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

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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 Na\textsubscript{2}S, GSSG, K\textsubscript{2}S\textsubscript{2}, Na\textsubscript{2}S\textsubscript{2}O\textsubscript{8}, 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 H\textsubscript{2}S 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

INTRODUCTION

Ethanol metabolism is catalyzed by aldehyde dehydrogenase (ADH), microsomal ethanol oxidizing system (MEOS) (cytochrome P450SE1) or catalase. 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\textsuperscript{+}-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 sulphydryl 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 cambylation of cysteine residue in the active site (Koppaka et al., 2012). On the other hand, daidzin, an active isoflavone 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 (H\textsubscript{2}S) and products of its oxidation: inorganic polysulfides (H\textsubscript{2}S\textsubscript{n}) and hydroperoxides (RSSH), that means compounds containing reactive sulfane sulfur. RSS can be also obtained from natural exogenous sources especially from garlic-derived organosulfur compounds, i.e. diallyl trisulfide (DATS) and H\textsubscript{2}S-releasing molecules. There are many studies documenting pharmacological effects of H\textsubscript{2}S and garlic-derived sulfane sulfur compounds (Toolehy & 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, K₂S₃, Na₂S₃O₆, 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 (K₂S₃) sodium sulfide, sodium thiosulfate (Na₂S₃O₆), glutathione (GSH), glutathione disulfide (GSSG), dihydroxyacid (DHLA), lipoic acid (LA), dithiothreitol (DTT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle, glyceraldehyde-3-phosphate dehydrogenase-3-phosphate (GAP), p-phenylenediamine, propionaldehyde as well as 4-methylpyrazole, rotenone, NAD⁺, NADH, ATP, and other sulfur 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 thiocyanate, 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 NH₄Cl, 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 cyanide in alkaline solution to form thiocyanate, which reacts with ferric ions (Fe³⁺) yielding a red complex. Formaldehyde stabilizes the complex by the reaction with cyanide excess.

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

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 275 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 25 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 thiocyanate, which reacts with ferric ions (Fe³⁺) yielding a red complex. Formaldehyde stabilizes the complex by the reaction with cyanide excess.

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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.

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again incubated for 10 min at room temperature. Then, the samples were centrifuged at 13,400 × g for 5 min and fluorescence was measured at wavelengths: A = 600 nm and A = 623 nm. The bound sulfane sulfur was evaluated from a standard curve for 100 μM Na₂S and was expressed in nmoles 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 Krakow (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 sodium phosphate buffer (pH 8.2), NAD homogenate RAX T8 homogenizer. 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 Na₂S₂O₇ led only to a slight inhibition of its activity, while hydroperoxysulfide GSSH suppressed activity of γALDH by nearly 50% compared to the control value. Polysulfide K₂S₃, which is a rich source of sulfur fuel, in the used concentration inhibited γALDH very strongly (by 96.8% compared to the control activity) (Fig. 1A).

![Figure 1A](image1)

**Figure 1A.** Incubation of γALDH with Na₂S and Na₂S₂O₇ led only to a slight inhibition of its activity, while hydroperoxysulfide GSSH suppressed activity of γALDH by nearly 50% compared to the control value.

**Figure 1B.** The effect of various garlic-derived thiosulfates (diallyl sulfide, DAS; diallyl disulfide, DADS and diallyl trisulfide, DATS) on the activity of γALDH.

![Figure 1B](image2)

**Figure 1B.** 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, K₂S₃) or hydroperoxysulfides (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, K₂S₃ 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 γALDH by RSS and its purification on Amicon Ultra Centrifugal Filters after the treatment

The mixtures containing γALDH 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 K₂Sₓ 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 K₂Sₓ and GSSH samples, respectively.

Figure 2. The ability of various reductants (DTT, GSH and DHLA) to reverse RSS-induced inhibition of ALDH activity. γALDH was incubated with 1 mM GSSH, K₂Sₓ (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 γALDH with sulfur agents, estimated by the cyanolysis method. (A) after K₂Sₓ treatment; (B) after Na₂S, GSSG and GSSH treatment (each at 2 mM concentration).

Figure 4. The effect of K₂Sₓ on: (A) the level of protein-bound sulfur and (B) enzymatic activity of γALDH compared to the control (untreated) enzyme. ***p<0.001 compared to the control enzyme.

Figure 5. The effect of GSSH, GSSG and Na₂S on: (A) the level of protein-bound sulfur and (B) the activity of γALDH compared to the control (untreated) enzyme. Na₂S, GSSH (obtained by mixing GSSG and Na₂S) and GSSG were used at 2 mM concentration. **p<0.01; ***p<0.001 compared to the control sample.
Inhibition of aldehyde dehydrogenase by sulfur compounds

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

Figure 4 presents the level of protein-bound sulfur in purified samples of γALDH (Fig. 4A) and γALDH activity (Fig. 4B) after incubation with K₂S₂. A significant amount of protein-bound sulfur was detected in K₂S₂-treated γALDH, which was dependent of its concentration. It was accompanied by the complete inhibition of γALDH activity after incubation with the polysulfide.

The results illustrating the effect of GSSH, Na₂S and GSSG on the level of ALDH-bound sulfur are presented in Fig. 5A. A significant amount of protein hydrosulfides was detected only after the treatment of γALDH with GSSH. The activity of the enzyme was completely inhibited in this case (Fig. 5B). After treatment with GSSG, the activity of γALDH 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.

Modification of GAPDH by RSS and its purification on Amicon Ultra Centrifugal Filters after treatment with various sulfur agents

GAPDH as a reference enzyme was incubated with various RSS and was purified using Amicon Ultra Centrifugal Filters 10 K. Figure 6 presents the sulfane sulfur contents in filtrates after each rinsing. Like in the case of ALDH, the high content of sulfane sulfur was detected after incubation with GSSH. Due to a smaller size of filter pores, more rinsing was needed to purify GAPDH when compared to ALDH.

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

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 hydrosulfide formation. Moreover, in comparison to the experiment with ALDH, GAPDH was less sensitive to this modification.

The effect of DATS administration on ALDH activity in the rat liver

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<0.01). 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 Kₘ 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 K$_2$S$_3$) and hydropersulfides (GSSH) had the strongest influence, while the effects of Na$_2$S$_2$O$_3$ and Na$_2$S 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 DHILA 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 DHILA, while GHS 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 DHILA is implicated in the restoration of oxidatively inhibited ALDH.

In the present study samples of ALDH after treatment with K$_2$S 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, K$_2$S 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 Na$_2$S 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 GSSG 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 Na$_2$S (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 i$p$ 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 administrated DATS in comparison to the control animals. The inhibitory effect of DATS in vivo (administered also by i$p$ 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 experiments, 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 acetdehyde 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 antioxidant-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 cytoskeleton Kelech-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 oxidative 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 cytoprotective 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). Sulforaphane and other natural iso-thiocyanates activate ALDH via activation of Nrf2 probably by oxidation of the key Cys residues in Keap1. Our study showed the inhibition of ALDH by the used sulfur compounds, among others by DATS. However, the influence of these compounds on the Keap1/Nrf2/ARE pathway was not studied in our paper. We can only speculate that modification of –SH groups into corresponding –SSH residues in this case did not lead to dissociation of the Keap1-Nrf2 complex. This modification is reversible and can be much milder than oxidation by sulforaphane 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 corresponding carboxylic acids. Inhibition of ALDH by sulfuric acid compounds (i.e. DATS derived from garlic) would lead to accumulation of these toxic aldehydes. These findings may have important implications for alcoholism, 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, like Parkinson’s and Alzheimer’s disease. Apart from acetaldehyde metabolism, ALDH2 is involved in oxidation 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 accumulates 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 between 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) indicated 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 demonstrated that ALDH activity could be regulated by RSS and in this case, the addition of a sulfate 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 investigations to clarify the mechanism of ALDH inhibition observed here are needed. Our results also suggested that polysulfides (DATS, K₂S₃) or hydropersulfides (GSSH), rather than H₂S 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 regarding this manuscript.

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