ACTA ABP BIOCHIMICA POLONICA

Vol. 65, No 1/2018
125–132
https://doi.org/10.18388/abp.2017_2324

Regular paper

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 hydropersulfides. 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_2S, GSSH, K_2S_x, $Na_2S_2O_3$, 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_2S but polysulfides or hydropersulfides were the oxidizing species responsible for this modification. This process was easily reversible by reducing agents. After the treatment with polysulfides or hydropersulfides 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

Received: 20 September, 2017; revised: 21 December, 2017; accepted: 28 December, 2017; available on-line: 09 March, 2018

✉e-mail: miciek@cm-uj.krakow.pl

*The results were presented at: X Parnas Conference "Molecules in the Living Cell and Innovative Medicine"; Wrocław, Polska; 10–12.07.2016 as a poster (Aldehyde dehydrogenase is inactivated by S-sulfhydration *in vitro* and *in vivo* – *Acta Biochim Pol* vol. 63. Suppl.1/2016, p.17)

Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; DATS, diallyl trisulfide; DHLA, dihydrolipoic acid; DTT, dithiothreitol; GSH, glutathione; RSS, reactive sulfur species

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 (MEOS) (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 isoflavon 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 hydropersulfides (–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_2S) and products of its oxidation: inorganic polysulfides (H_2S_n) and hydropersulfides (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_2S-releasing molecules. There are many studies documenting pharmacological effects of H_2S and garlic-derived sulfane sulfur compounds (Toohey & 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 Na_2S, GSSH, K_2S_x, $Na_2S_2O_3$, 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 (K_2S_x) sodium sulfide, sodium thiosulfate ($Na_2S_2O_3$), glutathione (GSH), glutathione disulfide (GSSG), dihydrolipoic acid (DHLA), lipoic acid (LA), dithiothreitol (DTT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle, glyceraldehyde-3-phosphate (GAP), p-phenylenediamine, propionaldehyde as well as 4-methylpyrazole, rotenone, NAD^+ and Folin-Ciocalteau reagent were provided by Sigma-Aldrich Chemical Company (Poznań, Poland). Diallyl sulfides (DAS, DADS and DATS) were purchased from LKT Laboratories, Inc. (Minnesota, USA). All other reagents were of analytical grade and were obtained from Polish Chemical Reagent Company (POCh, Gliwice, Poland)

The effect of various sulfur species on yALDH activity. All enzyme-containing mixtures were incubated at a temperature of 25°C. ALDH (6 mg/ml) in 50 mM sodium phosphate buffer (pH 8.0) was preincubated with various sulfur species (K_2S_x, $Na_2S_2O_3$, Na_2S, GSSH obtained by mixing GSSG with Na_2S and different diallyl sulfides) with or without a reducing agent (DTT, DHLA, GSH) for 15 min. Immediately thereafter, ALDH activity was determined in every sample.

The effect of various sulfur species on GAPDH activity. GAPDH (0.5 mg/ml) in 50 mM Tris-HCl buffer, pH 8.2 was incubated analogically as yALDH with GSSH and GSSG for 15 min. The control GAPDH was incubated only with the buffer. The protein-bound sulfur content and enzyme activity were then assayed.

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 pippeted 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 (Fe^{3+}) 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 NH_3, 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 Fe^{3+} 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 $FeCl_3$ 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 $FeCl_3$ 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 × *g* for 5 min and fluorescence was measured at wavelengths: A_{ex}=600 nm and A_{em}=623 nm. The bound sulfane sulfur was evaluated from a standard curve for 100 μM Na_2S and was expressed in nmoles of Na_2S 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 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 $mM^{-1}cm^{-1}$ 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 yALDH activity

First, the effect of different potential organic and inorganic sulfur containing compounds on the activity of yALDH was assayed. The obtained results are presented in Fig. 1. Incubation of yALDH with Na_2S and $Na_2S_2O_3$ led only to a slight inhibition of its activity, while hydropersulfide GSSH suppressed activity of yALDH by nearly 50% compared to the control value. Polysulfide K_2S_x, which is a rich source of sulfane sulfur, in the used concentration inhibited yALDH very strongly (by 96.8% compared to the control activity) (Fig. 1A).

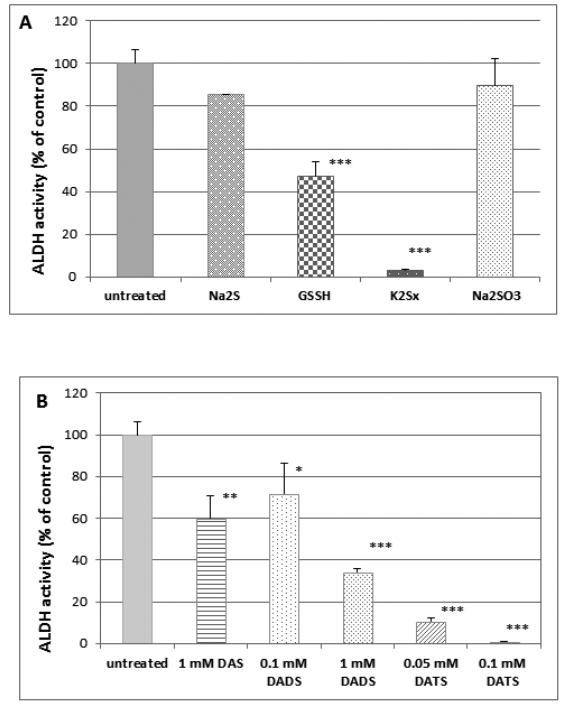

Figure 1. The effect of various reactive sulfur species on the activity of yALDH.
The samples of yALDH were incubated at room temperature for 15 min, then the activity was determined using propionaldehyde as the substrate. (A) Na_2S, GSSH (obtained by mixing GSSG and Na_2S) and $Na_2S_2O_3$ were used at 1 mM concentration while K_2S_x 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 yALDH. 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 yALDH activity at 0.05 mM and 0.1 mM concentrations. DADS inhibited activity of yALDH too but to a lesser extent and the effect of DAS was the weakest (Fig. 1B).

The results suggested that the activity of yALDH could be regulated by sulfur agents which in this case led to the inhibition of the enzyme. Moreover, it was clearly demonstrated that not H_2S but polysulfides (DATS, K_2S_x) or hydropersulfides (GSSH) were the oxidizing species mainly responsible for yALDH inhibition.

The effect of various reducing agents on reversibility of yALDH inhibition

The reversibility of sulfane sulfur-induced yALDH inhibition was studied using various reducing agents including DTT, GSH and DHLA. yALDH was incubated with the most effective sulfur agents (GSSH, K_2S_x 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 yALDH 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

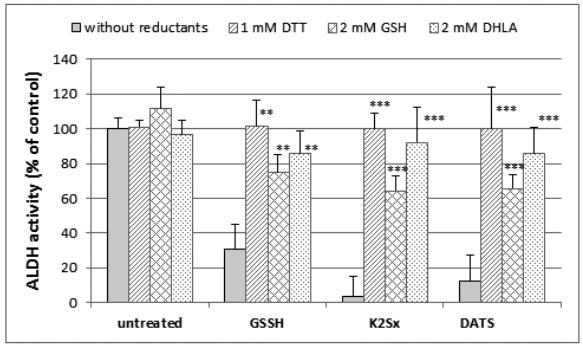

Figure 2. The ability of various reductants (DTT, GSH and DHLA) to reverse RSS-induced inhibition of ALDH activity.
yALDH was incubated with 1 mM GSSH, K_2S_x (0.35 mg/ml) or 0.05 mM DATS, together with the respective reducer. The activity of the enzyme was measured and compared with the sample without 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.

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

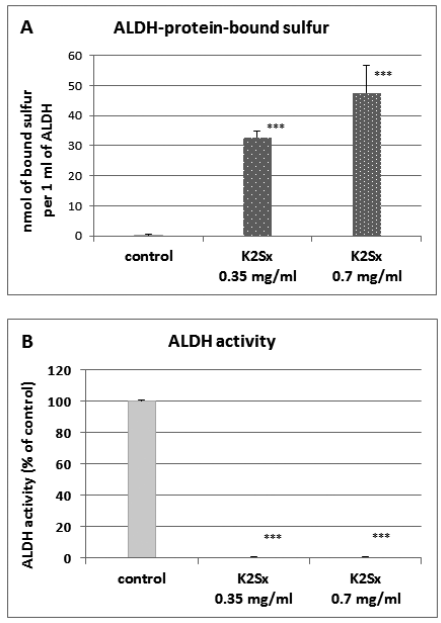

Figure 4. The effect of K_2S_x on: (A) the level of protein-bound sulfur and (B) enzymatic activity of yALDH compared to the control (untreated) enzyme. ***p<0.001 compared to the control enzyme.

-presented in Fig. 3. The high content of sulfane sulfur was detected in filtrate after incubation with K_2S_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_2S_x and GSSH samples, respectively.

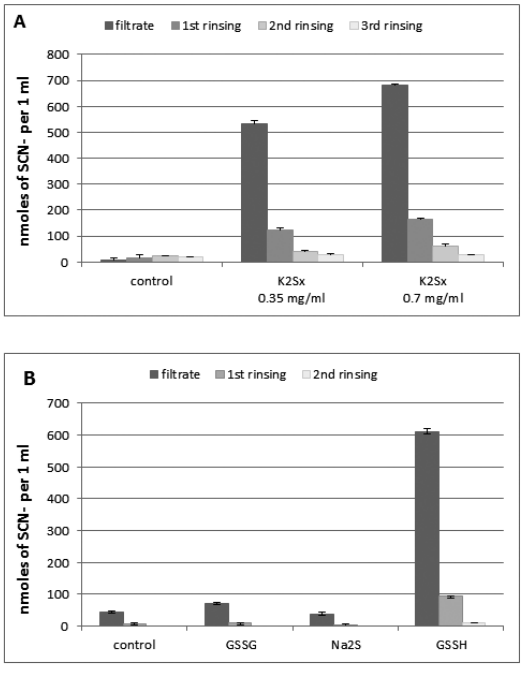

Figure 3. The contents of free sulfane sulfur in filtrates after incubation of yALDH with sulfur agents, estimated by the cyanolysis method.
(**A**) after K_2S_x treatment; (**B**) after Na_2S, GSSG and GSSH treatment (each at 2 mM concentration).

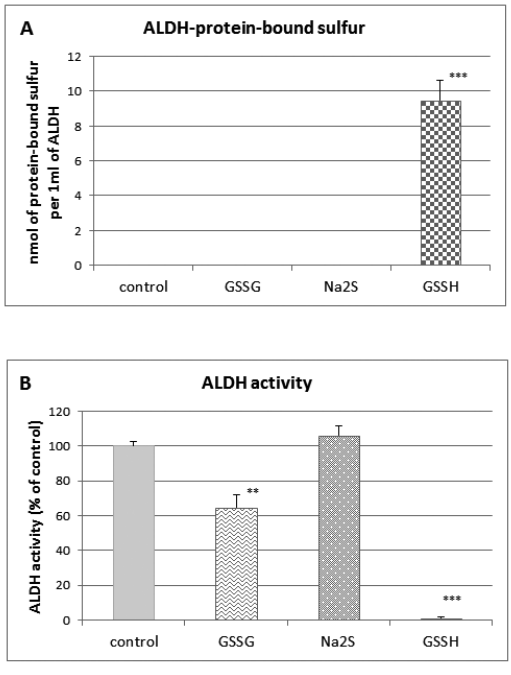

Figure 5. The effect of GSSH, GSSG and Na_2S on: (A) the level of protein-bound sulfur and (B) the activity of yALDH compared to the control (untreated) enzyme. Na_2S, GSSH (obtained by mixing GSSG and Na_2S) and GSSG were used at 2 mM concentration. **p<0.01; ***p< 0.001 compared to the control sample.

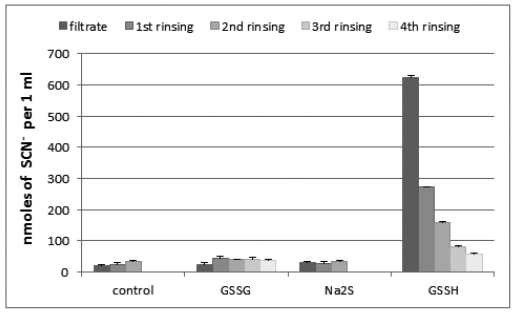

Figure 6. The contents of free sulfane sulfur in filtrates after incubation of rabbit muscle GAPDH with sulfur agents (GSSG and GSSH) estimated by the cyanolysis method.

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

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_2S_x. A significant amount of protein-bound sulfur was detected in K_2S_x-treated yALDH, which was dependent of 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_2S and GSSG on the level of ALDH-bound sulfur are presented in Fig. 5A. A significant content of protein hydropersulfides 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.

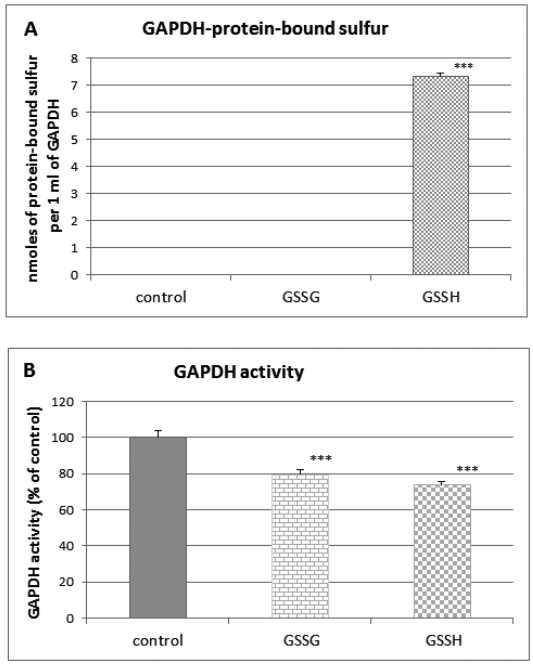

Figure 7. The effect of GSSH and GSSG on: (A) the level of protein-bound sulfur and (B) activity of rabbit muscle GAPDH compared to the control (untreated) enzyme. GSSH (obtained by mixing GSSG and Na_2S) and GSSG were used at 2 mM concentration.

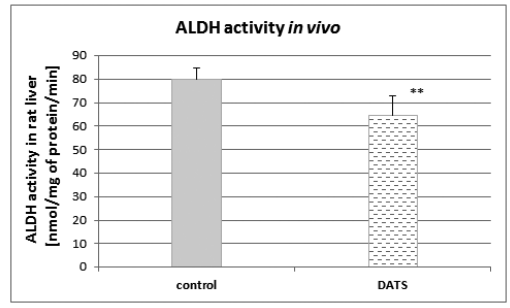

Figure 8. The effect of DATS dissolved in corn oil and injected *ip* at a dose of 25 mg per kg to the rats for 7 successive days on the activity of ALDH in the liver. Data represent the mean of 5 animals ± S.D. **p<0.01 significantly different from control group.

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 hydropersulfide 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=0.007). 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 yALDH, but polysulfides (DATS and K_2S_x) and hydropersulfides (GSSH) had the strongest influence, while the effects of $Na_2S_2O_3$ and Na_2S 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 yALDH 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 K_2S_x 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_2S_x and GSSH treatment significantly increased protein-bound sulfur content (Figs. 4A, 5A). It was accompanied by a complete loss of yALDH 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 yALDH 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_2S 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 Na_2S (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 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 Kelch-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 isothiocyanates 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 sulfane sulfur 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 (Wiliams *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 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 investigations to clarify the mechanism of ALDH inhibition observed here are needed. Our results also suggested that polysulfides (DATS, K_2S_x) or hydropersulfides (GSSH), rather than H_2S, 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.

Acknowledgements

This work was supported by statutory funds of the Faculty of Medicine Jagiellonian University Medical College, Kraków, Poland (No. K/ZDS/005886).

REFRENCES

Chen CH, Ferreira JC, Gross ER, Mochly-Rosen D (2014) Targeting aldehyde dehydrogenase 2: new therapeutic opportunities. *Physiol Rev* **94**: 1–34. http://doi: 10.1152/physrev.00017.2013

Chen Z, Foster MW, Zhang J, Mao L, Rockman HA, Kawamoto T, Kitagawa K, Nakayama KI, Hess DT, Stamler JS (2005) An essential role for mitochondrial aldehyde dehydrogenase in nitroglycerin bioactivation. *Proc Natl Acad Sci USA* **102**: 12159–12164

Edenberg HJ (2007) The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Res Health* **30**: 5–13

Greiner R, Pálinkás Z, Bäsell K, Becher D, Antelmann H, Nagy P, Dick TP (2013) Polysulfides link H2S to protein thiol oxidation. *Antioxid Redox Signal* **19**: 1749–1765. http://doi: 10.1089/ars.2012.5041

Grimsrud P, Xie H, Griffin T, Bernlohr D (2008) Oxidative stress and covalent modification of protein with bioactive aldehydes. *J Biol Chem* **283**: 21837–21841. http://doi: 10.1074/jbc.R700019200

Hao PP, Chen YG, Wang JL, Wang XL, Zhang Y (2011) Meta-analysis of aldehyde dehydrogenase 2 gene polymorphism and Alzheimer's disease in East Asians. *Can J Neurol Sci* **38**: 500–506

Iciek M, Bilska-Wilkosz A, Górny M, Sokołowska-Jeżewicz M, Kowalczyk-Pachel D (2016) The effects of different garlic-derived allyl sulfides on anaerobic sulfur metabolism in the mouse kidney. *Antioxidants* **5**: pii: E46

Iciek M, Kowalczyk-Pachel D, Bilska-Wilkosz A, Kwiecień I, Górny M, Włodek L (2015) S-sulfhydration as a cellular redox regulation. *Biosci Rep* **36**: e00304. http://doi: 10.1042/BSR20150147

Iciek M, Kowalczyk-Pachel D, Kwiecień I, Dudek M (2012) Effects of different garlic-derived allyl sulfides on peroxidative processes and anaerobic sulfur metabolism in mouse liver. *Phytother Res* **26**: 425–431. http://doi: 10.1002/ptr.3572

Jarosz AP, Wei W, Gauld JW, Auld J, Özcan F, Aslan M, Mutus B (2015) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is inactivated by S-sulfuration *in vitro*. *Free Radic Biol Med* **89**: 512–521. http://doi:10.1016/j.freeradbiomed.2015.09.007

Ju Y, Untereiner A, Wu L, Yang G (2015) H2S-induced S-sulfhydration of pyruvate carboxylase contributes to gluconeogenesis in liver cells. *Biochim Biophys Acta* **1850**: 2293–2303. http://doi: 10.1016/j.bbagen.2015.08.003

Kim SH, Kaschula CH, Priedigkeit N, Lee AV, Singh SV (2016) Forkhead Box Q1 is a novel target of breast cancer stem cell inhibition by diallyl trisulfide. *J Biol Chem* **291**: 13495–13508. http://doi: 10.1074/jbc.M116.715219

Koppaka V, Thompson DC, Chen Y, Ellermann M, Nicolaou KC, Juvonen RO, Petersen D, Deitrich RA, Hurley TD, Vasiliou V (2012) Aldehyde dehydrogenase inhibitors: a comprehensive review of the pharmacology, mechanism of action, substrate specificity, and clinical application. *Pharmacol Rev* **64**: 520–539. http://doi: 10.1124/pr.111.005538

Liu Y, Yamanaka M, Abe-Kanoh N, Liu X, Zhu B, Munemasa S, Nakamura T, Murata Y, Nakamura Y (2017) Benzyl isothiocyanate ameliorates acetaldehyde-induced cytotoxicity by enhancing aldehyde

dehydrogenase activity in murine hepatoma Hepa1c1c7 cells. *Food Chem Toxicol* **108**: 305–313. http://doi: 10.1016/j.fct.2017.08.016

Lowry OH, Rosenbrough NJ, Farr AL, Randall RI (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275

Luo XJ, Liu B, Ma QL, Peng J(2014) Mitochondrial aldehyde dehydrogenase, a potential drug target for protection of heart and brain from ischemia/reperfusion injury. *Curr Drug Targets* **15**: 948–955. http://doi:10.2174/1389450115666140828142401

Mikami Y, Shibua N, Kimura Y, Nagahara N, Ogasawara Y, Kimura H (2011) Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. *Biochem J* **439**: 479–485. http://doi: 10.1042/BJ20110841.

Motohashi H, Yamamoto M (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med* **10**: 549–557

Módis K, Ju Y, Ahmad A, Untereiner AA, Altaany Z, Wu L, Szabo C, Wang R (2016) S-Sulfhydration of ATP synthase by hydrogen sulfide stimulates mitochondrial bioenergetics. *Pharmacol Res* **113**: 116–124. http://doi:10.1016/j.phrs.2016.08.023

Moon KH, Kim BJ, Song BJ(2005) Inhibition of mitochondrial aldehyde dehydrogenase by nitric oxide-mediated S-nitrosylation. *FEBS Lett* **579**: 6115–6120. http://doi:10.1016/j.febslet.2005.09.082

Mustafa AK, Gadalla MM, Sen N, Kim S, Mu W, Gazi SK, Barrow RK, Yang G, Wang R, Snyder SH (2009) H2S signals through protein S-sulfhydration. *Sci Signal* **2**: ra72. http://doi: 10.1126/scisignal.2000464

Ogasawara Y, Isoda S, Tanabe S (1994) Tissue and subcellular distribution of bound and acid labile sulfur, and the enzymic capacity for sulfide production in the rat. *Biol Pharm Bull* **17**: 1535–1542

Orywal K, Jelski W, Werel T, Szmitkowski M (2017) The activity of class I, II, III and IV alcohol dehydrogenase isoenzymes and aldehyde dehydrogenase in the sera of bladder cancer patients. *Acta Biochim Pol* **64**: 81–84. http://doi: 10.18388/abp.2016_1289

Orywal K, Szmitkowski M (2017) Alcohol dehydrogenase and aldehyde dehydrogenase in malignant neoplasms. *Clin Exp Med* **17**: 131–139. doi: 10.1007/s10238-016-0408-3

Pang JJ, Barton LA, Chen YG, Ren J (2015) Mitochondrial aldehyde dehydrogenase in myocardial ischemia-reperfusion injury: from bench to bedside. *Sheng Li Xue Bao* **67**: 535–544. http://doi: 10.13294/j.aps.2015.0067

Paul BD, Snyder SH (2012) H_2S signaling through protein sulfhydration and beyond. *Nat Rev Mol Cell Biol* **13**: 499–507. http://doi: 10.1016/j.niox.2014.01.002.

Sladek NE (2003) Human aldehyde dehydrogenases: Potential pathological, pharmacological, and toxicological impact. *J Biochem Mol Toxicol* **17**: 7–23. http://doi: 10.1002/jbt.10057

Song BJ, Abdelmegeed MA, Yoo SH, Kim BJ, Jo SA, Jo I, Moon KH (2011) Post-translational modifications of mitochondrial aldehyde dehydrogenase and biomedical implications. *J Proteomics* **74**: 2691–2702. http://doi: 10.1016/j.jprot.2011.05.013

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

Toohey JI, Cooper AJ (2014) Thiosulfoxide (sulfane) sulfur: new chemistry and new regulatory roles in biology. *Molecules* **19**: 12789–12813. http://doi: 10.3390/molecules190812789.

Tottmar SO, Pettersson H, Kiessling KH (1975) The subcellular distribution and properties of aldehyde dehydrogenases in rat liver. *Biochem J* **135**: 577–586

Ushida Y, Talalay P (2013) Sulforaphane accelerates acetaldehyde metabolism by inducing aldehyde dehydrogenases: relevance to ethanol intolerance. *Alcohol Alcohol* **48**: 526–534. doi: 10.1093/alcalc/agt063

Wenzel P, Hink U, Oelze M, Schuppan S, Schaeuble K, Schildknecht S, Ho KK, Weiner H, Bachschmid M, Münzel T, Daiber A (2007) Role of reduced lipoic acid in the redox regulation of mitochondrial aldehyde dehydrogenase (ALDH-2) activity. Implications for mitochondrial oxidative stress and nitrate tolerance. *J Biol Chem* **282**: 792–799

Wey MC, Fernandez E, Martinez PA, Sullivan P, Goldstein DS, Strong R (2012) Neurodegeneration and motor dysfunction in mice lacking cytosolic and mitochondrial aldehyde dehydrogenases: implications for Parkinson's disease. *PLoS One* **7**: e31522. http://doi: 10.1371/journal.pone.0031522

Williams TI, Lynn BC, Markesbery WR, Lovell MA (2006) Increased levels of 4-hydroxynonenal and acrolein, neurotoxic markers of lipid peroxidation, in the brain in Mild Cognitive Impairment and early Alzheimer's disease. *Neurobiol Aging* **27**: 1094–1099

Wood JL (1987) Sulfane sulfur. *Methods Enzymol* **143**: 25–29

Yao L, Fan P, Arolfo M, Jiang Z, Olive MF, Zablocki J, Sun HL, Chu N, Lee J, Kim HY, Leung K, Shryock J, Blackburn B, Diamond I (2010) Inhibition of aldehyde dehydrogenase-2 suppresses cocaine seeking by generating THP, a cocaine use-dependent inhibitor of dopamine synthesis. *Nat Med* **16**: 1024–1028. http://doi: 10.1038/nm.2200.