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Subchronic exposure to ethyl tertiary butyl ether resulting in genetic damage in Aldh2 knockout mice

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ABSTRACT

Ethyl tertiary butyl ether (ETBE) is biofuel additive recently used in Japan and some other countries. Limited evidence shows that ETBE has low toxicity. Acetaldehyde (AA), however, as one primary metabolite of ETBE, is clearly genotoxic and has been considered to be a potential carcinogen. The aim of this study was to evaluate the effects of ALDH2 gene on ETBE-induced genotoxicity and metabolism of its metabolites after inhalation exposure to ETBE. A group of wild-type (WT) and Aldh2 knockout (KO) C57BL/6 mice were exposed to 500 ppm ETBE for 1–6 h, and the blood concentrations of ETBE metabolites, including AA, tert-butyl alcohol and 2-methyl-1,2-propanediol, were measured. Another group of mice of WT and KO were exposed to 0, 500, 1750, or 5000 ppm ETBE for 6 h/day with 5 days per weeks for 13 weeks. Genotoxic effects of ETBE in these mice were measured by the alkaline comet assay, 8-hydroxyguanine DNA-glycosylase modified comet assay and micronucleus test. With short-term exposure to ETBE, the blood concentrations of all the three metabolites in KO mice were significantly higher than the corresponding concentrations of those in WT mice of both sexes. After subchronic exposure to ETBE, there was significant increase in DNA damage in a dose-dependent manner in KO male mice, while only 5000 ppm exposure significantly increased DNA damage in male WT mice. Overall, there was a significant sex difference in genetic damage in both genetic types of mice. These results showed that ALDH2 is involved in the detoxification of ETBE and lack of enzyme activity may greatly increase the sensitivity to the genotoxic effects of ETBE, and male mice were more sensitive than females.

1. Introduction

Ethyl tertiary butyl ether (ETBE) is now used in automobile fuel as an oxygen-containing compound in some developed countries, in order to increase oxygen content of gasoline and reduce the emission of pollutants from automobiles. Inhalation of automobile exhaust emissions is a major pathway for exposure to ETBE by the general public. Another pathway is exposure from water contaminated with ETBE due to its mobility (van Wezel et al., 2009). Inhalation is an important way of occupational exposure among workers. With the extensive production and widespread use of ETBE, the possible adverse effects of ETBE exposure have become a public concern.

Limited evidences show that ETBE is nontoxic or has low toxicity (McGregor, 2007). However, methyl tertiary butyl ether (MTBE) which is the ethyl analog of ETBE, has been reported to have toxic potential. For example, oral exposure to MTBE caused dose-related increases of lymphoma, leukemias, and testicular Leydig cell cancers in rats (Belpoggi et al., 1995), and inhalation exposure to MTBE resulted in an increase of liver tumors in mice (Burleigh-Flayer et al., 1992). In addition, tertiary butyl alcohol (TBA), an oxidative metabolite of both MTBE and ETBE, has been reported to induce renal tumors in male rats (Takahashi et al., 1993). As shown in Fig. 1, MTBE is composed of tert-butyl group and methoxyl group, and ETBE tert-butyl group and ethoxyl group, so, the similarity in

Abbreviations: ETBE, ethyl tertiary butyl ether; AA, acetaldehyde; ALDH2, aldehyde dehydrogenase2; WT, wild-type; KO, knockout; TBA, tert-butyl alcohol; MPD, 2-methyl-1,2-propanediol; MTBE, methyl tertiary butyl ether; IARC, International Agency for Research on Cancer; hOGG1, 8-hydroxyguanine DNA-glycosylase; MN-RETs, micronucleated reticulocytes; MN-NCE, micronuclei in the mature normochromatic erythrocyte.

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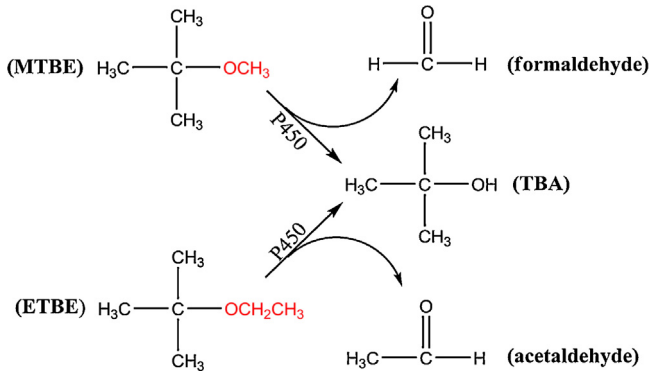

Fig. 1. Structural diagram of MTBE and ETBE and their primary metabolites. Formaldehyde is classified into group 1 as carcinogenic to humans by IARC. Acetaldehyde is classified into group 2B as a possibly carcinogenic to humans by IARC. Furthermore, IARC considers that acetaldehyde associated with consumption of alcoholic beverages is carcinogenic to humans (group 1).

their chemical structures between ETBE and MTBE suggests that the toxicological properties of ETBE may be similar to those of MTBE.

It is clear that more studies are needed to evaluate the overall toxicity of ETBE, especially the potential genotoxic effects. Another important reason is that acetaldehyde (AA) shown in Fig. 1, the primary metabolite of ETBE, is clearly genotoxic and has been considered to be a potential carcinogen in humans and experimental animals by the International Agency for Research on Cancer (IARC) Working Group (Baan et al., 2007). Because AA is also the primary oxidative metabolite of ethanol, there has long been considerable interest in AA metabolism. AA is rapidly metabolized to acetic acid via NAD-linked aldehyde dehydrogenase2 (ALDH2) (Zorzano and Herrera, 1990; Rashkovetsky et al., 1994). ALDH2 enzyme activity involved in the metabolism of AA are subject to genetic polymorphisms, and the mutant ALDH2 shows extremely low or no activity due to an amino acid substitution of lysine for glutamine at position 487 of the protein following a base change G→A (Seitz and Stickel, 2007). A recent study showed that the blood concentration-time curve for AA in *ALDH2*2* homozygotes is 220- and 4.7-fold greater than those of *ALDH2*1* homozygotes and the heterozygotes, respectively, during 130 min post-ingestion (Chen et al., 2009). Epidemiologic studies of cancer indicate that *ALDH2*1/2* heterozygotes who consume ethanol develop upper aerodigestive tract cancer, particularly the oesphageal cancer as a high-risk group (Yokoyama et al., 1998, 2002, 2006; Matsuo et al., 2001). Moreover, functional studies also support these conclusions regarding *ALDH2* polymorphisms and their reference to cancer risk. The habitual drinkers with *ALDH2*1/2* are associated with the higher levels of AA-derived DNA adducts (Matsuda et al., 2006) and DNA strand breaks (Weng et al., 2010), and increased frequencies of sister chromatid exchanges (Morimoto and Takeshita, 1996) and micronuclei (Ishikawa et al., 2007) in peripheral lymphocytes compared with ALDH2*1/1. Furthermore, IARC (2012) considers that acetaldehyde associated with consumption of alcoholic beverages is carcinogenic to humans (group 1). It is worth mentioning that these reports were mainly based on data collected from Asia population. There are about 30–50% of the East Asians bearing the variant allele of *ALDH2* (*ALDH2*2*) (Brennan et al., 2004). These data suggest that it is possible that the *ALDH2* variant allele may increase the risk of health effects in ETBE-exposed individuals, especial in East Asians.

There were no data available regarding *Aldh2* polymorphisms related to the metabolism and genetic toxicology of ETBE before we conducted this study. We used *Aldh2* knockout mice as a model of ALDH2-deficient humans to analyze the effects of *Aldh2* polymorphisms on the metabolites of ETBE, including AA, TBA and 2-methyl-1,2-propanediol (MPD), and further to clarify the influence of *Aldh2* polymorphisms on the genotoxic effects of ETBE exposure.

2. Materials and methods

2.1. Animals and reagents

During the experiment, we followed the guidelines for the care and use of laboratory animals set forth by our Institutional Animal Care and Use Committee of Japan National Institute of Occupational Safety and Health. *Aldh2* wild type (WT) of C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan) and the *Aldh2* knockout (KO) mice of the same line were generated as described previously (Kitagawa et al., 2000). ETBE was purchased from Tokyo Kasei (>97% pure; Tokyo, Japan). MicroFlow^{PLUS} Kit (Mouse blood) was purchased from Litron Laboratories (Rochester, NY), containing mouse anti-CD71 antibody, mouse platelet antibody, diluents solution, RNase solution, propidium iodide (PI) solution, and biological standards (positive control samples, negative control samples, CD71-setup samples, and fixed malaria-infected bio-standard blood samples).

2.2. Blood concentration of AA, TBA and MPD

In experiment #1, 20 KO and 20 WT C57BL/6 mice of both sexes, 8 weeks old, were used. The mice were exposed to 500 ppm ETBE by single inhalation exposure for 1–6 h, and then sacrificed at 1, 2 and 4 h from start of exposure and at 0, 2, 4, 6, 8 and 24 h after the 6 h exposure. At each time point, a blood sample (0.1 ml) was collected from mice under anesthesia with pentobarbital, into 20 ml-head space vial including 0.1 ml 0.6 N perchloric acid to prevent reaction with the protein, and then neutralized with 6 M-KOH. Metabolites of ETBE, AA, TBA, and MPD in blood were measured by head space gas chromatography with mass selective detector (G1888: Agilent Technologies Japan, Ltd., Tokyo, Japan; GC HP6890 series and MSD HP5973: Hewlett-Packard Japan, Ltd., Tokyo, Japan) fitted with a separation column (DB-624, 60 m × 0.25 mm × 1.4 μm, Agilent Technologies Japan, Ltd., Tokyo, Japan). Samples were injected in the splitless mode and eluted from the column at 35 °C with a flow rate of 1 ml/min using helium as the carrier gas. Sodium sulfate was added to the vials for salting out. For MPD analysis, samples were heated under hydrochloric acid at 90 °C for 30 min to transform dehydrate to iso-butylaldehyde (Amberg et al., 1999) and sodium chloride was used for the salting out in this assay. The area under the curve (AUC) of the blood AA, TBA and MPD concentrations were calculated by the trapezoidal method from the exposure start to 24 h after exposure.

2.3. Thirteen-week inhalation exposure to ETBE

In experiment #2, there were 3 exposure groups and a control group for each sex and genetic type, with five mice in each group. Inhalation exposures were conducted in stainless steel chambers (Sibata Scientific Technology, Tokyo, Japan). The target ETBE-exposure concentrations of 500 ppm (low), 1750 ppm (middle) and 5000 ppm (high) were monitored using a gas chromatograph (Shimadzu GC-7A, Kyoto) and adjusted with flow meters to a constant target value ± 5% throughout the exposure. The control group was exposed to filtered air only. These mice were exposed for 6 h per day, 5 consecutive days per week, for 13 weeks. Mean exposure concentrations/13 weeks were expressed as mean values ± SD (ppm). WT male mice: 0 ppm, 500 ± 24.7 ppm, 1757.2 ± 101.1 ppm, and 4999.0 ± 239.3 ppm; KO male mice: 0 ppm, 498.4 ± 26.1 ppm, 1753.6 ± 102.8 ppm, and 4979.2 ± 305.7 ppm; WT female mice: 0 ppm, 499.2 ± 25.9 ppm, 1758.6 ± 97.8 ppm, and 4984.9 ± 302.9 ppm; KO female mice: 0 ppm, 498.2 ± 26.0 ppm, 1752.8 ± 101.5 ppm, and 4976.6 ± 303.9 ppm.

2.4. The alkaline comet assay

Mice were sacrificed under anesthesia 20–24 h after the last ETBE exposure. Blood samples from the postcaval vein were collected into coded tubes containing an anticoagulant EDTA and kept on ice before the analysis of DNA damage. We carried out the alkaline comet assay according to the method described by Singh et al. (1988) with slight modifications. Briefly, 5 μl of fresh whole blood was gently mixed with 200 μl of 1% molten low melting agarose (Sigma) at 38 °C and immediately 30 μl pipetted onto a 20 well CometSlide (Trevigen, Gaithersburg, MD). After placing slides flat in refrigerator (4 °C) for 15 min, the slides were treated as described by Singh et al., 1988. The electrophoresis time was 15 min under 21 V (1 V/cm) and approximately 250 mA using a Trevigen's CometAssay Electrophoresis System (Trevigen). After electrophoresis, slides were neutralized by flooding with three changes of neutralization buffer (0.4 mol/l Tris, pH 7.5) for 5 min with each wash. Each circle of the slides were then stained with 50 μl SYBR Green I (Trevigen) for 5 min. Slides were scored using a Comet IV capture system (Perceptive Instruments, Suffolk, England) and 100 cells were scored per sample. The tail intensity (TI), defined as the percentage of DNA migrated from the head of the comet into the tail and considered to be the most useful parameter in the comet assay (Collins et al., 2008), were measured for each nucleus scored.