Is aldehyde dehydrogenase inhibited by sulfur compounds? *In vitro* and *in vivo* studies*

Małgorzata Iciek, Magdalena Górny, Anna Bilska-Wilkosz and Danuta Kowalczyk-Pachel

Chair of Medical Biochemistry, Jagiellonian University, Medical College Kraków, Poland

Aldehyde dehydrogenase (ALDH) catalyzes the critical step of ethanol metabolism, i.e. transformation of toxic acetaldehyde to acetic acid. It is a redox sensitive protein with the key Cys in its active site. Recently, it has been documented that activity of some proteins can be modified by sulfur-containing molecules called reactive sulfur species leading to the formation of hydroperoxides. The aim of the present study was to examine whether ALDH activity can be modified in this way. Studies were performed *in vitro* using yeast ALDH and various reactive sulfur species, including Na₂S, GSSH, K₂S₂, Na₂S₂O₂, and garlic-derived allyl sulfides. The effect of garlic-derived trisulfide on ALDH activity was also studied *in vivo* in the rat liver. The obtained results clearly demonstrated that ALDH could be regulated by sulfur species which inhibited its enzymatic activity. The results also suggested that not H₂S but polysulfides or hydroperoxides were the oxidizing species responsible for this modification. This process was easily reversible by reducing agents. After the treatment with polysulfides or hydroperoxides the level of protein-bound sulfur increased, while the activity of the enzyme dramatically decreased. Moreover, the study demonstrated that ALDH activity was inhibited *in vivo* in the rat liver after garlic-derived trisulfide administration. This is the first study reporting the regulation of ALDH activity by sulfane sulfur species and the results suggest that it leads to the inhibition of the enzyme.

**Key words:** aldehyde dehydrogenase, reactive sulfur species, sulfane sulfur

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**INTRODUCTION**

Ethanol is metabolized in the human body in two phases. The first phase involves its oxidation to formaldehyde by three pathways catalyzed by alcohol dehydrogenase (ADH), microsomal ethanol oxidizing system (MÉOS) (cytochrome P4502E1) or catalase. All three oxidation reactions lead to the formation of very toxic acetaldehyde, which is next metabolized by aldehyde dehydrogenase (ALDH). When ethanol intake is moderate, the major route of ethanol metabolism in the liver is through ADH and ALDH. Mammalian ALDH exists as the NAD+-dependent family of isoenzymes divided into several classes with different substrate specificity and expression level in various tissues (Edenberg, 2007; Orywal et al., 2017). Of all ALDH isoenzymes, the mitochondrial ALDH2 plays the major role in human acetaldehyde metabolism while the others metabolize a variety of substances. ALDH2 transforms highly toxic acetaldehyde to nontoxic acetate and this is the rate-limiting step in ethanol metabolism. In ALDH2 enzyme-deficient individuals, a significant amount of acetaldehyde is rapidly accumulated even after ingestion of a moderate amount of alcohol (Hao et al., 2011). Moreover, metabolic role of ALDH2 has been investigated in nitroglycerin bioactivation (Chen et al., 2005) and in cocaine addiction (Yao et al., 2010). Recently, a significant role of ALDH2 has emerged also in preventing numerous pathologies. ALDH2 dysfunction may contribute to cardiovascular diseases, diabetes, neurodegenerative diseases, stroke, cancer and aging (Chen et al., 2014; Orywal & Szmitkowski, 2017).

The human ALDH2 is a redox sensitive protein and the Cys302 sulfhydryl group in its active site plays an essential role in its activity. The well-known inhibitor of ALDH2, disulfiram used in the treatment of alcohol abuse, irreversibly inactivates the enzyme by carbamylation of cysteine residue in the active site (Koppaka et al., 2012). On the other hand, daidzin, an active isoflavonoid identified in the root and flowers of Kudzu, acts as a potent reversible competitive inhibitor of ALDH2 and leads to the accumulation of acetaldehyde and toxic effects (Koppaka et al., 2012; Chen et al., 2014).

Recently, it has been suggested that protein –SH groups can be modified by sulfur-containing molecules called reactive sulfur species (RSS) influencing the protein’s activity. This is a kind of reversible oxidation of –SH groups to hydroperoxides (–SSH). Many proteins have been documented to be modified through this process which is regarded as a part of cellular redox regulation and fulfills an important signaling role (Paul & Snyder, 2012; Greiner et al., 2013). Some of them are activated and others are inhibited through this process (Iciek et al., 2015; Ju et al., 2015; Módis et al., 2016).

RSS can be created endogenously during cysteine metabolism, and include hydrogen sulfide (H₂S) and products of its oxidation: inorganic polysulfides (H₃S₄) and hydroperoxides (RSSH), that means compounds containing reactive sulfane sulfur sulfur. RSS can be also obtained from natural exogenous sources especially from garlic-derived organosulfur compounds, i.e. diallyl trisulfide (DATS) and H₂S-releasing molecules. There are many studies documenting pharmacological effects of H₂S and garlic-derived sulfane sulfur compounds (Toohely & Cooper, 2014; Iciek et al., 2015) but such studies regard-
ing ALDH activity are lacking. They would be very interesting due to the role of ALDH2 not only in ethanol metabolism but also in other above-mentioned aspects. The ALDH-activating potential of sulfur compounds can be helpful during alcohol intake. In addition, the activation of ALDH2 would be effective in prevention of cardiovascular diseases and stroke. On the other hand, the ability of exogenous RSS to inhibit ALDH2 can be useful in cancer studies, where accumulation of toxic aldehydes leads to death of cancer cells. In the light of the various physiological roles of ALDH2, it seems that studies of the influence of RSS on its activity are important and useful both for biochemists and pharmacologists.

The aim of the present study was to examine whether ALDH2 activity can be modified by RSS. We used yeast-derived ALDH (yALDH) in all in vitro studies due to its high homology to human ALDH2. Various reactive sulfur species including Na₂S, GSSH, K₂S, Na₂S₂O₃, and garlic-derived allyl sulfides (DAS, DADS, DATS) were used to investigate their effect on ALDH activity. Since our in vitro studies demonstrated that the yALDH activity was inhibited by RSS, then the potential of some reducing agents (DTT, GSH, DHLA) to reverse persulfidation was examined. Next, in some cases, the level of protein-bound sulfur was estimated. We also performed a comparative study using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the regulation of which by S-sulfhydration is well established. Moreover, we assayed the activity of ALDH in the liver of rats after ip treatment with DATS and we found that indeed the ALDH activity was decreased after DATS treatment vs. control animals. Altogether, our results suggest that ALDH is inhibited by sulfane sulfur compounds.

**MATERIALS AND METHODS**

Chemicals and reagents. Purified yeast ALDH, potassium (poly)sulfide (K₂S), sodium sulfide, sodium thiosulfate (Na₂S₂O₃), glutathione (GSH), glutathione disulfide (GSSG), dihydrodipic acid (DHLA), lipoic acid (LA), dithiothreitol (DTT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle, glyceraldehyde-3-phosphate (GAP) and 275 µl of 50 mM Tris-HCl buffer pH 8.2 with 5 mM EDTA were pipetted into a cuvette. The reaction was initiated by the addition of 50 µl of the yALDH sample into the cuvette and absorbance change at 340 nm was monitored by the increase in NADH concentration.

**Purification of yALDH/GAPDH after incubation with RSS.** After the incubation of yALDH/GAPDH with sulfur species the mixtures were transferred to Amicon Ultra Centrifugal Filters (Merck KGaA, Darmstadt, Germany) (30 K for ALDH and 10 K for GAPDH). The samples were next centrifuged at 8000 rpm for 5 min. Then, the filter device was transferred to a new tube and it was rinsed with buffer two or more times to remove excess of sulfur agents. After centrifugation, sulfane sulfur was assayed in each filtrate to verify the presence of sulfane sulfur compounds. The control sample of yALDH/GAPDH (without sulfur agents) was washed with buffer and centrifuged in the same way. After the last centrifugation, the concentrated enzyme samples were diluted with buffer to the initial volume of 0.5 ml and then were used for determination of protein-bound sulfur content as well as enzyme activity.

**yALDH activity assay.** 710 µl of 50 mM sodium phosphate buffer (pH 8.0), 200 µl of 5 mM NAD⁺ and 40 µl of 1 mM propionaldehyde were pipetted into a cuvette. The reaction was initiated by the addition of 50 µl of the yALDH sample into the cuvette and absorbance change at 340 nm was monitored for 1.5 min at 25°C to calculate the rate of NADH production and to compare it with the control sample containing only yALDH without sulfur agents. Data are presented as the percentage relative to control (100%).

**GAPDH activity.** 500 µl of 10 mM NAD⁺, 110 µl of 0.1 M potassium phosphate, 100 µl of glyceraldehyde-3-phosphate (GAP) and 273 µl of 50 mM Tris-HCl buffer pH 8.2 with 5 mM EDTA were pipetted into a cuvette. Reaction was initiated by the addition of 50 µl of enzyme sample with a sulfur agent or with the buffer (control sample). The enzymatic activity of GAPDH was monitored by the increase in NADH concentration measured at 340 nm. The effect of GSSH and GSSG on the activity of GAPDH was presented as the percent of control (untreated enzyme).

**Sulfane sulfur determination.** The content of compounds with this reactive kind of sulfur was determined as was described previously (Wood, 1987) based on the reaction of cyanolysis. Persulfides, polysulfides and other sulfane sulfur-containing compounds react with cyanide in alkaline solution to form thioceyanate, which reacts with ferric ions (Fe³⁺) yielding a red complex. Formaldehyde stabilizes the complex by the reaction with cyanide excess.

Briefly, to 100 µl of filtrate, 80 µl of 1 M NH₄, 720 µl of distilled water and 100 µl of 0.5 M KCN were added and mixed thoroughly. The samples were incubated at 37°C for 5 min and 20 µl of 38% formaldehyde solution and 200 µl of the Goldstein reagent containing Fe³⁺ cation was added. The absorbance was measured at a wavelength λ=460 nm. The whole pool of sulfane sulfur was evaluated from a standard curve for 1 mM KSCN and was expressed in nmoles of SCN⁻ per 1 ml of solution.

**Protein-bound sulfur estimation.** The level of sulfane sulfur bound to proteins (as persulfides) was assayed by the modified method of Ogasawara and coworkers (1994). In this method, sulfide ions released from protein persulfides by DTT reduction react with p-phenylenediamine in the presence of FeCl₃ yielding a fluorescent dye thionine.

Briefly, to 125 µl of yALDH/GAPDH solution, 125 µl of borate buffer (pH=9.0) and 250 µl of 20 mM DTT were added. The mixture was incubated at 37°C for 10 min and then 10 µl of 0.1 M NaOH, 400 µl of 12.5 mM p-phenylenediamine and 100 µl of 40 mM FeCl₃, in 6 M HCl were added. This reaction mixture was
again incubated for 10 min at room temperature. Then, the samples were centrifuged at 13,400 x g for 5 min and fluorescence was measured at wavelengths: λex=600 nm and λem=623 nm. The bound sulfane sulfur was evaluated from a standard curve for 100 μM Na₂S and was expressed in nmol of Na₂S per 1 ml of enzyme solution.

Animals and treatment. Experimental protocols involving the use of laboratory animals were approved by the Ethics Committee for Animal Research in Kraków (94/VIII/2011). The experiments were carried out on male Wistar rats weighing approximately 250 g. The animals were divided randomly into two groups of six animals each. Diallyl trisulfide (DATS) was dissolved in corn oil and administered i.p. at a dose of 25 mg/kg in the total volume of 0.3 ml to one of the animal groups for successive 7 days. Control rats (second group of animals) received 0.3 ml of vehicle (corn oil) in the same way. On the 8th day of experiment the rats were sacrificed by decapitation, the livers were collected, placed in liquid nitrogen and stored at −80°C until ALDH activity test was performed.

Preparation of liver homogenates. The frozen livers were weighed and homogenates were prepared by homogenization of 1 g of the tissue in 4 ml of 0.1 M phosphate buffer, pH 7.4 using an IKA-ULTRA-TURRAX T8 homogenizer.

Determination of ALDH activity in the rat liver homogenate. The assay mixture contained liver homogenate, sodium phosphate buffer (pH 8.2), NAD⁺, EDTA, 4-methylpyrazole and rotenone. The reaction was initiated by the addition of propionaldehyde as a substrate. 4-Methylpyrazole was added to inhibit alcohol dehydrogenase, and rotenone to inhibit mitochondrial NADH oxidase. The blank sample in which the homogenate was omitted was run simultaneously. The activity of ALDH was calculated using the molar extinction coefficient of NADH of 6.22 mM⁻¹cm⁻¹ at 340 nm with the use of a modified protocol published earlier (Tottmar et al., 1975).

Specific activity of the enzyme was expressed as nmol of NADH produced per 1 mg of protein per 1 min. The protein content was measured using the method of Lowry and coworkers (Lowry et al., 1951).

Statistical analysis. Results from in vitro study are presented as the mean ± standard deviation (S.D.) of three replicates. Statistical calculations were carried out with the STATISTICA 13.0 computer program using a one-way ANOVA followed by the Tukey post-hoc test. Data from the experiment on animals were analyzed statistically by Student’s t-test and are presented as the mean ± S.D. for each group of animals. For all data, the values of p<0.05 were considered as statistically significant.

RESULTS

The effect of various sulfur agents on γALDH activity

First, the effect of different potential organic and inorganic sulfur containing compounds on the activity of γALDH was assayed. The obtained results are presented in Fig. 1. Incubation of γALDH with Na₂S and Na₂S₃O₃ led only to a slight inhibition of its activity, while hydropersulfide GSSH suppressed activity of γALDH by nearly 50% compared to the control value. Polysulfide K₃S₅ which is a rich source of sulfane sulfur, in the used concentration inhibited γALDH very strongly (by 96.8% compared to the control activity) (Fig. 1A).

Figure 1. The effect of various reactive sulfur species on the activity of γALDH.

The samples of γALDH were incubated at room temperature for 15 min, then the activity was determined using propionaldehyde as the substrate. (A) Na₂S, GSSH (obtained by mixing GSSG and Na₂S) and Na₂S₃O₃ were used at 1 mM concentration while K₃S₅ at a concentration of 0.35 mg/ml. (B) All diallyl sulfides were dissolved in DMSO and then diluted with the buffer. *p<0.05; **p<0.001; ***p<0.001 compared to the untreated ALDH.

Figure 1B presents the effect of various garlic-derived allyl sulfides (diallyl sulfide, DAS; diallyl disulfide, DADS and diallyl trisulfide, DATS) on the activity of γALDH. DAS lacks sulfane sulfur, DATS is a sulfane sulfur-containing compound, while DADS can be isomerized to sulfane sulfur-containing thiosulfoxide. It was observed that DATS showed a very strong inhibitory effect on γALDH activity at 0.05 mM and 0.1 mM concentrations. DADS inhibited activity of γALDH too but to a lesser extent and the effect of DAS was the weakest (Fig. 1B). The results suggested that the activity of γALDH could be regulated by sulfur agents which in this case led to the inhibition of the enzyme. Moreover, it was clearly demonstrated that not H₂S but polysulfides (DATS, K₃S₅) or hydropersulfides (GSSH) were the oxidizing species mainly responsible for γALDH inhibition.

The effect of various reducing agents on reversibility of γALDH inhibition

The reversibility of sulfane sulfur-induced γALDH inhibition was studied using various reducing agents including DTT, GSH and DHLA. γALDH was incubated with the most effective sulfur agents (GSSH, K₃S₅ and DATS) together with a respective reductant, and the activity was measured and compared with the sample without the reducer. Simultaneously, the effect of reducing agents on γALDH activity in the absence of sulfur agents was assayed. The results presented in Fig. 2 showed that 100% of enzyme activity was restored when DTT was added to the incubation milieu. Other reductants used in twice higher concentration than DTT also diminished the inhibitory effect of sulfur species but to a
lesser extent. The effect of DHLA as a reversible agent was stronger than GSH’s (Fig. 2).

These results suggested that RSS-induced inhibition of ALDH is a reversible process but the presence of physiological reducers (GSH and DHLA) could be insufficient to restore the full activity of ALDH.

Modification of yALDH by RSS and its purification on Amicon Ultra Centrifugal Filters after the treatment

The mixtures containing yALDH and sulfur reagents after the incubation were purified to remove the excess of sulfur agents using Amicon Ultra Centrifugal Filters. Each filtrate was tested for sulfane sulfur presence by the cyanolysis method (Wood 1987) and the results were presented in Fig. 3. The high content of sulfane sulfur was detected in filtrate after incubation with K$_2$S$_x$ depending on its concentration and after GSSH treatment. Sulfane sulfur concentration decreased sharply with each rinsing. After 3rd and 2nd rinsing the content of free sulfane sulfur was comparable to control samples in K$_2$S$_x$ and GSSH samples, respectively.

Figure 2. The ability of various reductants (DTT, GSH and DHLA) to reverse RSS-induced inhibition of ALDH activity.

yALDH was incubated with 1 mM GSSH, K$_2$S$_x$ (0.35 mg/ml) or 0.05 mM DATS, together with the respective reducer. The activity of the enzyme was measured and compared with the sample without the reducer. Simultaneously, the effect of reducing agents on ALDH activity in the absence of sulfur compounds was assayed (untreated). **p<0.05; ***p<0.001 compared to the sample with RSS without the reductant.

Figure 3. The contents of free sulfane sulfur in filtrates after incubation of yALDH with sulfur agents, estimated by the cyanolysis method. (A) after K$_2$S$_x$ treatment; (B) after Na$_2$S, GSSG and GSSH treatment (each at 2 mM concentration).

Figure 4. The effect of K$_2$S$_x$ on: (A) the level of protein-bound sulfur and (B) enzymatic activity of yALDH compared to the control (untreated) enzyme. ***p<0.001 compared to the control enzyme.

Figure 5. The effect of GSSH, GSSG and Na$_2$S on: (A) the level of protein-bound sulfur and (B) the activity of yALDH compared to the control (untreated) enzyme. Na$_2$S, GSSH (obtained by mixing GSSG and Na$_2$S) and GSSG were used at 2 mM concentration. **p<0.01; ***p<0.001 compared to the control sample.
Figure 4 presents the level of protein-bound sulfur in purified samples of yALDH (Fig. 4A) and yALDH activity (Fig. 4B) after incubation with K$_2$S$_x$. A significant amount of protein-bound sulfur was detected in K$_2$S$_x$-treated yALDH, which was dependent on its concentration. It was accompanied by the complete inhibition of yALDH activity after incubation with the polysulfide.

The results illustrating the effect of GSSH, Na$_2$S and GSSG on the level of ALDH-bound sulfur are presented in Fig. 5A. A significant content of protein hydroperoxysulfides was detected only after the treatment of yALDH with GSSH. The activity of the enzyme was completely inhibited in this case (Fig. 5B). After treatment with GSSG, the activity of yALDH was decreased to 65% of the control but it was not accompanied by an increase in protein-bound sulfur content. This effect was probably connected with modification by S-glutathionylation.

Figure 6. The contents of free sulfane sulfur in filtrates after incubation of rabbit muscle GAPDH with sulfur agents (GSSG and GSSH) estimated by the cyanolysis method.

The effect of various sulfur species on the level of protein-bound sulfur and the activity of yALDH

The significant content of protein-bound sulfur was detected after treatment of GAPDH with GSSH (Fig. 7A). Both tested sulfur compounds (GSSG and GSSH) decreased the activity of GAPDH but only by 20–30% (Fig. 7B). The drop in GAPDH activity after GSSG treatment, similarly to ALDH, can be explained by S-glutathionylation. The decrease in the activity together with the rise in protein-bound sulfur after GSSH treatment suggests hydroperoxysulfide formation. Moreover, in comparison to the experiment with ALDH, GAPDH was less sensitive to this modification.

The effect of various RSS on the level of protein-bound sulfur and activity of GAPDH

In order to verify the inhibiting effect of sulfane sulfur species on ALDH activity in vivo, DATS dissolved in corn oil was administered ip to rats. The activity of ALDH was estimated in the rat liver after DATS treatment and compared to the control rats which received ip corn oil. The obtained results are presented in Fig. 8. The activity of ALDH in the liver of rats after administration of DATS was statistically significantly decreased in comparison to control animals ($p<0.001$). It suggests that in in vivo conditions DATS administration can lead to the inhibition of ALDH activity.

DISCUSSION

Mutations of some isozymes of the human ALDH superfamily are associated with inborn anomalies leading to altered aldehyde metabolism and in the consequence to some pathologies (Sladek, 2003). Among the isozymes, mitochondrial ALDH2 is a low $K_m$ enzyme responsible for the metabolism of acetaldehyde and lipid peroxides,
such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE). Moreover, it was documented that ALDH2 was implicated in nitroglycerin bioactivation and cocaine addiction (Chen et al., 2005; Yao et al., 2010). Studies on animal models revealed that the accumulation of toxic aldehydes after myocardial or cerebral ischemia/reperfusion (I/R) injury, such as HNE and MDA, was prevented by ALDH2. The ALDH2 activator Alda-1 demonstrated protective effects on heart and brain I/R injuries (Luo et al., 2014). All these facts and other published data show that ALDH2 is an important enzyme playing many physiological roles.

ALDH2, especially under oxidative/nitrosative stress is susceptible to many various posttranslational modifications with participation of its –SH groups, such as oxidation, S-nitrosylation and S-glutathionylation (Song et al., 2011). It is well documented that this enzyme is reversibly inhibited through S-nitrosylation of its cysteine groups in the presence of various NO donors (Moon et al., 2005).

In the present study, we demonstrated for the first time that ALDH activity could be regulated by sulfane sulfur species. This subtle modification connected with transformation of sulfhydryl group to persulfide led to inhibition of the enzyme. All tested sulfur compounds produced an inhibitory effect on the activity of γALDH, but polysulfides (DATS and K₂Sₓ) and hydropersulfides (GSSH) had the strongest influence, while the effects of Na₂SₓOₓ and Na₂S alone were very slight (Fig. 1). It confirmed the suggestions of some authors that persulfide formation (called S-sulfhydration) is caused by the oxidizing agents, such as sulfane sulfur-containing species (Toohey, 2011). Interestingly, thiosulfate was not an efficient source of sulfur for this process, although one of its sulfur atoms has properties of sulfane sulfur. It means that only sulfane sulfur in the form of persulfides or polysulfides can oxidize –SH groups of γALDH leading to the inhibition of the enzyme.

The hydropersulfide formation is a reversible modification which can be reverted by reducing agents. In the present study, DTT restored the total activity of ALDH treated with poly- or persulfides, while DHLA and then GSH (at twice as high concentration as DTT) also caused its activation but less effectively (Fig. 2). It seems to be consistent with other studies showing that dithiols are most efficient in reducing persulfides (Mikami et al., 2011). Wenzel and coworkers (2007) investigated the effect of various oxidants on ALDH activity and possibilities of its restoration using yeast enzyme. In their study, ALDH activity inhibited by superoxide, peroxynitrite or nitroglycerin was restored by dithiol compounds, such as DTT or DHLA, while GSH had only a minor effect. Because DTT is a synthetic compound and endogenous GSH could not fully reactivate ALDH, it seems that in physiological conditions DHLA is implicated in the restoration of oxidatively inhibited ALDH.

In the present study samples of ALDH after treatment with K₂Sₓ and GSSH were purified using Amicon Ultra Centrifugal Filters to remove poly- or persulfide excess, and the content of protein-bound sulfur was assayed. The obtained results suggested that ALDH does not contain persulfide groups, however, K₂Sₓ and GSSH treatment significantly increased protein-bound sulfur content (Figs. 4A, 5A). It was accompanied by a complete loss of γALDH activity (Figs. 4B, 5B). It is worth mentioning that the concentration of sulfur compounds used in this experiment, especially GSSH, was twice as high as in the first experiment (Fig. 1), so its effect on γALDH activity was stronger than previously observed.

The GSSG-induced inhibition of ALDH was not connected with the increase in protein-bound sulfur because it was caused by another thiol modification, namely S-glutathionylation. The lack of protein-bound sulfur after treatment with Na₂S confirmed that not sulfides but polysulfides or persulfides were responsible for modification and inhibition of ALDH.

GAPDH, the enzyme reported earlier to undergo S-sulfhydration, was used in our study as a reference protein. The results presented in Fig. 7 showed that GSSH decreased GAPDH activity. Study of Mustafa and coworkers (Mustafa et al., 2009) reported that S-sulfhydration of GAPDH led to augmentation of its catalytic activity. On the other hand, recently Jarosz and coworkers (2015) showed a decrease in GAPDH activity caused by polysulfide, similarly as in our study. GSSG lowered GAPDH activity (Fig. 7B) which was connected with S-glutathionylation, like in the case of ALDH. Incubation of GAPDH with GSSH led to an increase in protein-bound sulfur (persulfide formation) (Fig. 7A), exactly like in the case of ALDH. Generally, it seems that GAPDH in our study was less sensitive to modification by RSS than ALDH. It was observed in the case of modification by GSSG (S-glutathionylation) and especially in the case of modification by GSSH. It can result from the fact that GAPDH has less Cys residues per one mole than ALDH and it is partially oxidized. In many reports, GAPDH was reduced with DTT before experiments to obtain fully reduced –SH groups. Moreover, in our study, in the opposite to ALDH experiments, the protein-bound sulfur was estimated after the reaction of GAPDH with Na₂S (not shown) which suggested that some of its –SH groups were reversibly oxidized.

To verify whether ALDH is modified in a similar manner in in vivo conditions, we performed a pilot study using rats that were injected ip with DATS at a dose of 25 mg/kg b.w. for successive 7 days. We chose this dose and duration of the experiment based on our previous studies performed on mice (Iciek et al., 2012; Iciek et al., 2016). ALDH activity was estimated in the rat liver and compared to the control animals. The obtained results revealed a statistically significant decrease in ALDH activity in the liver of rats that were administered DATS in comparison to the control animals. The inhibitory effect of DATS in vivo (administered also by ip injection) on ALDH activity was demonstrated also in the mouse kidney (Iciek et al., 2016). It suggests that the modification of –SH groups may be responsible for this effect, but a detailed study is needed to verify this hypothesis.

Some previous studies demonstrated that ALDH activity could be regulated by other organosulfur compounds, namely isothiocyanates isolated from broccoli. It was documented in in vitro study using murine hepatoma cells as well as in in vivo studies performed on mice (Liu et al., 2017; Ushida & Talalay, 2013). In the latter experiment, mice were fed isothiocyanate for 7 days before a single administration of ethanol and then ALDH activity and expression (mRNA level) were estimated. The results of this study showed that isothiocyanate induced ALDH activity and significantly increased acetaldehyde metabolism (Ushida & Talalay, 2013). Similar results were obtained by Liu and coworkers (2017) who suggested that isothiocyanate derived from cruciferous vegetables was a potent inducer of total as well as mitochondrial fraction of ALDH in murine hepatoma cells. Both these papers insinuated that the increase in ALDH activity was connected with activation of the Keap1/Nrf2/ARE pathway which can be induced by a variety of small molecules including isothiocyanates. The transcription factor Nrf2
is a central agent involved in the regulation of antioxid-
ant-responsive element containing genes that are often
activated in response to oxidative stress. In normal non-
stress conditions, Nrf2 exists in the form bound with cy-
toskeleton Kelel-like ECH associated protein 1 (Keap1)
and this Keap1-Nrf2 complex is degraded by ubiquitin-
proteasome system (Grimsrud et al., 2008). Two Cys
residues in intervening region (IVR) of this protein play
a key role in repressive activity of Keap1. Under oxida-
tive stress, reactive oxygen species or electrophiles break
the bonds between Keap1 and Nrf2. As the effect, Nrf2
is accumulated in the nucleus where it activates many cy-
toprotective genes. It was documented that modification
of -SH groups in Cys residues can lead to dissociation
of Nrf2 and its translocation to the nucleus (Motohashi
& Yamamoto, 2004). Sulfuraphane and other natural iso-
thiocyanates activate ALDH via activation of Nrf2 prob-
ably by oxidation of the key Cys residues in Keap1. Our
study showed the inhibition of ALDH by the used sul-
fur compounds, among others by DATS. However, the
fluence of these compounds on the Keap1/Nrf2/ARE
pathway was not studied in our paper. We can only spec-
ulate that modification of -SH groups into corre-
ponding –SSH residues in this case did not lead to disso-
ciation of the Keap1-Nrf2 complex. This modification is
reversible and can be much milder than oxidation by sul-
furaphane but this issue needs to be clarified. Thus,
the observed biological effects on ALDH activity in the
studies mentioned above and in our study are different.
ALDH is involved in metabolism of aldehydes to
responding carboxylic acids. Inhibition of ALDH by
sulfuric compounds (i.e. DATS derived from gar-
ic) would lead to accumulation of these toxic aldehydes.
These findings may have important implications for al-
coholism, nitroglycerin bioactivation, cocaine addiction
as well as for East Asian people, many of whom show
a decreased ALDH2 activity due to the ALDH2 gene
mutation (Edenberg, 2007). The mitochondrial ALDH2 is
regarded as a crucial enzyme involved in protecting the
heart from oxidative stress (Chen et al., 2014; Pang et al.,
2015). Some studies also indicated a significant role of
ALDH2 in development of neurodegenerative diseases,
lke Parkinson’s and Alzheimer’s disease. Apart from
acetdehyde metabolism, ALDH2 is involved in oxida-
tion of other toxic aldehydes converting them to non-
toxic metabolites. 3,4-Dihydroxyphenylacetaldehyde
(DOPAL) is a dopamine metabolite in the brain and its accumulation can induce parkinsonism (Wey et al., 2012).
Another reactive aldehyde, HNE is formed as a result of oxidation of membrane lipids in the brain. It accumu-
lates in the hippocampal regions of patients with early
Alzheimer’s disease (Williams et al., 2006). The protective
role of ALDH2 in Alzheimer’s disease was confirmed by
epidemiological studies which showed a correlation be-
tween the incidence of this disease and inactive ALDH2 in
Asian patients (Hao et al., 2011). On the other hand,
the positive aspect of ALDH inhibition is that it can
be used in cancer therapy, because the accumulation of
toxic aldehydes promotes death of cancer cells. The
study of Kim and coworkers (Kim et al., 2016) indicat-
ed that the inhibition of ALDH activity was one of the
mechanisms by which Dats suppressed the growth of
breast cancer cells in vitro and in vivo.

CONCLUSIONS
All results obtained from in vitro study clearly demon-
strated that ALDH activity could be regulated by RSS
and in this case, the addition of a sulfane sulfur atom
to the Cys residue led to the inhibition of the enzyme.
However, we are aware that the results presented here
should be treated as preliminary study and detailed inves-
tigations to clarify the mechanism of ALDH inhibi-
tion observed here are needed. Our results also suggest-
ed that polysulfides (DATS, K,S, or hydrosulfides
(GSSH), rather than H,S were the oxidizing species
responsible for observed inhibition. Moreover, it seems
that ALDH is more sensitive to modification by RSS
than GAPDH, another protein, the activity of which is
regulated in this way. The present studies also showed
that DATS inhibited ALDH activity in the rat liver, which
suggests that this modification occurs also in vivo
under the influence of RSS.

Conflict of Interest
The authors do not have any conflict of interest re-
garding this manuscript.

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REFERENCES
Chen CH, Ferreira JG, Gross ER, Monchy-Rosen D (2014) Targeting
aldehyde dehydrogenase 2: new therapeutic opportunities. Physiol
Chen Z, Foster MW, Zhang J, Mao L, Rockman HA, Kawamoto T,
Kitagawa K, Nakayama KI, Hess DT, Stamler JS (2005) An essen-
tial role for mitochondrial aldehyde dehydrogenase in nitroglycerin
bioactivation. Proc Natl Acad Sci U S A 102: 12159–12164
Edenberg HJ (2007) The genetics of alcohol metabolism: role of al-
cohol dehydrogenase and aldehyde dehydrogenase variants. Alcohol
Health Res 30: 5–13
Greiner R, Pålkinas Z, Büsell K, Becker D, Antelmann H, Nagy P,
Grimsrud P, Xie H, Griffin T, Bernlohr D (2008) Oxidative stress and
equilvalent modification of protein with bioactive aldehydes. J Biol
of aldehyde dehydrogenase 2 gene polymorphism and Alzheimer’s
Icek M, Bilska-Wilkosz A, Górný M, Sokoloska-Żejewicz M, Kow-
alezyk-Pachel D (2016) The effects of different garlic-derived allyl
sulfides on anarobic sulfur metabolism in the mouse kidney. Anti-
nocicept. 5: pp: E46
Icek M, Kowalezyk-Pachel D, Bilska-Wilkosz A, Kwiecień I, Górný
Icek M, Kowalezyk-Pachel D, Kwiecień I, Dudek M (2012) Effects of
different garlic-derived allyl sulfides on peroxidative processes and
anarobic sulfur metabolism in mouse liver. Physiopath Res 26: 425–
Jarosz AP, Wei W, Gauld JW, Auld J, Özcan F, Aslan M, Mutus B,
icke 3-phosphate dehydrogenase (GAPDH) is in-
http://doi:10.1016/j.freeradbiomed.2015.09.007
tion of pyruvate carboxylase contributes to gluconeogenesis in liver
bba.2015.08.015
head Box Q1 is a novel target of breast cancer stem cell inhibition
10.1074/jbc.M116.715219
Koppaka V, Thompson DC, Chen Y, Ellermann M, Nicolaou KC, Ju-
Aldehyde dehydrogenase inhibitors: a comprehensive review of the
pharmacology, mechanism of action, substrate specificity, and clin-
111.005538
Liu Y, Yamanaaka M, Abe-Kanoh N, Liu X, Zhu B, Munemasa S, Na-
kamura T, Murata Y, Nakamura Y (2017) Benzyl isothiocyanate
ameliorates acetaldehyde-induced cytotoxicity by enhancing aldehyde


Toohey JI (2011) Sulfur signaling is the agent sulfide or sulfane? Anal Biochem 413: 1–7


