

ORIGINAL ARTICLE

Sulforaphane Accelerates Acetaldehyde Metabolism by Inducing Aldehyde Dehydrogenases: Relevance to Ethanol Intolerance

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Abstract — **Aims:** Many East Asians are highly intolerant to even modest alcohol consumption. These individuals accumulate acetaldehyde, the primary metabolite of ethanol, because of a genetic polymorphism of aldehyde dehydrogenase (ALDH) that metabolizes acetaldehyde to nontoxic acetate. The aim of these studies is to upregulate ALDH by dietary means, thereby reducing acetaldehyde toxicity. **Methods:** Sulforaphane [SF, 1-isothiocyano-4-(methylsulfinyl)butane] derived from its glucosinolate precursor contained in cruciferous vegetables and related inducers of the Keap1/Nrf2/ARE pathway were assessed for their potencies to induce ALDH in murine hepatoma Hepa1c1c7 cells. Inducer potencies for ALDH were compared with those for NQO1, a prototypical cytoprotective enzyme present downstream of the Keap1/Nrf2/ARE pathway. SF (5 or 20 µmol/day) was fed to CD-1 mice for 7 days prior to a single administration of ethanol, and then ALDH induction in organs and pharmacokinetics of acetaldehyde was examined. **Results:** In addition to SF, other electrophiles, including many Michael reaction acceptors, induce ALDH. Potencies of these agents as inducers parallel their activities in inducing NQO1, and are also dependent on Nrf2. In mice, *in vivo*, feeding of SF induced tissue ALDH and dramatically increased (doubled) the rate of elimination of acetaldehyde arising from the administration of ethanol. **Conclusion:** SF and other edible phytochemicals may ameliorate the alcohol intolerance of individuals who are polymorphic with respect to ALDH.

INTRODUCTION

It is well known that many East Asians including Chinese, Japanese and Koreans are highly sensitive to alcohol, and even small doses of ethanol evoke the unpleasant effects of flushing, nausea, headache and tachycardia (Yoshida *et al.*, 1984; Eng *et al.*, 2007). Whereas modest consumption of ethanol is believed to be beneficial, large doses can cause many serious medical problems (Crabb *et al.*, 2004). In the body, alcohol dehydrogenase (ADH) rapidly converts ethanol to its primary metabolite, acetaldehyde, which is mutagenic and carcinogenic, and is largely responsible for the above-mentioned symptoms and associated health damage. Acetaldehyde is then further metabolized to nontoxic acetate by aldehyde dehydrogenase (ALDH), predominantly mitochondrial ALDH2 (Impraim *et al.*, 1982). Polymorphism of ALDH is widespread among East Asians, in whom about 40% of the population have a mutation of ALDH2 (ALDH2*2) resulting in much reduced or absent enzyme activity and the accumulation of acetaldehyde. This paper describes dietary methods for inducing ALDHs to counteract the toxic effects of acetaldehyde accumulation.

ALDH polymorphism results in increased vulnerability to developing cancer and other serious diseases (Harada *et al.*, 1981). Particularly, the risk of esophageal squamous cell (ESC) cancer, common among middle-aged men in Japan, is often associated with the polymorphism of ALDH2 (Brooks *et al.*, 2009; Oze *et al.*, 2011). Thus, the risk of ESC cancer is dramatically increased in individuals with ALDH2*2 who drink alcohol compared to those with normal type of ALDH2 (Yokoyama *et al.*, 2003). Therefore, in addition to smoking, acetaldehyde accumulation after alcohol consumption contributes to carcinogenesis.

A recent population-based prospective cohort study of 38,700 middle-aged Japanese men (Yamaji *et al.*, 2008)

provided possible insight into protection against damage caused by over-consumption of alcohol. An increase in consumption of fruits and vegetables by 100 g/day reduced the hazard ratio for esophageal carcinoma by 11%, and importantly, higher intake of cruciferous vegetables was strongly associated with a significant further decrease in the risk by 56% ($P < 0.02$).

Many epidemiological studies have suggested that cruciferous vegetables have beneficial health effects because they contain glucosinolate precursors of isothiocyanates which interact with various functional groups of proteins due to their electrophilic properties (Talalay *et al.*, 2003; Higdon *et al.*, 2007; Fahey *et al.*, 2012). The isothiocyanate sulforaphane (SF) that was isolated from broccoli is a prominent example (Zhang *et al.*, 1992). SF has attracted widespread attention for two decades since it protects aerobic cells against carcinogens, toxic DNA-damaging electrophiles and oxidants by induction of a network of cytoprotective phase 2 enzymes and by suppressing inflammatory responses (Juge *et al.*, 2007; Guerrero-Beltrán *et al.*, 2010; Elbarbry and Elrody, 2011). Major chemoprotective effects of SF are mediated by the transcriptional upregulation of the Keap1/Nrf2/ARE pathway and other anti-inflammatory mechanisms that regulate COX2, iNOS and macrophage migration inhibitory factor (MIF) (Liu *et al.*, 2008; Baird and Dinkova-Kostova, 2011; Healy *et al.*, 2011; Suganuma *et al.*, 2011). The Keap1/Nrf2/ARE pathway does not operate at its maximal capacity under basal conditions, but it can be induced by a wide variety of small molecules (Talalay *et al.*, 1995, 2003; Dinkova-Kostova *et al.*, 2010; Kensler *et al.*, 2013). This strategy mobilizes natural compounds to protect cells against stress, and reduces the risk of developing cancer and chronic degenerative diseases. We establish here that ALDH expression is under major control of transcriptional factor Nrf2, and that ALDH activities can be substantially increased by SF and related inducers of the

Keap1/Nrf2/ARE pathway in animal cells and organs, and mice *in vivo*. This strategy has the potential to protect humans who have low or absent ALDH activity and are intolerant to alcohol.

MATERIALS AND METHODS

Chemicals and materials

Sulforaphane was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other isothiocyanates were from LKT Laboratories (St. Paul, MN, USA). 4-(Rhamnopyranosyloxy)benzyl isothiocyanate from *Moringa oleifera* was a gift from J.W. Fahey. Triterpenoid TP-225 (Dinkova-Kostova *et al.*, 2005) was a gift of M.B. Sporn (Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH, USA). Celastrol was from Cayman Chemical (Ann Arbor, MI, USA). Bis(benzylidene)acetones, HBB-2 and HBB-4 were synthesized (Dinkova-Kostova *et al.*, 1998). The tricyclic bis(cyano-enone), TBE-31, was synthesized by Tadashi Honda (Honda *et al.*, 2007; Dinkova-Kostova *et al.*, 2010), and was a gift from M. B. Sporn. Flavonoids were from Indofine Chemical Company (Hillsborough, NJ, USA).

Cell culture

All cell lines were grown in 5% CO₂ at 37°C. Murine hepatoma cell line, Hepa1c1c7, from the American Type Culture Collection was cultured in α -MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). Mouse embryo fibroblasts (MEFs) derived from day 13.5 embryos of wild-type or Nrf2-knockout C57BL/6 mice were cultured in Iscoves Modified Dulbecco's Medium (with L-glutamine) supplemented with 10% (v/v) heat-inactivated FBS.

Animal experiments

All animal experiments were performed in compliance with National Institutes of Health Guidelines and those of the Animal Care and Use Committee of the Johns Hopkins University (MO11M123). First, 15 female, 8- to 9-week-old CD-1 mice (Charles River Laboratories, Wilmington, MA, USA) were used to assess the effects of dose of SF on enzyme activities and mRNA expression of ALDHs. All mice were fed a low inducer-containing diet (AIN-76A) for 7 days, and were randomly assigned to three groups, control, low- and high-SF groups ($n=5$ in each group). They received basal diet, 5 or 20 μ mol SF per 3 g diet, respectively. After 7 days, mice were euthanized and their livers, forestomachs, glandular stomachs and proximal small intestines were harvested, frozen in liquid N₂ and stored at -80°C until analyzed. A small portion of each tissue was stored in RNA-stabilizing solution (RNAlater, Sigma, St. Louis, MO, USA) at 4°C until total RNA extraction.

Second, 18 female CD-1 mice were acclimatized on the AIN76A, divided into two groups, control ($n=9$) and SF ($n=9$), and then received AIN76A or 20 μ mol SF per 3 g AIN76A diet for 7 days, respectively. After fasting overnight, they were gavaged with 35% (v/v) ethanol (2.0 g ethanol/kg body weight). Before, and 0.5, 1, 2, 3, 4, and 6 h later, 20 μ l of blood was collected by nicking the tail vein, and used to determine blood ethanol and acetaldehyde levels.

Assay of ALDH activity

Hepa1c1c7 cells or MEFs were seeded on 6-cm plates at 6.0×10^5 or 1.2×10^6 cells/plate, incubated for 24 h at 37°C in 5% CO₂ and then treated with serial dilutions of inducers dissolved in acetonitrile or DMSO. Final concentrations of the solvents were <0.5 or 0.1% (v/v), respectively. After 48 h, cells were washed twice with PBS, scraped from the plates and homogenized in micro-homogenizers at 4°C. The homogenates were centrifuged at $3000 \times g$ for 15 min at 4°C, and ALDH activities were measured fluorometrically in the supernatant fractions in 96-well opaque plates by modification of the method of Koivula *et al.* (1975). The assay mixture (190 μ l) contained 70 mM sodium pyrophosphate buffer (pH 8.0), 1.67 mM pyrazole, 1.33 mM NAD and supernatant fraction. The assay was started by addition of 10 μ l of 90 mM propionaldehyde, bringing the total volume to 200 μ l. The initial velocity of NADH generation was measured (λ_{ex} 340 nm and λ_{em} 460 nm) at 25°C for 10 min in a microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). Blank reaction rates without propionaldehyde were also determined. ALDH velocity was normalized to the protein concentration and expressed as change in nanomoles of NADH formed per min per mg of protein.

To determine ALDH activities of cytosolic/microsomal and mitochondrial fractions individually, cells or harvested tissues were homogenized at 4°C in 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1 mM 2-mercaptoethanol and centrifuged at $750 \times g$ for 15 min to remove nuclei and cell debris. Supernatant fractions were then centrifuged twice at $12,000 \times g$ for 20 min to obtain the cytosolic/microsomal and the sedimented mitochondrial fractions. Mitochondrial fractions were suspended in 0.25 M sucrose, diluted with one-half volume of 1% sodium bicarbonate containing 1% sodium deoxycholate (w/v) and sonically disrupted in a Branson Sonicator at 4°C for five 2-min periods before assays.

Assay of NAD(P)H-quinone acceptor oxidoreductase 1 activity

Hepa1c1c7 cells were seeded in 96-well plates at 10^4 cells per well, grown for 24 h and then exposed to serial dilutions of inducers for 48 h. NAD(P)H-quinone acceptor oxidoreductase 1 (NQO1) activities were determined with menadione as substrate by the Prochaska assay (Prochaska and Santamaria, 1988; Fahey *et al.*, 2004). Cytosolic/microsomal fractions of tissues were subjected to similar NQO1 assays.

Analysis of mRNA expression of ALDHs

Total RNA was extracted from Hepa1c1c7 cells that had been exposed to serial dilutions of inducers for 24 h, or from RNA-stabilized tissues from mice with TRIzol reagent (Invitrogen, Grand Island, NY, USA), followed by reverse transcription into cDNA (iScript, Bio-Rad, Hercules, CA, USA). The cDNA was subjected to quantitative real-time PCR analysis by using SYBR Green fluorescence (POWER SYBR Mastermix, Applied Biosystems, Carlsbad, CA, USA) with 7000 Sequence Detection System (Applied Biosystems). All primers used in this study were designed based on previous reports (Levi *et al.*, 2009). Relative expressions of individual mRNAs for ALDH were normalized to β -actin as endogenous

control, and the expression rate was calculated by the comparative $\Delta\Delta C_t$ method.

Determinations of ethanol and acetaldehyde levels in mouse blood

These were determined enzymatically by modification of the method of Tottmar *et al.* (1978). Whole blood samples (20 μ l) were obtained by incisions of mouse tail veins, and were immediately mixed with 200 μ l of 4% (v/v) perchloric acid in sealed tubes. After incubation at 4°C for 10 min, the tubes were centrifuged at 3000 \times g for 10 min, and the pH values of the supernatant fractions were adjusted to 7.5–8.0 by adding 20 μ l of 3 M K_2CO_3 in new tubes. After removing precipitated perchlorate by centrifugation at 3000 \times g for 10 min, supernatant fractions were stored in sealed tubes at 4°C until assayed.

Diluted supernatant fractions were added to each well of 96-well plates containing in a final volume of 200 μ l: 0.5 M glycine-NaOH buffer (pH 9.0), 0.6 mM NAD and 53 units/ml of ADH (purified ADH from yeast, Sigma, St. Louis, MO, USA). Blood ethanol levels were calculated from the rate of NADH generation for 5 min measured with a microplate fluorometer (λ_{ex} 340 nm and λ_{em} 460 nm) with ethanol standards.

Acetaldehyde was measured in 10-mm quartz fluorescence cuvettes in an assay mixture containing in a final volume of 2.33 ml: 50 mM sodium pyrophosphate (pH 8.8), 0.1 mM pyrazole, 0.1 mM NAD and 0.2 unit/ml of purified ALDH (potassium-activated from baker's yeast, Sigma) with a luminescence spectrophotometer (Model LS 50, Perkin Elmer, Waltham, MA, USA). Before and 5 min after addition of 170 μ l of the supernatant fraction, intensities were determined (λ_{ex} 340 nm and λ_{em} 460 nm). The acetaldehyde level in blood was calculated from rate of change in fluorescence intensity by using acetaldehyde standard curves corresponding to ranges of 1.56–50 μ M in blood.

Statistical analysis

All values are means \pm SEM. Differences between groups were determined by Student's *t*-test or Dunnett's *post hoc* test after a one-way ANOVA.

RESULTS

Sulforaphane increases total ALDH activity in Hepa1c1c7 cells

We first measured the total ALDH activities of lysates of Hepa1c1c7 murine hepatoma cells with propionaldehyde as substrate. Treatment of these cells with a series of concentrations (0.3, 1.0 and 3.0 μ M) of SF for 48 h induced total ALDH activity. Significant increases in activity were observed in cells exposed to 0.3 μ M SF, similar to the concentration required to activate many Nrf2-dependent genes (Fig. 1A). These increases were both SF concentration- and time-dependent (Fig. 1B), but did not occur immediately, suggesting that SF induces ALDH transcriptionally but does not activate it directly.

Chemical structures and ALDH inducer potencies in Hepa1c1c7 cells

We assayed a wide range of compounds belonging to eight structurally very different chemical classes previously shown to be inducers of phase 2 cytoprotective genes (Table 1). Of 20 compounds examined, 15 chemical agents including isothiocyanates such as SF, flavonoids and triterpenoid Michael acceptors increased total ALDH specific activity in Hepa1c1c7 cells. Inducer potencies were expressed as concentrations required to double the basal specific enzyme activities (CD values).

Inducer potencies of compounds for ALDH and NQO1 in Hepa1c1c7 cells

Next, inducer potencies of the above-described compounds for ALDH and the prototypical phase 2 enzyme NQO1 were compared by determining CD values for inducing ALDH and NQO1 activities (Table 1). The CD values ranged from the most potent triterpenoid TP-225 (CD values of 0.0016 μ M for ALDH and 0.00038 μ M for NQO1) to the stilbene resveratrol, the least potent compound assessed in the present study. Interestingly, in Hepa1c1c7 cells the compounds were uniformly 5–10 times more potent as inducers of NQO1 than of ALDH. The reasons for these differences are unclear, but other studies (not shown) indicate that the relative potencies of

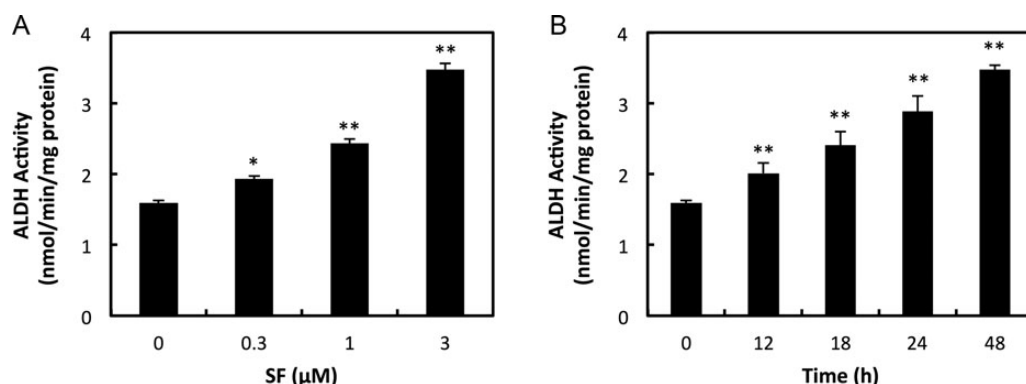
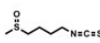
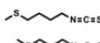
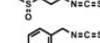

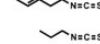
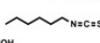
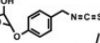
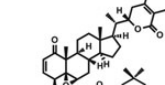
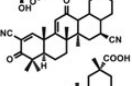
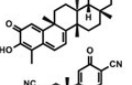
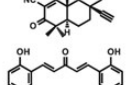
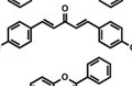
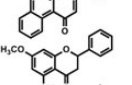
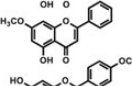
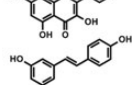
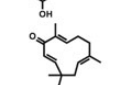


Fig. 1. Effect of SF on total ALDH activity in Hepa1c1c7 cells. The cells were treated with a series of concentrations of SF (0.3, 1.0 or 3.0 μ M) for 48 h (A), or with 3.0 μ M SF for 0, 12, 18, 24 or 48 h (B). ALDH activity was determined in supernatant fractions of cell homogenates with propionaldehyde as substrate. The values are normalized for protein concentration. Means \pm SEM are shown ($n = 8$). $P < 0.05$ (*), $P < 0.01$ (**), for differences from control values.

Table 1. Potencies of chemically distinct classes of compounds for induction of ALDH and NQO1 in Hepa1c1c7 cells, expressed as CD (concentrations required to double) values

Chemical class, compound and structure	CD, μ M in Hepa1c1c7 cells	
	ALDH	NQO1
Isothiocyanates		
Sulforaphane [1] ^a 	2.31	0.22
Erucin [2] 	1.61	0.65
Iberin [3] 	13.0	2.01
Benzyl isothiocyanate 	>30	>30
Phenethyl isothiocyanate 	>10	>10
Propyl isothiocyanate 	>100	>100
Hexyl isothiocyanate 	>100	>100
4-(Rhamnopyranosyloxy)benzyl -isothiocyanate; 4RBITC [4] 	3.04	0.14
Steroids		
Withaferin A [5] 	1.98 ^b	0.19
Triterpenoids		
2-Cyano-3,12-dioxooleane-1,9(11)-dien -28-onitrile; TP-225 [6] 	0.0016	0.00038
Celastrol [7] 	0.94	0.24
Tricyclic (cyano enones)		
Tricyclic bis(cyano-enone); TBE-31 [8] 	0.032	0.0019
Bis(benzylidenes)		
Bis(2-hydroxybenzylidene)acetone; HBB-2 	>10	0.27
Bis(4-hydroxybenzylidene)acetone; HBB-4 [9] 	47.0	16.9
Flavonoids		
β -Naphthoflavone [10] 	0.60	0.023
Pinostrobin [11] 	4.6	0.67
Tectochrysin [12] 	7.0	0.4
Kaempferide [13] 	27.8	6.4
Stilbenes		
Resveratrol [14] 	121 ^b	>150
Sesquiterpenes		
Zerumbone [15] 	3.53	0.6

^aNumbers in brackets are linked with Fig. 2.^bExtrapolated values.

compounds vary greatly among cell types, and thus may be attributable to differences in the metabolism rather than fundamental molecular mechanisms of the inducers in different cells. The relationship of potencies for induction of ALDH and NQO1 was also analyzed by displaying the CD values of 15 compounds in Hepa1c1c7 cells as a double logarithmic plot (Fig. 2). A highly significant linear correlation was observed over more than five orders of magnitude of potency ($r^2 = 0.94$). This result strongly suggests that induction of ALDH by phase 2 inducers operates through the same mechanism as induction of NQO1, i.e. the Keap1/Nrf2/ARE pathway.

Participation of Nrf2 in induction of ALDH by SF

SF was a much less potent inducer of total ALDH in C57B/6 MEFs than in Hepa1c1c7 cells. Notably, ALDH activity

increased dose-dependently upon treatment with SF in WT MEFs, whereas SF had no effect on ALDH induction in Nrf2^{-/-} MEFs (Fig. 3), establishing that Nrf2 is clearly involved in the induction of ALDH by SF and probably by other inducers.

Effects of phase 2 inducers on individual ALDHs

Three representative phase 2 inducers with radically different structures (the isothiocyanate SF, the triterpenoid TP-225 and the flavonoid β -naphthoflavone) were examined for their capacities to induce individual ALDHs in Hepa1c1c7 cells, with special emphasis on cytosolic ALDH1A1 and mitochondrial ALDH2, which are principally involved in acetaldehyde metabolism. After 48 h, ALDH activities in both fractions were dose-dependently increased up to 3-fold (Fig. 4). Moreover, dose-dependent elevations of mRNA expression of *Aldh1a1*

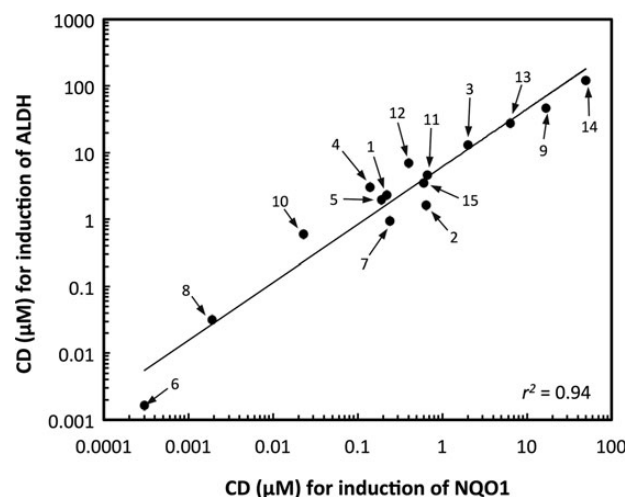


Fig. 2. Comparison of potencies of 15 inducers of NQO1 and ALDH in Hepa1c17 cells. The concentrations required to double (CD) both enzymatic specific activities were determined for each agent and are plotted as a double logarithmic graph. The CD values for the following inducers (chemical structures in Table 1) are shown: [1] sulforaphane, [2] erucin, [3] iberin, [4] 4-(rhamnopyranosyloxy)benzyl isothiocyanate (4RBITC), [5] withaferin A, [6] 2-cyano-3,12-dioxooleane-1,9(11)-dien-28-onitrile (TP-225), [7] celastrol, [8] tricyclic bis(cyano-enone) (TBE-31), [9] bis(4-hydroxybenzylidene) acetone (HBB-4), [10] β -naphthoflavone, [11] pinostrobin, [12] tectochrysin, [13] kaempferide, [14] resveratrol, [15] zerumbone. Correlation coefficient $r^2 = 0.94$.

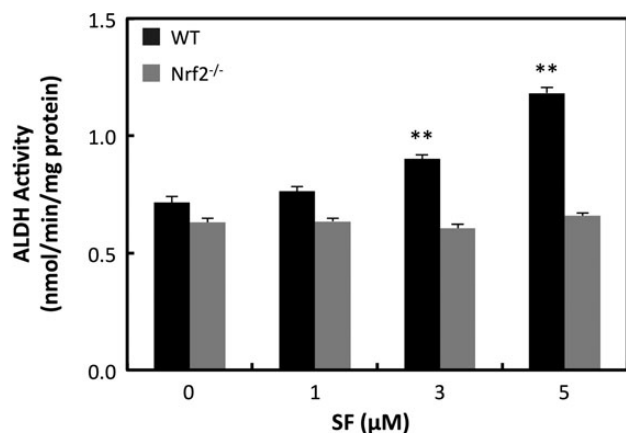


Fig. 3. Effect of SF on ALDH activities in WT and Nrf2^{-/-} mouse embryonic fibroblasts (MEF). These embryonic fibroblasts were treated with a range of concentrations of SF for 48 h. ALDH activities were determined in supernatant fractions of cell homogenates with propionaldehyde as substrate. Means \pm SEM are shown ($n = 8$). $P < 0.05$ (*), $P < 0.01$ (**), for differences from control values.

and *Aldh2* were observed in Hepa1c17 cells treated with the three compounds (not shown).

Antioxidant response element consensus sequences in ALDH genes

We examined whether the established antioxidant response element (ARE) core sequence present in phase 2 cytoprotective genes [RTGA(S/Y)nnnGCR (where R = A or G, S = C or G, Y = C or T and n = any nucleotide)] is also present in

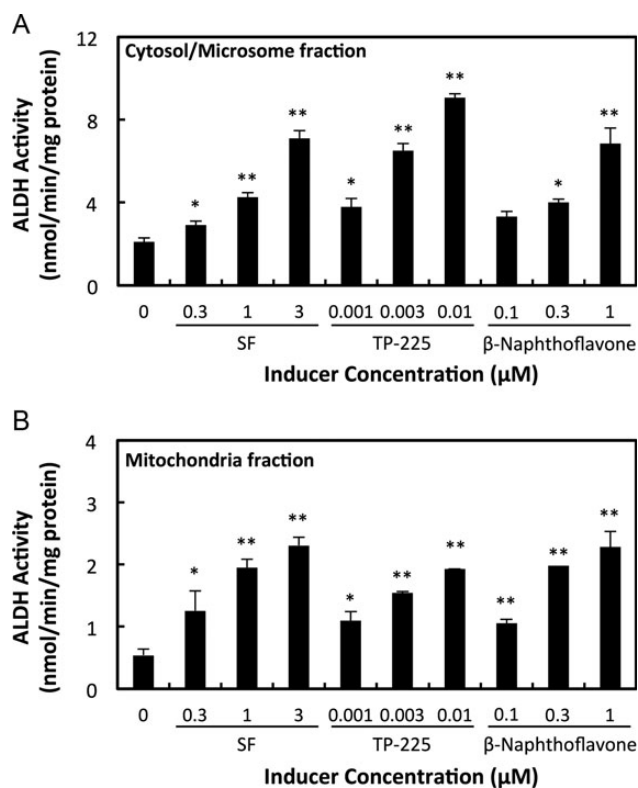


Fig. 4. Effects of various types of inducers on ALDH activities in subcellular fractions of Hepa1c17 cells. The cells were treated with representative phase 2 inducers belonging to distinct chemical classes: sulforaphane, an isothiocyanate; TP-225, a triterpenoid; β -naphthoflavone, a flavonoid for 48 h (see structures in Table 1). Cells were homogenized and fractionated into cytosolic/microsomal and mitochondrial fractions. ALDH activities were determined with propionaldehyde as substrate. Means \pm SEM are shown ($n = 4$). $P < 0.05$ (*), $P < 0.01$ (**), for differences from control values.

ALDH genes. Previous reports showed that induction of both *Aldh1a1* and *Aldh3a1* is probably mediated by the Keap1/Nrf2/ARE pathway because ARE core consensus sequences were identified in the 5-flanking region of their genes (Sreerama and Sládek, 2001; Abdullah *et al.*, 2012). Search for the ARE core consensus sequences by the procedure of Abdullah *et al.* disclosed at least two perfectly matched (8/8) sequences within 2000-bp of 5-flanking region of *Aldh2* (in the ENSEMBL mouse project website). These sequences, GTGACcagGCG and ATGAGacaGCA, are situated 82–92 bp and 1364–1374 bp upstream of the putative transcription start site, respectively. Both ARE sequences are categorized as class 4 enhancers because they do not contain embedded activator protein 1-binding sites TGASTCA (Hayes *et al.*, 2010). Additionally, >10 similar sequences (scored 5/8–7/8) were found in the region, indicating that induction of *Aldh2* as well as *Aldh1a1* and *Aldh3a1* is also likely to be mediated by the Keap1/Nrf2/ARE pathway.

Effects of SF on ALDH activities in mouse organs

To evaluate the potential of SF for inducing ALDH *in vivo*, we measured ALDH activities in cytosolic/microsomal and mitochondrial compartments and mRNA expression of ALDHs in liver, forestomach, glandular stomach and proximal small intestine of female CD-1 mice. The animals were fed SF

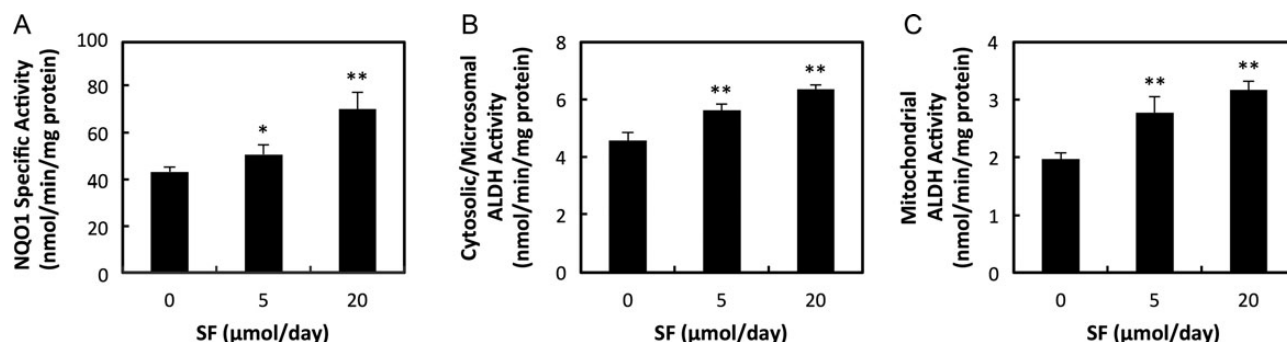


Fig. 5. Effects of SF on enzyme activities of NQO1 and ALDH in mouse livers. Female CD-1 mice received basal diet, 5 or 20 μmol SF per 3 g diet, respectively, for 7 days. Their livers were harvested, homogenized and fractionated into cytosolic/microsomal and mitochondrial fractions. NQO1 activity in the cytosolic/microsomal fraction was determined with menadione as substrate (A). ALDH activities in both fractions were determined with propionaldehyde as substrate (B and C). Means \pm SEM are shown ($n = 5$). $P < 0.05$ (*), $P < 0.01$ (**), for differences from control values.

Table 2. Effects of SF on mRNA levels of ALDH genes in mouse organs

Gene	SF ($\mu\text{mol/day}$)	Expression level (ratio to liver in control mice)		
		Liver	Forestomach	Glandular stomach
<i>Aldh1a1</i>	0 (control)	1.00 \pm 0.05 ^a	0.14 \pm 0.02	0.066 \pm 0.005
	5	1.72 \pm 0.19	0.27 \pm 0.04	0.099 \pm 0.005
	20	2.54 \pm 0.18	0.32 \pm 0.02	0.102 \pm 0.009
<i>Aldh2</i>	0 (control)	1.00 \pm 0.13 ^a	0.050 \pm 0.001	0.028 \pm 0.001
	5	1.82 \pm 0.14	0.070 \pm 0.002	0.038 \pm 0.002
	20	2.15 \pm 0.11	0.076 \pm 0.003	0.046 \pm 0.002
<i>Aldh3a1</i>	0 (control)	1.00 \pm 0.06 ^b	14700 \pm 325	4920 \pm 529
	5	1.75 \pm 0.25	26000 \pm 3880	14900 \pm 1870
	20	3.53 \pm 0.33	34000 \pm 4250	34600 \pm 4300

The comparative $\Delta\Delta\text{Ct}$ method was used to compare mRNA levels in organ collected from CD-1 mice that received diets containing SF (0, 5 or 20 $\mu\text{mol/day}$) for 7 days. β -Actin was used as an endogenous control for all target genes, and values are represented as the relative fold change in the mRNA levels to liver in control mice. Means \pm SEM are shown ($n = 5$).

^aThe basal expression levels of *Aldh1a1* and *Aldh2* in liver were higher than those in both parts of stomach.

^bThe basal expression level of *Aldh3a1* in liver was very much lower than that in stomach.

either at low doses (5 μmol) or high doses (20 μmol) per 3 g of diet, respectively, for 7 days. Importantly, there were no significant differences in body weight and food intake among these groups throughout the feeding period. In agreement with previous studies, intake of SF for 7 days resulted in a dose-dependent increase in NQO1 activities in liver, both parts of the stomach and proximal small intestine up to 1.8-fold in the high-dose group (Fig. 5A). High doses of SF significantly increased ALDH activities by 1.4- and 1.5-fold in cytosolic/microsomal and mitochondrial fractions, respectively, from liver, which are most important in metabolizing acetaldehyde derived from ethanol (Fig. 5B and C), and where basal activities of ALDH were actually higher than those from other organs. Also, intake of SF dose-dependently increased ALDH activities in both compartments in other organs including both parts of stomach and proximal small intestine. Interestingly, the highest susceptibility to SF was observed in glandular stomach where cytosolic/microsomal and mitochondrial ALDH activities were increased up to 4.7- and 5.1-fold, respectively (not shown).

We next analyzed mRNA expression of ALDH genes in mouse organs. Of note, although SF increased the expression

levels of ALDH genes such as *Aldh1a1*, 2 and 3a1 in all organs examined, the basal expression levels of these genes varied considerably among organs (Table 2). Expression of *Aldh1a1* and *Aldh2* messengers was much higher in liver than in other organs, whereas that of *Aldh3a1* was observed in both parts of stomach but only minimally in liver and proximal small intestine. In the liver, expression levels of *Aldh1a1* and 2 were increased by 2.5- and 1.8-fold, respectively, which possibly contributed to increased ALDH activities in cytosolic/microsomal and mitochondrial fractions from mouse livers. In stomach, *Aldh3a1* was strikingly induced by 2.3-fold (forestomach) and 6.5-fold (glandular stomach) as well as *Aldh1a1* and 2, with lower expression in stomach than in liver. In agreement with cytosolic/microsomal ALDH activity, the highest induction of *Aldh3a1* was observed in glandular stomach, which indicated that ALDH3A1 might largely reflect the activity in glandular stomach. In proximal small intestine, basal expression of ALDH genes was low, and induction was not prominent. These results suggest that SF is a highly potent inducer of ALDHs *in vivo* and protects against aldehyde toxicities by enhancing aldehyde metabolism.

Effects of SF on blood ethanol and acetaldehyde levels in mice

We measured changes in blood ethanol and acetaldehyde levels in CD-1 mice that were fed 20 μmol of SF per 3 g diet per day for 7 days before a single oral administration of ethanol (2.0 g/kg body weight).

In control mice, the blood ethanol and acetaldehyde levels were increased to about 30 mM and 22 μM at 1 to 2 h after ethanol gavage, respectively. Thereafter, ethanol levels fell rapidly, but acetaldehyde was still detectable in blood at 6 h after ethanol gavage (Fig. 6). Feeding SF for 7 days slightly affected the ethanol level only in the descending phase of the blood ethanol curve, and there was no significant difference between the area under the curve (AUC) of control and SF-fed mice. In contrast, prior feeding of SF markedly reduced the increase in blood acetaldehyde level by 30% in the ascending phase, and it remained at the lower level to the end of the experiment. Blood AUC for acetaldehyde was significantly lower in the SF-fed mice compared with those fed control diet. Pharmacokinetic analysis with a first order model showed that elimination of blood acetaldehyde was strongly accelerated in SF-fed compared with that in control mice with half-lives of 1.77 ± 0.12 and 3.43 ± 0.23 h, respectively (elimination rate

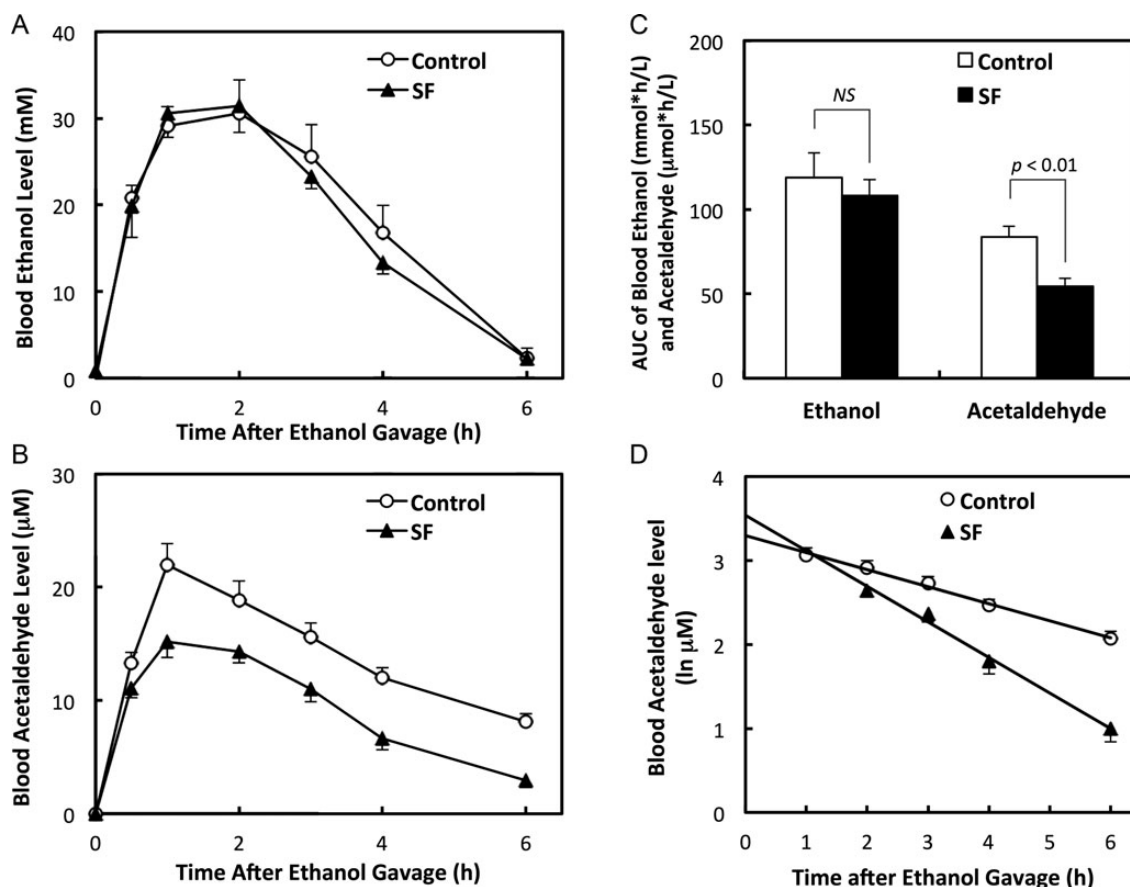


Fig. 6. Effects of SF on ethanol and acetaldehyde pharmacokinetics after single-dose ethanol administration. After feeding basal or SF-containing diet (20 $\mu\text{mol}/3$ g/day) for 7 days ($n = 9$ in each group), female CD-1 mice received a single dose of ethanol (2.0 g/kg body weight) by gavage. Just before and 0.5, 1, 2, 3, 4 and 6 h after gavage, blood was collected by nicking the tail vein. Perchlorate-treated blood samples were subjected to enzymatic determination of ethanol and acetaldehyde. Time course of changes in blood ethanol levels (A) and acetaldehyde levels (B). Calculated areas under the curve (AUC) for blood ethanol and acetaldehyde levels (C). Semi-log plot of blood acetaldehyde levels for pharmacokinetic analysis (D). Means \pm SEM are shown.

constants, k : 0.40 ± 0.03 and $0.21 \pm 0.02 \text{ h}^{-1}$, $P < 0.01$; initial blood levels, C_0 : 32.38 ± 2.4 and $28.62 \pm 2.1 \mu\text{M}$, *not significant*). We conclude that doubling of the rate of acetaldehyde elimination by SF feeding results from enhancement of acetaldehyde metabolism through induction of ALDH in liver and other organs by SF.

DISCUSSION

Total ALDH activity was measured in cell homogenates to assess the potencies of SF and various other phase 2 inducers of ALDH. The human genome contains at least 17 genes that are members of ALDH superfamily (Vasiliou *et al.*, 2004), and the total ALDH activity measured in the present study includes activities of multiple ALDH enzymes. We focused on enzyme activities of ALDH1A1 and 2 (coded by *Aldh1a1* and *Aldh2*) that are known to be principally responsible for metabolism of acetaldehyde in cytosol and mitochondria. Evaluation of 20 phase 2 inducers belonging to more than eight chemical classes demonstrated that their potencies for the induction of ALDH were correlated with those for the induction of NQO1. Additionally, increase of the total ALDH activity by SF was not observed in *Nrf2*^{-/-} MEFs, which

strongly suggests that induction of ALDHs is regulated by the Keap1/Nrf2/ARE pathway. These findings are in agreement with previous genomic and proteomic analyses with WT and *Nrf2*^{-/-} mice (Lee *et al.*, 2003; Cho *et al.*, 2005; Shen *et al.*, 2005; Nair *et al.*, 2006; Kitteringham *et al.*, 2010; Abdullah *et al.*, 2012), in which up-regulations of the basal and induced expression levels of ALDH1A1 (*Aldh1a1*) and ALDH2 (*Aldh2*) in WT mice were consistently observed. Furthermore, a previous report in which markedly increased mortality associated with liver failure was observed in *Nrf2*^{-/-} mice given ethanol doses that were tolerated by WT mice also supports our findings (Lamé *et al.*, 2008). Toxicity of acetaldehyde was postulated as one of the causes of serious damage in *Nrf2*^{-/-} mice, because ALDH enzyme activity was significantly lower and consequently acetaldehyde levels in the liver were much higher in *Nrf2*^{-/-} mice than in WT animals. In the present study, we confirmed that phase 2 inducers with different structures increased gene expressions of *Aldh1a1* and *Aldh2* in Hepa1c1c7 cells, and intake of SF increased ALDH activities in both cytosol and mitochondria in mouse livers concomitantly with gene expressions of *Aldh1a1* and *Aldh2*. In this connection, it will be of interest to examine the response to SF of mutant cells with *Aldh2* +/+, +/- and -/- genotypes.

In conclusion, inductions of ALDHs including ALDH1A1 and ALDH2, as well as a network of phase 2 cytoprotective enzymes, are regulated by the Keap1/Nrf2/ARE pathway. It is therefore very likely that a variety of natural products will have the potential of protecting individuals who are alcohol intolerant against acetaldehyde toxicity (Talalay and Fahey, 2001).

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