The Pathogenesis of Alcohol-Induced Airflow Limitation in Acetaldehyde Dehydrogenase 2-Deficient Mice

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Keywords

Acetaldehyde · Alcohol-induced asthma · ALDH2-deficient mice · Ethanol · Histamine

Abstract

Background: In Japanese patients, alcohol-induced asthma is attributed to elevated plasma concentrations of acetaldehyde following alcohol consumption because of an acetaldehyde dehydrogenase 2 gene (*ALDH2*) polymorphism. The resulting increase in plasma histamine concentrations seems to trigger the onset of asthma symptoms. However, the specific pathogenic mechanism underlying this response remains unclear. ALDH2-deficient mice were therefore generated to investigate the pathogenesis of alcohol-induced asthma.

Methods: ALDH2-deficient mice were generated using embryonic stem cells that were derived from C57BL/6 mice. The resulting mice were backcrossed into the BALB/c mice background. Exon 1 of *ALDH2* was replaced with the *Neo* cassette. Pure ethanol was orally administered to ALDH2-deficient and wild-type mice, and the plasma concentrations of ethanol, acetaldehyde, and histamine, in addition to enhanced pause (Penh) values, were determined and compared between the 2 groups. **Results:** We established an ALDH2-deficient mouse line to compare responses between wild-type and ALDH2-

deficient mice receiving orally administered ethanol. The results showed that the plasma concentrations of acetaldehyde ($p < 0.0001$) and histamine ($p < 0.005$) were significantly higher, and the Penh values ($p < 0.01$) were significantly greater in the ALDH2-deficient mice, although plasma ethanol levels were not different. **Conclusions:** We studied the pathogenesis of alcohol-induced asthma using ALDH2-deficient mice. The results demonstrated that alcohol intake resulted in an increase in acetaldehyde levels, and a subsequent increase in histamine levels, which induced airway constriction. Alcohol consumption is known to be an important factor that exacerbates bronchial asthma, and studies investigating this factor are useful for the treatment of patients with alcohol-induced asthma.

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Introduction

Approximately half of Japanese asthma patients have experienced an exacerbation of the symptoms of asthma after drinking alcohol. This condition is referred to as alcohol-induced asthma [1]. Consumed ethanol is metabolized to acetaldehyde in the liver, and acetaldehyde is then further converted to nontoxic acetic acid by acetaldehyde

Fig. 1. Targeting strategy and PCR genotyping of ES cell DNA. **a** The *ALDH2* exon1 was replaced with a *Neo* cassette using homologous recombination. In the targeting construct, a 2.4-kb 5' fragment and a 7.8-kb 3' fragment were used as the short and long homologous arms, respectively. Arrows indicate the primers that were used for 5' homologous recombination (f1, r1). **b** PCR was performed using the *Neo* cassette-specific primers r1 and f1, which corresponded to the short arm (producing a 3.0-kb fragment from the knockout [KO] allele). f1 primer: 5'-CCTAAGAATGTCTCAGACCAGCTAT-3'; r1 primer: 5'-CTTCCTC-GTGCTTTACGGTATC-3'.

dehydrogenase 2 (ALDH2) [2, 3]. ALDH2 is a member of the ALDH superfamily, which comprises 9 major families. ALDH2 is localized in the mitochondria and plays a role in the NAD⁺-dependent irreversible oxidation of a wide range of aliphatic and aromatic aldehydes into carboxylates [3]. Previous human studies have demonstrated that there is a correlation between the *ALDH2* gene polymorphism and alcohol-induced asthma [4, 5]. Polymorphism-dependent abnormal ALDH2 activity is thought to induce airway constriction in alcohol-induced asthma by elevating the plasma levels of acetaldehyde following the consumption of alcohol. Impaired ALDH2 activity leads to high peak levels of acetaldehyde and to histamine release [1, 4, 6]. Ethanol itself has been reported to cause bronchodilation and suppress airway hyperresponsiveness [7, 8], and inhaling acetaldehyde has been shown to stimulate airway hyperresponsiveness in asthma patients [9]. Furthermore, Myou et al. [10, 11] have reported that acetaldehyde inhalation induces histamine-

mediated airway constriction in humans and guinea pigs. This effect was also observed in a study in which acetaldehyde was intravenously injected [12]. However, the molecular mechanisms underlying alcohol-induced asthma are not fully understood. In vivo experimental studies using animal models of a variety of diseases have been useful for exploring the mechanisms underlying many biological processes. In this study, we generated ALDH2-deficient mice and assessed whether they exhibited a phenotype resembling alcohol-induced asthma including elevated plasma acetaldehyde and histamine levels, following ethanol consumption.

Material and Methods

Generation of ALDH2-Deficient Mice

In the mouse genome, the gene *ALDH2* consists of 13 exons and 12 introns and has a full length of approximately 26 kb [13]. A targeting vector was created by replacing a 2.1-kb region con-

Fig. 2. Southern blot analysis of genomic DNA obtained following the homologous recombination of ES cells. Genomic DNA obtained from ES cells after homologous recombination was digested with *HindIII* (or *NdeI*) and then hybridized with the external 5' probe or 3' probe. The 5' probe detected a 4.2-kb wild-type (WT) band and a 5.4-kb knockout (KO) band in the KO allele. The 3' probe detected an 11.3-kb WT band and a 10.9-kb KO band in the KO allele.

taining the first exon, which includes the initiation codon of *ALDH2*, and a *Neo* cassette (Fig. 1a). This vector was linearized and transfected into C57BL/6 embryonic stem (ES) cells via electroporation, and the G418-resistant cell clones were isolated. To confirm the homologous recombination of the target gene in the transfected ES cells, genomic DNA was extracted from the isolated cells, and analyzed using PCR with primers that were homologous to specific sequences within the region of homologous recombination. A 3.0-kb band was detected in some clones (Fig. 1b). To further confirm these results, genomic DNA was extracted from the

ES cells, digested using restriction enzymes, and analyzed using Southern blot analysis with probes that were homologous to specific sequences within the region of homologous recombination. Genomic DNA was digested using either *HindIII* or *NdeI* and then hybridized with 2 probes, a 5' probe and a 3' probe, that were specific to the 5' and 3' ends, respectively, of the region of homologous recombination. The 5' probe detected a 4.2-kb band in the wild-type allele and a 5.4-kb band in the knockout allele. The 3' probe detected an 11.3-kb band in the wild-type allele and a 10.9-kb band in the knockout allele (Fig. 2). In addition, the *HindIII*-digested

Fig. 3. The clones in lanes 2 and 4 possess a *Neo* resistance gene-containing DNA fragment that inserted into the intended genomic region. The clones in lanes 1 and 3 possess a *Neo* resistance gene-containing DNA fragment that inserted into a site located outside the intended genomic region (all of the clones in lanes 1–4 are *Neo*-resistant clones). *Neo*-R, *Neo* cassette-specific primer; KO, knockout; WT, wild type.

genomic DNA was hybridized using a probe that was specific to the *Neo* resistance gene within the region of homologous recombination. A 5.4-kb band was also detected in some clones (Fig. 3). These results indicate that homologous recombination occurred as intended, and resulted in ES cells that contained the correct insertion sequence in the appropriate target region of the gene. ES cells that were found to have undergone homologous recombination were injected into BALB/c mouse blastocysts to generate chimeric (F0) mice, which were identified by their coat color. The F0 male mice were crossed with C57BL/6J female mice to produce F1 transgenic (*ALDH2*^{+/−}) mice. The genotypes of the mice were confirmed based on the detection of a 3.0-kb band on PCR using the same primer set that was used for the PCR analysis of the transfected ES cells (Fig. 4a). Next, male and female *ALDH2*^{+/−} mice were crossed to establish *ALDH2*-deficient (*ALDH2*^{−/−}) mice. The genotypes of these mice were confirmed using PCR with primers that were homologous to specific sequences within the region of homologous recombination. The results revealed a single 2.2-kb band for *ALDH2*^{+/+} mice, both a 2.2-kb and an approximately 650-bp band for *ALDH2*^{+/−} mice, or a single band of approximately 650 bp for *ALDH2*^{−/−} mice (Fig. 4b). *ALDH2*-deficient mice were maintained by mating the same *ALDH2*-deficient mice amongst each other. These mice were used to monitor plasma ethanol and acetaldehyde concentrations and to measure enhanced pause (Penh) values, as described below.

Furthermore, to achieve a genetic background that was closer to that of BALB/c mice, in which allergic reactions are easier to induce [14], *ALDH2*-deficient male mice were crossed with BALB/cCrSlc female mice (Japan SLC, Inc.), and the resulting *ALDH2*^{+/−} male and female progeny were then crossed with each other. The resulting *ALDH2*-deficient mice were used to monitor plasma histamine concentrations, as described below.

All experiments performed were conducted with the prior approval of the Animal Experiment Committee at UNITECH Co., Ltd. (approval No. AGR SHI-140901K-AS).

Blood Biochemistry Tests

Plasma ethanol and acetaldehyde concentrations were monitored in wild-type female mice (aged 8–10 weeks, C57BL/6Jcl; CLEA Japan, Inc.) and *ALDH2*-deficient female mice (aged 10–18 weeks). The mice were administered an intraperitoneal dose of 0.5% carboxymethyl cellulose that was followed 1 h later by a 10 mL/kg dose of 25% ethanol. The doses were administered via gavage. Some of the mice were anesthetized via an intraperitoneal injection with 10% Somnopentyl (pentobarbital sodium) so that their entire blood volume could be collected from the heart using heparinized needles. This procedure was performed either before dosing or at 1 or 2 h after dosing. The collected blood was centrifuged at 10,000 rpm for 5 min at 4°C to obtain plasma samples. To determine plasma ethanol concentrations, 50 µL of plasma was mixed with 400 µL of ice-cold 0.33 mol/L perchloric acid, and the solution was centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was assayed using an ethanol test kit (F-kit Ethanol; J.K. International Inc., 176290). To determine plasma acetaldehyde concentrations, plasma samples were mixed with an equal amount of ice-cold 1.0 mol/L perchloric acid, and the solution was centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was then collected and neutralized to a pH of approximately 7 using 1.0 mol/L potassium hydroxide, and this solution was centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was then assayed using an acetaldehyde test kit (F-kit Acetaldehyde; J.K. International Inc., 668613).

Plasma histamine concentrations were monitored in wild-type female mice (aged 10–18 weeks, BALB/cCrSlc, Japan SLC, Inc.)

Fig. 4. Generation of ALDH2-knockout (KO) mice. **a** PCR genotyping of tail DNA extracted from F1 heterozygous mice. The primer was the same as that used in the PCR genotyping of ES cells. **b** PCR genotyping of tail DNA extracted from F2 heterozygous mice utilizing the *Neo* cassette-specific primer *Neo*-R (5'-CTTCCTCGT-GCTTTACGGTATC-3') for the mutant allele (658 bp) as well as a genomic 5' primer (WT-R: 5'-TACTTCACAACCAC-GTTCTTTCAT-3') and the same 3' primer (WT-F: 5'-TGTAGTCAATGAGACAG-CAAGTAGC-3') to identify the wild-type (WT) allele (2,263 bp). MK, midkine.

and 32-week-old ALDH2-deficient female mice. The mice were administered a 10 mL/kg dose of 25% ethanol via gavage. Plasma histamine concentrations were determined before dosing and at 15, 30, 60, and 120 min after dosing. Blood samples were drawn from the caudal vein of restrained mice without anesthesia using heparinized hematocrit capillary tubes (TGL Inc.). To determine plasma histamine concentrations, whole-blood samples were centrifuged at 15,000 rpm for 3 min at room temperature, and the isolated plasma was analyzed using a histamine test kit (Bertin Pharma).

Penh Measurement

Penh values were measured in wild-type male mice (C57BL/6J, 16 weeks old), NC/Nga mice (NC/NgaTndCrlj, 8–9 weeks old; Charles River Laboratories International, Inc.), and ALDH2-deficient mice (15 weeks old). The mice were administered a 10 mL/kg dose of 25% ethanol via gavage, and the Penh values were then analyzed using a respiratory function analyzer (BUXCO Japan, Inc.). The Penh analysis was performed at 1 min intervals for 10 min before dosing and at 1, 2, 3, 4, 5, and 8 h after dosing. Penh values were calculated as the mean of the measurements taken 6–10 min before dosing (measurements taken after 1–5 min were not used).

Statistical Analysis

The data obtained from the blood biochemistry tests are expressed as the mean $\pm$ standard deviation, and the data from the Penh analysis are reported as the mean $\pm$ standard error. Biochemical data were analyzed using the Student *t* tests or the Welch *t* test, while the Penh data were analyzed using single-factor ANOVA and the Tukey-Kramer post hoc test. A *p* value <0.05 was considered to indicate statistical significance. These statistical analyses were conducted using Statcel 3 (OMS Publishing, Saitama, Japan).

Results

Plasma Ethanol and Acetaldehyde Concentrations after Oral Ethanol Challenge

Plasma ethanol and acetaldehyde concentrations were monitored in wild-type and ALDH2-deficient mice following the oral administration of ethanol. There was no difference in plasma ethanol concentrations between the wild-type and ALDH2-deficient mice (Fig. 5a). Plasma acetaldehyde concentrations were significantly higher in the ALDH2-deficient mice than in the wild-type mice at 1 h post-dose (0.0020 ± 0.0004 g/L in wild-type [$n = 3$] vs. 0.0086 ± 0.0004 g/L in ALDH2-deficient [$n = 3$], $p < 0.001$, Student *t* test) and at 2 h post-dose (0.0014 ± 0.0001 g/L in wild-type [$n = 3$] vs. 0.0088 ± 0.0001 g/L in ALDH2-deficient [$n = 3$], $p < 0.05$, Welch *t* test) (Fig. 5b). Pre-dose plasma acetaldehyde concentrations were also significantly higher in ALDH2-deficient mice than in wild-type mice (0.0017 ± 0.0001 g/L in wild-type [$n = 3$] vs. 0.0026 ± 0.0002 g/L in ALDH2-deficient [$n = 3$], $p < 0.01$, Student *t* test) (Fig. 5b).

Plasma Histamine Concentrations after Oral Ethanol Challenge

Plasma histamine concentrations were monitored in wild-type and ALDH2-deficient mice after oral ethanol administration. Plasma histamine concentrations were significantly higher in ALDH2-deficient mice than in

Fig. 5. Plasma ethanol (a) and plasma acetaldehyde (b) concentrations after oral ethanol challenge. Mean $\pm$ SD.

wild-type mice at 15 min post-dose ($3,195 \pm 940 \mu\text{M}$ in wild-type [$n = 3$] vs. $12,158 \pm 3,869 \mu\text{M}$ in ALDH2-deficient [$n = 3$], $p < 0.05$, Student *t* test), 30 min post-dose ($3,380 \pm 2,533 \mu\text{M}$ in wild-type [$n = 3$] vs. $17,092 \pm 2,862 \mu\text{M}$ in ALDH2-deficient [$n = 3$], $p < 0.005$), 60 min post-dose ($2,509 \pm 1,200 \mu\text{M}$ in wild-type [$n = 3$] vs. $15,552 \pm 1,639 \mu\text{M}$ in ALDH2-deficient [$n = 3$]; $p < 0.005$), and 120 min post-dose ($3,329 \pm 806 \mu\text{M}$ in wild-type [$n = 3$] vs. $12,025 \pm 4,971 \mu\text{M}$ in ALDH2-deficient [$n = 3$], $p < 0.05$) (Fig. 6a).

Penh Values after Oral Ethanol Challenge

The Penh analysis was performed on wild-type and ALDH2-deficient mice after oral ethanol administration. Penh values were significantly higher in ALDH2-deficient mice than in wild-type mice at 1 h post-dose (0.4815 ± 0.044 in wild-type [$n = 5$] vs. 1.7615 ± 0.3016 in ALDH2-deficient [$n = 5$], $p < 0.01$, Tukey-Kramer post hoc test), 2 h post-dose (0.4636 ± 0.0782 in wild-type vs. 1.179 ± 0.2037 in ALDH2-deficient, $p < 0.01$), 5 h post-dose (0.5045 ± 0.0255 in wild-type vs. 0.6531 ± 0.0149 in

ALDH2-deficient; $p < 0.05$), and 8 h post-dose (0.4443 ± 0.0294 in wild-type vs. 0.7057 ± 0.0726 in ALDH2-deficient, $p < 0.05$) (Fig. 6b). Next, ethanol was administered to nonimmunized NC mice to compare the extent of airway constriction between NC and ALDH2-deficient mice. Penh values were significantly higher in NC mice than in wild-type mice before ethanol was administered (0.4582 ± 0.0111 in wild-type [$n = 5$] vs. 0.6980 ± 0.0302 in NC [$n = 5$], $p < 0.01$, Tukey-Kramer post hoc test), at 5 h post-dose (0.5045 ± 0.0255 in wild-type vs. 0.8460 ± 0.050 in NC, $p < 0.01$) and at 8 h post-dose (0.4444 ± 0.0294 in wild-type vs. 0.7821 ± 0.0512 in NC, $p < 0.01$). However, the phenotype was more prominent in the ALDH2-deficient mice before ethanol administration (0.6980 ± 0.0302 in NC [$n = 5$] vs. 0.5496 ± 0.0351 in ALDH2-deficient [$n = 5$], $p < 0.01$, Tukey-Kramer post hoc test), at 1 h post-dose (0.8970 ± 0.1917 in NC vs. 1.7615 ± 0.3016 in ALDH2-deficient, $p < 0.05$) and at 5 h post-dose (0.8460 ± 0.050 in NC vs. 0.6531 ± 0.0149 in ALDH2-deficient, $p < 0.01$) (Fig. 6b).

Fig. 6. Plasma histamine concentrations (**a**) and Penh values (**b**) after oral ethanol challenge. Mean $\pm$ SD.

Discussion

ALDH2 is the primary enzyme that converts acetaldehyde into acetic acid during alcohol metabolism [2, 3]. To examine whether the ALDH2-deficient mice generated in this study exhibited metabolic aberrations in the conversion of acetaldehyde to acetic acid, we monitored plasma ethanol and acetaldehyde concentrations in wild-type and ALDH2-deficient mice after oral ethanol administration. There was no difference in plasma alcohol concentrations between the wild-type and ALDH2-deficient mice, but plasma acetaldehyde concentrations were significantly higher in the ALDH2-deficient mice than in the wild-type mice. These results indicate that ALDH2 plays a major role in the conversion of acetaldehyde during the process of the metabolic breakdown of orally administered ethanol, and that this process is impaired in ALDH2-deficient mice. In addition, pre-dose plasma acetaldehyde concentrations were significantly higher in ALDH2-deficient mice than in wild-type mice. This suggests that while acetaldehyde metabolism is not related

to ethanol consumption, it was also defective in our ALDH2-deficient mice. Based on our previous studies, abnormal ALDH2 activity induces airway constriction following alcohol consumption by increasing plasma acetaldehyde levels, which causes the subsequent release of histamine from mast cells or basophils [1, 4]. Next, to verify that ALDH2-deficient mice demonstrate a phenotype similar to the one we previously reported, we monitored plasma histamine concentrations and Penh values after the oral administration of ethanol in wild-type and ALDH2-deficient mice. The ALDH2-deficient mice demonstrated significantly higher plasma histamine concentrations and Penh values than the wild-type mice. These results indicate that excess histamine release occurs as a result of the increase in plasma acetaldehyde concentrations that was observed in the ALDH2 mice. NC/Nga (NC) mice, which are often used as an animal model for naturally occurring dermatitis, have been shown to develop asthma-like symptoms as a result of ovalbumin immunization [15]. When we orally administered ethanol to non-immunized NC mice to compare the extent of airway

constriction between NC and ALDH2-deficient mice, we found that the Penh values were significantly higher in the NC mice than in the wild-type mice. This indicates that even more severe airway obstruction was induced in ALDH2-deficient mice than in NC mice. Additionally, these results indicate that both allergic response pathways and ALDH2 deficiency play an important role in the pathogenesis of alcohol-induced asthma. Previous studies in humans and in vitro systems have demonstrated that drinking alcohol causes acetaldehyde levels to increase, which leads to an increase in the release of histamine from mast cells or basophils, and the consequential induction of airway constriction [1, 16]. Studies have also indicated that administering antihistamines could prevent alcohol-induced asthma in humans [17, 18]. These findings suggest that excess histamine release plays an important role in alcohol-induced asthma. Further studies are required to explore the mechanisms underlying this process, including how histamine localizes to target organs, the molecular mechanism associated with the actions of histamine, the roles of the nonhistamine chemical mediators that are released from mast cells and basophils, and the characteristics of the immune system response in these patients [19, 20]. Future potential research topics include the study of sensitized ALDH2-deficient mice and an assessment of the inhibitory effects caused by various agents.

In summary, in Japanese patients, alcohol-induced asthma is thought to be triggered by an increase in plasma

acetaldehyde concentration following the consumption of alcohol. This increase is triggered because of abnormal ALDH2 activity that results from a gene polymorphism [4, 5]. We produced ALDH2-deficient mice that showed elevated plasma acetaldehyde levels and increased airway obstruction after they were administered an oral dose of ethanol, and we demonstrated that symptoms resembling alcohol-induced asthma were easily induced in this mouse model system. The ALDH2-deficient mouse system established in this study is therefore expected to substantially contribute to future progress in in vivo research studies of alcohol-induced asthma.

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Disclosure Statement

The authors have declared that they have no conflict of interest.

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