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ORIGINAL ARTICLE

Effects of Neuroactive Amino Acids Derivatives on Cardiac and Cerebral Mitochondria and Endothelial functions in Animals Exposed to Stress

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ABSTRACT

Introduction: To study the effects of glufimet, a new derivative of glutamic acid, and phenibut, a derivative of γ-aminobutyric acid (GABA), on cardiac and cerebral mitochondria and endothelial functions in animals following exposure to stress and inducible nitric oxide synthase (iNOS) inhibition. **Methods:** Rats suspended by their dorsal cervical skin fold for 24 hours served as the immobilization and pain stress model. Arterial blood pressure was determined using a non-invasive blood pressure monitor. Mitochondrial fraction of heart and brain homogenates were isolated by differential centrifugation and analysed for mitochondrial respiration intensity, lipid peroxidation (LPO) and antioxidant enzyme activity using polarographic method. The concentrations of nitric oxide (NO) terminal metabolites were measured using Griess reagent. Hemostasis indices were evaluated. Platelet aggregation was estimated using modified version of the Born method described by Gabbasov et al., 1989. **Results:** The present study demonstrated that stress leads to an elevated concentration of NO terminal metabolites and LPO products, decreased activity of antioxidant enzymes, reduced mitochondrial respiratory function, and endothelial dysfunction. Inhibition of iNOS by aminoguanidine had a protective effect. Phenibut and glufimet inhibited a rise in stress-induced nitric oxide production. This resulted in enhanced coupling of substrate peroxidation and ATP synthesis. The reduced LPO processes caused by glufimet and phenibut normalized the endothelial function which was proved by the absence of average daily blood pressure (BP) elevation episodes and a significant increase in platelet aggregation level. **Conclusion:** Glufimet and phenibut restrict the harmful effects of stress on the heart and brain possibly by modulating iNOS activity.

KEYWORDS: Immobilization and pain stress, iNOS, Mitochondrial dysfunction, Lipid peroxidation, Endothelial dysfunction

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality globally. Today, stress is considered one of the major factors contributing to heart damage and CVD [1, 2, 3, 4].

A long-term and intense exposure to stress can bring about an excessive cell response which may be destructive and cause various pathologies [5]. The increased adaptation reaction and higher metabolic rates are due to increased secretion of catecholamines and glucocorticoids which is associated with a leak of electrons from the mitochondrial respiratory chain and their interaction with molecular oxygen promoting free radical formation [6].

A number of oxidative metabolic pathways and numerous redox transmitters and centers, potentially capable of one-electron reduction of oxygen to superoxide anion radical, a precursor of other reactive oxygen species (ROS), are localized in mitochondria. ROS are produced at Complexes I and III of mitochondrial respiratory chain. ROS generated by mitochondria increase intracellular oxidative stress arising out of the imbalance between generation of free radicals and defense mechanisms against them. A higher rate of ROS production and lower activity of the antioxidant system result in the damage to mitochondrial electron transport chain, a decrease in ATP synthesis and activity of ATP-dependent enzymes maintaining the cell membrane potential [7, 8, 9]. Cell membranes become depolarized and Ca^{2+} enters the mitochondrial matrix. The performance of Ca^{2+} pump evacuating ions from cells deteriorates due to ATP deficiency. Excessive ROS and Ca^{2+} in mitochondria promote activation of phospholipases which split fatty acids from phospholipids. All these factors induce uncoupling of oxidative phosphorylation, mitochondrial swelling, changes in

the mitochondrial membrane permeability involving the formation of permeability transition pore (PTP), further disturbance of ion homeostasis, and cell death [10].

Oxidative stress plays an important role in endothelial dysfunction (ED) development. A decreased level of biologically active nitric oxide (NO) produced by vascular endothelium, or its reduced bioavailability, which is associated with increased generation of ROS and the ability of NO to interact with them, is believed to be an essential link of this process. Nitric oxide reacts with superoxide anion ($O2^{*}$-) to form peroxynitrite (ONOO-), which is a weak agonist of guanylate cyclase; NO-induced relaxation deteriorates. Peroxynitrite can also disturb other enzyme systems maintaining vascular homeostasis, e.g., nitration and inactivation of prostacyclin synthase. Moreover, the synthesis of prostacyclin, a vasodilator, becomes impaired and endothelial dysfunction is aggravated. Peroxynitrite oxidizes tetrahydrobiopterin causing a decrease in endothelial inducible nitric oxide synthase (iNOS) activity which results in a decline in NO production and vasodilation and brings about a shift in the thrombogenic potential towards thrombus formation [11, 12].

Furthermore, the activation of iNOS produces NO which can easily convert to ONOO- and contributes to the pathogenesis of stress reactions [13].

These facts necessitate a search for agents restricting the negative effects of stress on mitochondrial performance and preventing iNOS activation, oxidative stress development and subsequent damage to organs and tissues. As is known, the GABA-ergic system is a stress-limiting system; it inhibits stress reactions at the central and peripheral levels. Earlier studies have demonstrated that GABA derivatives have an endothelioprotective action [14], inhibit lipid peroxidation (LPO), increase the activity of antioxidant enzymes [15], and improve mitochondrial functions [16]. Glutamate, which is a GABA precursor, is involved in various metabolic reactions; α-ketoglutarate, a Krebs cycle intermediate, is synthesized from it. Some authors claim that activation of glutamate receptors which are located on NO-ergic neurons, is one of the ways of regulating nitric oxide production [17, 18]. Moreover, there is evidence of close functional interaction between glutamate- and the NO-ergic system [19, 20].

The purpose of this study was to explore the effects of glufimet, a new derivative of glutamic acid, and phenibut, a derivative of γ-aminobutyric acid (GABA), on cardiac and cerebral mitochondria and endothelial functions in animals following exposure to stress and iNOS inhibition.

METHODS

Animals

The experiments involved 56 female Wistar rats weighing 200-250g. When stressed, they were at the diestrum stage. The animals were obtained from the FGUP Laboratory Animal Bank Rappolovo (Leningrad region) and kept in vivarium conditions of Volgograd State Medical University (VolgSMU).

The animals were cared for according to the Principles of the Expedient Laboratory Practice, the National Standard of the Russian Federation GOST P-53434-2009, and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, 1986. The protocol of the experimental study was approved by the Regional Independent Ethics Committee (SE Volgograd Medical research Centre) (protocol № 198-2014 of 25.04.2014).

The animals were divided into 7 groups with 8 animals in each group: 1 – normal control group (intact rats); 2 – negative control group – stressed animals which received 0.9% saline solution following the same regimen as in the experimental groups; 3 and 4 – experimental groups in which glutamic acid derivatives were administered intraperitoneally, glufimet at a dose of 29 mg/kg [21], phenibut at a dose of 50 mg/kg [22], respectively; 5 – group of rats receiving aminoguanidine, an iNOS inhibitor, intraperitoneally, at a dose of 50 mg/kg [23]; 6 and 7 – groups of female rats where glufimet and phenibut were administered, respectively, in combination with iNOS inhibition. Glufimet and phenibut were synthesized by the department of organic chemistry of Herzen State Pedagogical University, St Petersburg, Russia. The techniques of synthesizing glufimet and phenibut have been described in a number of publications [24, 25]. The investigated compounds and

the inhibitor were administered as a single dose ten minutes prior to suspending the animals.

Study Design

After the rats had been exposed to stress for 24 hours, their arterial pressure was measured, after which they were narcotized and their blood was collected to assess the total concentration of nitrite and nitrate ions and hemostasis parameters. Their heart and brain were eviscerated to evaluate the level of NO metabolites, the intensity of LPO processes and mitochondrial respiratory function.

Exposure to Stress

Animals were suspended by their dorsal cervical skin fold with a Kocher's clamp for 24 hours to induce immobilization and pain stress [26].

Measurement of Blood Pressure (BP)

Average daily arterial blood pressure was registered 30 minutes before and after the suspension with a non-invasive blood pressure monitor (Kent Scientific Corporation, Canada). The rats previously spent one hour a day for three days in plastic cases to adjust to a BP measurement procedure.

Isolation of Mitochondria and Assessment of Their Functional Condition

After blood samples were taken, the heart (1g) and brain (1g) were eviscerated from the narcotized rats (chloral hydrate 400 mg/kg). The organs were washed in an ice-cold saline and homogenized in ice-cold medium containing 300mM sucrose (Sigma, USA), 220mM mannitol (Sigma, USA), 10mM EDTA (Fluka Analytical, Czech Republic), 100mM tris, (Sigma, USA) (pH 7.4) in a Potter glass homogenizer in the ratio of 1:5. The homogenates were then cooled and centrifuged at 600 G for 10 minutes to sediment debris and intact cells. The supernatant was centrifuged again for 20 minutes (8000 G). The supernatant was then used to subsequently determine the concentration of

terminal metabolites. The sediment was resuspended and used as mitochondrial fraction [27], in which respiration intensity, lipid peroxidation products and antioxidant enzyme activities were determined. Respiration intensity was assessed by polarographic method with Clark's electrode (Expert – 01 Econiks device, Econika, Russia), after various substrates (succinate, malate/glutamate (Sigma, USA)) had been added.

A mitochondrial suspension (1 mg protein) was added to 1 ml of assay buffer (300 mM sucrose, 10mM KCl (Reachim, Russia), 5 KH_2PO_4 (Reachim, Russia), 1mM EDTA, 1.2 mM $MgCl_2$ (Reachim, Russia), 5 tris -HCl pH 7.4, at 33°C in a sealed chamber equipped with a magnetic stirrer. Oxygen consumption was measured in the presence of 0.5mM malate/0.5mM glutamate or 1mM succinate as substrates.

The mitochondrial respiration rate was expressed in nmol O_2/mg protein/min and calculated in the following metabolic conditions: V_3 – respiration coupled with phosphorylation in the presence of 0.2 мM ADP, V_4 – respiration after the depletion of added ADP, and respiratory control as a V_3/ V_4 ratio [28].

Determination of Lipid Peroxidation Products and Antioxidant Enzyme Activity in Heart and Brain Mitochondria

The concentrations of LPO products, conjugated dienes (CD) and diketones were expressed in optical density units using Placer's technique modified by Ushkalova [29]. The concentration of malondialdehyde (MDA) was determined from its reaction with thiobarbituric acid using the method described by Stalnaya and Garishvili, 1977 [30]. Glutathione peroxidase (GlP) activity was evaluated by measuring the depletion of reduced glutathione reacting with 5.5'dithiobis-(2-nitrobenzoic acid) [31]; catalase activity assessment employed a technique based on hydrogen peroxide capacity to form a stained complex with ammonium molybdate [32]; total superoxide dismutase (SOD) activity was determined by monitoring the inhibition of quercetin oxidation [33]. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA).

Determination of Terminal Metabolites of Nitric Oxide in Serum, Heart and Brain Homogenates

The concentration of nitric oxide terminal metabolites, nitrite and nitrate ions in serum, heart and brain homogenates were determined using a modified method described by Metelskaya and Gumanova, 2005 [34] which involves a single-stage measurement with Griess reagent.

Determination of Platelet Aggregation Level and Indices of Plasma-coagulation Hemostasis

Blood samples were collected from the abdominal aorta and analysed for the indices of plasma-coagulation link of hemostasis and platelet aggregation in all groups. Blood was stabilized with 3.8% sodium citrate solution at a ratio of 9:1. Hemostasis indices which include prothrombin time (PT), fibrinogen (FG), and activated partial thromboplastin time (APTT), were measured using a programmable mechanical and optical detection coagulometer (Minilab 701) and assay kits (NPO RENAM, Russia). Platelet aggregation was determined using the method described by Gabbasov et al., (1980) [35] on a two-channel laser analyzer (Biola, Moscow, Russia). Platelet aggregation was induced by ADP at a final concentration of 5 µM.

Statistical Processing of Data

The findings were analysed with Statistica 6.0 software which involved the parametric Student's t-test to compare paired samples and the Newman-Keuls test for multiple comparisons. The Shapiro-Wilk test was used to determine normal distribution.

RESULTS

NO Metabolites in Serum, Heart and Brain Homogenates

The concentrations of NO terminal metabolites in the serum, heart and brain homogenates in the animals exposed to immobilization and pain stress were 44.0%, 40.2% and 23.3% higher ($p \leq 0.05$) than those in the intact rats, respectively while administration of glufimet caused a 27.2% and 33.5% decline in the serum and the heart, respectively ($p \leq 0.05$) (Figure 1). In the animals receiving phenibut, the levels of nitrite and nitrate ions were significantly lower i.e. by 37.7% in the serum ($p \leq 0.05$) and by 24.7% in the heart ($p \leq 0.05$) as compared to the control stressed animals. When iNOS was inhibited, the concentration of nitric oxide terminal metabolites in the serum was lower by 42.8%, in the heart homogenates by 42.4% and in the brain by 16.1% ($p \leq 0.05$) as compared to the negative control. In the stressed animals treated with glufimet in combination with aminoguanidine, the concentration of nitric oxide terminal metabolites was not significantly different from the animals with inhibited iNOS. However, the nitric oxide terminal metabolites level was lower than that in the animals exposed to stress: in the serum by 33.2%, in the heart by 36.0% ($p \leq 0.05$).

When iNOS was inhibited, stressed animals which received phenibut showed a lower level of serum NO metabolites by 46.9% ($p \leq 0.05$), and by 14.6% in the heart compared to the negative controls (Figure 1).

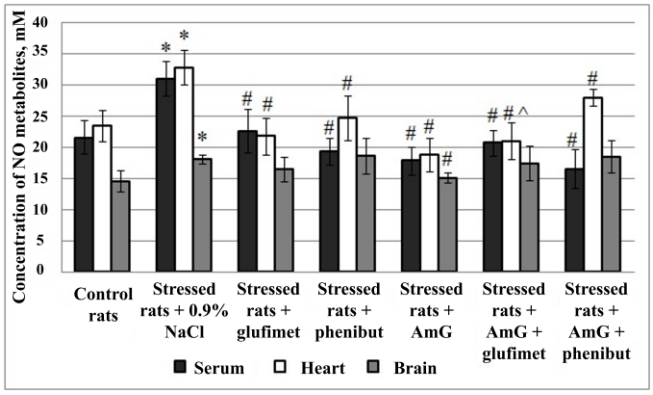

Figure 1 Concentrations of nitric oxide terminal metabolites in serum, heart and brain of intact rats (control group); stressed rats treated with saline (negative control group), stressed rats treated with glufimet, phenibut and aminoguanidine plus glufimet and phenibut (n = 8, mean ± SD).
Note: AmG – aminoguanidine, NO metabolites – total concentration of nitrite and nitrate ions
* - the changes are significant in relation to the intact group animals (Student's t-test, $p \leq 0.05$);
# - the changes are significant in relation to the control group of stressed female rats (Newman-Keuls test, $p \leq 0.05$);
^ - the changes are significant in relation to the group of stressed animals receiving aminoguanidine (Newman-Keuls test, $p \leq 0.05$).

Concentration of LPO Products and Antioxidant Enzyme Activity in Heart and Brain Mitochondria

The concentrations of lipid peroxidation products in heart and brain mitochondria in the stressed animals were significantly higher than in the intact female rats which demonstrates that lipid peroxidation processes are intensified as a result of exposure to immobilization and pain (Table 1). The administration of glufimet caused a considerable decrease in conjugated dienes, diketones and MDA in the studied organs of the stressed rats as compared to the negative control animals, with or without iNOS inhibition. Rats receiving phenibut showed less pronounced decrease in the level of LPO products in the heart and a more noticeable decrease in the brain as compared to the animals receiving glufimet. The concentrations of LPO products were significantly lower in the rats following inhibition of iNOS in contrast to the rats subjected to stress.

Table 1 Changes in the level of LPO products and antioxidant enzyme activity in the heart and brain mitochondria of intact and stressed rats (M± σ)

Animal groups	Organ	CD, D_{233}/mg protein	Diketones, D_{278}/mg protein	MDA, mM/mg protein	SOD, IU/mg protein	GlP, mM GSH/min/ mg protein	Catalase, mg H_2O_2/min/ mg protein
Intact (n=8)	Heart	2.78 ± 0.22	0.75 ± 0.05	7.1 ± 1.3	51.3 ± 6.7	51.6 ± 9.0	13.0 ± 2.0
	Brain	2.10 ± 0.18	0.43 ± 0.04	12.6 ± 1.7	22.6 ± 4.9	16.2 ± 2.6	8.1 ± 1.2
Stress + Saline solution (n=8)	Heart	3.14 ± 0.18* (12.9%)	0.81 ± 0.05* (8.0%)	9.3 ± 0.8* (31.0%)	34.6 ± 5.3* (-32.5%)	29.0 ± 4.2* (-43.8%)	9.0 ± 0.7* (-30.8%)
	Brain	2.48 ± 0.23* (18.1%)	0.51 ± 0.06* (18.6%)	17.3 ± 1.1* (37.3%)	19.9 ± 3.0 (-11.9%)	12.3 ± 1.9* (-24.1%)	8.2 ± 2.1 (1.2%)
Stress + glufimet (n=8)	Heart	2.51 ± 0.3# (-20.1%)	0.68 ± 0.05# (-16.0%)	7.9 ± 0.8# (-15.0%)	39.6 ± 5.6 (14.4%)	34.5 ± 5.5 (19.0%)	13.1 ± 1.0# (45.5%)
	Brain	2.05 ± 0.23# (-17.3%)	0.44 ± 0.04# (-13.7%)	12.7 ± 2.2# (-26.6%)	22.5 ± 3.8 (13.1%)	15.6 ± 3.5 (26.8%)	8.8 ± 1.6 (7.3%)
Stress + phenibut (n=8)	Heart	2.85 ± 0.12# (-9.2%)	0.73 ± 0.07# (-9.9%)	8.3 ± 0.5 (-10.7%)	51.4 ± 6.2# (48.5%)	54.0 ± 7.1# (86.0%)	12.5 ± 1.7# (38.9%)
	Brain	1.67 ± 0.22# (-32.3%)	0.39 ± 0.06# (-23.5%)	11.1 ± 2.5# (-35.8%)	28.3 ± 5.2# (42.2%)	18.0 ± 3.1# (46.3%)	8.6 ± 1.6 (4.9%)
Stress + aminoguanidine (n=8)	Heart	3.13 ± 0.25 (-0.3%)	0.77 ± 0.03 (-4.9%)	8.5 ± 1.0 (-8.3%)	44.7 ± 3.5# (29.2%)	39.9 ± 7.6# (37.6%)	11.9 ± 1.2# (32.2%)
	Brain	1.73 ± 0.19# (-30.2%)	0.42 ± 0.04# (-17.6%)	14.9 ± 1.4 (-13.9%)	27.1 ± 3.8# (36.2%)	16.2 ± 3.1 (31.7%)	8.0 ± 1.9 (-2.4%)
Stress + aminoguanidine + glufimet (n=8)	Heart	2.99 ± 0.11 (-4.5%)	0.71 ± 0.05# (-7.8%)	8.5 ± 0.8 (0.0%)	39.6 ± 6.9 (-11.4%)	42.5 ± 6.2# (6.5%)	10.7 ± 0.8# (-10.1%)
	Brain	1.69 ± 0.29# (-2.3%)	0.39 ± 0.03# (-7.1%)	11.4 ± 2.1#^ (-23.5%)	29.9 ± 4.5# (10.3%)	18.0 ± 4.3# (11.1%)	7.4 ± 1.0 (-7.5%)
Stress + aminoguanidine + phenibut (n=8)	Heart	3.01 ± 0.12 (-3.8%)	0.72 ± 0.05# (-6.5%)	7.4 ± 0.8# (-12.9%)	44.8 ± 6.7# (0.2%)	34.6 ± 6.8 (-13.3%)	9.3 ± 1.6^ (-21.8%)
	Brain	1.95 ± 0.17# (12.7%)	0.44 ± 0.04# (4.8%)	15.5 ± 1.4 (4.0%)	25.4 ± 4.4 (-6.3%)	15.9 ± 3.0 (-1.8%)	8.0 ± 1.3 (0.0%)

The table shows the percentage of changes in the indicators.

- in the group of stressed animals in relation to the intact group rats;
- in the groups of animals which received glufimet, phenibut, aminoguanidine in relation to the group of stressed rats;
- in the groups of animals which received glufimet and phenibut when iNOS was blocked in relation to the group of rats which received aminoguanidine.

* - the changes are significant in relation to the intact group animals (Student's t-test, $p \leq 0.05$);
\# - the changes are significant in relation to the control group of stressed female rats (Newman-Keuls test, $p \leq 0.05$);
^ - the changes are significant in relation to the group of stressed animals receiving aminoguanidine (Newman-Keuls test, $p \leq 0.05$).

Excessive NO production and its conversion to peroxynitrite can account for these changes (Table 1).

The data of the present study also showed that immobilization and pain stress resulted in decreased activity of antioxidant enzymes compared to controls. The activity of SOD, GlP and catalase in the heart and brain of the animals receiving aminoguanidine was significantly higher than in the stressed animals of the control group. The rats which received phenibut showed considerable differences in these indices as compared to the stressed animals (Table 1).

Respiratory Function of Heart and Brain Mitochondria

In the rats exposed to immobilization and pain, enhanced lipid peroxidation intensity caused damage to cardiac and cerebral mitochondria which was manifested as a rise in oxygen uptake in the V_4 condition described by Chance (The classic oxygen electrode experiments to determine mitochondrial bioenergetic function were developed by Chance and Williams) [36] (by 28.8% and 32.5%, respectively, when malate was used as a substrate); it increased by 37.4% and 18.2%, respectively, when succinate was added, as compared to the intact animals ($p \leq 0.05$) (Figure2).

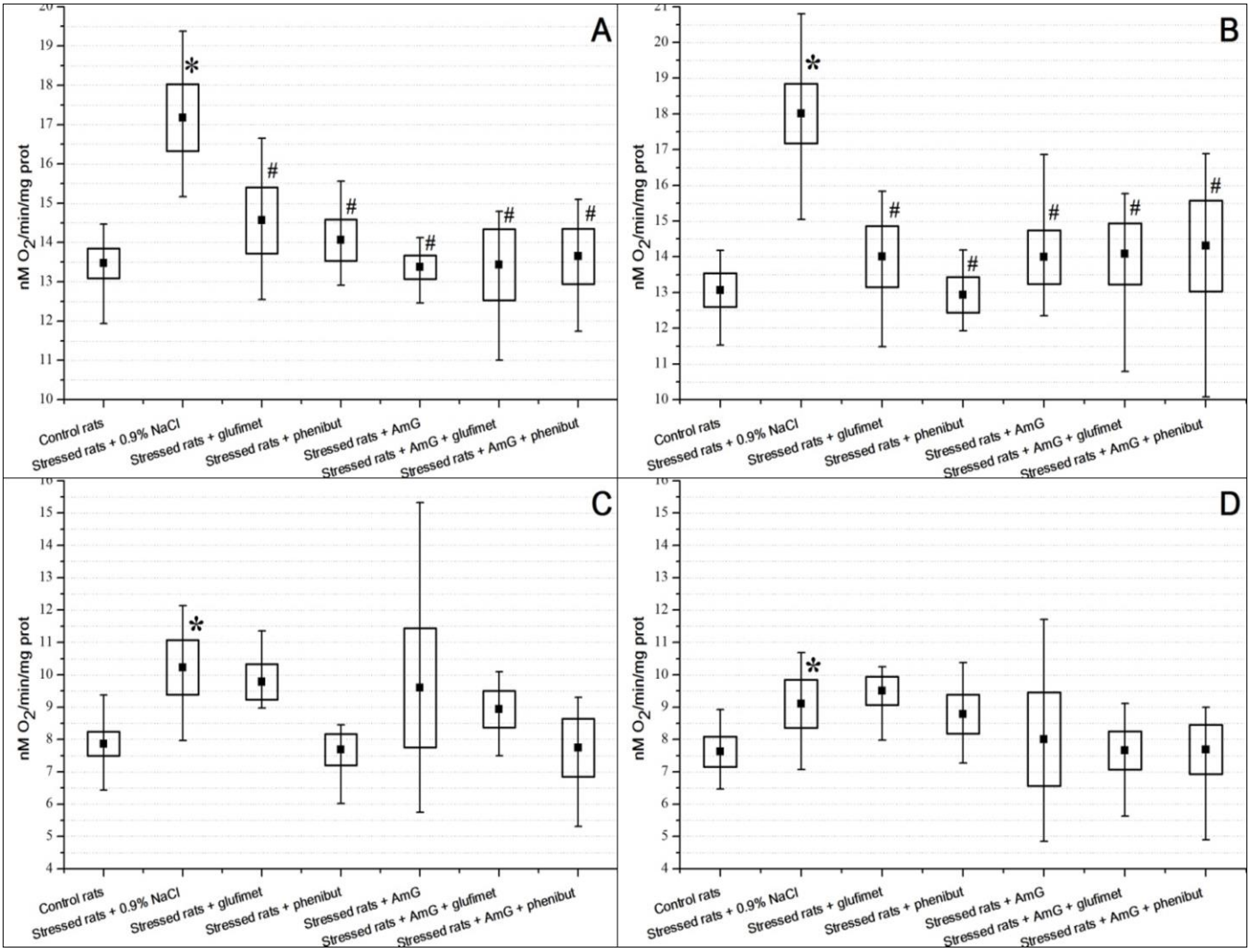

Figure 2 Non-stimulated rate of oxygen consumption (V_4) in heart and brain mitochondria of intact rats (control group); stressed rats treated with saline (negative control), stressed rats treated with glufimet, phenibut; and aminoguanidine plus glufimet and phenibut. (n = 8; mean ± SD).
The figure demonstrates the mean values of the baseline oxygen consumption rate indices together with standard variation limits and maximum and minimum value limits expressed in nmol O_2/min/mg protein for the V_4 condition (according to B. Chance). The measurements were made when malate/glutamate (A, C) and succinate (B, D) were used as substrates for respiratory chain in heart (A, B) and brain (C, D) mitochondria.
The changes are statistically significant according to the Newman-Keuls test, $p \leq 0.05$:
* - in relation to the group of intact animals;
# - in relation to the stressed animals of the control group.

In the rats treated with glufimet, malate-dependent rate of oxygen consumption by heart mitochondria in the V_4 condition was 14.1% lower; and when succinate was used, it was 22.2% lower as compared to the stressed rats ($p \leq 0.05$). The administration of phenibut caused a 17.1% decrease in V_4 of heart mitochondria when malate was used as an oxidation substrate; and by 28.3% when succinate was used ($p \leq 0.05$). The inhibition of iNOS in the animals subjected to stress reduced non-stimulated respiration of heart mitochondria by 21.1% ($p \leq 0.05$) when malate was used and by 22.2% ($p \leq 0.05$) when succinate was used, respectively. The administration of glufimet and aminoguanidine without ADP (V_4), when both substrates (malate and succinate) were used in the stressed animals, caused a significant decrease in the rate of oxygen consumption in heart mitochondria as compared to V_4 of the stressed animals in the negative control group. A similar trend was observed in the stressed animals which received both phenibut and aminoguanidine (Figure 2). When respiration was stimulated by the addition of ADP into a polarographic cell (V_3 condition), the rate of oxygen consumption by heart mitochondria of the stressed animals was 17.1% lower than that of the intact rats with malate as a substrate; and it was 20.7% lower with succinate ($p \leq 0.05$)(Figure3).

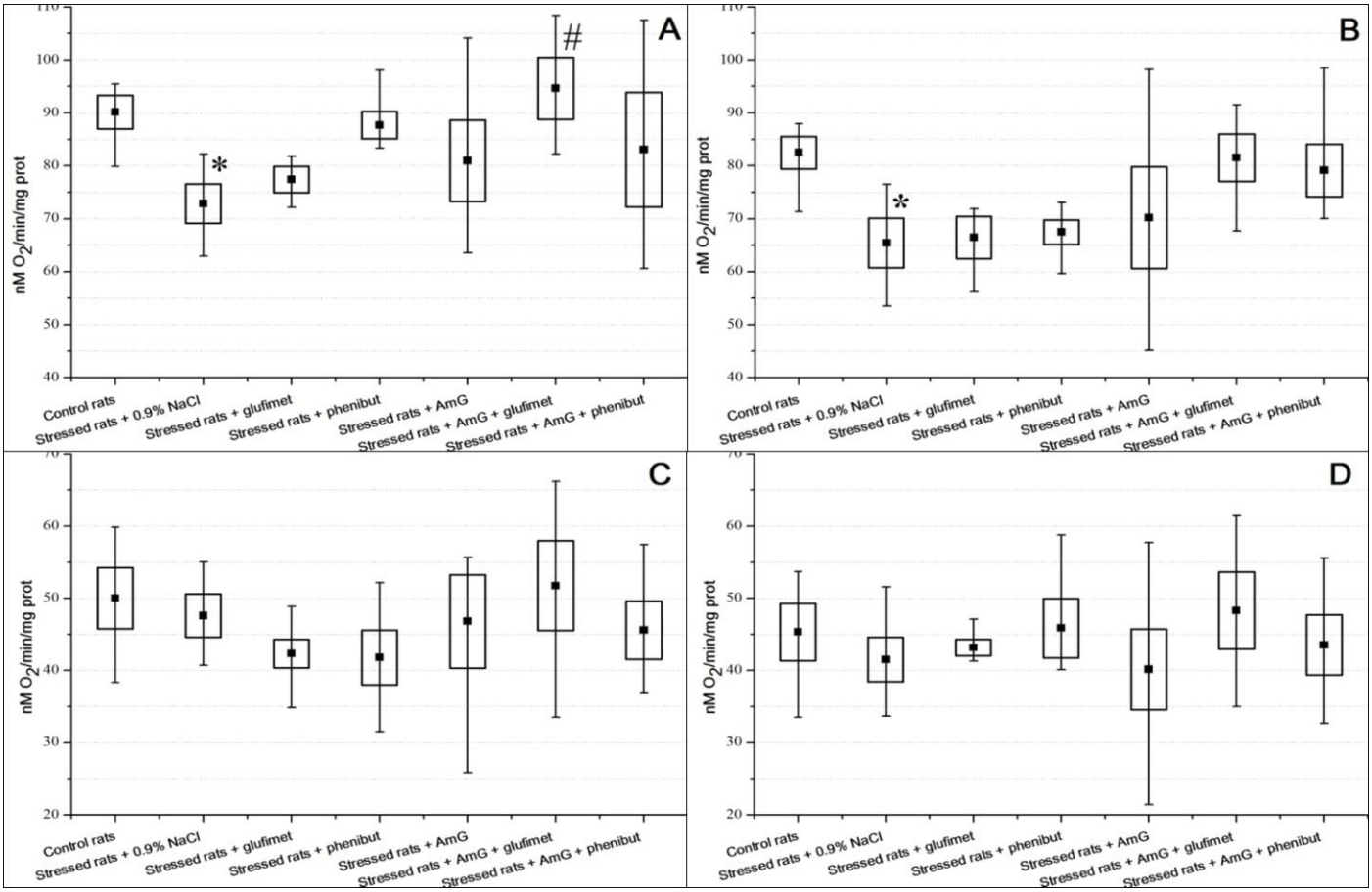

Figure 3 Rate of oxygen consumption in heart and brain mitochondria of intact rats (control group); stressed rats treated with saline (negative control), stressed rats treated with glufimet, phenibut and aminoguanidine plus glufimet and phenibut after ADP is added (V_3) (n = 8; mean ± SD).
The figure demonstrates the mean values of the baseline oxygen consumption rate indices together with standard variation limits and maximum and minimum value limits expressed in nmol O_2/min/mg protein for the V_3 condition (according to B. Chance). The measurements were made when malate/glutamate (A, C) and succinate (B, D) were used as substrates for respiratory chain in heart (A, B) and brain (C, D) mitochondria.
The changes are statistically significant according to the Newman-Keuls test, $p \leq 0.05$:
* - in relation to the group of intact animals;
# - in relation to the stressed animals of the control group.

The respiratory control ratio (V_3/V_4), which is the ratio of stimulated to non-stimulated respiration, significantly decreased in the heart and brain when malate and succinate were used as compared to the intact rats ($p \leq 0.05$) (Table 2). This index was significantly higher for heart mitochondria of all groups compared to negative control ($p \leq 0.05$). Rats treated with glufimet after iNOS inhibition had a significantly lower respiratory control ratio than those that only received aminoguanidine ($p \leq 0.05$). The brain mitochondria of the animals of the experimental groups did not show any significant differences in the parameters studied (Table 2).

Average Daily Arterial Blood Pressure

The level of average daily BP did not change significantly in the intact rats, but in the stressed animals of the control group, this index was 14.0% higher than the baseline level. In the stressed animals of other experimental groups, the average daily BP did not show any significant changes (Table 3).

Platelet Aggregation and Plasma-coagulation Hemostasis Indices

As is known, stress activates the sympathetic nervous system, increases release of catecholamines from the sympathetic nerve endings and adrenal medulla, enhances platelet aggregation and blood clotting ability which may bring about thromboembolic complications. This study has also demonstrated that exposure to stress causes a significant increase in platelet aggregation, a decline in APTT and PT, and a rise in fibrinogen concentration in comparison with the intact animals ($p \leq 0.05$) (Table 4). Rats receiving glufimet did not show any rise in platelet aggregation. Increased APTT and decreased fibrinogen concentrations in the plasma were registered as compared to the stressed rats ($p \leq 0.05$).

A similar trend was registered in the animals which received phenibut. The inhibition of inducible NO-synthase prevented a rise in platelet aggregation level. The compounds under study had no effect on platelet aggregation when inducible NO-synthase was inhibited. APPT, prothrombin time and fibrinogen concentration in the female rats of this group and those receiving aminoguanidine, glufimet and phenibut did not show any significant differences from the stressed rats(Table4).

Table 2 Changes in the respiratory control ratio in heart and brain mitochondria in the intact and stressed animals (M± σ)

Animal groups	Heart		Brain	
	Malate / glutamate	Succinate	Malate / glutamate	Succinate
Control rats (n=8)	6.7 ± 0.9	6.4 ± 0.7	6.4 ± 1.2	5.9 ± 1.1
Stressed rats + 0.9% NaCl (n=8)	4.3 ± 0.6* (- 35.8%)	3.6 ± 0.6* (- 43.7%)	4.8 ± 1.0* (- 25.0%)	4.7 ± 0.9* (-20.3%)
Stressed rats + glufimet (n=8)	5.3 ± 0.3# (23.3%)	4.8 ± 0.9# (33.3%)	4.9 ± 0.5 (2.1%)	4.8 ± 0.5 (2.1%)
Stressed rats + phenibut (n=8)	6.2 ± 0.5# (44.2%)	5.2 ± 0.5# (44.4%)	5.3 ± 0.7 (10.4%)	5.3 ± 0.9 (12.8%)
Stressed rats + AmG (n=8)	6.1 ± 0.8# (41.9%)	5.1 ± 1.3# (41.7%)	5.2 ± 0.7 (8.3%)	5.4 ± 0.7 (14.9%)
Stressed rats + AmG + glufimet (n=8)	7.1 ± 0.4# (16.4%)	5.9 ± 1.2 (15.7%)	5.9 ± 0.7# (13.5%)	6.3 ± 0.7 (16.7%)
Stressed rats + AmG + phenibut (n=8)	6.1 ± 0.5# (0.0%)	5.5 ± 0.6# (7.8%)	5.9 ± 0.5# (13.5%)	5.6 ± 1.0 (3.7%)

Note: AmG – aminoguanidine
The table shows the percentage of changes in the indicators.

- in the group of stressed animals in relation to the intact group rats;
- in the groups of animals which received glufimet, phenibut, aminoguanidine in relation to the group of stressed rats;
- in the groups of animals which received glufimet and phenibut when iNOS was blocked in relation to the group of rats which received aminoguanidine.

* - the changes are significant in relation to the intact group animals (Student's t-test, $p \leq 0.05$);
# - the changes are significant in relation to the control group of stressed female rats (Newman-Keuls test, $p \leq 0.05$);
^ - the changes are significant in relation to the group of stressed animals receiving aminoguanidine (Newman-Keuls test, $p \leq 0.05$).

Table 3 Changes in the increase in average daily BP in the intact and stressed animals (M± σ)

Animal groups	Baseline average daily BP	Average daily BP after stress	% of increase
Control rats (n=8)	100.4 ± 3.6	99.8 ± 2.9	— 0.6 ± 4.7
Stressed rats + 0.9% NaCl (n=8)	101.7 ± 3.2	120.4 ± 3.9	18.4 ± 4.4*
Stressed rats + glufimet (n=8)	103.1 ±2.9	103.2 ± 3.1	0.1 ± 3.6#
Stressed rats + phenibut (n=8)	102.8 ± 3.0	102.1 ± 2.4	— 0.7 ± 4.4#
Stressed rats + AmG (n=8)	103.7 ± 6.8	101.0 ± 12.7	1.1 ± 5.8#
Stressed rats + AmG + glufimet (n=8)	104.5 ± 2.8	101.3 ± 7.4	— 3.1 ± 5.9#
Stressed rats + AmG + phenibut (n=8)	103.3 ± 5.7	99.3 ± 8.3	— 3.8 ± 6.5#

Note: AmG – aminoguanidine
* - the changes are significant in relation to the intact group animals (Student's t-test, $p \leq 0.05$);
# - the changes are significant in relation to the control group of stressed female rats (Newman-Keuls test, $p \leq 0.05$).

Table 4 Changes in platelet aggregation degree and hemostasis indices in the intact and stressed animals (M± σ)

Animal groups	Studied index			
	Platelet aggregation degree, IU	APTT, s	Prothrombin time, s	Fibrinogen, g/l
Control rats (n=8)	25.0 ± 2.5	16.3 ± 0.9	23.2 ± 1.9	3.3 ± 0.3
Stressed rats + 0.9% NaCl (n=8)	31.1 ± 3.5* (24.4%)	13.1 ± 0.5* (-19.6%)	18.6 ± 0.9* (-19.8%)	5.3 ± 0.2* (60.6%)
Stressed rats + glufimet (n=8)	23.6 ± 3.2# (-24.1%)	13.9 ± 0.3# (6.1%)	18.2 ± 0.9 (-2.1%)	4.6 ± 0.3# (-13.2%)
Stressed rats + phenibut (n=8)	23.7 ± 2.5# (-23.8%)	14.2 ± 0.7# (8.4%)	19.7 ± 1.0 (5.9%)	4.8 ± 0.4 (-9.4%)
Stressed rats + AmG (n=8)	24.5 ± 2.8# (-21.2%)	12.9 ± 0.8 (-1.5%)	18.2 ± 1.6 (-2.1%)	4.9 ± 0.2 (-7.5%)
Stressed rats + AmG + glufimet (n=8)	22.3 ± 2.6# (-9.0%)	13.6 ± 0.7 (5.4%)	19.2 ± 2.9 (5.5%)	5.0 ± 0.6 (2.0%)
Stressed rats + AmG + phenibut (n=8)	23.7 ± 1.6# (-3.3%)	14.0 ± 0.5#^ (8.5%)	18.1 ± 1.4 (-0.5%)	4.8 ± 0.4 (-2.0%)

Note: AmG – aminoguanidine
The table shows the percentage of changes in the indices.

- in the group of stressed animals in relation to the intact group rats;
- in the groups of animals which received glufimet, phenibut, aminoguanidine in relation to the group of stressed rats;
- in the groups of animals which received glufimet and phenibut when iNOS was blocked in relation to the group of rats which received aminoguanidine.

* - the changes are significant in relation to the intact group animals (Student's t-test, $p \leq 0.05$);
# - the changes are significant in relation to the control group of stressed female rats (Newman-Keuls test, $p \leq 0.05$);
& - the changes are significant in relation to the group of stressed animals receiving aminoguanidine (Newman-Keuls test, $p \leq 0.05$).

DISCUSSION

The present study showed that immobilization and pain stress brought about a rise in the concentration of nitric oxide metabolites which may have resulted from enhanced expression and activity of inducible NO synthase (iNOS). Moreover, the results show that the level of the primary and secondary LPO products increased in heart and brain mitochondria while antioxidant activity decreased. Concurrently, a rise in oxygen consumption by heart and brain mitochondria when ADP had not been added, and a decrease in stimulated respiration rate were observed which indicates that respiration and oxidative phosphorylation were uncoupled. This may be due to damage to respiratory complexes caused by reactive oxygen and nitrogen species. The resulting electron escape from the respiratory chain intensified LPO processes, caused further damage to mitochondria, provoked a decrease in ATP synthesis and energy deficiency in cells which led to cell dysfunction and death. Our study has demonstrated that there was a significant rise in BP and platelet aggregation level, a decrease in APTT and PT, and an increase in fibrinogen concentration in the plasma of the stressed female rats as compared to the intact rats. This fact is consistent with the recently published data and proves that endothelial vasodilating and antithrombogenic functions are depressed [37, 38].

Inhibition of iNOS normalized the mitochondrial functional condition and reduced the intensity of LPO processes. This effect is likely to result from decreased synthesis of NO by iNOS which is activated in stress [13]. Decreased reactive nitrogen species (RNS) caused a decline in the endothelial dysfunction severity, did not elevate the average daily BP and normalized platelet aggregation capacity. Plasma hemostasis indices did not show any significant differences as compared to the negative control rats.

Glufimet inhibited a rise in nitric oxide (NO) production when the animals were subjected to stress. When iNOS was inhibited, the concentration of terminal metabolites in the animals which received the compound did not differ significantly from the animals treated with aminoguanidine. A decrease in NO promoted closer coupling of substrate oxidation processes and ATP synthesis, mainly, in heart mitochondria which was manifested as an increase in respiratory control ratio in contrast to the stressed control groups which received saline solution and aminoguanidine. Decreased NO concentration is likely to reduce peroxynitrite formation, restrict enzyme damage and electron escape from the mitochondrial respiratory chain, and depress the intensity of LPO processes which may contribute to the normalization of endothelial function. This is implicated in the lack of average daily BP elevation episodes and a significant decrease in platelet aggregation level as compared to the stressed rats.

The chemical composition of glufimet includes gamma-aminobutyric and glutamic acids, phenibut and glycine (Figure 4) which makes it possible to assume that it has a multifactorial stress-protective action. This action may result from the modulation of GABA- and NO-ergic stress-limiting systems.

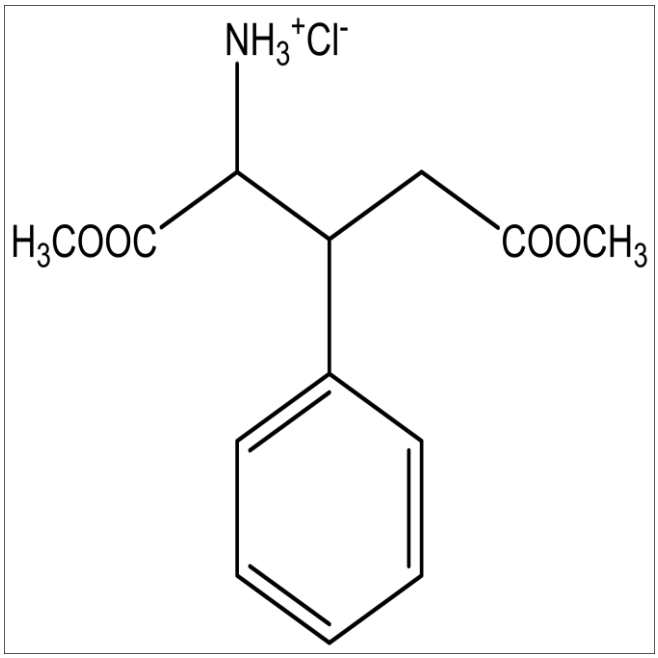

Figure 4 Dimethyl hydrochloride 3-phenylglutamic acid (glufimet)

Similar changes of the studied indices were registered in the animals which received phenibut (Figure 5). The revealed stress-protective effects of this compound are likely to be due to the activation of GABA-ergic system as it is a GABA-mimetic agent [39]. Moreover, our previous studies have demonstrated that phenibut has a pronounced cardioprotective action in stress-related myocardial

damage which is implicated in a significant increase in myocardial contraction and relaxation rate, left ventricular pressure, heart rate and maximum intensity of structural functions in response to a volume loading test (increased preload), adrenoreactivity test, and maximal isometric leading test (increased afterload). A significantly smaller increase in the above-mentioned indices in the stressed animals receiving phenibut was registered following the inhibition of non-selective NOS L-NAME which may indicate that the NO-ergic stress-limiting system is involved in restricting the harmful effect of stress on the heart [40].

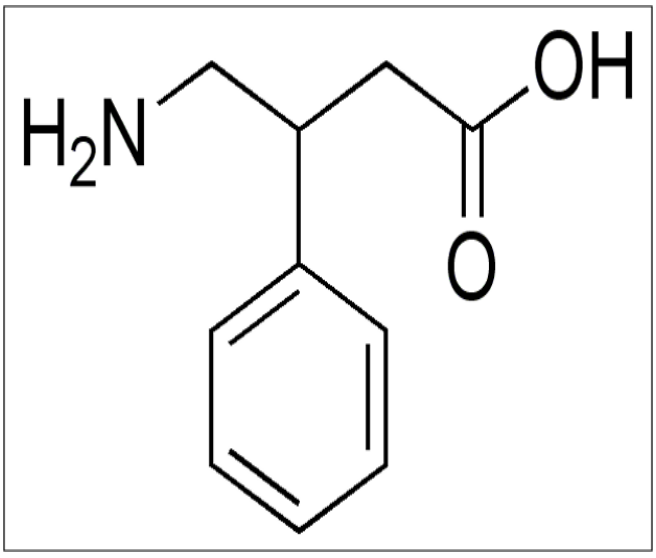

Figure 5 γ-amino-β-phenylbutyric acid (phenibut)

When aminoguanidine, an iNOS inhibitor, was administered, the studied compounds tended to inhibit the intensity of stress reactions which was manifested as reduced concentrations of LPO primary and secondary products, enhanced activity of antioxidant enzymes and respiratory control ratio. It was also implicated in improved coupling of respiration and phosphorylation in heart and brain mitochondria, and lower severity of endothelial dysfunction. The findings make it possible to assume that glufimet and phenibut stimulate neuronal NOS, whose activity decreases when exposed to stress [41]. This leads to production of baseline NO required for the NO-ergic system to exert its stress-protective effects.

CONCLUSION

The study demonstrated that GABA and glutamic acid derivatives restrict the harmful effects of stress on the heart and brain and that the stress protective effects may involve modulation of iNOS activity.

Conflict of Interest

Authors declare none.

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