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Short communication

Biological actions of lipoic acid associated with sulfane sulfur metabolism

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Abstract:
This work aimed to investigate the effect of lipoic acid (LA) on sulfane sulfur (S^*) level and rhodanese activity in rat tissues. *In vitro* studies conduced so far have indicated that dihydrolipoic acid serves as an S^* acceptor in the rhodanese-catalyzed S^* transfer. This study revealed a significant increase in S^* level and rhodanese activity in the heart, liver and kidney homogenates from rats that had previously been treated intraperitoneally with LA. It demonstrated for the first time *in vivo* that biological activity of lipoate might be connected with anaerobic cysteine metabolism to S^* and with rhodanese activity.

Key words:
lipoic acid, sulfane sulfur, rhodanese, reactive oxygen species

Introduction

Lipoic acid (1,2-dithiolane 3-pentanoic acid, LA) and its reduced form dihydrolipoic acid (DHLA) are naturally occurring compounds, with one chiral center. The R-form of LA is the only enantiomer synthesized in biological systems. LA has long been known as a coenzyme of multienzymatic mitochondrial complexes catalyzing oxidative decarboxylation of α-ketoacids, but the present investigations are focused on its pharmacological properties. Therapeutic action of LA is based on unique antioxidant properties of LA/DHLA system, which possesses one of the lowest standard biological redox potentials ($E^{0'}$ = –0.29V). Thus, DHLA is able to reduce not only reactive oxygen species (ROS) but also oxidized forms of other antioxidants. For this reason, it is called an antioxidant of antioxidants [29]. It is worth remembering that LA has antioxidant properties, as well. The reaction of LA with hydroxyl radical, for example, yields lipoic acid cation radical, which is transformed into LA by other antioxidants that can be regenerated by DHLA [25]. Exogenous LA can be reduced to DHLA by several enzymes, including mitochondrial lipoamide dehydrogenase, which utilizes NADH, and cytoplasmic NADPH- dependent reductases: glutathione reductase and thioredoxin reductase [2]. Owning to its easy absorption from the gastrointestinal tract and ability to cross the blood-brain barrier,

exogenous LA can reach the majority of tissues. Moreover, LA and DHLA are easily soluble both in fats and water, which is a unique feature among antioxidants. Other properties of lipoic acid, apart from the antioxidant function, comprise: modulation of mitogen-activated protein kinase activity [36], reduction of production of inflammatory mediators [43], lowering of endothelin expression [25], effect on secondary messengers of the nuclear factor β (NF- β) and peroxisome proliferator-activated receptors (PPAR) activation cascade [30, 34, 35], and implication in the regulation of carbohydrate and lipid metabolism [5, 8, 25].

At present, LA is a medication recommended in many countries at high doses (up to 1800 mg/24 h) in diabetic neuropathy [4]. Studies currently in progress support its use in the treatment of other diseases (cardiovascular, neurodegenerative, autoimmune diseases, cancer, AIDS) [5, 7, 11, 28, 39, 41].

Rhodanese (thiosulfate : cyanide sulfotransferase EC 2.8.1.1), described by Lang in 1933 [38], has been found in most of mammalian tissues, in frogs, birds and plants, in some insects and in several microorganisms. Rhodanese in humans occurs mainly in mitochondria, and its highest activity was detected in the liver [21, 37]. Rhodanese catalyzes sulfane sulfur (S^*) transfer to different acceptors. S^* is a labile reactive sulfur atom in the 0 or –1 oxidation state, covalently bound to another sulfur atom. Outer sulfur atom of thiosulfate ($S{=}SO_3^{2-}$) and elemental sulfur (S_8) also have these properties [20]. S^*-containing compounds are endogenous metabolites formed in mammalian cells by cysteine (Cys) desulfuration catalyzed by cystathionine γ-lyase (CSE) and 3-mercaptopyruvate transferase (MPST) [20, 40]. Rhodanese metabolizes such substrates as thiosulfate, thiosulfonates, organic and inorganic persulfides and a trisulfide – thiocystine. The role of sulfane sulfur acceptor can be fulfilled by cyanide, sulfate (IV), sulfinates and thiols [20, 40]. The reactions of S^* transfer from different donors (e.g. thiosulfate) to different acceptors (e.g. cyanide), catalyzed by rhodanese, involve the formation of an unstable sulfane sulfur-containing hydropersulfide with –SH group of Cys-$_{247}$, located in the active center of the enzyme.

$$S^* = SO_3^{-2} + \text{Rhodanese-Cys}_{247}\text{-SH} \rightarrow SO_3^{-2} + \text{Rhodanese-Cys}_{247}\text{-S-S*H}$$

thiosulfate

$$\text{Rhodanese-Cys}_{247}\text{-S-S*H} + CN^- \rightarrow \text{Rhodanese-Cys}_{247}\text{-SH} + SCN^-$$

thiocyanate (rhodanate)

Until recently, it was assumed that the main biological function of rhodanese consists in the transformation of toxic cyanide to nontoxic thiocyanate, with the use of outer sulfur atom of thiosulfate according to the above reaction scheme [12]. Currently, other aspects of the biological role of rhodanese are emphasized, as well. S^* transfer to thiol groups of receptor and enzymatic proteins leads to the formation of hydropersulfides and/or trisulfides that often changes activity of these proteins. Therefore, this is another, besides S-thiolation and S-nitrosylation, mechanism of covalent modification of protein –SH groups [20]. The role of S^* transferred by rhodanese in the formation of iron-sulfur center of iron-sulfur proteins has also been suggested [13, 14].

It is probable that LA/DHLA system plays a significant role in these processes, since it is known from *in vitro* studies that DHLA serves as a S^* acceptor in rhodanese-catalyzed reactions. S^*-containing dihydrolipoic acid hydropersulfide, formed in this reaction, releases S^* in the form of hydrogen sulfide (H_2S) and the oxidized form of lipoate, i.e. LA, is produced concomitantly (Fig. 1). First papers addressing this issue, cited until today, were published in 1960s [37, 38, 44, 45]. Those studies were conducted *in vitro*, as already mentioned, but well-documented data from *in*

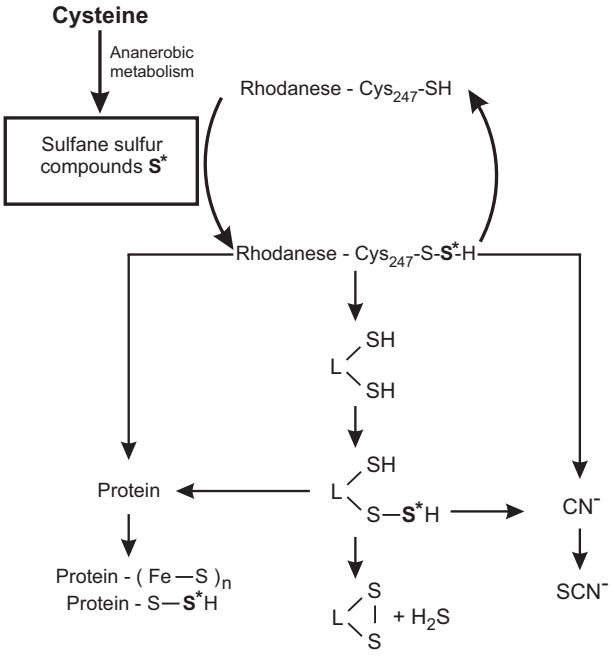

Fig. 1. A putative mechanism of lipoic and rhodanese cooperation *in vivo* in sulfane sulfur transfer reactions, according to [13], modified

vivo studies of this subject are missing. Therefore, we first posed a question whether LA has any effect *in vivo* on S^* level and rhodanese activity. To answer this question, we administered LA to rats and then we assayed S^* level, rhodanese activity, ROS and malondialdehyde (MDA) as a marker of lipid peroxidation in brain, heart, liver and kidney homogenates. In our studies, we used the formulation Neurex, which contains a racemic mixture of LA as a pharmacologically active substance and is used for treatment of diabetic neuropathy.

Materials and Methods

Animals

The experiments were carried out on male Wistar rats (180–250 g). Animals were housed in plastic cages in a room at a constant temperature of 20 ± 2°C with natural light-dark cycle. They had free access to standard pellet diet and water. Groups consisted of 6 animals each. All experiments were conducted according to guidelines of the Animal Use and Care Committee of the Jagiellonian University.

Drugs and compounds

The drug LA was a gift from Hexal A.G. (Holzkirchen, Germany). Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Fluka (Busch, Switzerland). 1,1',3,3'-tetraethoxypropane, dichlorofluorescein and 2'7'-dichlorohydrofluorescein diacetate (DCFH-DA) were obtained from Sigma Chemical Company (Steinheim, Germany). Potassium cyanide was purchased from Merck Chemical Company (Mannheim, Germany). All the other reagents were of analytical grade and were obtained from Polish Reagent Company (P.O.C.H., Gliwice, Poland).

Experimental groups

α-LA was given intraperitoneally once a day for 8 days at a dose of 35 mg/kg (LA35 group) or twice a day for 8 days at a dose of 50 mg/kg (LA100 group). Control groups were treated with saline in a volume of 0.5 ml. Subsequently, animals were sacrificed and the livers, brains, hearts, kidneys were removed, washed in 0.9% NaCl, placed in liquid nitrogen and stored at –70°C until biochemical tests were performed.

Determination of reactive oxygen species in rat tissues

2'7'-Dichlorohydrofluorescein diacetate (DCFH-DA) was deesterified in homogenates to dichlorohydrofluorescein, and was then oxidized to fluorescent dichlorofluorescein by ROS [9]. Briefly, 10 µl of a tissue sample were added to 900 µl of 0.1 M phosphate buffer (pH 7.4) and 10 µl of DCFH-DA. Mixtures were incubated in a water bath (37°C) for 30 min and centrifuged at 12,000 × g for 8 min. Fluorescence was measured with a Hitachi F-2000 fluorescence spectrometer at an excitation of 488 nm and an emission of 525 nm. Reactive oxygen species were evaluated from a standard curve prepared with dichlorofluorescein.

Determination of lipid peroxidation level in rat tissues (malondialdehyde level)

The levels of MDA were measured using the thiobarbituric acid (TBA) spectrophotometric assay with 1,1',3,3'-tetraethoxypropane as a standard [27]. Briefly, 250 µl of a tissue sample were added to 250 µl of distilled water, 500 µl of 15% TCA and 500 µl of 0.37% TBA. TCA and TBA solutions were prepared in 0.25 M HCl. The samples were heated in a boiling water bath for 10 min. After cooling, the samples were centrifuged at 10,000 × g for 10 min. The absorbance of the supernatant was measured at 535 nm.

Determination of sulfane sulfur level in rat tissues

The level of S^* in homogenates was determined by cold cyanolysis [50]. To 100 µl of liver homogenate 80 µl of 1 M NH_3, 720 µl of distilled water and 100 µl of 0.5 M KCN were added. The samples were incubated at room temperature for 45 min. Then 20 µl of 38% formaldehyde and 200 µl of Goldstein's reagent [$Fe(NO)_3$ + HNO_3+ H_2O] were added. After centrifugation at 12,000 × g for 10 min, the absorbance at 460 nm was determined. A standard curve was prepared with 1 mM KSCN.

Determination of rhodanese activity in rat tissues

Enzymatic activity of rhodanese was determined according to Sörbo [31]. Assay mixture containing 100 μl of homogenate diluted 100 times, 125 μl of 60 mM thiosulfate, 125 μl of 47.5 mM potassium phosphate buffer pH 7.4 and 75 μl of 71.5 mM cyanide was incubated at room temperature for 5 min. The reaction was stopped by addition of formaldehyde. Reaction product, i.e. thiocyanide and ferric ions formed colored complex, whose absorbance was measured spectrophotometrically at 460 nm. The activity of the enzyme was expressed in μmoles of the product (evaluated from a standard curve for thiocyanide) formed during 1 min of incubation per 1 mg of protein.

Determination of protein in rat tissues

The proteins were measured using Lowry's method [24]. This method is based on the reaction of peptide bonds and aromatic amino acid residues of proteins with Folin-Ciocalteau reagent (a mixture of phosphotungustic acid and phosphomolibdenic acid) in alkaline environment in the presence of cupric ions. Copper (II) ions, bound to protein tyrosine and tryptophan residues, reduce the above acids to oxides. Absorbance was measured at 500 nm. A 1% solution of bovine albumin was used to prepare a standard curve.

Statistical analysis

The results are presented as the mean ± SEM for each group. Statistically significant differences between groups were calculated using one-way analysis of variance (ANOVA). The criterion for significance was set at $p < 0.05$.

Results and Discussion

The present studies indicated that LA at both doses 35 mg/kg/24 h and 100 mg/kg/24 h [LA(35) and LA(100) groups, respectively] significantly lowered ROS concentration in all tissues under study and reduced lipid peroxidation, as measured by MDA level in the heart, liver and kidney (Fig. 2A–D). This result is concordant with the expectations since investigations of many research centers have confirmed powerful ROS scavenging properties of both LA and DHLA. For further detailed information about antioxidant properties of LA and its metabolites, the reader is referred to other papers [2, 5, 8, 25, 29].

It is noteworthy that the LA-induced decrease in MDA level was not observed in the brain in any of the dose groups LA(35) or LA(100). Likewise, LA treatment did not change S^* level and rhodanese activity in the brain (Fig. 2A), while these values in the liver and kidney were elevated in LA(100) group (Fig. 2B–D). However, it should be remembered that brain metabolism differs substantially from metabolism of other organs. ROS generating and scavenging reactions in the brain are very intense due to high oxygen consumption. For instance, human brain, which constitutes 2% of body weight, consumes 20% of oxygen taken in by the body [15]. The brain in highly protected not only from exogenous prooxidants but also from antioxidants, as presently demonstrated for LA. It also should not be overlooked that the brain is a very heterogeneous organ, with numerous morphologically and metabolically differentiated cells. This was confirmed by our previous studies, which revealed that LA regulated in a different manner the glutathione defense system in the striatum and prefrontal cortex [6].

Interestingly, in the heart, LA at 35 mg had no effect on S^* level, but increased rhodanese activity. In contrast, its dose of 100 mg elicited the opposite effect, namely, it elevated S^* level and had no influence on rhodanese activity (Fig. 2B). It is not excluded that S^* is the main source of hydrogen sulfide (H_2S) in the heart. It is formed in rhodanese-catalyzed reaction with DHLA as a S^* acceptor (Fig. 1). However, this problem requires further studies.

H_2S in the cell is formed mostly from Cys in the reaction catalyzed by cystathionine β-synthase (CBS) and CSE. Biological action of physiological H_2S concentrations includes hypotension, vasodilatation, potassium channel activation, inhibition of vascular muscle cell proliferation, activation of brain NMDA receptors [3, 23]. Studies on some animal models have indicated that H_2S deficit could contribute to development of arterial hypertension [3, 23]. Decreased CSE gene expression and H_2S concentration was demonstrated in the animal model of hypertension induced by chronic nitric oxide synthase (NOS) inhibitor treatment [19, 23, 46]. However, the mechanism of mutual relationships between NO and H_2S has not

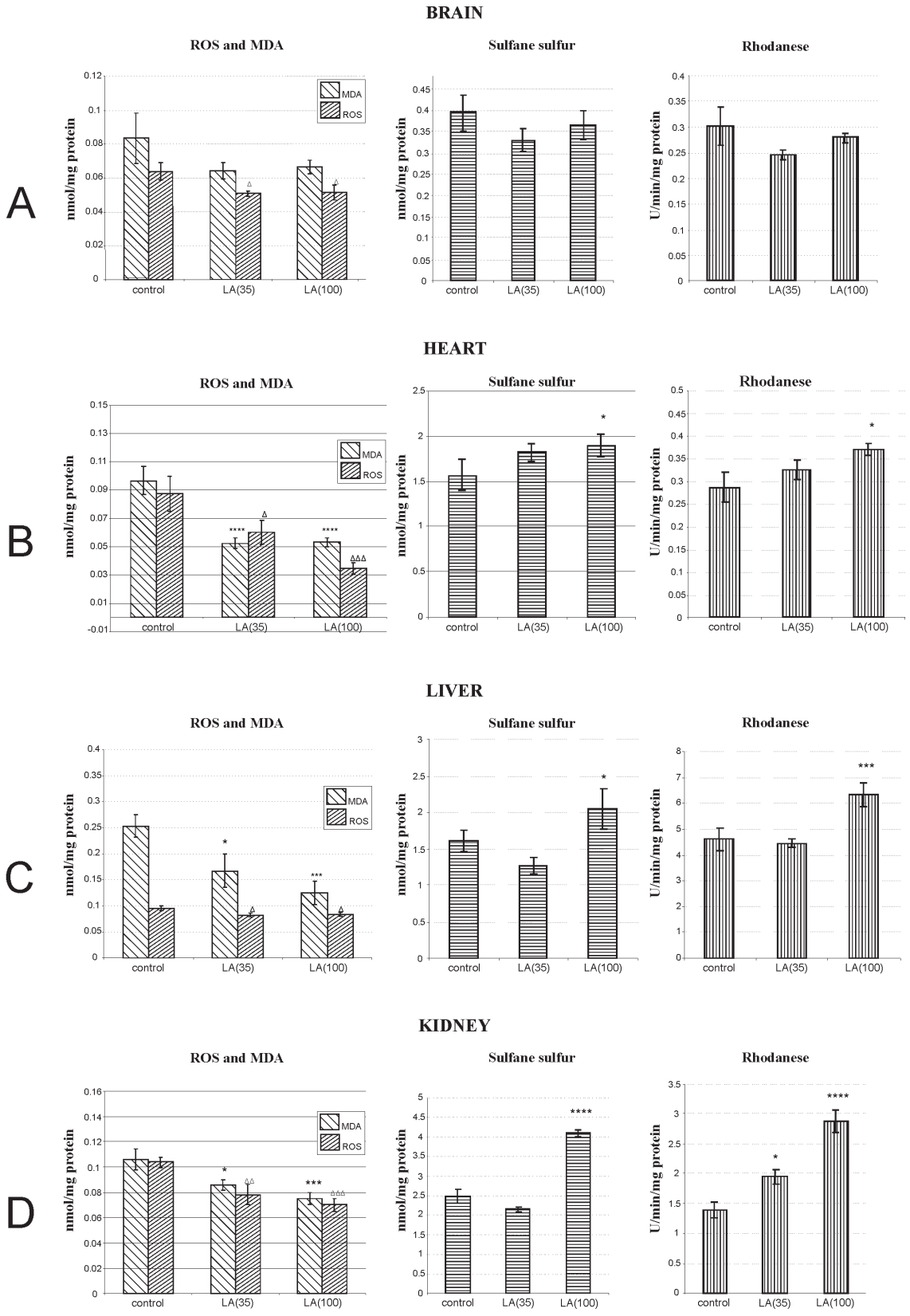

Fig. 2. The effect of LA (35 mg/kg/24 h – LA(35) and 100 mg/kg/24 h – LA(100); *ip*) administration on the levels of ROS, MDA, S^* and the activity of rhodanese in the rat tissues: **A** – brain, **B** – heart, **C** – liver, **D** – kidney. Symbols indicate significance of differences, $^{\Delta}$ $p < 0.05$; $^{\Delta\Delta}$ $p < 0.02$; $^{\Delta\Delta\Delta}$ $p < 0.01$; $^{\Delta\Delta\Delta\Delta}$ $p < 0.001$ *vs.* control of ROS, * $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$; **** $p < 0.001$ *vs.* control of MDA, S^* and activity of rhodanese

been fully elucidated. It is obvious that lowering of H_2S level can result from diminished CBS and CSE activities, but it should be noted that this increases homocysteine (Hcy) level. Hcy can react with NO to yield S-nitrosohomocysteine. This process is both beneficial and harmful. On the one hand, S-nitrosylation protects cells from atherogenic Hcy action, but concomitantly depletes NO level. A currently popular hypothesis suggests a possibility of NOS modification by Hcy, directing this pathway towards ROS formation from arginine (Arg) instead of NO [42].

Mutual relationships between metabolic pathways of Hcy and Arg, precursors of H_2S and NO, respectively, appear also very interesting. Arg methylation leads through S-adenosylmethionine to asymmetric N_G, N_G-dimethylarginine and N_G-monomethylarginine, that are NOS inhibitors. On the other hand, it is known that S-adenosylmethionine is an activator of CBS that participates in H_2S synthesis [42].

It is known that NO increases CSE activity and amount of H_2S formed in the cardiovascular system, furthermore, NO donors were shown to escalate vasodilating action of H_2S [46, 51].

In the light of the above data, it seems that NO level is positively correlated with H_2S level. On the other hand, our previous research indicated that activity of rhodanese, participating in sulfane sulfur transport, could be suppressed by S-nitrosylation of the -SH group of Cys-247, located in a rhodanese active center [22], which seems to be equivalent to the blockade of H_2S formation in the reaction catalyzed by rhodanese with DHLA as an S^* acceptor (Fig. 1).

As our knowledge is fragmentary and still not complete and data are often discrepant, it is difficult to propose a concise and reliable hypothesis regarding mutual relationships between NO and H_2S and their biological significance. We hope that further studies will allow for revealing more details of these, undoubtedly complex but fascinating relations, that will open new therapeutic perspectives for, mostly, cardiovascular diseases.

S^* has also properties of a ROS scavenger [20, 40]. The highest activity of enzymes involved in S^* biosynthesis and transport has been found in the liver and kidney [20, 26, 33]. A significant increase in rhodanese activity and S^* level in LA(100) group was observed just in the liver and kidney (Fig. 2C–D), and the largest increase *vs.* control group was seen in the kidney (Fig. 2D). Although it is assumed that metabolic profile of the kidney is generally close to that of the liver, the role of the kidney in Cys metabolism seems to be exceptional. Renal cells were demonstrated to contain the highest, incomparable with any other organ, amounts of Cys [26]. For instance, Cys level in the liver is 0.08 μmol/g, whereas the respective value in the kidney is 0.65 μmol/g [1].

Cys is a toxic amino acid and its physiological concentration is very low compared with other amino acids. Thus, it is an interesting and unresolved problem why Cys does not show toxic effects in the kidney, where its physiological concentration is almost 8 times higher than in the liver, and what role Cys plays in the kidney [1, 32]. The kidney is characterized also by the highest level of S^*, what was confirmed by our studies (Fig. 2D). Moreover, a crucial role of the kidney in Cys metabolism is corroborated by the fact that plasma levels of sulfur-containing amino acids are changed the most compared to other amino acids in terminal renal failure. Plasma Cys and Hcy concentrations in these patients drastically increase [48, 49], what is accompanied by a drop in S^*. Hemodialysis further enhances this decline in comparison with healthy people [48, 49]. It should be remembered that more than 80% of Hcy in this organ is metabolized *via* transsulfuration, which suggests that the kidney can produce significant amounts of H_2S [17, 23]. Noteworthy, γ-glutamyl transpeptidase (γ-GT) activity in the kidney is almost 900 times higher than in the liver, which suggests that extracellular biodegradation of glutathione is an important function of the kidney [47]. In addition, the kidney is responsible for maintaining physiological plasma Cys level. This is a not always clearly emphasized function that the kidneys fulfill in the body [18]. Our previous studies also demonstrated that the kidney differently reacted to acivicin (AC), a γ-GT inhibitor, than the liver. The inhibition of this enzyme caused a much higher rise in renal Cys and S^* concentrations. Conversely, activity of CSE, the enzyme responsible for S^* and H_2S biosynthesis, drastically increased in the liver, but not in the kidney, after AC treatment [unpublished data]. An interest in S^* in patients with renal diseases is also related to the elevated cyanide level. Toxic action of cyanide is caused by inactivation of cytochrome oxidase and respiratory chain blockade, accompanied by augmented ROS generation and peroxidative damage. Therefore, both S^* donors and antioxidants can protect cells from CN^- toxicity. LA appears to fulfill both these functions, because this compound activates rhodanese and S^*, and has antioxidant properties. It was

confirmed by our studies and literature data demonstrating a beneficial effect of LA in certain types of human glomerulonephritis [10].

In conclusion, it should be emphasized that in our studies LA action was dose-dependent. LA at 35 mg/kg /24 h significantly decreased ROS in all tissues under study, lowered MDA level in the heart, liver and kidney and increased rhodanese activity in the kidney. On the other hand, LA(100) group had a significantly lowered ROS and MDA in all studied tissues and increased S^* and rhodanese activity in the heart, liver and kidney. This indicates that the higher LA dose was more efficient in this case. It appears to confirm clinical studies, in which diabetic neuropathy patients (program ALADIN) were treated with LA at 1200, 600 and 100 mg, achieving the highest percentage of positive responses (drop in plasma level of glucose and lipid peroxidation products, and improved sensation in lower limbs) in LA-treated groups at the highest dose (1200 mg) *vs.* placebo group [52]. However, this problem seems to be much more complicated, namely, depending on the dose, LA can even act oppositely. It was confirmed by studies of Dovinowa et al. [16] on cultures of the L1210 mouse leukemia cells. They showed that LA at low concentration acts as a growth factor and at a higher concentration acts as an antiproliferative agent. Certainly, this problem is very interesting and requires further studies.

Despite numerous literature reports published in the last decade and dealing with pharmacological properties of LA, the mechanism of its action has not been fully elucidated and not always can be explained by its antioxidant properties. The present work is one of a few examining *in vivo* a relationship between biological role of LA and S^* metabolism. Although this problem requires further studies, the results presented in this paper indicate that LA increases S^* level and rhodanese activity in the rat heart, liver and kidney. This suggests that its influence on S^* metabolism and rhodanese activity is one of putative mechanisms of biological action of LA. This means that it can elicit an indirect effect as well on regulatory as antioxidant action of S^* in rat tissues.

The results of our studies seem to be particularly relevant to the kidney, since S^* level and rhodanese activity rose after LA the most significantly in this organ (Fig. 2D). It can be expected that discovering more details of sulfur metabolism in the kidney can offer some practical benefit to the therapy of patients suffering from renal and urinary tract diseases.

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