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Original Article

The effect of NaCl on the level of reduced sulfur compounds in rat liver. Implications for blood pressure increase*

Wpływ iniekcji NaCl na poziom zredukowanych związków siarki w wątrobie szczura. Konsekwencje dla rozwoju nadciśnienia

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C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: It is commonly known that excessive salt intake is a risk factor of hypertension. Currently, there is an increasing interest in reduced reactive sulfur species (RSS), mainly H_2S and sulfane sulfur (SS) as new gasotransmitters showing vasorelaxant properties. The aim of the present study was to determine the effect of repeated administration of low sodium chloride dose included in physiological saline on blood pressure, on the level of RSS and activity of enzymes involved in their biosynthesis in the rat.

Methods: Two separate experiments were carried out on male Wistar rats: one with intravenous injections of saline and the second one with intraperitoneal saline injections. Blood pressure was measured during the experiment. The level of RSS and other biochemical assays were conducted in the rat liver, because of an intense cysteine metabolism to RSS in this organ.

Results: Intravenous administration of saline induced a significant increase in systolic blood pressure while intraperitoneal injections showed only a tendency towards increasing blood pressure. The RSS (H_2S and SS) level as well as the activity of the main enzyme responsible for their production in the liver of animals after iv saline injections were decreased. Animals injected with physiological saline by ip route did not reveal any statistically significant differences in SS, H_2S, and activities of sulfurtransferases, although a tendency to decrease in the RSS was observed.

Conclusions: The repeated iv saline injection induced a slight hypertension accompanied by disturbances in anaerobic cysteine metabolism in the rat liver.

Key words: sodium chloride • hypertension • reactive sulfur species • sulfane sulfur • hydrogen sulfide

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Abbreviations: **CSE** – cystathionine γ-lyase; **DADS** – diallyl disulfide; **DATS** – diallyl trisulfide; **DBP** – diastolic blood pressure; **GSH** – glutathione; **MST** – 3-mercaptopyruvate sulfurtransferase; **NPSH** – non-protein sulfhydryl groups; **ROS** – reactive oxygen species; **RSS** – reactive sulfur species; **SBP** – systolic blood pressure; **SS** – sulfane sulfur; **TST** – rhodanese.

INTRODUCTION

Hypertension is one of the most important factors contributing to cardiovascular disease. Globally, nearly 1 billion people suffer from high blood pressure, which is responsible for chronic heart failure, heart attacks and strokes [19,20]. Hypertension risk factors, apart from overweight and lack of physical activity, include high sodium content in the diet. It should be remembered that our ancestors consumed minimal amounts of NaCl present in natural food, i.e. <1g per week [26]. Unfortunately, present-day foods, especially commercially available, contain marked amounts of NaCl which may lead to serious health problems. Many reports documented the relationship between the incidence of hypertension and a sodium intake [26]. Studies in chimpanzees, which share 98.8% nucleotide sequence homology with humans, demonstrated that and increase in NaCl intake to 10-15 g/day, which is the case in the population of industrialized countries, led to hypertension in animals [3]. One of the proposed mechanisms contributing to hypertension is connected with Na^+K^+ ATPase inhibition which raises the blood pressure [16].

Recently, much attention has been devoted to hydrogen sulfide (H_2S) as a new gaseous signal transmitter, which, like nitric oxide (NO), is implicated in blood pressure regulation and possesses hypotensive properties [41]. H_2S, regarded previously as a toxic gas, plays significant physiological and regulatory roles [17,42]. It is produced from L-cysteine in various mammalian tissues, including the nervous system, heart, liver, kidney and blood vessels. Its production in liver is catalyzed mainly by cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MST). H_2S is a weak acid, which can undergo a two-stage dissociation to HS – and trace amounts of S^2- [17,42]. In the literature, the term hydrogen sulfide signifies usually the sum of all forms, which in physiological conditions contains principally: H_2S (20%), HS – (80%) and S^{2-} (below 1%). Sulfane sulfur (SS) is a sulfur atom occurring in the 0 or – 1 oxidation state covalently bound to another sulfur atom. Sulfur with such properties is present in thiosulfate, hydropersulfides (R-SSH), tiosulfonates, polysulfides, polythionates and elemental sulfur (Fig. 1) [13,39]. The enzymes taking part in the formation of compounds containing SS include mainly CSE and MST, while the function of rhodanese (TST) is to transport SS from donors (i.e. tiosulfate, persulfides) to acceptors (i.e. thiols). SS-containing compounds (mainly RSSH) are regarded as a storage form of H_2S, due to the possibility of its release in response to a physiological signal [18, 38]. SS binding to protein Cys residues, which leads to the formation of hydropersulfides, is called protein S-sulfhydration (or sulfuration) [11]. Some studies revealed that many liver proteins are sulfhydrated under physiological conditions [29].

H_2S and products of its oxidation (inorganic polysulfides HSS_nH, thiosulfate $S_2O_3^{2-}$) as well as other compounds containing sulfane sulfur (SS) belong to the pool of compounds containing reduced sulfur which have been attributed a regulatory and antioxidant role and were included among the so-called reactive sulfur species (RSS) (Fig. 1) [7, 27].

The hypotensive effect of H_2S or SS-containing compounds is thought to be primarily mediated through ATP-sensitive potassium channels (K_{ATP}) [21]. Closure of K_{ATP} channels reduces membrane potential, thus causing the constriction of blood vessels and an increase in blood pressure. H_2S relaxes vascular smooth muscles by activating K_{ATP}. Mustafa et al. found that the vasodilatory effects of H_2S or SS were connected with Cys-S-sulfhydration and subsequent hyperpolarization of K_{ATP} channels, which led to vasodilation and a drop in blood pressure (Fig. 1) [30]. It is also documented that H_2S dilates blood vessels in synergy with another important gaseous signal molecule, namely NO [9].

Protein – and non-protein thiols (NPSH) are redox buffers and a reservoir of reductive power in cells. The thioltripep-
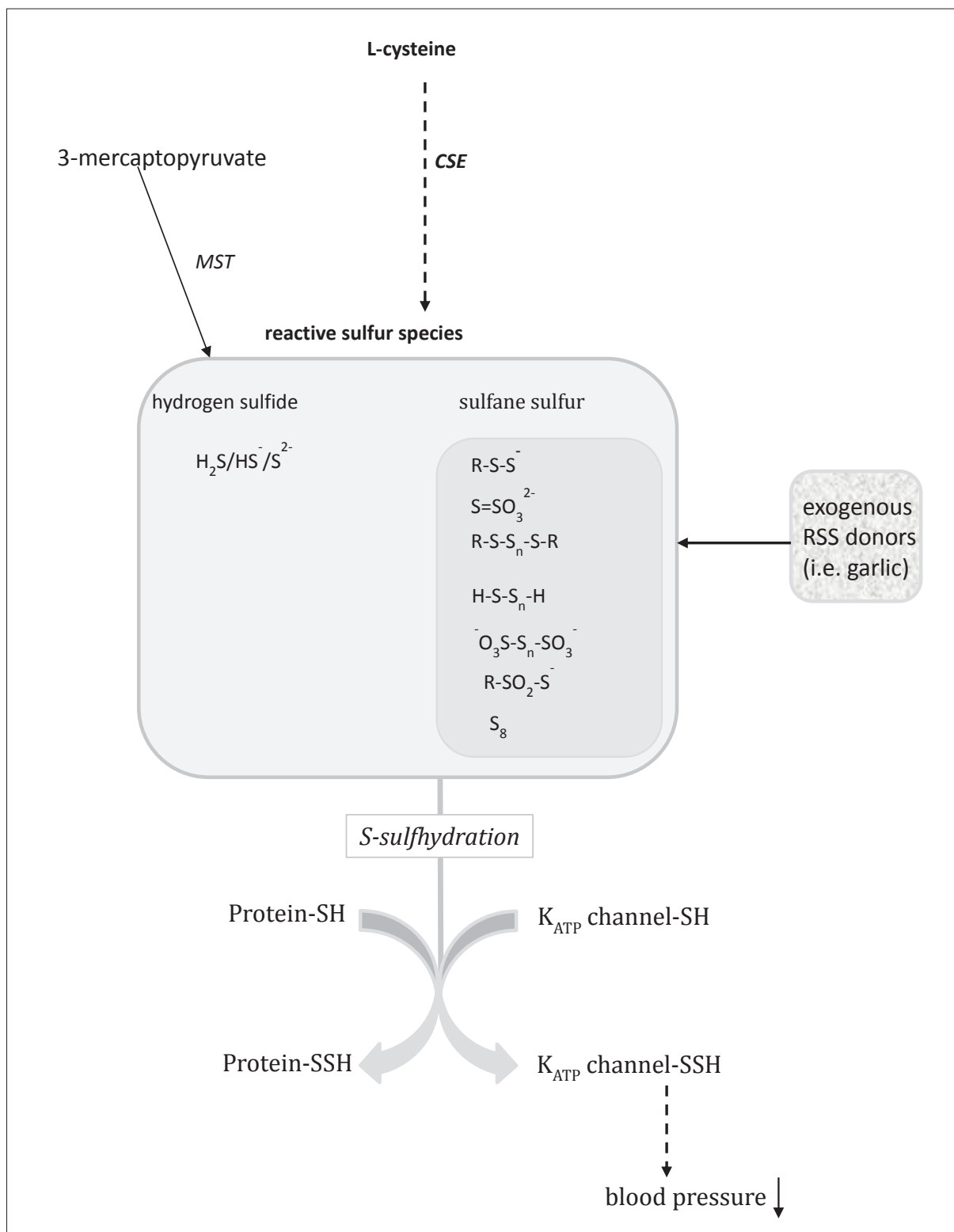
L-cysteine

3-mercaptopyruvate

CSE

MST

reactive sulfur species

hydrogen sulfide

$H_2S/HS^-/S^{2-}$

sulfane sulfur

$R\text{-}S\text{-}S^-$

$S=SO_3^{2-}$

$R\text{-}S\text{-}S_n\text{-}S\text{-}R$

$H\text{-}S\text{-}S_n\text{-}H$

$^-O_3S\text{-}S_n\text{-}SO_3^-$

$R\text{-}SO_2\text{-}S^-$

S_8

exogenous RSS donors (i.e. garlic)

S-sulfhydration

Protein-SH

K_{ATP} channel-SH

Protein-SSH

K_{ATP} channel-SSH

blood pressure ↓

Fig. 1. Reactive sulfur species and their influence on regulatory processes

tyde glutathione (GSH), quantitatively the major thiol in cells, and cysteine (Cys) are the most important NPSH in cells. There is increasingly more evidence for the existence of an extensive intracellular thiol redox signaling called "thiolstat" [15]. Changes in this dynamic equilibrium are linked with signal perception and transduction. Interestingly, it has been postulated that hypertension may be a result of a disruption in redox signaling [23].

In the light of the above facts, it can be expected that NaCl-induced hypertension may be associated with disturbances in the synthesis of H_2S and SS-containing compounds and in thiol redox status. Recent studies have reported that in 2010 the NaCl consumption was around 10 g per person per day [31], while according to the World Health Organization (WHO) recommendation, the daily NaCl intake should be less than 5 g [44]. A 500 ml dose of physiological saline (0.9% NaCl) commonly used for injections contains 4.5 g of NaCl, (which represents almost the whole recommended daily dose). Hence, it can be expected that such an amount of salt added to normal of high salt content in the diet can contribute to the development of arterial hypertension.

For this reason, the aim of the present study was to determine the effect of a low dose of NaCl contained in physiological saline on the blood pressure and on the level of reduced RSS (H_2S and SS) and the activity of enzymes involved in their synthesis and transport (CSE, MST, TST) as well as on the NO level. Biochemical studies were conducted in the liver, because it is the organ where anaerobic cysteine metabolism leading to RSS synthesis is the most intense and the activity of their biosynthetic enzymes is the highest.

MATERIALS AND METHODS

Animals and treatments

The study was conducted in accordance with the rules of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Experimental protocols involving the use of laboratory animals were approved by the Ethics Committee for Animal Research in Krakow (number 153/2012). All efforts were made to minimize the number and suffering of the animals used.

The experiments were carried out on male Wistar rats (body weight 150 – 200 g). The animals were housed in constant temperature facilities exposed to 12:12 h light-dark cycle and maintained on a standard pellet diet (containing 0.5% NaCl), tap water was given *ad libitum*. Two separate experiments were carried out at different times: one with intravenous injection of saline and the second with intraperitoneal saline injection. For technical reasons, intravenous injections were administered for a relatively short time (7 days), whereas intraperitoneal injections were prolonged to 39 days. In each experiment, the rats were randomly assigned to two different treatment groups of six animals each.

Experiment 1

Group 1A – intravenous injections of 0.9 % NaCl in a volume of 1 ml to the tail vein for 7 days

Group 1B – normal rats that were pricked to the tail without injections (in order to expose them to similar stress as during injection) for 7 days

Experiment 2

Group 2A – intraperitoneal injections of 0.9 % NaCl (0.3 ml for 21 days and 1ml for the next 18 days)

Group 2B – intraperitoneal injections of sterile water (0.3 ml for 21 days and 1ml for the next 18 days)

Blood pressure measurements

Blood pressure (BP) was determined using a non-invasive BP measurement system for rodents LE 5007 Panlab Harvard Apparatus. For this purpose, for a period of 5 days the rats were habituated to the touch of a human hand and to the measuring tubes. Pressure measurements were made according to the NIBP method (Non-Invasive Blood Pressure), which is based on the technique used to measure blood pressure in humans. The measurement was conducted on rats closed in measuring tubes based on the measurement of the pulse in the tail artery. For each rat, one measurement session comprised 5 readings of systolic and diastolic pressure and the arithmetic mean was calculated.

Livers sampling

After the conclusion of each experiment (i.e. on the 8th day for the iv experiment and on the 40th day for the ip experiment) rats were killed by decapitation, the livers were collected and stored at – 80⁰C until biochemical experiments were conducted.

Preparation of tissue homogenate

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 IKA-ULTRA-TUR-RAX T8 homogenizer. Liver homogenates were next used for assay the levels of SS, H_2S, nitric oxide (NO) and the activity of CSE, MST and rhodanese (TST).

Chemicals

Thiosulfate, formaldehyde, zinc diacetate, sodium sulfite were obtained from the Polish Chemical Reagent Company (P.O.Ch, Gliwice, Poland). Dithiothreitol (DTT), N-ethylmaleimide (NEM), b-nicotinamide adenine dinucleotide reduced form (NADH), 3-methyl-2-benzo-thiazolinone hydrazone (MBTH), pyridoxal 5'-phosphate (PLP), homoserine, mercaptopyruvic acid sodium salt, p-phenylenediamine, potassium cyanide (KCN), trichloroacetic acid (TCA) and lactic dehydrogenase (LDH) were provided by Sigma Chemical Co (St. Louis, MO, USA).

BIOCHEMICAL ANALYSES

Determination of sulfane sulfur (SS) level

The level of the compounds containing SS was determined by the Wood method (1987) based on cold cya-

nolysis and colorimetric detection [43]. SS-containing compounds react with cyanide in alkaline solution at room temperature yielding thiocyanates, which react with ferric ions resulting red ferric thiocyanates, which are determined at 460 nm. Briefly, to the 200 μl of liver homogenate, 80 μl of 1M NH_3, 620 μl of distilled water and 100 μl of 0.5M KCN were added, mixed thoroughly and incubated at room temperature for 45 min. Then 20 μl of 38% formaldehyde was added (for stabilization of ferric thiocyanate by reaction with cyanide excess). Then 200 μl of Goldstein's reagent ($Fe(NO_3)$+ HNO_3 +H_2O) was added and the samples were centrifuged at 12 000 x g for 10 min. The supernatant was carefully collected and absorbance was measured at 460 nm. A standard curve was prepared for 1mM KSCN. The level of SS was expressed in μmoles of SCN – per 1 g of wet tissue.

Determination of hydrogen sulfide (H_2S) level

H_2S level was estimated using modification of method of Shen et al. [35]. In this method in alkaline environment, equilibrium of H_2S is shifted towards formation of sulfide, which is trapped by zinc acetate and then determined by reaction with p-phenylenediamine in the presence of ferric ion. The product of this reaction, thionine is assayed fluorometrically. Briefly, to 250 μl of 1% zincacetate, 125 μl of liver homogenate, 125 μl of borate buffer, pH 9.0 were added and incubated at 37°C for 10 min. Then, 400 μl of 12.5 mM p-phenylenediamine and 100 μl of 40 mM $FeCl_3$ in 6M HCl were added. After 10 min of incubation at room temperature, samples were centrifuged at 12 000 x g for 5 min, and fluorescence of supernatants was measured (E_{ex}=600 nm, E_{em}=623 nm). Concentration of H_2S were calculated from calibration curve prepared for 1 μM thionine.

Determination of γ-cystathionine γ-lyase (CSE) activity

Enzymatic activity of CSE was determined according to Matsuo and Greenberg [24] with modifications. L-homoserine was used as a substrate, and pirydoxal phosphate (PLP) was a coenzyme. α-Ketobutyric acid formed from L-homoserine was assayed using 3-methyl-2-benzo-thiazolinone hydrazone (MBTH) according to the method of Soda [36]. 50 μl of 0.2 M of L-homoserine and 650 μl of 0.1M phosphate buffer, pH 7.4, were added to 50 μl of 1mM PLP. The reaction was started by adding 250 μl of liver homogenate diluted 50 x with phosphate buffer and the reaction mixtures were incubated at 37°C for 30 min. Then, 250 μl of 50% TCA was added and centrifuged at 12,000 x g for 10 min. 500 μl of the supernatant was transferred to 500 μl of 1M acetate buffer, pH 5.0, and 200 μl of 0.1% MBTH was added. The reaction mixtures were mixed and incubated at 50°C for 30 min. After cooling, absorbance was measured at 320 nm and the amount of the product was calculated from a standard curve prepared from 2 mM α-ketobutyric acid. The activity of enzyme was expressed as nmoles of the product formed during 1 min per mg of protein.

Determination of 3-mercaptopyruvate sulfutransferase (MST) activity

The activity of MST was determined by measuring the amount of pyruvate formed during 15-minute incubation at 37°C in accordance with the method of Valentine and Frankenfeld [40]. In this method, the sulfur atom from 3-mercaptopyruvate (MP) is transferred by MST yielding pyruvate, which, in the next step, is reduced to lactate with LDH and NADH. This method utilizes the difference in absorption at 340 nm between NADH and NAD^+, which corresponds to the amount of the pyruvate formed. The reaction mixture contained 250 μl of 0.12 M phosphate buffer, pH 8.0, 50 μl of 0.5 M Na_2SO_3, 50 μl of 10.15 M DTT, 50 μl of 0.1 M MP, 50 μl of liver homogenate diluted 100 x with 0.1 M phosphate buffer, pH 7.4 and 50 μl distilled water and was incubated at 37°C for 15 min. Then, 250 μl of 1.2 M $HClO_4$ was added and the mixture was centrifuged at 3000 x g for 10 min, subsequently 100 μl of the obtained supernatant was added to 1.2 ml of 0.12 M phosphate buffer, pH 8.0 and 100 μl of 0.1 M NEM and 50 μl of 7 mM NADH. After first absorption measurement at 340 nm, 5 μl of LDH was added and the second measurement was carried out after 1 min after LDH addition. The amount of pyruvate was calculated from the standard curve prepared for 1mM pyruvate. The activity of the MST was expressed as μmoles of product formed during 1 min per mg of protein.

Determination of rhodanese (TST) activity

TST activity was determined by Sorbo's method [37] using thiosulfate as a substrate. The reaction involves the transfer of sulfane sulfur atom of thiosulfate to cyanide yielding thiocyanate formation. The amount of thiocyanate is measured colorimetrically using the reaction with ferric ions. Briefly, assay mixture containing 100 μl of liver homogenate diluted 100 x with phosphate buffer, pH 7.4, 100 μl of distilled water, 125 μl of 0.25 M thiosulphate, 125 μl of 0.2 M KH_2PO_4 and 75 μl of 0.5 M KCN was incubated at room temperature for 5 min. Then, 125 μl of 38% formaldehyde and 600 μl of Goldstein's reagent ($Fe(NO_3)$+ HNO_3 +H_2O) were added. After centrifugation at 12 000 x g for 10 min, absorbance of the color complex was measured at 460 nm. A standard curve was prepared for 2.5 mM KSCN and the activity of TST was expressed as μmoles of product formed during 1 min per mg of protein.

Determination of non-protein thiols (NPSH) level

Determination of NPSH level is based on Ellman's method in which dithionitrobenzoic acid (DTNB) is reduced by –SH groups to product with intensive yellow color, which is measured spectrofotometrically [34]. In order to dermine the NPSH, liver homogenate was deproteinized by addition of cold TCA (475 μl of homogenate and 25 μl of 50% TCA), samples were thoroughly mixed and then centrifuged at 12 000 x g for 10

min at +4°C. The obtained supernatant was next used for determination of NPSH. To 850 μl of 0.2 M phosphate buffer, pH 8.2, 100 μl of 6 mM DTNB and 50 μl of supernatant were added. Absorbance was measured at 412 nm 1 min after supernatant addition. The total content of NPSH was calculated from a standard curve prepared for 1 mM GSH and expressed in μmoles per gram of liver.

Determination of nitric oxide (NO) level

The level of NO was assayed spectrophotometrically using the "Nitric oxide colorimetric assay" manufactured by Roche.
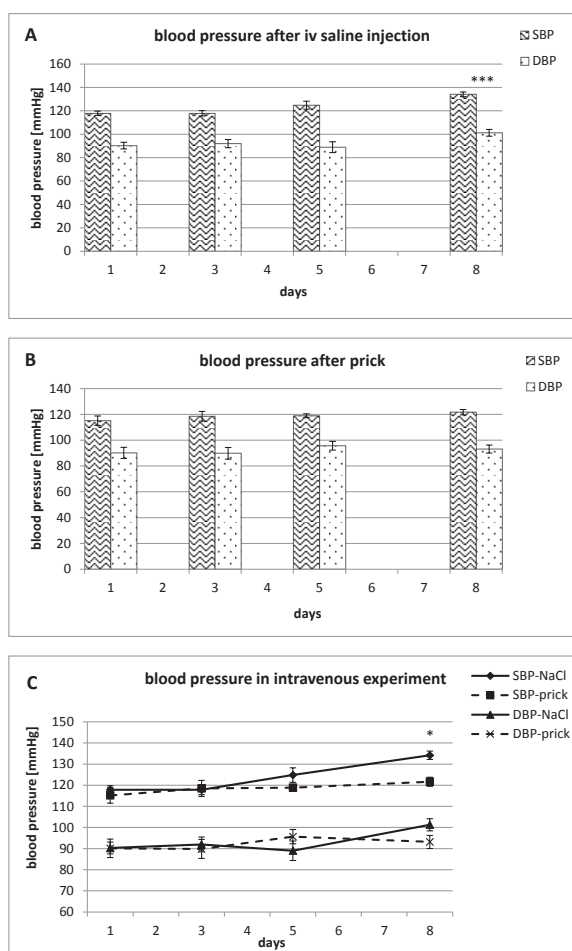
Fig. 2. Changes in blood pressure of rats after intravenous injection of 0.9 % NaCl; A – Blood pressure in rats after intravenous injections of 0.9% NaCl in comparison to the first day of experiment, B – Blood pressure in control rats in comparison to the first day of experiment. These rats were pricked in the tail without injections, C – Blood pressure in rats after intravenous injections of 0.9% NaCl compared to the control animals in each time point. The measurement of systolic (SBP) and diastolic (DBP) blood pressure was based on pulse rate measurement on the tail artery every 2 or 3 days. Five readings of SBP and DBP were taken and values represent the mean ± SEM. *p<0.05 statistically significant difference
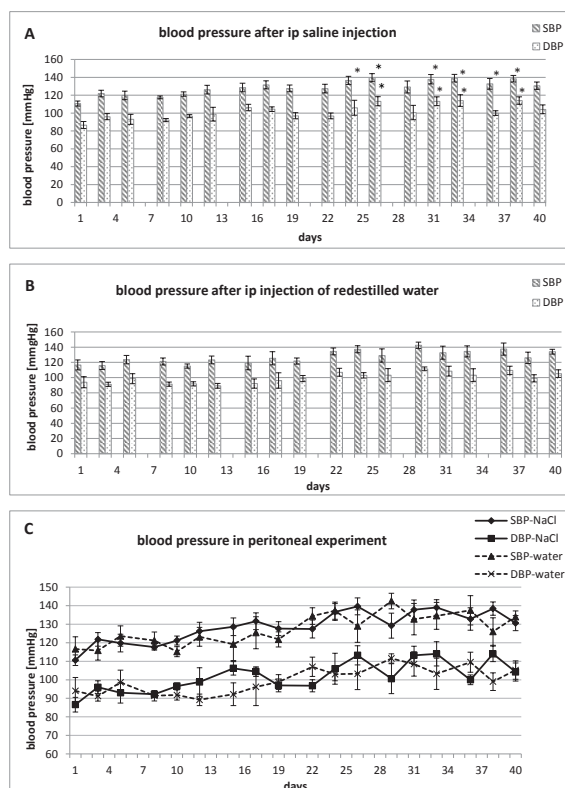

Fig. 3. Changes in blood pressure of rats after intraperitoneal injection of 0.9 % NaCl; A – Blood pressure in rats administered ip of 0.9% NaCl in comparison to the first day of experiment, B – Blood pressure in control rats administered ip of redistilled water in comparison to the first day of experiment, C – Blood pressure in rats after intraperitoneal injections of 0.9% NaCl compared to the control animals in each time point. The measurement of systolic (SBP) and diastolic (DBP) blood pressure was based on pulse rate measurement on the tail artery every 2 or 3 days. Five readings of SBP and DBP were taken and values represent the mean ± SEM. *p<0.05 statistically significant difference

Determination of protein content

Protein content was assayed using Lowry's et al. method [22], which is based on the reaction of peptide bonds and aromatic amino acid residues of proteins with Folin-Ciocalteau reagent in alkaline environment in the presence of cupric ions.

Statistical analysis

The results are presented as the means ± SEM. Statistical significance of differences for biochemical assays was determined using Student's t-test. However, the significance of differences for blood pressure measurements was analyzed by a one – and two-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison test. The differences were considered statistically significant when p < 0.05.
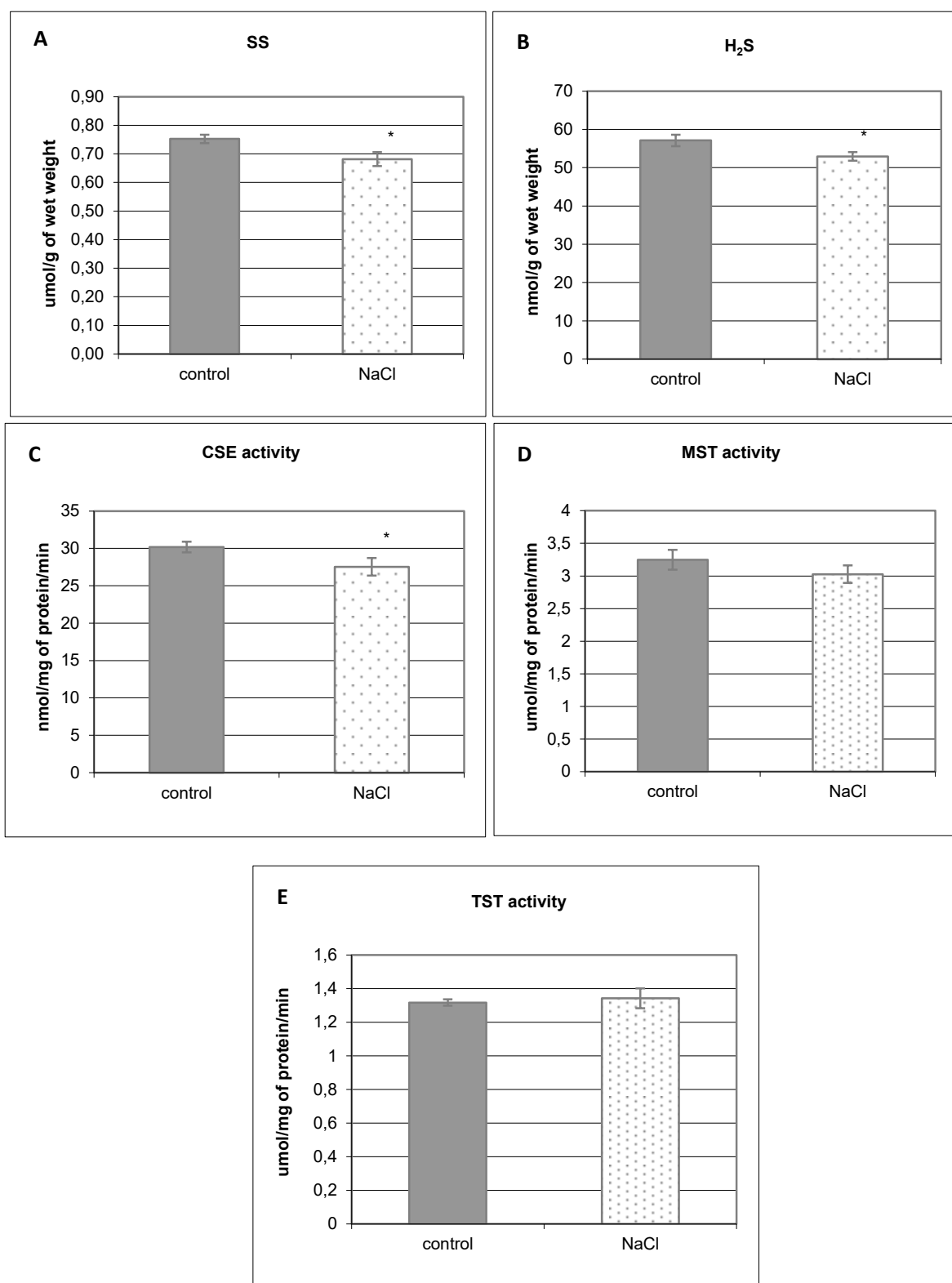
Fig. 4. Effect of intravenous injection of 0.9% NaCl on the level of sulfane sulfur (A), hydrogen sulfide (B) and on the activities of γ-cystathionase, CSE (C), 3-mercaptopuryvate sulfurtransferase, MST (D) and rhodanese TST, (D) in rat liver in comparison to the control (pricked) rats. Values represent the mean ± SEM. Statistically significant difference compared with the control *p<0.05
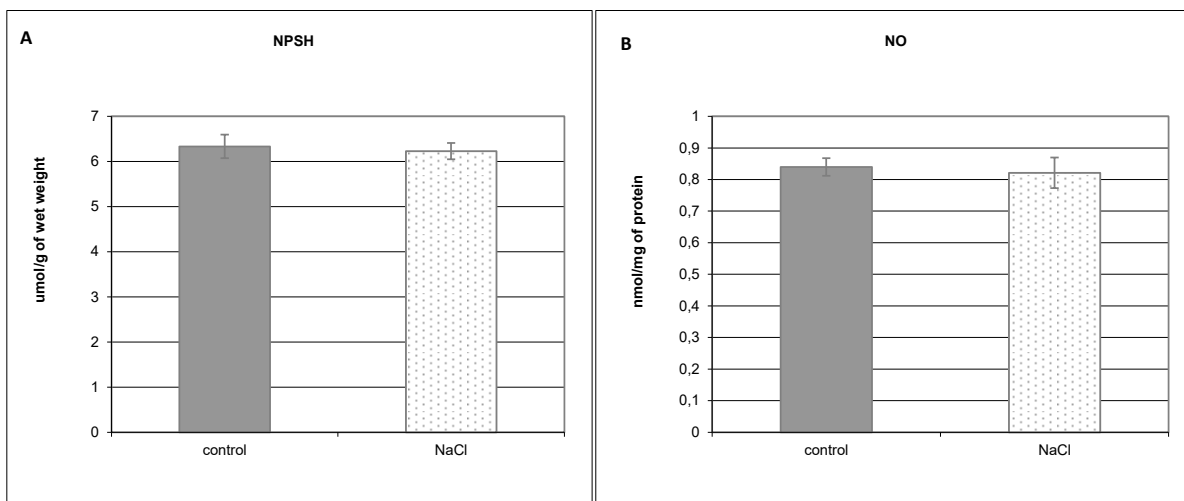
Fig. 5. Effect of intravenous injection of 0.9% NaCl on the level of non-protein sulfhydryl groups (NPSH) (A) and nitric oxide (NO) (B) in rat liver in comparison to the control (pricked) rats. Values represent the mean ± SEM
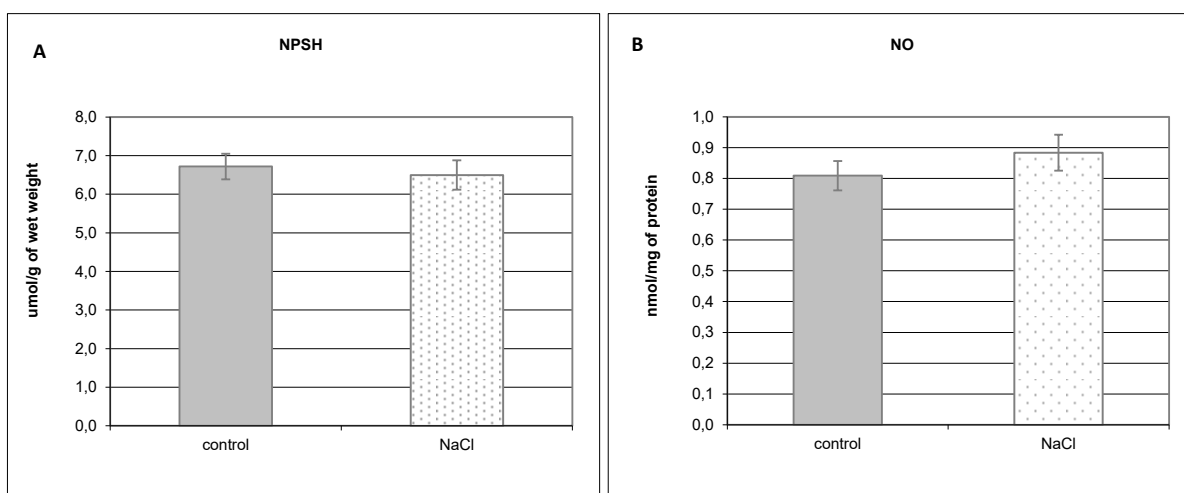

Fig. 7. Effect of intraperitoneal injection of 0.9% NaCl on the level of non-protein sulfhydryl groups (NPSH) (A) and nitric oxide (NO) (B) in rat liver in comparison to the control (injected with redistilled water) rats. Values represent the mean ± SEM

RESULTS

The effect of injections of 0.9% NaCl on blood pressure

After intravenous administration of 1 ml of 0.9% NaCl to the animals for 7 days, on the 8th day an increase in blood pressure was observed in comparison to the first day (before saline injection). Systolic blood pressure increased by 13.7% and the increase was statistically significant whereas diastolic pressure rose by 12.2% and the difference was not statistically significant (Fig. 2A). In the control group i.e. in rats which were only pricked (stressed like the injected animals), blood pressure did not change statistically significantly within 7 days compared to the values before experiment (day 1) (Fig. 2B).

In addition, a two-way ANOVA was used to assess the differences in blood pressure between pricked (control) and saline-injected animals in each time point. These results revealed also statistically significant increase in systolic blood pressure on the 8th day of the experiment (Fig. 2C).

In the experiment with intraperitoneal saline injection, animals were administered initially 0.3 ml of saline for 21 days and then the volume of saline was increased to 1 ml. These studies demonstrated a significant increase in SBP (by 16.8-26.2%) and DBP (by 30-31.6%) when compared with the first day (Fig. 3A). Blood pressure in control animals given *ip* injections of sterile water also increased in comparison to the first day, however to a lesser degree (SBP by 15.2-21.8% and DBP by 13.8-16.4%) but differences were not statistically significant (Fig. 3B). A two-way ANOVA
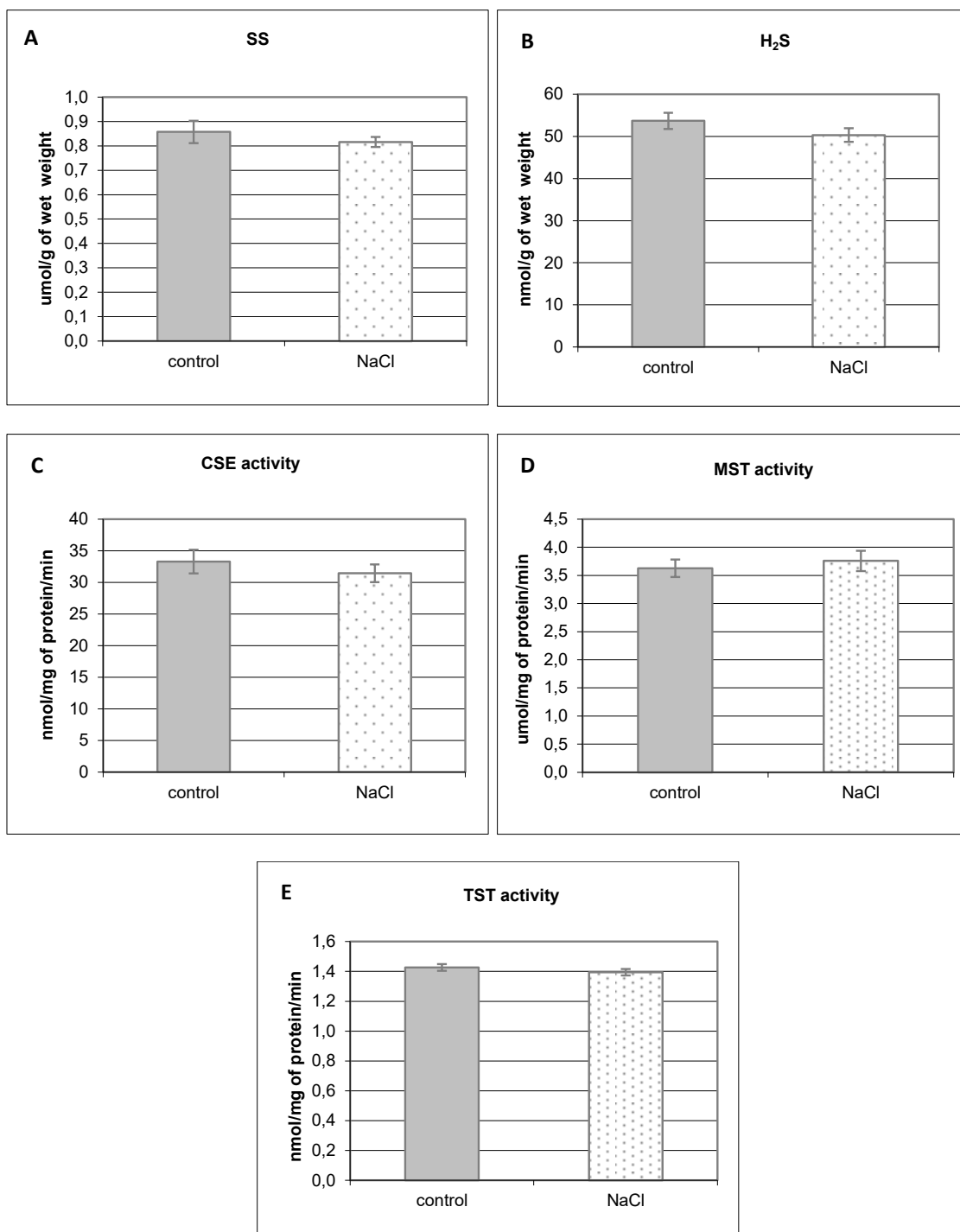
Fig. 6. Effect of intraperitoneal injection of 0.9% NaCl on the level of sulfane sulfur (A), hydrogen sulfide (B) and on the activities of γ-cystathionase, CSE (C), 3-mercapto-sufurtransferase, MST (D) and rhodanese TST, (E) in rat liver in comparison to the control (injected with redistilled water) rats. Values represent the mean ± SEM

showed no significant differences between blood pressure of animals receiving ip water injection (control) and animals injected ip with 0.9% NaCl in each time point (Fig. 3C).

The effect of 0.9% NaCl injections on anaerobic cysteine metabolism in the rat liver

Biochemical studies on the livers of animals injected iv 1 ml of physiological saline for 7 days demonstrated a statistically significant decrease in the SS and H_2S level vs. control animals which were only pricked (90.6% and 92.7% of initial value, respectively) (Fig. 4A,B). It was accompanied by a significance reduction of the CSE activity (91.1% of control value) (Fig. 4C) while the decrease in MST activity was not statically significant (93%) (Fig. 4D). There were no significant differences in TST activity (responsible mostly for transfer of sulfane sulfur atom to different acceptors) (Fig 4E) or in NO and NPSH levels in the livers of animals after intravenous injection of 0.9% NaCl (Fig. 5 A, B).

When 0.9% NaCl was administered intraperitoneally for 39 days, no statistically significant differences were seen in hepatic SS, H_2S, NO and NPSH levels or in the activity of sulfurtransfereases (Fig. 6, 7). The SS and H_2S levels in saline-injected animals were slightly reduced compared with animals receiving ip injections of redistilled water (Fig. 6 A, B) which was accompanied by a slight decrease in CSE activity (Fig. 6 C) but these reductions were not significant. Likewise, ip physiological saline injections did not cause changes in MST or TST activities (Fig. 6 D, E) or NPSH levels (Fig. 7 A). On the other hand, NO level increased in the liver of animals injected 0.9% NaCl vs. animals injected redistilled water; however, this increase was not statistically significant (Fig. 7 B).

Discussion

The relationship between high NaCl consumption (excessive sodium intake) and the development of hypertension is commonly known and discussed [19]. Physiological saline is an isotonic fluid widely used for intravenous injections and in animal studies, it is most often believed to be a "neutral" solvent administered to control groups. In the present studies, for the first time an attempt was made to explain whether the dose of sodium contained in physiological saline administered regularly can contribute to an increase in blood pressure. The studies were conducted in two independent models of saline injections in animals, i.e. via intravenous and intraperitoneal route. The aim of these experiments was to investigate the effect of repeated saline injections not only on blood pressure but also on anaerobic cysteine metabolism in the rat liver yielding RSS (mainly H_2S and SS).

The results suggest that even the dose of sodium contained in physiological saline, if administered repeatedly, can contribute to an increase in blood pressure, but the effect depends on the route of administration. In the present experiment, 7-day intravenous admin-

istration of 1 ml of 0.9% NaCl statistically significantly increased SBP compared with blood pressure on the first day of the experiment (Fig.2A) and with control animals on the 8th experimental day (Fig. 2C). In this experiment, a significant effect of iv saline injection on DBP was not observed. Earlier studies and meta-analyses have also shown a strong positive association between sodium intake and SBP with much less effect on DBP. Simultaneously, it was shown that sodium restriction led to a significant reduction in SBP [6]. In the case of intraperitoneal injections, the effect of physiological saline on blood pressure was much less conspicuous. Initial injections of 0.3 ml of 0.9% NaCl did not cause significant changes in blood pressure. However, when the volume of saline was increased to the dose of 1 ml administered ip for 26 days, it did influence SBP and DBP vs. the value measured in this group on the first experimental day. However, analysis of blood pressure in these animals vs. control group receiving redistilled water did not confirm statistically significant changes (Fig. 3C). Therefore, in this case, only a tendency towards SBP and DBP increase could be observed. It is not excluded that the tendency towards an increase in blood pressure in control animals, caused probably by increased fluid volume due to ip saline injections, could have contributed to the lack of significant differences vs. control animals. However, a slight SBP increase observed in animals injected with water on 28th-40th experimental day was not statistically significant vs. the first day of the experiment, in contrast to saline-treated animals.

In the experiment with intravenous saline injections, the control group was not injected water because of the risk of hemolysis. This fact could have some impact on differences in fluid volume in the circulation between both groups of animals; however, for the above reasons, it could not be avoided. Further, literature data on the effect of salt on blood pressure combined with the changes in RSS in the liver, observed in this experiment, speak for the effect of salt.

Much evidence confirms the association between high sodium intake and increase in blood pressure [6]; however, the physiological mechanisms underlying NaCl-induced changes in blood pressure are not completely understood. Moreover, there are evidences that elevated dietary sodium, even in the absence of its influence on blood pressure has adverse effects on target organs (heart, kidney and even brain) [19]. However, no information in the literature was found on the impact of the sodium dose contained in physiological saline administered repeatedly on blood pressure and sulfur metabolism in the liver.

Recently, researchers' attention has been focused on hypotensive effects of RSS, mainly H_2S and SS [41,42]. The liver is thought to be the main source of H_2S and SS in the circulation since the liver is characterized by the most intense anaerobic cysteine metabolism [47]. This is the reason why we conducted biochemical assays in this

organ. The level of compounds with reduced sulfur, i.e. H_2S and SS in the livers of animals after intravenous saline administration was reduced (Fig. 4A, B), which was accompanied by a drop in the activity of CSE, the main enzyme responsible for RSS production in the liver. Intravenous saline injections did not significantly affect MST and TST activity. MST is the enzyme responsible for H_2S production from 3-mercaptopyruvate [18]. The lack of saline-induced changes in MST activity confirms that CSE plays the main role in RSS production in the liver. On the other hand, TST is involved in reactive SS transport, although other proteins (e.g. albumins) can also fulfill this function. The decrease in RSS level indicates that saline may disturb thiol regulation based on H_2S and SS in the liver. Studies have revealed that many liver proteins are sulfhydrated (forming protein-SSH) under physiological conditions including GAPDH, $β$-tubulin and actin [30]. Other studies indicate that S-sulfhydration of cardiac proteins is involved in H_2S-dependent cardiac relaxation in the frog and the rat [25]. SS bound to proteins is regarded as the storage form of H_2S, which can be released in response to physiological signals. The decrease in SS level in the liver induced by intravenous saline injection, observed by us, suggests that H_2S is released from ProteinSSH. On the other hand, the decline in free H_2S in the liver can be explained by its probable release to the circulation. H_2S produced in cytosol of hepatocytes can be released to the circulation where in the protein-bound form, it is transported to other tissues. It is not excluded that it can constitute a defense mechanism against harmful effects of salt, because RSS circulating in blood can be used as sulfhydrating agents for ATP-sensitive potassium (K_{ATP}) channels in response to increased blood pressure. Significance of H_2S produced in the liver for keeping normal blood pressure was confirmed by the studies in CSE gene knockout mice. The absence of CSE activity led to the inhibition of H_2S generation, then to closure of K_{ATP} channels and finally to the increase in blood pressure in hepatic vessels and reduction of H_2S level in plasma and in the heart [45].

Under physiological conditions, free H_2S level is low compared with SS-containing compounds. When H_2S synthesis is enhanced, its excess is stored in the form of SS-containing hydropersulfides, which also constitutes a redox regulatory mechanism. Some studies demonstrated that H_2S exerted antioxidant action on the cardiovascular system by inhibiting reactive oxygen species (ROS) production [28]. Therefore, reduction of H_2S and SS level in the liver observed in this study after iv saline injection could contribute to an increase in ROS level. However, the absence of changes in NPSH level, which represents the main cellular antioxidant (GSH), did not confirm disturbances in redox balance after saline injection (Fig. 5A). The present studies also did not reveal the effect of saline on the second, beside H_2S, important gasotransmitter involved in blood pressure regulation, namely NO (Fig. 5B). No effect of sodium supplementation on blood NO concentration was also demonstrated in a randomized study in untreated (pre) hypertensive patients. Sodium supplementation did not affect the

NO level in these patients vs. placebo group [5]. On the other hand, some studies in normotensive animals suggest that a reduction in vascular NO is a key factor in the pressure-independent effect of dietary salt [2]. In that case, the decrease in NO level is strongly associated with the increased ROS level leading not only to scavenging of NO but also to the disruption of some signaling pathways that mediate its production. In present experiments, an increase in SBP was observed after iv sodium injection with no effect on NO or ROS levels.

The present studies revealed that even the sodium doses contained in physiological saline, if used for repeated infusions, influenced the RSS pool, and tended to increase blood pressure. It is suggested that the influence of physiological saline is not fully "neutral", so in experimental studies, if possible, control animals should be administered water and not saline. This effect of physiological saline should be taken into consideration especially in salt-sensitive patients when they need a long-term infusion of physiological saline.

With the aim of increasing hepatic RSS level, reduced by high doses of NaCl, and decreasing blood pressure, supplementation of H_2S donors can be considered. Research is currently under way to find the therapeutic uses of RSS-based medication on salt-induced hypertension. Although H_2S itself is not currently used clinically, slow-release H_2S donors, such as GYY4137, S-diclofenac, S-naproxen or combined NO/H_2S-releasing aspirin are tested at present [4,8,33]. Thiosulfate, the H_2S oxidation product containing SS, is also used as an RSS donor. Thiosulfate is already in clinical use for the treatment cyanide poisoning [46]. Due to rapid breakdown by gastric acids, thiosulfate must be administered intravenously, making its use difficult. Fortunately, some dietary components, especially garlic (Allium sativum) are good, safe and accessible sources of RSS. Garlic, known for its medicinal use since antiquity, possesses antibacterial, antivirus, anticancer, cholesterol lowering, and hypotensive properties [12,32]. These beneficial properties of garlic are attributed to sulfur components, e.g. diallyl disulfide (DADS) and diallyl trisulfide (DATS). Benavides et al. have demonstrated that vasoactive effect of garlic is connected in physiological conditions with the transformation of diallyl polysulfides to the SS-containing hydropersulfides, which react with thiols to release H_2S [1]. Earlier studies demonstrated that DADS and DATS could be a good source of SS for liver cells and could stimulate the CSE activity [14]. It appears that garlic consumption is particularly important for the elderly, because RSS levels in the body decrease with age [10], while cardiovascular problems, especially hypertension, increase. For this reason, reducing dietary salt intake and increasing the consumption of natural RSS-rich dietary supplements provide significant health benefits particularly for the aging human population.

Summing up, the repeated iv saline injection induced a slight hypertension accompanied by disturbances in anaerobic cysteine metabolism in the liver. However, in

the case of repeated ip saline injections, the effect on blood pressure and biochemical assays in the liver was much weaker and insignificant. It can be concluded that even sodium dose delivered with physiological saline, if administered repeatedly, can contribute to increased blood pressure, but the impact depends on the route of administration.

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