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INFLUENCE OF N-ACETYLCYSTEINE ON BIOACTIVATION OF NITROGLYCERIN TO NITRIC OXIDE AND S-NITROSOTHIOLS IN THE LIVER AND BRAIN OF MICE

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Three-day nitroglycerin (NTG) administration at progressively increasing doses caused a drop in the liver S-nitrosothiol (SNT) and malonyldialdehyde (MDA) concentrations below the control levels. It suggests that NTG administered in this way, exhibits antioxidant activity due to releasing the biologically active SNT and nitric oxide (NO). On the other hand, in the brain, NTG did not influence SNT concentrations, but slightly elevated NO formation. N-acetylcysteine (NAC) given jointly with NTG substantially stimulated NTG bioactivation to the biologically active NO and SNT as well in the liver as in the brain. It was accompanied by a rise in non-protein sulfhydryl thiol (NPSH) level and additional suppression of lipid peroxidation in hepatocytes. Therefore, is seems that the combined administration of NTG and thiols or other antioxidants is very much justified not only because of their influence on the vascular endothelial cells but also on such organs as the liver and brain.

Key words: *N-acetylcysteine, nitroglycerin, nitric oxide, S-nitrosothiols*

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Abbreviations: DTNB – *5,5'-dithio-bis(2-nitrobenzoic acid),* γ-GT – *γ-glutamyl transpeptidase,* GST – *glutathione S-transferase,* MDA – *malonyldialdehyde,* NAC – *N-acetylcysteine,* NTG – *nitroglycerin,* NPSH – *non-protein sulfhydryl thiols,* OTC – *2-oxo-thiazolidine-4-carboxylic acid,* SNT – *S-nitrosothiols*

INTRODUCTION

Nitric oxide (NO) is a highly reactive molecule, participating in maintaining homeostasis in the cardiovascular, nervous and immune systems. Nitroglycerin (NTG) and other organic nitrates are used as drugs, when endogenous biosynthesis of NO from arginine is insufficient. They are administered in a variety of diseases, such as angina pectoris, myocardial infarction, pulmonary hypertension, cardiac insufficiency, coronary angioplasty and fibrinolysis [1, 18].

One of the adverse reactions developing during the therapy with organic nitrates is hemodynamic tolerance [10]. It consists in a gradual decrease in therapeutic efficiency in the course of the treatment with NTG, due to a drop in a level of biologically active NO, which is generated from it. Long-term NTG treatment is also accompanied by an enhancement of peroxidative processes in the vascular endothelial cells [8, 23]. It leads to the elevated formation of superoxide radical anion ($O_2^{\bullet-}$), whose reaction with NO causes a loss of the biologically active NO and generation of toxic peroxynitrite ($ONOO^-$) [15].

Thiols fulfill important, albeit not fully elucidated function in the reductive biotransformation of organic nitrates to pharmacologically active NO and S-nitrosothiols (SNT) [16]. They allow also for the transport and storage of NO in the form of SNT [34], and act as antioxidants [31]. To prevent development of tolerance to NTG, its doses are recommended to be increased in the course of the treatment [29] and thiols should be administered simultaneously [6, 16]. A majority of studies on the contribution of thiols to bioactivation of NTG has been conducted on the vascular endothelial cells [32]. Our present study was designed to investigate NTG bioactivation in the liver and brain of mice. After the administration of increasing doses of NTG with or without N-acetylcysteine (NAC), we determined the liver levels of non-protein sulfhydryl thiols (NPSH), NO and SNT, and also malonyldialdehyde (MDA), which is an indicator of peroxidative processes. At the same time, activities of glutathione S-transferase (GST), an enzyme participating in NTG biodegradation [25, 37], and γ-glutamyl transpeptidase (γ-GT), responsible for biodegradation and biosynthesis of glutathione (GSH) [13] were assayed. The aim of these investigations was to shed some light on the course and effects of the process of NTG bioactivation in the absence and presence of NAC in cell other than the vascular endothelial cells.

MATERIALS and METHODS

Female Swiss mice were assigned to four groups, 8 mice each. Control group 1 was administered intraperitoneally (*ip*) 0.3 ml of physiological saline for 3 consecutive days. The animals in group 1 received NTG at gradually increasing doses of 0.01, 0.02 and 0.03 mg/kg (which corresponded to 20, 40 and 60 μl of 1 mg % solution of NTG in 0.3 ml of physiological saline) once a day for 3 days. The mice in group 2 were administered NAC at 250 mg/kg (5 mg in a volume of 0.3 ml) for 3 days. The animals belonging to group 3 were given injections of NAC (5 mg in a volume of 0.3 ml) and the increasing doses of NTG (0.01, 0.02 and 0.03 mg/kg, as in group 1) for 3 days. After 3 days, the mice were killed by decapitation, the isolated organs were placed in liquid nitrogen and stored at –70°C until assayed.

Reagents

N-Acetyl-L-cysteine, 5,5'-dithio-bis(2-nitrobenzoic acid), 2,3,diaminonaphthalene (DAN), mercury chloride, thiobarbituric acid, 1,1'3,3'-tetrahydroxypropane, sulfanilamide, N-1-naphthyl-ethylenediamine, glycylglycine, 1-chloro-2,4-dinitrobenzene were obtained from Sigma Chemical Company (Deisenhofen, Germany). NTG was purchased from PLIVA (Poland), trichloroacetic acid (TCA) was from Ubichem plc. Sigma Chemical Co., while L-glutamyl-4-nitroanilide was from Boehringer Mannheim GmbH (Germany). The remaining chemicals were purchased from POCh (Gliwice, Poland). NO assay kit "Nitrite/nitrate colorimetric method" was purchased from Roche.

Preparation of tissue homogenate

Homogenates of the tissues were prepared by homogenization of 1 g of tissue in 4 ml of 0.1 M

phosphate buffer pH 7.4 at a temperature of +4°C and 9500 rpm using IKA-ULTRA-TURRAX T25 homogenizer.

Determination of non-protein sulfhydryl groups [22]

In this assay based on Ellman's method, 5,5'-dithio-bis(2-nitrobenzoic acid), was reduced by NPSH in TCA extract to 2-nitro-5-mercaptobenzoic acid. This product was characteristic because of its yellow color. The reference solution contained the same components except for supernatant (TCA extract). For the estimation of NPSH, 50 µl of TCA extract and 100 µl of 6mM DTBN were added in succession to 800 µl of 0.2M phosphate buffer pH 8.2, and the absorbance was measured at 412 nm. The amount of 2-nitro-5-mercaptobenzoic acid formed in this reaction was determined from the standard curve prepared for the 1 mM reduced glutathione solution in 2.5% TCA and expressed in micromoles per mg of protein.

Determination of S-nitrosothiol level using fluorometric method of Marzinzig et al. [19]

In this method, DAN is nitrosated to 2,3-naphthotriazole (NAT), a fluorescent product, which allows for determination of nitrates III (NO_2^-), the products of NO oxidation in biological samples. Because SNT are degraded in the presence of mercury ions (Hg^{2+}), the difference between NO_2^- level in the presence and absence of mercury ions allows for establishing the SNT level in plasma. In order to determine the SNT level in the examined homogenates, 50 µl of homogenate was added to 700 µl of redistilled water and 250 µl of DAN reagent mixture. DAN reagent mixture was prepared by mixing 1.11mM $HgCl_2$ and 15.8 mM DAN in 0.62 M HCl at 1:4 ratio. In parallel experiments (without Hg^{+2}), 250 µl of 15.8 mM DAN in 0.62 M HCl was added. The samples were incubated at room temperature in the dark for 10 min. To stop the reaction progress, the samples were alkalized to pH ~11.5–12.0 by adding 50 µl of 10 M NaOH. Fluorescence measurements were performed at excitation wavelength of 365 nm and emission wavelength of 410 nm. The SNT level was calculated according to standard curve obtained for the solution prepared by mixing 10 µM L-cysteine with 10 µM $NaNO_2$.

Determination of lipid peroxidation level as a malonyldialdehyde level [26]

This is a method based on the reaction of thiobarbituric acid (TBA) with some products of lipid peroxidation in acidic environment at increased temperature. The product that is formed is pink in color, which enables its spectrophotometric determination. The determination procedure consisted in the addition of 250 µl of distilled water, 500 µl of 15% TCA, 500 µl of 0.37% TBA to 250 µl of homogenate. TBA and TCA solutions were prepared in 0.25 M HCl. The samples were heated in boiling water bath for 10 min. After cooling, the samples were centrifuged at 4500 rpm for 10 min. Absorbance of the supernatant was measured at 535 nm. The MDA concentration was calculated according to standard curve prepared from 1,1',3,3'-tetraethoxypropane.

Determination of nitrite level (as a measure of NO level) [12]

The level of nitrites (the final products of NO metabolism), was determined using Roche's test "Nitric oxide colorimetric assay" (Cat. No. 1 756 281). The test is based on the following reaction: nitrites + sulfanilamide + N-(1-naphthyl)-ethylenediamine dihydrochloride yield colored diazo product, whose absorption was measured at 540 nm.

The mixture of 500 µl of redistilled water and 250 µl of homogenate were placed in water-bath at 100°C for 15 min to stop enzymatic processes. After cooling 30 µl of Carrez I (0.36 M $K_4[Fe(CN)_6]\cdot 3H_2O$) and 30 µl of Carrez II (1 M $ZnSO_4\cdot 7H_2O$) were added. Next the samples were alkalized to pH 8.0 by adding 4 µl of 10 M NaOH and centrifuged at 10000 × g before use. For nitrite determination, 100 µl of supernatant and 54 µl of redistilled water were placed in microplate wells. In a blank sample, redistilled water was added instead of supernatant. After 30 min of incubation at room temperature, absorbance was measured at 540 nm. Next 50 µl of sulfanilamide and the same volume of N-(1-naphthyl)-ethylenediamine dihydrochloride were added to each well. After mixing microplates were allowed to stand in dark for 15 min and absorbance was again measured at 540 nm. The results were calculated according to standard curves obtained for solutions of sodium nitrite (6–600 µM), using the change in absorbance measured before and af-

ter incubation with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride.

Determination of glutathione S-transferase activity [3]

GST catalyzes reaction between glutathione and 1-chloro-2,4-dinitrobenzene, yielding colored conjugate, 2,4-dinitrophenyl-S-glutathione, which shows absorbance maximum at a wavelength of 340 nm. An increase in absorbance after 1 min of incubation was used as a measure of reaction progress. The activity of the enzyme was calculated on the basis of millimolar absorbance index of the formed conjugate, which is 9600 $mM^{-1} \times cm^{-1}$.

Procedure: the 50 μl of 20 mM GSH solution, the same volume of 20 mM CDNB solution and 50 μl of the homogenate diluted 50 times were added to 850 ml of 0.1 M phosphate buffer pH 6.5. Thirty and 90 s afterwards, an absorbance at 340 nm was measured against the control sample, containing phosphate buffer instead of the homogenate. A difference between an increase in absorbance in the test sample and control sample was a measure of the enzyme's activity. Activity of the enzyme was expressed in millimoles of the conjugate formed within 1 min under the experimental conditions applied in this study.

Determination of L-γ-glutamyl transpeptidase activity [27]

In this method [27] the colorless substrate L-γ-glutamyl-p-nitroanilide was enzymatically converted to p-nitroaniline. The reaction mixture contained 5 μM of L-γ-glutamyl-p-nitroanilide and 10 μM of $MgCl_2$ dissolved in 0.9 ml of 111 mM Tris-HCl buffer, pH 9 and was incubated for 5 min at 37°C with 0.1 ml of liver or brain homogenates. The reaction was stopped by adding 1 ml of 1.5 M acetic acid. The probes were centrifuged at 12000 × g for 5 min, and the absorbance of p-nitroaniline that developed during 5 min was measured at 410 nm. The enzyme activity was expressed in μmoles of the product (evaluated from a standard curve for p-nitroaniline) formed during 1 min of incubation per 1 mg of protein.

Protein determination

The protein content was measured according to the method of Lowry et al. [17] using bovine serum albumin as a standard.

Statistical analysis

The results were presented as means of eight assays ± SD, and statistical significance of the differences was evaluated using analysis of variance and one way ANOVA test. The difference was considered statistically significant when $p < 0.05$.

RESULTS

In the present study, the increasing doses of NTG were administered *ip* alone or simultaneously with NAC for 3 consecutive days, and subsequently, the levels of NO, SNT, NPSH and MDA, as well as the activities of γ-GT and GST were determined in the liver and brain of mice.

The results obtained in the liver

Three-day administration of increasing doses of NTG caused statistically significant decrease in SNT level and slight but significant increase in NO concentration (Fig. 1, group 1). It was accompanied by statistically significantly diminished MDA level. NPSH level and γ-GT activity (Fig. 1 and 2, group 1) remained similar to the values observed in the control group. When NAC was administered together with NTG (Fig. 1, group 3), the levels of

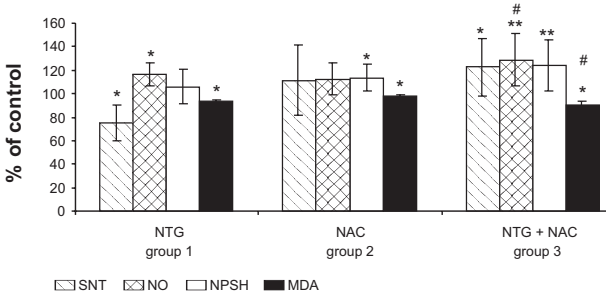

Fig. 1. Influence of progressively increasing doses of nitroglycerin (NTG) (0.01, 0.02, 0.03 mg/kg) administered *ip* alone for three consecutive days (group 1), NTG at the same doses given jointly with N-acetylcysteine (NAC) (at a dose of 250 mg/kg) (group 3), and NAC alone (250 mg/kg) (group 2) on the liver levels of S-nitrosothiols (SNT), nitric oxide (NO), non-protein sulfhydryl thiols (NPSH) and malonyldialdehyde (MDA), expressed as a percent of the control. Control values were as follows: SNT 0.053 ± 0.010 nmoles/mg of protein, NO 2.271 ± 0.218 nmoles/mg of protein, NPSH 24.37 ± 1.553 nmoles/mg of protein, MDA 0.105 ± 0.001 μmoles/mg of protein. * statistically significant vs. the control group at $p < 0.05$, ** statistically significant vs. the control group at $p < 0.01$, $^{\Delta}$ statistically significant vs. the group 1 (NTG) at $p < 0.05$, $^{\Delta\Delta}$ statistically significant vs. the group 1 (NTG) at $p < 0.01$, $^{\#}$ statistically significant vs. the group 2 (NAC) at $p < 0.05$

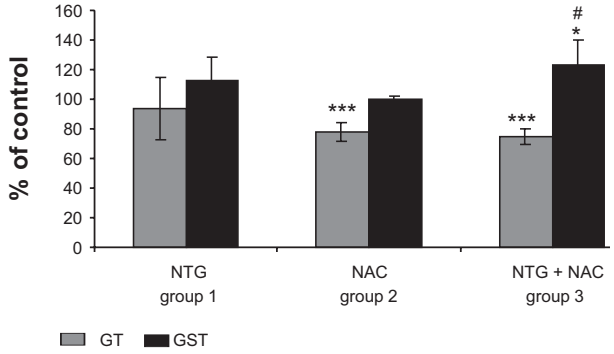

Fig. 2. Influence of progressively increasing doses of nitroglycerin (NTG) (0.01, 0.02, 0.03 mg/kg) administered *ip* alone for three consecutive days (group 1), NTG at the same doses given jointly with N-acetylcysteine (NAC) (at a dose of 250 mg/kg) (group 3), and NAC alone (250 mg/kg) (group 2) on the liver activity of γ-glutamyl transferase (γ-GT) and glutathione S-transferase (GST), expressed as a percent of the control group. Activity of γ-GT was expressed in μmoles of p-nitroaniline formed within 1 min per 1 mg of protein, while activity of GST was presented as mmoles of S-conjugate formed within 1 min per 1 mg of protein. Control values were: γ-GT 0.094 ± 0.005 μmoles/mg of protein, GST 0.497 ± 0.021 mmoles/mg of protein. * statistically significant vs. the control group at $p < 0.05$, *** statistically significant vs. the control group at $p < 0.001$, $^{\Delta\Delta}$ statistically significant vs. the group 1 (NTG) at $p < 0.01$, $^{\Delta\Delta\Delta}$ statistically significant vs. the group 1 (NTG) at $p < 0.001$, $^{\#}$ statistically significant vs. the group 2 (NAC) at $p < 0.05$

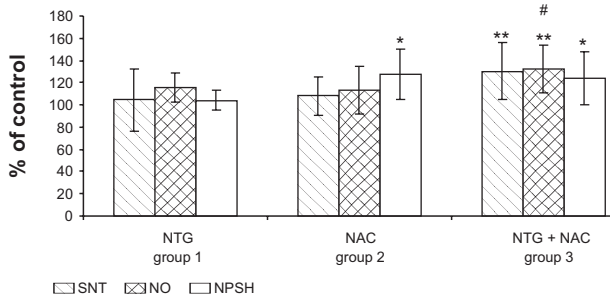

Fig. 3. Influence of progressively increasing doses of nitroglycerin (NTG) (0.01, 0.02, 0.03 mg/kg) administered *ip* alone for three consecutive days (group 1), NTG at the same doses given jointly with N-acetylcysteine (NAC) (at a dose of 250 mg/kg) (group 3), and NAC alone (250 mg/kg) (group 2) on the levels of S-nitrosothiols (SNT), nitric oxide (NO) and non-protein sulfhydryl thiols (NPSH) in the mice brain, expressed as a percent of the control value. Control values were: SNT 0.312 ± 0.068 nmoles/mg of protein, NO 0.512 ± 0.175 nmoles/mg of protein, NPSH 17.452 ± 2.958 nmoles/mg of protein. * statistically significant vs. the control group at $p < 0.05$, ** statistically significant vs. the control group at $p < 0.01$, $^{\Delta}$ statistically significant vs. the group 1 (NTG) at $p < 0.05$, $^{\#}$ statistically significant vs. the group 2 (NAC) at $p < 0.05$

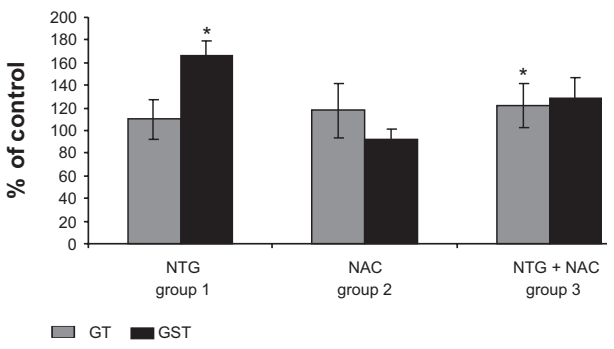

Fig. 4. Influence of progressively increasing doses of nitroglycerin (NTG) (0.01, 0.02, 0.03 mg/kg) administered *ip* alone for three consecutive days (group 1), NTG at the same doses given jointly with N-acetylcysteine (NAC) (at a dose of 250 mg/kg) (group 3), and NAC alone (250 mg/kg) (group 2) on the activity of γ-glutamyl transferase (γ-GT) and glutathione S-transferase (GST) in the mice brain, expressed as a percent of the control group. Activity of γ-GT was expressed in μmoles of p-nitroaniline formed within 1 min per 1 mg of protein, while activity of GST was presented as mmoles of S-conjugate formed within 1 min per 1 mg of protein. Control values were: activity of γ-GT: 0.133 ± 0.022 $\mu M \times mg^{-1} \times min^{-1}$, and activity of STG: 0.125 ± 0.026 $mM \times mg^{-1} \times min^{-1}$. * statistically significant vs. the control group at $p < 0.05$, $^{\Delta}$ statistically significant vs. the group 1(NTG) at $p < 0.05$

SNT, NPSH and NO in the liver significantly rose, as did the activity of GST (an enzyme, participating in NTG biodegradation), while MDA level and γ-GT activity were lowered (Fig. 1 and 2, group 3). It indicates that the concomitant administration of NTG and thiols, such as NAC, causes significant enhancement of the bioactivation, i.e. the level of pharmacologically active SNT and NO rises, which is accompanied by strengthening of antioxidant defense (an increase in NPSH level) and by decreasing of peroxidative processes (MDA). It fully confirms beneficial effect of thiols on pharmacological activity of NTG also in the mouse liver. On the other hand, administration of NAC alone (Fig. 1, group 2) did not show statistically significant influence on endogenous NO and SNT biosynthesis. The treatment with NAC alone led to an elevation of NPSH level and to lowering of γ-GT activity in comparison with the control group (Fig. 1 and 2, group 2).

The results obtained in the brain

The treatment with increasing doses of NTG alone did not cause any statistically significant changes in SNT and NPSH levels in the brain (Fig. 3, group 1),

and only slight rise in NO concentration and significant rise in GST activity were noted (Fig. 3 and 4, group 1). Joint administration of NTG with NAC (Fig. 3, group 3) induced substantial and significant increase in SNT, NPSH and NO levels and γ-GT activity in the brain. It suggests activating effect of thiols on the level of biologically active NO and SNT formed from NTG also in this organ.

DISCUSSION

Development of tolerance to NTG and other organic nitrates is a serious pharmacological problem since it decreases efficiency of their action and shortens a duration of therapy with these drugs. Tolerance to NTG is accompanied by a drop in the level of thiols, i.e. cysteine and glutathione in the vascular endothelial cells [4, 5]. On the basis of these findings, Needleman et al. presented so-called "thiol deficiency" theory [24] to explain causes of this tolerance. It was confirmed by the studies indicating that thiol compounds abolished NTG tolerance [11]. Meanwhile, NAC was shown to enhance efficiency of NTG both in tolerant and non-tolerant rats [6]. Moreover, the treatment with cysteine precursor, 2-oxo-thiazolidine-4-carboxylic acid (OTC) led to different effects in non-tolerant animals in comparison with tolerant ones. In the former, this compound increased thiol concentration in the vascular endothelial cells [5, 6], similarly as in the liver cells [21], while in tolerant animals, an increase in cysteine and GSH level was not observed [5, 6]. These results led to a suggestion that most probably NTG disturbed thiol homeostasis in the vascular endothelial cells. It was also noticed that, in contrast to another cysteine precursor NAC, OTC did not promote NTG effects [6]. In the case of such cysteine precursor as OTC, cysteine can be released only as the result of enzymatic reaction, i.e. it occurs only intracellularly [36]. For this reason, the observed activating influence of NAC has been suggested to be due to its extracellular action [6]. As under physiological conditions, concentration of thiols in the interstitial fluid surrounding the vascular endothelial cells is much higher than in plasma, it is suspected that NAC administration can imitate such conditions [20].

In this study, we investigated bioactivation of NTG to SNT and NO, with concomitant determination of the levels of non-protein thiols and lipid peroxidation in the liver and brain of mice. Its goal was to elucidate the course of NTG bioactivation in the presence or absence of NAC in the cells other than the vascular endothelial cells. The obtained results indicated that NTG administered at increasing doses for 3 days caused statistically significant decrease in the liver SNT level, accompanied by significant drop in lipid peroxidation (MDA) (Fig. 1, group 1). The combined treatment with NTG and NAC raised the levels of the biologically active NO and SNT and further lowered lipid peroxidation in the mouse liver (Fig. 1, group 3). This observation is surprising because it indicates that NTG, administered at the dosage and schedule used in this study, exhibits antioxidant effect in the liver. Therefore, NTG activity in this organ differs from the data obtained with the endothelial cells, where NTG induced ROS formation [23]. This observation can be explained only by antioxidant action of NO [28], released as a result of NTG biodegradation, and SNT generated in this process [30], i.e. it occurs at the cost of biologically active molecules. NTG injected alone (Fig. 2, group 1) elevated non-significantly the liver activity of GST, an enzyme participating in its biodegradation to NO and SNT [25, 37]. However, NAC given alone and together with NTG (Fig. 2, group 2 and 3) inhibited liver γ-GT activity, and did not show any significant effect on GST activity. It also did not affect endogenous NO and SNT biosynthesis, but only slightly diminished MDA level (Fig. 1, group 2). It indicates that NAC suppresses γ-glutamyl cycle, involved in biosynthesis and biodegradation of GSH. Glutathione does not cross cell membrane, so its extracellular biodegradation, in which γ-GT participates, is indispensable for securing cysteine for intracellular GSH formation [13]. The administration of NAC, which is a cysteine precursor, from which this amino acid is produced as a result of enzymatic deacetylation, provides sufficient cysteine supply to the cells [7], that probably contributes to the inhibition of γ-glutamyl cycle.

In the brain, the combined treatment with NTG and NAC (Fig. 3, group 3) led also to statistically significant increase in SNT, NPSH and NO concentration, but in this organ, γ-GT activity was elevated (Fig. 4, group 3). It indicates that simultaneous administration of NTG and NAC causes a rise in the levels of the therapeutically important SNT and NO, but also demonstrates an improvement of antioxidant status of the brain, which is evidenced by an elevated level of NPSH. NAC administered

jointly with NTG takes over antioxidant defense and participates in NTG bioactivation to NO and SNT.

NAC is a drug used for detoxification of such substances, as paracetamol [2]. It is a precursor of cysteine, which is essential for GSH biosynthesis and takes direct part in antioxidant reactions [14, 33]. Although it is commonly accepted that NAC toxicity is low, it cannot be entirely excluded [35], besides, NAC was demonstrated to cause adverse effects [9]. Therefore, search for new thiols, capable of increasing NTG efficacy and free of adverse reactions is highly recommended.

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