQ might be a cytotoxic and genotoxic metabolite of PCP.

However, the mechanisms of genotoxicity and mutagenesis induced by TCHQ remain unclear. In this study, we examined the

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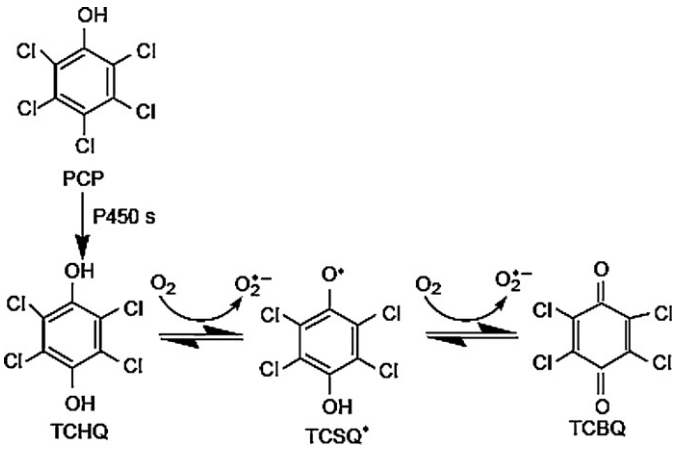

Fig. 1. Diagrams of pentachlorophenol, TCHQ, and THCQ metabolites.

genotoxicity of TCHQ by using comet assays to detect DNA breakage and formation of TCHQ-DNA adducts. Then, we further verified the mutagenesis using the pSP189 shuttle vector in A549 human lung carcinoma cells. From our results, TCHQ could significantly induce DNA breakage and formation of DNA adducts. Additionally, DNA sequencing analysis of TCHQ-induced mutations revealed that 85.36% were single base substitutions, 9.76% were single base insertions, and 4.88% were large fragment deletions. More than 80% of the base substitutions occurred at G:C base pairs, and nucleotides 129, 141 and 155 in the *supF* gene of plasmid pSP189 were identified as hotspots for TCHQ-induced mutations. We conclude that TCHQ induces sequence-specific DNA mutations at high frequencies, which provides a potential mechanism for its mutagenesis. Therefore, the safety of PCP usage should be seriously considered.

2. Materials and methods

2.1. Chemicals and plasmids

TCHQ ($C_{11}H_{10}N_4O_4$, FW 262.23, CAS no. 6804-07-5; 98%) (Provided by Dr. Ben-zhan Zhu) was primarily purchased from Sigma–Aldrich Company, dissolved in acetone, then diluted in serum- and antibiotic-free medium at the desired concentrations. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-1-thiogalactoside (IPTG) were obtained from Sigma. Dulbecco's MEM cell culture medium (DMEM) was obtained from Invitrogen, and fetal bovine serum (FBS) was obtained from Gibco. The *DpnI* restriction endonuclease was purchased from New England Biolabs, and the Qiagen PreSpin Plasmid Kit was purchased from Qiagen.

The pGL2 plasmid (Promega, Madison, WI, USA) carries a luciferase gene driven by the simian virus 40 (SV40) promoter and enhancer sequences, resulting in strong luciferase expression in many types of mammalian cells. The pSV-β-galactosidase plasmid (Promega, Madison, WI, USA) is designed as a positive control vector for monitoring transfection efficiencies of mammalian cells.

The shuttle vector pSP189 [16] and the *Escherichia coli* strain MBM7070 were kindly provided by Dr. Michael M. Seidman (National Institute on Aging, National Institutes of Health, Baltimore, MD, USA). The pSP189 vector contains the *supF* mutagenic target, a tyrosine amber-suppressor tRNA gene flanked by the ampicillin gene and the bacterial origin of replication. The vector also carries an SV40 replication origin and large T-antigen gene.

2.2. Cells and cell culture

This study employed two types of cells: A549 (ATCC, #CCL-185) and Vero (ATCC, #CCL-81) cells. A549 cells were derived from a human lung carcinoma, and Vero cells were initiated from the kidney of a normal adult African green monkey. Both cell lines were provided by the Center for Cell Resources at the Shanghai Institutes for Life Sciences/Cell bank of China Center for Type Culture Collection, Chinese Academy of Sciences (CTCCCAS). A549 and Vero cell lines were routinely cultured in RPMI-1640 medium and DMEM medium (Invitrogen, USA) respectively. All media was supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin, and all cultures were maintained at 37 °C in a humidified mixture of 5% CO_2 and 95% air.

2.3. Single cell gel electrophoresis (comet assay)

The extent of DNA damage caused by TCHQ was determined using the comet assay as described below. After treatment with TCHQ, cells were suspended in 1% low-melting point agarose (LMPA) at a density of 10^6 cells/mL. The cell suspension was then pipetted onto traditional glass microscope slides that were pre-coated with 0.5% normal-melting point agarose (NMP). After addition of a third layer of agarose (1% LMPA), slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100 v/v at pH 10). Slides were then placed in a horizontal electrophoresis tank with freshly prepared cold electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) for 30 min at 4 °C and subjected to electrophoresis (20 V) for 15 min. Slides were then washed three times with 0.4 M Tris (pH 7.5) to remove excess running buffer. Afterwards, slides were stained with ethidium bromide (20 μg/mL) and scored by fluorescence microscope with an excitation filter of 515–560 nm and a barrier filter of 590 nm. DNA damage levels were evaluated according to the head and tail features of the cells.

2.4. Detection of DNA single-strand breaks (SSB)

Formation of DNA single-strand breaks was measured by conversion of supercoiled ΦX174 DNA into a nicked, open circular form. A standard reaction contained 0.3 μg of supercoiled plasmid DNA, 25 mM MOPS or phosphate buffer (pH 7.0) and the indicated concentrations of TCHQ in a total volume of 25 μl. After incubation at 37 °C for 60 min, samples were placed on ice and loaded onto 1% agarose TAE gels. DNA bands were stained with ethidium bromide and analyzed using an Alpha-Innotech FluorChem™ FC2 gel screening system.

2.5. Treatment of DNA with TCHQ in vitro

30 μg pSP189 DNA or pGL2 DNA were treated with TCHQ at distinct concentrations (10, 25, 50, 100, 200, 300 and 500 μM) and 0.1% acetone in 0.5 mL of TE buffer [10 mM Tris–HCl and 1 mM EDTA (pH 7.8)]. The reaction was carried out at 37 °C for either 0.5 or 1 h in the dark. After incubation, the plasmids were precipitated by ethanol, filtered through SUPER™-02 (Takara, Japan) to remove excess TCHQ, and redissolved in TE buffer at a concentration of 0.1 μg/μL and stored at −20 °C. The TCHQ-modified pGL2 plasmid was used for host cell reactivation assays and the TCHQ-modified pSP189 was used for *SupF* mutagenesis assays.

2.6. Host cell reactivation assay

We used a previously described host cell reactivation assay to evaluate DNA damage repair capabilities [17,18]. Briefly, TCHQ-modified pGL2 plasmid (1.8 μg) (for monitoring repair of TCHQ-DNA adducts) and pSV-β-galactosidase plasmid (0.2 μg) (for monitoring transfection efficiency) were co-transfected into cells (cultured in a 35 mm dish at a density of 3×10^5 cells/dish) by using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfection procedure has been previously described in detail [19]. For repair of TCHQ-DNA adducts, transfected cells were incubated for another 8 and 24 h, as indicated. Luciferase and β-galactosidase activities were determined by the Luciferase Assay System and the β-galactosidase Enzyme Assay System (Promega, Madison, WI, USA), respectively, according to the provided standard protocols. The DNA repair capability was evaluated by the removal of TCHQ-DNA adducts, which correlates with the recovery of luciferase activity. Relative luciferase activity was defined as [(luciferase activity/β-galactosidase activity) in cells transfected with TCHQ-modified pGL2/(luciferase activity/β-galactosidase activity) in cells transfected with un-modified pGL2] × 100%.

2.7. SupF mutagenesis assay

The pSP189 shuttle vector carries a *supF* gene as a mutation target for studying mutagenesis in mammalian cells [16]. The mutagenicity of the TCHQ-modified pSP189 was determined as previously described [20] with some modifications. Briefly, pSP189 (5 μg) with or without TCHQ modification was transfected into A549 cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). After a 48 h period to allow repair of TCHQ-DNA adducts and replication of plasmids, pSP189 plasmids were rescued from the cells by the Promage Plasmid Purification Kit, and then digested with *DpnI* (Promage, 20 U) to remove any parental DNA introduced by transfection.

The recovered plasmid DNA was transformed into *E. coli* MBM7070 bacteria using the calcium chloride method. *E. coli* MBM7070 carries an amber-mutated *lacZ* gene as an indicator of *supF* compensation from the pSP189 plasmid. Transformed *E. coli* were then assayed for ampicillin resistance and mutations in the *supF* gene by plating on agar plates containing ampicillin (100 μg/mL), X-gal (50 mg/mL), and IPTG (200 mg/mL) and incubating at 37 °C for 24 h. *E. coli* carrying plasmids with *supF* mutations formed white or light blue colonies, whereas *E. coli* carrying plasmids with a functional *supF* gene formed blue colonies. All colonies were counted and mutation frequencies were calculated. Plasmid DNA samples were extracted and purified from the above white and light blue colonies that were derived from spontaneous or TCHQ-induced mutants, respectively. The resulting DNA sequences of 47 mutants, including six from control groups, were determined using di-deoxy sequencing with