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Diepoxybutane induces the formation of DNA–DNA rather than DNA–protein cross-links, and single-strand breaks and alkali-labile sites in human hepatocyte L02 cells

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ABSTRACT

1,3-Butadiene (BD) is an air pollutant and a known carcinogen. 1,2,3,4-Diepoxybutane (DEB), one of the major in vivo metabolites of BD, is considered the ultimate culprit of BD mutagenicity/carcinogenicity. DEB is a bifunctional alkylating agent, being capable of inducing the formation of monoalkylated DNA adducts and DNA cross-links, including DNA–DNA and DNA–protein cross-links (DPC). In the present study, we investigated DEB-caused DNA cross-links and breaks in human hepatocyte L02 cells using comet assay. With alkaline comet assay, it was observed that DNA migration increased with the increase of DEB concentration at lower concentrations (10–200 μM); however, at higher concentrations (200–1000 μM), DNA migration decreased with the increase of DEB concentration. This result indicated the presence of cross-links at >200 μM, which was confirmed by the co-treatment experiments using the second genotoxic agents, *tert*-butyl hydroperoxide and methyl methanesulfonate. At 200 μM, which appeared as a threshold, the DNA migration-retarding effect of cross-links was just observable by the co-treatment experiments. At <200 μM, the effect of cross-links was too weak to be detected. The DEB-induced cross-links were determined to be DNA–DNA ones rather than DPC through incubating the librated DNA with proteinase K prior to unwinding and electrophoresis. However, at the highest DEB concentration tested (1000 μM), a small proportion of DPC could be formed. In addition, the experiments using neutral and weakly alkaline comet assays showed that DEB did not cause double-strand breaks, but did induce single-strand breaks (SSB) and alkali-labile sites (ALS). Since SSB and ALS are repaired more rapidly than cross-links, the results suggested that DNA–DNA cross-links, rather than DPC, were probably responsible for mutagenicity/carcinogenicity of DEB.

1. Introduction

1,3-Butadiene (BD), a colorless gas, is a petrochemical that is manufactured in high volume and used primarily in industrial production of synthetic rubber and plastics. As an air pollutant, it is listed as one of 188 "Air Toxic" by the U.S. Environmental Protection Agency (EPA). It has been classified as a human carcinogen by EPA in 2002 [1] and by the International Agency for Research on Cancer (IARC) in 2008 [2]. The environmental sources of BD include automobile exhaust, cigarette smoke, and exhaust from biomass burning [1]. Thus, the presence of BD at low levels (0.1–10 ppb) in ambient air in urban locations is ubiquitous [1].

BD itself is not a direct-acting carcinogen; its carcinogenicity stems from the in vivo metabolites [3]. The initial biotransformation step of BD is cytochrome P450-mediated oxidation to form 3,4-epoxy-1-butene (EB) [4]. EB can be further metabolized to 1,2,3,4-diepoxybutane (DEB) [4,5] or 3-butene-1,2-diol (BDD) [4,6]. Hydrolysis of DEB by epoxide hydrolase or oxidation of BDD by cytochrome P450 produces 3,4-epoxy-1,2-butanediol (EBD) [4,7].

EB, DEB, and EBD, which can react with DNA to form a variety of adducts due to their electrophilic nature [8], are all direct-acting

Abbreviations: AGT, O^6-alkylguanine DNA-alkyl transferase; ALS, alkali-labile sites; BD, 1,3-butadiene; BDD, 3-butene-1,2-diol; DEB, 1,2,3,4-diepoxybutane; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPC, DNA–protein cross-links; DSB, double-strand breaks; EB, 3,4-epoxy-1-butene; EBD, 3,4-epoxy-1,2-butanediol; FA, formaldehyde; FBS, fetal bovine serum; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; ICL, interstrand cross-links; MMS, methyl methanesulfonate; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PI, propidium iodide; PK, proteinase K; RCE, relative cloning efficiency; SSB, single-strand breaks; tBHP, *tert*-butyl hydroperoxide; %Tail DNA, DNA percentage in the tail.

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mutagens/carcinogens [9,10], with DEB being the most potent one. It has been reported that DEB is 50- to 100-fold more mutagenic than EB and 100- to 200-fold more mutagenic than EBD [9,10]. Therefore, DEB is considered to be the ultimate metabolite that is responsible for BD mutagenicity/carcinogenicity [11]. As a result, DEB has long been the focus in the studies of mutagenicity/carcinogenicity of BD.

DEB is a bifunctional alkylating agent [12] and readily reacts with nucleic acid bases, nucleosides, and calf thymus DNA to form a variety of monoalkylated adducts [13–16] and cross-links [17–19]. Monoalkylated adducts are generally predominant, especially at higher DEB concentrations [19]. However, the formation of monoalkylated adducts cannot explain the enhanced genotoxic potency and distinct mutational spectrum of DEB, since EB and DEB both induce similar types and numbers of DNA adducts [14,20]. Consequently, strong mutagenicity/carcinogenicity of DEB is usually attributed to its cross-linking capability [3,19]. Therefore, it is very important to scrutinize the ability of DEB to form cross-links.

DEB is capable of generating two types of cross-links, DNA–DNA and DNA–protein cross-links (DPC). Since 1960s DEB has been known to induce DNA–DNA cross-linking [21]. The first DNA–DNA cross-linking product detected in vivo was 1,4-*bis*-(guan-7-yl)-2,3-butanediol [19,22]. Afterwards, another cross-linking product, 1-(aden-1-yl)-4-(guan-7-yl)-2,3-butanediol, was detected in livers of female B6C3F1 mice exposed to 625 ppm BD for two weeks [23]. However, 1,4-*bis*-(guan-7-yl)-2,3-butanediol was the predominant cross-linking product formed in vivo [22,24]. On the other hand, DEB can also induce the formation of DPC [25]. The formation of DPC induced by DEB was first found with alkaline elution assay in 1989 [26]. In 2004, it was observed that the genotoxicity of DEB was enhanced in the presence of O^6-alkylguanine DNA-alkyl transferase (AGT), suggesting that DEB induced DNA–AGT cross-links [27]. The formation of DNA–AGT cross-links was confirmed later by other authors and the structures of the cross-links were determined [28]. In addition, DEB could also induce the formation of cross-linking products of DNA with other proteins, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [29] and histones [30]. Most recently, in a proteomic study 39 human proteins were identified to form covalent cross-linking products with DNA in the presence of DEB [31].

While it is clear that DEB can induce the formation of both DNA–DNA cross-links and DPC, it is still unknown whether the two types of cross-links are formed in similar yields in vivo, and if not, which one is formed predominantly. This is an important issue because it may be critical to understand the biological effects of DEB, and it may also affect the choice of the biomarkers for BD exposure [22]. It could be difficult, however, to investigate the issue on animals exposed to BD, because the yield of DEB in animals was extremely low [32]. An alternative is to investigate the issue in mammalian cells. However, so far, few studies have been performed at the cellular level. In 1996, Kligerman et al. investigated the cytogenetic effects of EB and DEB in rat and mouse splenocytes and failed to find any statistically significant DNA-damaging effects as measured by comet assay [33]. Recently, Cemeli and colleagues investigated genotoxicity of EB, DEB, and styrene oxide in human lymphocytes using the comet assay and observed the cross-linking effect of DEB at 1000 μM [34].

Several techniques can be used to investigate the cross-linking effect of a chemical at the cellular level. One such technique is comet assay, or the single-cell gel electrophoresis (SCGE) [35], which is a simple and sensitive technique to assess DNA damage at the level of the single cell. In alkaline comet assay, cells embedded in agarose on a microscope slide are lysed by detergents, and the resulted DNA is subjected to unwinding and electrophoresis under strongly alkaline condition (pH > 13) to cause migration. A genotoxic agent induces strand breaks, thus leading to an increase in DNA migration. The DNA migration can be quantitated through staining DNA with a fluorescence dye and analyzing the results under a fluorescence microscope. The formation of cross-links will retard DNA migration; a decrease in DNA migration thus indicates the presence of cross-links. DNA–DNA cross-links and DPC can be distinguished by incubating lysed cells with proteinase K (PK) prior to unwinding and electrophoresis [35,36]. Exposure to PK can reduce or eliminate DPC (thus partly or completely restoring DNA migration), while having no effect on DNA–DNA cross-links. In addition, comet assay can also be used to detect other types of DNA lesions. For example, neutral and weakly alkaline comet assays can be used to examine the presence of double-strand breaks (DSB) [37–39] and single-strand breaks (SSB)/alkali-labile sites (ALS) [35,40], respectively.

In the present study, we set out to investigate the DNA cross-linking capability of DEB and to determine the type of DEB-induced cross-links in human hepatocyte L02 cells using comet assay. We also examined if DEB induced DSB, SSB, and ALS using neutral and weakly alkaline comet assays.

2. Materials and methods

2.1. Materials

Racemic DEB (CAS No. 1464-53-5) was obtained from Alfa Aesar. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (GIBCO®). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Triton X-100, agarose, propidium iodide (PI), etoposide, methyl methanesulfonate (MMS), and PK were purchased from Sigma–Aldrich. Other reagents, which were of analytical reagent grade, were obtained from Sinopharm Chemical Reagent Company (Shanghai, China). Co-60 γ-ray radiation (60 Gy) was carried out at 15.8 Gy/min in the Second Military Medical University, Shanghai, China. Fresh DEB stock solution in water was prepared just before experiments to avoid hydrolysis. Etoposide was dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution at 5 mM.

2.2. Cell culture

The immortalized human normal hepatocyte cell line L02 was the gift of Professor Ping-Kun Zhou (Beijing Institute of Radiation Medicine, Beijing, China). The cell line has been used in many studies to evaluate the biological effects of xenobiotics and also to investigate the cellular signaling pathways [41–46]. Cells were cultured as monolayers in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2 and routinely passaged by trypsinization when nearly confluent. When cells grew to ~80% confluence, the culture medium was discarded, and FBS-free medium containing the chemical tested was supplemented. Cells were then incubated at 37 °C for specified time.

2.3. Cytotoxicity

Cytotoxicity was assessed with MTT assay. Cells were treated with different concentrations of DEB for specified time, and then the medium was removed and a solution of 10 μl MTT (5 mg/ml) in 90 μl FBS-free DMEM per well was added. After incubation at 37 °C for 4 h, the medium was discarded and 100 μl DMSO per well was added. The plates were shaken at ambient temperature for 3 min, and the optical density at 490 nm was recorded.

Relative cloning efficiency (RCE) as a measure for long-term survivability was determined as follows: cells (200 for most experiments, but 2000 for DEB at 200 μM, and 5000–10,000 for DEB at 500, 800, and 1000 μM) were plated into petri dishes. The next day cells were treated with FBS-free fresh medium containing the chemical tested at 37 °C for 1 h. After ten days, colonies were fixed, stained, and counted. Survival rate was determined relative to the corresponding control.

2.4. Comet assay

2.4.1. Alkaline comet assay

Alkaline comet assay was performed according to the recommended procedure [35]. About 30,000 cells in 30 μl DMEM were mixed with 140 μl low melting point agarose (1%) and were added to frosted glass slides precoated with normal melting point agarose. Cells were lysed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10; 1% Triton X-100 and 10% DMSO were added fresh) at 4 °C for 1 h. The slides were placed in electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH > 13) at 4 °C for 40 min, and then electrophoresis was carried out at 25 V (~300 mA) for 20 min. After neutralization with 400 mM Tris (pH 7.5), DNA was stained with 40 μl PI (5 μg/ml) and analyzed using a fluorescence microscope (Olympus BX-51, Japan).