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Aldh2 Knockout Mice Were More Sensitive to DNA Damage in Leukocytes due to Ethyl Tertiary Butyl Ether Exposure

Zuquan WENG[1], Megumi SUDA[1], Katsumi OHTANI[1], Nan MEI[2], Toshihiro KAWAMOTO[3], Tamie NAKAJIMA[4] and Rui-Sheng WANG[1]*

[1]National Institute of Occupational Safety and Health, 6–21–1 Nagao, Kawasaki 214-8585, Japan
[2]Department of Genetic and Molecular Toxicology, National Center for Toxicological Research, Jefferson, AR, USA
[3]Department of Environmental Health, University of Occupational and Environmental Health, Kitakyushu, Japan
[4]Department of Occupational and Environmental Health, Nagoya University, Nagoya, Japan

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Abstract: To clarify the genotoxicity of ethyl tertiary butyl ether (ETBE), a gasoline additive, male and female C57BL/6 mice of *Aldh2*+/+ and *Aldh2*–/– genotypes, aged 8 wk, were exposed to 0, 500, 1,750, or 5,000 ppm ETBE for 6 h/day, 5 d per week for 13 wk. DNA damage in leukocytes was measured by the alkaline comet assay and expressed quantitatively as Tail Intensity (TI). For male mice, TI was significantly higher in all three groups exposed to ETBE than in those without exposure within *Aldh2*–/– mice, whereas within *Aldh2*+/+ mice, TI increased only in those exposed to 5,000 ppm of ETBE as compared with mice without exposure. For female mice, a significant increase in TI values was observed in the group exposed to 5,000 ppm of ETBE as compared with those without exposure within *Aldh2*–/– mice; TI in *Aldh2*–/– mice exposed to 1,750 and 5,000 ppm was significantly higher than in *Aldh2*+/+ mice without exposure. TI did not significantly increase in any of the groups exposed to ETBE within female *Aldh2*+/+ mice. Based on the results we suggest that *Aldh2*–/– mice are more sensitive to DNA damage caused by ETBE than *Aldh2*+/+ mice and that males seem more susceptible to this effect than females.

Key words: Ethyl tertiary butyl ether, Genotoxicity, *Aldh2* knockout mice, Comet assay, Leukocyte, Aldehyde dehydrogenase, Acetaldehyde

Ethyl tertiary butyl ether (ETBE) is used as a gasoline additive in order to reduce the emission of carbon monoxide, hydrocarbons, polycyclic aromatics, oxides of nitrogen and particulate carbon from the exhaust gases without lowering the octane number[1]. However, the extensive production and increasing use of ETBE may cause environmental pollution, resulting in an increase in the potential exposure for not only occupational workers but also the general population. Currently, there are several reports indicating that ETBE has low toxicity or is non-toxic[2], but the information about the health effects of ETBE does not yet meet the scientific needs.

ETBE is metabolized to tertiary butyl alcohol (TBA) and acetaldehyde[3]. The intermetabolite acetaldehyde is considered to be a potential carcinogen in humans and experimental animals[4]. Acetaldehyde is rapidly catalyzed by aldehyde dehydrogenase2 (ALDH2)[5, 6] and this may explain the minimal or non-toxic effects of ETBE observed in some studies[2]. However, about 40% of the East Asians have no or low activity of ALDH2[6].

*To whom correspondence should be addressed.
E-mail: wang@h.jniosh.go.jp

In the present study, *Aldh2* knockout mice were used as a model of ALDH2-deficient humans to clarify the influence of *Aldh2* gene polymorphisms on the genotoxic effects of ETBE exposure.

Male and female *Aldh2* knockout (*Aldh2*–/–) and wild-type C57BL/6 (*Aldh2*+/+) mice, 8 wk old, were used. *Aldh2*+/+ mice were purchased from Charles River Japan (Yokohama, Japan) and the *Aldh2*–/– mice were generated as described previously[7]. 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 the Japan National Institute of Occupational Safety and Health. The study design consisted of 3 exposure groups and a control group for each of the sexes and genetic types, with five mice in each group. ETBE was purchased from Tokyo Kasei (>97% pure). Inhalation exposures were conducted in stainless steel chambers (Sibata Scientific Technology, Tokyo). The target exposure concentrations of ETBE, 500 ppm (low), 1,750 ppm (middle) and 5,000 ppm (high), were monitored using a gas chromatograph (Shimadzu GC-7A, Kyoto) during the exposure hours. The control group was exposed to filtered air only. Mice were exposed for 6 h per day, 5 consecutive days per week, for 13 wk.

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.*[8] with slight modifications. Briefly, 5 μl of fresh whole blood were gently mixed with 200 μl of 1% molten low melting agarose (Sigma) at 38°C and 30 μl samples were immediately pipetted onto a 20-well CometSlide (Trevigen, Gaithersburg, MD, USA). After placing slides flat in a refrigerator (4°C) for 15 min, the slides were treated as described by Singh *et al*[8]. The electrophoresis time was 15 min under 21 V (1 V/cm) and approximately 250 mA using a Trevigen 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 of 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 which is considered to be the most useful parameter in the comet assay[9], was measured for each nucleus scored.

All data were analyzed using the SPSS statistical package (SPSS, Chicago, USA). A two-way analysis of variance (ANOVA) test was used to evaluate the effects of genotypes and doses of ETBE. Comparisons among multiple groups were made using the one-way ANOVA test. If the results of ANOVA were statistically significant, Dunnett's post hoc test was performed to determine which exposure groups were different from the control group. The level of statistical significance was set at $p<0.05$.

The effects of ETBE in *Aldh2* genotypes on DNA damage in leukocytes as assessed by TI values are shown in Fig. 1 for male and female mice. Two-way ANOVA showed that both genotypes and dose of ETBE significantly affected TI values in male mice ($F=113.53$, and 17.87, respectively, $p<0.001$). One-way ANOVA followed by Dunnett's post hoc test indicated that TI values were significantly higher in all four groups of *Aldh2*–/– mice as compared with the

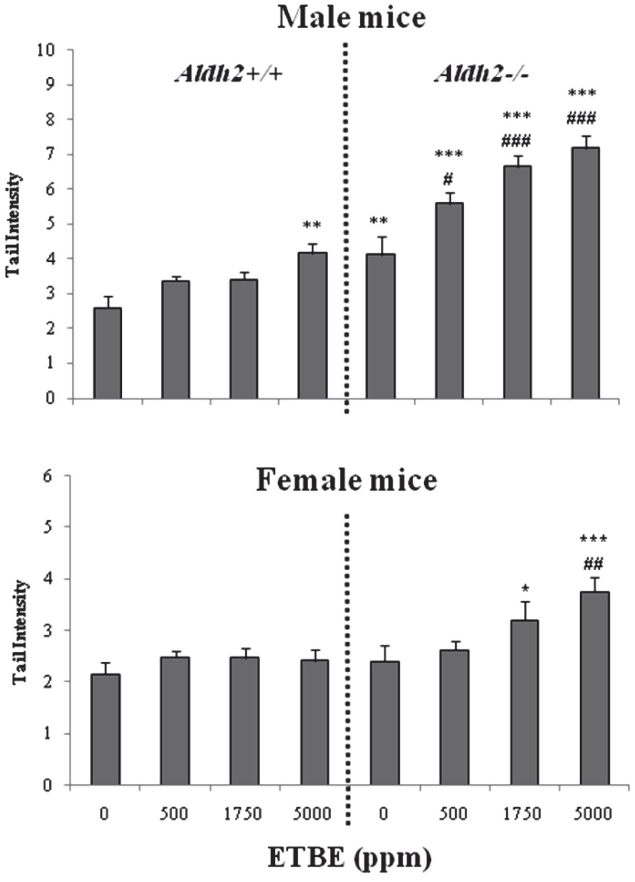

Fig. 1. Effects of ETBE on Tail Intensity in leukocytes in *Aldh2* genotypes of male and female mice (Mean values with standard errors).
*$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared with the *Aldh2*+/+ group without ETBE exposure (Dunnett's post hoc test).
#$p<0.05$, ##$p<0.01$, ###$p<0.001$ as compared with the *Aldh2*–/– group without ETBE exposure (Dunnett's post hoc test).

group of *Aldh2*+/+ mice without exposure to ETBE. Within the *Aldh2*+/+ mice groups, the TI value in the 5,000 ppm ETBE group was significantly increased by 60% as compared with mice without ETBE exposure. Within the *Aldh2*–/– mice groups, on the other hand, TI values in the 500, 1,750 and 5,000 ppm ETBE groups were increased by 40%, 60% and 70%, respectively, as compared with mice without exposure; the differences between the each of exposure groups and those without exposure were statistically significant.

For female mice, two-way ANOVA analysis gave results which were similar to those for male mice, i.e., both genotypes and the dose of ETBE significantly increased TI values (F=11.20, and 3.69, respectively, $p<0.05$). One-way ANOVA followed by Dunnett's post hoc test indicated that TI values were significantly increased in the 1,750 and 5,000 ppm groups of *Aldh2*–/– mice as compared with *Aldh2*+/+ mice without exposure to ETBE. Within the *Aldh2*+/+ mice groups, however, no significant differences in TI values were observed between each of the three groups exposed to ETBE and the controls without ETBE exposure. Within the *Aldh2*–/– mice groups, on the other hand, 5,000 ppm group showed a significant increase in the TI value (60%).

Chronic exposure to ETBE at a high concentration (5,000 ppm) induced genetic damage in leukocytes in *Aldh2*+/+ male mice. However, in mice lacking ALDH2 enzyme activity, the DNA damage was detectable even at the lowest dose of ETBE (500 ppm), suggesting that low ALDH2 activity due to genetic polymorphisms may greatly increase the risk of adverse health effects of ETBE. Previous experimental animal studies have shown that substantial damage in liver and kidney is caused by chronic exposure to ETBE at concentrations of 1,750 ppm or higher[10]. However, we suggest that the threshold of toxicity, should be lowered to 500 ppm.

There is evidence that the plasma concentration of acetaldehyde in the *ALDH2* variant homozygotes is several folds higher than in the *ALDH2* wild-type homozygotes in humans[5]. It was observed that blood acetaldehyde concentrations were several folds higher in *Aldh2*–/– mice than in *Aldh2*+/+ mice 1 h after the administration of ethanol gavages at multiple doses[11]. In addition, acetaldehyde has been proven to be genotoxic. For example, inhalation of acetaldehyde resulted in the occurrence of nasal adenocarcinomas and squamous cell carcinomas in rats and hamsters[12, 13]. Acetaldehyde induces DNA strand breaks as measured by the comet assay in human lymphocytes[14] and in neuronal cells of rat[15]. Moreover, variant allele *ALDH2*2* was significantly associated with an increased micronuclei frequency in habitual drinkers[16, 17]. Therefore, our observation of DNA damage induced by ETBE in leukocytes can be attributed primarily to acetaldehyde production after ETBE exposure and extensive accumulation of acetaldehyde in *Aldh2*–/– mice may have increased their sensitivity to this genetic damage. Thus the ALDH2 enzyme plays a critical role in the metabolism of ETBE and its consequent toxicity. In the present study, DNA damage detected by the alkaline comet assay could not have been repaired completely when the mice were sacrificed, i.e. 20–24 h after the cessation of ETBE exposure. As it is reported that acetaldehyde–protein reversible adducts in peripheral blood can be maintained for 48 h after drinking in subjects with variant allele *ALDH2*2*[18], it is possible that leukocytes were continuously attacked by acetaldehyde released from reservoir proteins in *Aldh2*–/– mice.

TBA is another primary metabolite of ETBE, which is believed to have no genotoxicity[2]. However, Yuan *et al.*[19] reported that TBA could form adducts with DNA in the mouse liver, lung, and kidney. In a more recent report, it was shown that TBA could induce nuclear DNA fragmentation as measured by the comet assay in Rat-1 cells[20]. Although the evidence for the carcinogenicity of TBA is unconvincing, it is possible that TBA may play, to some extent, a role in the genotoxic effect of ETBE. This needs to be clarified by further studies.

In female mice, no significant genotoxic effects were detected in the *Aldh2*+/+ groups exposed to ETBE. Even in *Aldh2*–/– female mice, DNA damage was only observed in the two groups exposed to the higher ETBE levels. These results suggest that there may be a sex difference in the genotoxicity of ETBE. Male rats were reported to be more sensitive to TBA toxicity than female ones[21]. Borghoff *et al.*[22] observed that TBA was cleared more slowly from the kidney in male rats than in female rats.

We also observed higher basic levels of DNA damage in *Aldh2*–/– than in *Aldh2*+/+ mice in the present study. The former group lacks of ALDH2 activity, which might have resulted in lower clearance of intrinsic aldehydes and other chemicals originated from foods, resulting in a higher basic level of DNA damage.

In summary, the present study indicates that ETBE has toxic effects on genetic materials in mice, and ALDH2 activity deficiency greatly increases the sensitivity, possibly through the accumulation of acetaldehyde in body. Inter-individual and sex differences in ETBE-induced adverse health effects represent a risk factor and should be taken into considerations in the hazard assessment of ETBE.

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