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Stimulation of mitochondrial hydrogen sulfide and glutathione production improves Frank-Starling response of the rat heart via NO-dependent pathway

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Complete List of Authors:	Goshovska, Yulia ; Bogomoletz Institute of Physiology National Academy of Sciences of Ukraine, Department of Blood Circulation Fedichkina, Raisa; Bogomoletz Institute of Physiology National Academy of Sciences of Ukraine, Circulation Korkach, Iulia; Bogomoletz Institute of Physiology National Academy of Sciences of Ukraine, Department of Blood Circulation Sagach, Vadim; Bogomoletz Institute of Physiology National Academy of Sciences of Ukraine, Department of Blood Circulation
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1 **Stimulation of mitochondrial hydrogen sulfide and glutathione production improves Frank-**
2 **Starling response of the rat heart via NO-dependent pathway**

3 Yulia V. Goshovska, Raisa A. Fedichkina*, Yulia P. Korkach, Vadym F. Sagach
4 Department of Blood Circulation, Bogomoletz Institute of Physiology, National Academy of Sciences of
5 Ukraine, Kyiv, Ukraine, +380442562000 (fax)

6 *Corresponding author: +380972563471 (tel), dobrovolska.raisa@gmail.com

7 ORCID: Yulia Goshovska <https://orcid.org/0000-0003-3308-9091>

8 Raisa Fedichkina <https://orcid.org/0000-0002-9051-427X>

9 Yulia Korkach <https://orcid.org/0000-0002-4697-2150>

10 Vadym Sagach <https://orcid.org/0000-0003-3801-6019>

11

12 **Abstract**

13 The Frank-Starling response of the heart is known to be mediated by NO signaling, which is regulated by
14 GSH and H₂S. We hypothesized that stimulation of endogenous H₂S or GSH synthesis improve Frank-
15 Starling response . Wistar male rats were injected with propargylglycine (PAG, 11.3mg/kg⁻¹,
16 40min,n=12), an inhibitor of H₂S producing enzyme (cystationine-γ-lyase) and L-cysteine (121mg/kg⁻¹,
17 30min,n=20), a precursor of H₂S and GSH. Pretreatment with PAG or L-cysteine separately slightly
18 improved P-V dependence of the isolated rat heart, but combination of PAG and L-cysteine (n=12)
19 improved heart contractile activity. H₂S content, cNOS activity, nitrate reductase activity and nitrite
20 content increased by 2, 3.83, 2.5 and 1.3 times in cardiac mitochondria as well as GSH and GSSG levels
21 increased by 2.24 and 1.86 times in heart homogenates of PAG+L-cysteine group comparing to control
22 (all P<0.05). Inhibition of glutathione with DL-buthionine-sulfoximine (22.2mg/kg⁻¹, 40min, n=6)
23 drastically decreased Frank-Starling response of the heart and prevented PAG+L-cysteine induced
24 increase of GSH and GSSG levels (BSO+PAG+L-cysteine,n=9). Inhibitor of NOS, N-nitro-L-arginine-
25 methylester hydrochloride (40min, 27mg/kg⁻¹), abolished positive inotropy induced by PAG+L-cysteine
26 pretreatment (L-NAME+PAG+L-cysteine,n=7). Thus, PAG+L-cysteine administration improves Frank-

27 Starling response with up-regulation of mitochondrial H₂S, glutathione and NO synthesis that might be
28 promising approach in treatment of myocardial dysfunction.

29 INTRODUCTION

30 Frank-Starling law is known as an ability of the myocardium to adjust force of contraction due to
31 increased ventricular filling and stretching of walls. That provides energy-saving mode of the heart
32 pumping function, coupling of venous return of blood with cardiac output and its changes under physical
33 activity. Fundamental studies revealed that the probability of crossbridge formation and myofilament
34 calcium affinity are the molecular basis of the Frank-Starling mechanisms (ALLEN and KENTISH, 1985;
35 Fabiato and Fabiato, 1975; Shiels and White, 2008). Prendergast et al showed that gas transmitter nitric
36 oxide (NO) is an important mediator of the Frank-Starling law: NO has ability to reduce the diastolic
37 rigidity and improve diastolic and systolic function of the heart (Prendergast et al., 1997). Later, NO-
38 guanylate cyclase-protein kinase G (NO-GC-PKG) pathway was considered as the main regulator of
39 intracellular calcium metabolism in the Frank-Starling mechanism (BELGE et al., 2005; Casadei and
40 Sears, 2003; Scotcher et al., 2016). Reduced NO production might be the main reason of deteriorated
41 pressure-volume (P-V) regulation in arterial hypertension, Parkinson's disease or other pathological
42 conditions, which also indicates the involvement of the NO system in the heterometric regulation of the
43 heart function (Dorofeieva et al., 2012; Talanov et al., 2009). At the same time, stimulation of NO
44 synthesis by catestatin protein increase Frank-Starling response not only in hypertensive but also in
45 normotensive rat heart (Angelone et al., 2015). Moreover, NO mediates adaptation to physical training:
46 isolated heart of trained rats demonstrated improved response to volume load, whereas L-NAME (N-
47 nitro-L-arginine methyl ester hydrochloride), an inhibitor of NO synthases, completely abolished the
48 positive effect of physical training (Shymans'ka et al., 2010). Thus, endogenous NO plays an exceptional
49 role in adaptation of myocardium to increased volume loads.

50 Recently, Altaany et al showed that NO production is greatly regulated by another gas transmitter
51 hydrogen sulfide (H₂S). The commonly used H₂S donor NaHS stimulated the phosphorylation of
52 endothelial NO synthase (eNOS) and enhanced NO (Altaany et al., 2013). H₂S synthesis is significantly

deteriorated in aging, hypertension, coronary heart disease (Jiang et al., 2005; Mys et al., 2020; Yan et al., 2004), which may be the cause of down regulated NO production, inefficacy of the NO-GC-PKG pathway and impaired heart function under these pathological conditions. Previously we showed that NaHS greatly increased the Frank-Starling response (Shymans'ka et al., 2012). However, the data concerning the role of endogenously produced H₂S in regulation of P-V dependence of the heart are lacking.

In cardiovascular system, H₂S is produced by cystathionin gamma-lyase (CSE) enzyme which uses amino acid L-cysteine as a substrate and the 3-mercaptopyruvate sulfurtransferase (3-MPST) localized mainly in mitochondria (Kamoun, 2004). Alternatively, L-cysteine can be used in other metabolic pathways. Glutamate cysteine ligase (GCL) synthesized γ -glutamylcysteine from L-cysteine and L-glutamate as a first step in glutathione synthesis. A tripeptide glutathione possess powerful antioxidant potential and ability to influence the proteins' function by the S-glutathionylation posttranslational modification (Mailloux and Treberg, 2016). It is known that overproduction of NO and reactive nitrosative species accelerates the S-glutathionylation (Xiong et al., 2011). Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Mieyal et al., 2008) and ryanodine receptors (Aracena-Parks et al., 2006) are considered to be activated by S-glutathionylation. Under oxidative stress, S-glutathionylation of eNOS leads to its uncoupling and an increase in production of superoxide anion radical instead of NO (Chen et al., 2010). In addition, S-glutathionylation of complex I of the respiratory chain increases ROS production. The superoxide rapidly transforms into peroxide and diffuses into the cytoplasm suggesting possible mechanism for the mitochondria redox signaling function. It is believed that mitochondrial uncoupling proteins UCP2 and UCP3 are S-glutathionylated in normal conditions. As soon as ROS production becomes massive, UCPs undergo de-S-glutathionylation and catalyze the proton leak decreasing proton gradient across the inner mitochondrial membrane and ROS production (Mailloux and Harper, 2011). Thus, glutathione acts as regulator of intracellular calcium concentration, membrane potential and muscle relaxation. All above mentioned processes point to the tight relationships between gas transmitters and glutathione in regulation of myocardial diastolic function.

79 The aim of this study was to evaluate the role of endogenous H₂S, glutathione and their interplay with NO
80 in the Frank-Starling response of the heart.

81

82 **MATERIAL AND METHODS**

83 **Animals and Drugs**

84 All experiments were carried out in compliance with the Directive of the Parliament of Ukraine
85 №3447-IV "On the protection of Animals from Cruel Treatment" (2006) and Directive 2010/63/EU of the
86 European Parliament and of the Council on the protection of animals used for scientific purposes
87 (22.09.2010). Animals were treated in accordance with the Canadian Council on Animal Care. The study
88 was approved by the Institutional Committee for Biomedical Research Ethics (protocol 4/20 from
89 22.05.20). Male Wistar rats were kept on a natural day-to-night cycle with free access to food and water.

90 The following groups were used: 1) control (n=21); 2) L-cysteine (n=20); 3) DL-propargylglycine
91 (PAG, n=12); 4) PAG + L-cysteine (n=12); 5) DL-buthionine-sulfoximine (BSO, n=6); 6) BSO + PAG +
92 L-cysteine (n=9); 7) L-NAME + PAG + L-cysteine (n=7).

93 All drugs were dissolved in 0.5 ml of water for injection and administered intraperitoneally. CSE
94 inhibitor PAG ("Sigma", USA) was administered 40 minutes before sacrificing in a dose of 11.3 mg·kg⁻¹.
95 L-cysteine ("Sigma", USA) was administered 30 minutes before sacrificing in a dose of 120 mg·kg⁻¹.
96 Glutathione inhibitor BSO ("Sigma", USA) was injected 40 minutes before sacrificing in a dose of 22.2
97 mg·kg⁻¹. NO inhibitor N-nitro-L-arginine methyl ester hydrochloride (L-NAME, "Sigma", USA) was
98 administered 40 minutes before sacrificing in a dose of 27 mg·kg⁻¹.

99 **Cardiodynamics of isolated rat heart**

100 Rats were anesthetized with intraperitoneal injection of 1,5 g·kg⁻¹ urethane. After thoracic surgery, hearts
101 were isolated, placed in ice-cold buffered Krebs-Henseleit solution and immediately connected to
102 Langendorff apparatus via aorta. Coronary vessels of isolated rat hearts were perfused at a constant
103 perfusion pressure of 80 mmHg with non-recirculating Krebs-Henseleit solution (in mmol L⁻¹): NaCl 118;
104 KCl 4.7; MgSO₄ 1.2; NaHCO₃ 24; KH₂PO₄ 1.2; glucose 10.0; CaCl₂ - 2.5, pH 7.4, 37°C. The perfusion

solution was constantly aerated by gas mixture 95% O₂ and 5% CO₂. The left ventricular developed pressure (LVDP), end-diastolic pressure (EDP) and the heart rate were measured using a latex balloon placed into the left ventricle and connected to the pressure transducer ("Mingograf", Elema, Sweden). Cardiodynamic parameters were recorded and analysed using Global Lab software.

The Frank-Starling response of isolated rat heart

At the beginning of experiment, the intraventricular balloon was stretched to establish end-diastolic pressure of 5-7 mmHg for evaluation of LVDP and coronary flow. Hearts with LVDP lesser than 70mmHg were not included into research. After 15-20 min of steady state, the volume of the balloon was decreased to 0 mmHg and the P-V dependence curve was generated by increasing the volume of the intraventricular balloon in several steps 34 µL each. The number of steps depended on functional reserves of the heart in every group and averaged 7. Cardiac function was assessed within 1 min from each volume increment, corresponding to the setting of a steady state. The complete assessment of the Frank-Starling response was accomplished within 10 min (Shymans'ka et al., 2010).

Glutathione assay

The measurements of oxidized (GSSG) and reduced glutathione (GSH) were performed in the heart homogenates with Ellman's reagent (Rahman et al., 2006). Briefly, hearts were washed with cold (+ 4°C) 0.9% KCl solution, weighed and homogenized in isolation buffer based on 0.1 M potassium phosphate buffer (KPE) with the addition of 5 mM EDTA (EDTA sodium salt) at pH = 7.5, 0.1% Triton X-100 and 0.6% sulfosalicylic acid. The ratio of isolation buffer and tissue was 1:9. The homogenized tissue was centrifuged (Allegra X-22R, Beckman Coulter, USA) at +4°C, 8,000g for 10 min. Next, the supernatant was leached into clean microtubes. An aliquot of supernatant for GSH measurement was immediately freezed (-20°C). For GSSG measurement, 1 ml of supernatant was mixed with 30 µl of 97% 2-vinylpyridine in KPE (1:10), stirred, and 60 µl of 98% triethanolamine in KPE (1:6) was added after an hour, stirred again and freezed. Measurements were performed using a Biosan HiPo MPP-96 microplate reader (Lithuania). 60 µl of 500 units glutathione reductase in KPE solution (1:150), 60 µl 0.8 mM

cofactor β - NADPH and 60 μ l 1.68mM dithiobisnitrobenzoic acid were added to initiate the reaction. Optical density was measured immediately and for 2 minutes each 30 seconds at 405 nm. The concentrations of GSH and GSSG were calculated according to linear regression equation obtained from the calibration curve of the standard GSH and GSSG solutions.

Isolation of cardiac mitochondria

Mitochondria isolation from heart tissue was performed using differential centrifugation (Dorofeyeva et al., 2012). Isolated hearts were washed with cold 0.9% KCl, chopped in 10 mL of isolation buffer (in mM: sucrose – 250, Tris-HCl – 25, EDTA – 1, pH 7.35) and minced with dounce homogenizer. Then, homogenates were centrifuged at 700g for 8 min (Allegra X-22R, Beckman Coulter, USA, 4°C). The supernatant was centrifuged at 11,000g for 16 min. The pellet (mitochondrial fraction) was resuspended in 5 mL of isolation buffer and centrifuged again at 11,000g for 16 min. The final pellet was resuspended in 1 mL of isolation buffer and used for H₂S and NO metabolites measurements. Mitochondrial protein content was determined by the Lowry method using bovine serum albumin as standard.

H₂S assay

The content of H₂S was determined by method described by Yan et al (Yan et al., 2004). , cardiac tissue was homogenized in a 10-fold volume (w/v) of 50 mM ice-cold potassium phosphate buffer (pH 6.8). 0.5 mL of 1% zinc acetate was added to the aliquot of the samples and incubated at 37.5 °C for 10 minutes. Then, 0.5 mL of 20 mM of N,N-dimethyl-p-phenylenediamine dihydrochloride in 7.2 M HCl and 0.5 mL of 30 mM FeCl₃ in 1.2 M HCl were added. After the incubation in the cold and dark for 10 min, 0.5 ml 10% trichloroacetic acid was added to the reaction mixture prior to the addition of 2.5 ml distilled water. Then, the optical density was measured at 670 nm. The H₂S concentration in the solution was determined by calibration curve of the standard NaHS solutions. The H₂S content was expressed in nmol·mg⁻¹ of protein.

NOS assay

The method for cNOS and iNOS activity was based on the ability of NOS to utilize the L-arginine as substrate with the production of L-citrulline (Chin et al., 1999; Salter et al., 1991). The sample aliquots containing 500-1000 mg of protein were mixed with reaction mixture (final concentrations: 1 mM NADPH, 2 mM L-arginine and 50mM KH_2PO_4 , 1 mM MgCl_2 , 2 mM CaCl_2 and incubated at 37°C for 60 min to determine the levels of summary NOS activity. Duplicate samples were incubated with addition of EGTA (5 mM) instead of CaCl_2 to determine the levels of Ca^{2+} -independent iNOS activity. Reaction was stopped by adding 300 μL of 2N HClO_4 . Then, the mixture was centrifuged (3,500 g for 10 min) and 1 mL of the supernatant was collected for L-citrulline measurement by the methods which included addition of urease, chromogenic solution (diacetyl monoxime, FeCl_3 , thiosemicarbazide), boiling at 100°C for 10 min and measurement of optical density at 492 nm. The concentration of L-citrulline was found from the calibration curves. Ca^{2+} -dependent NOS activity (cNOS) was calculated as: NOS activity in 2 mM Ca^{2+} (total NOS activity) - NOS activity in 5mM EDTA (iNOS activity). The activity of enzymes was expressed as picomoles of newly formed L-citrulline for 1 min per 1 mg of protein in the sample.

Nitrite assay

Nitrite content was determined by the method of Green with the freshly prepared Griess reagent (Green et al., 1982). Griess reagent was prepared by mixing equal volumes of 0.1% water solution of *N*-(1-naphthyl) ethylenediamine dihydrochloride with 1% sulfanilamide in 5% H_3PO_4 just before use. An aliquot of sample was mixed with Griess reagent in a proportion 1:1. After 5 min of incubation at room temperature, optical density at 546 nm was measured. The concentration of nitrite was determined from calibration curve of the standard NaNO_2 solutions.

Nitrate assay

Nitrate content was measured by the method based on the reduction of NO_3^- to NO_2^- by zinc (Pfeiffer et al., 1997). For the determination of the summary content of $\text{NO}_2^- + \text{NO}_3^-$ 1.0 ml aliquots of the samples at

pH 7.4 were mixed with 50 μL of zinc suspension ($100 \text{ mg}\cdot\text{ml}^{-1}$ in water). After 5 min centrifugation, 0.5 ml of the supernatants were sampled and mixed with equal volume of the Griess reagent; next, optical density at 546 nm was measured. For the determination of nitrate content, that of nitrite, established in parallel missing the reduction procedure, was subtracted.

Nitrate reductase activity assay

The method for nitrate reductase activity was measured as described earlier (Jansson et al., 2008) with some modification. Incubation media was prepared on phosphate buffer (pH 7.4) and contained 100 μL of the sample, 0.5 mM glutathione (GSH), 1 mM NADH, 300 μM NaNO_3 as a substrate. The mixture was incubated at 37°C for 60 min and reaction was stopped by adding 300 μL of 2N HClO_4 , centrifuged at 3,500 g for 10 min. 0.5 ml of supernatant was collected for nitrite measurement as described in the nitrite assay above (Green et al., 1982).

Measurement of superoxide radical (O_2^-) generation rate

The O_2^- was measured by oxidation of cytochrome c in 10 mmol/L HCl-Tris buffer (pH 7.4, $t=37^\circ\text{C}$) recording changes of absorbance of the sample containing mixture at $\lambda=550 \text{ nm}$ during 30 min. The amount of generated O_2^- was expressed in nmol per mg of protein per min using a molar extinction coefficient $\epsilon = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Kuthan et al., 1982).

Measurement of diene conjugates

The content of diene conjugates was determined spectrophotometrically by absorbance at $\lambda=232 \text{ nm}$ in heptanoic extracts of samples (Gavrilov V B et al., 1988). The amount of diene conjugates was determined using molar absorption coefficient $21,000 \text{ mol}^{-1}\cdot\text{cm}^{-1}$.

Measurement of malonic dialdehyde content

208 The method for malonic dialdehyde (MDA) determination was based on reaction of MDA with 2'-
209 thiobarbituric acid and formation of trimetin complex as red derivative that was detected at 532 nm
210 (Mihara and Uchiyama, 1978). The amount of MDA was determined using molar absorption coefficient
211 15,600 mol⁻¹·cm⁻¹).

212

213 **Statistical analysis**

214 Normal distributions of data were assessed using the Shapiro–Wilk test. The significance of differences
215 between groups was assessed by Kruskal-Wallis ANOVA test for multiple independent groups followed
216 by Mann-Whitney U-test. STATISTICA7.0 and MS Excel soft were applied for statistical analysis. All
217 data were presented as Mean ± S.E.M. P values less than 0.05 were significant.

218

219 **RESULTS**

220 **Improved Frank-Starling response of myocardium under CSE inhibition**

221 To find out the role of endogenous H₂S in Frank-Starling response of the heart, rats were administrated
222 with PAG as inhibitor of CSE or L-cysteine as precursor of H₂S synthesis. LVDP was increased at 135
223 µL and 170 µL of intraventricular balloon (all P <0.05 vs. control) in PAG pretreated group (Figure 1A).
224 The further increase of the balloon volume showed no increment but decrease of LVDP in PAG group.
225 The L-cysteine pretreated group showed significant increase in LVDP at balloon volume of 170 µL, 200
226 µL and 235 µL (all P<0.01 vs. control). To reveal the effect of L-cysteine independent from H₂S
227 production, administration of PAG in combination with L-cysteine was studied. PAG+L-cysteine showed
228 pronounced positive inotropy (Figure 1B) such as significantly increased LVDP beginning with 70 µL of
229 extension. At balloon volume of 135 µL, LVDP was 154 ± 5.2 mmHg in PAG+L-cysteine group (133.6 ±
230 6.7 mmHg in control, P <0.01) and remain as high to 235 µL (127±4.7 mmHg in PAG group, P<0.05;
231 108±3.4 mmHg in control, P<0.001). No significant changes were observed for EDP curves between all
232 groups; however, hearts from PAG+L-cysteine group exhibited the lesser stiffness of myocardium as the
233 smallest increment of EDP during volume loading procedure. Thus, combination of PAG and L-cysteine

showed cumulative effect on contractile function increasing increment of LVDP at the beginning of the volume load procedure and extending the plateau of LVDP curve at the end point.

H₂S and NO production in cardiac mitochondria under CSE inhibition

Since we used PAG as inhibitor of H₂S produced by CSE, theoretically, the P-V dependence should be lower than in normal conditions because of decreased content of H₂S as a relaxant agent. However, we showed that PAG+L-cysteine administration caused improved contractility of the heart, so we hypothesized that L-cysteine was used by other H₂S producing enzyme, 3-MPST, located in mitochondria. Additionally, we studied parameters of NO synthesis *de novo* and by reduction mechanism in cardiac mitochondria. Our results showed that H₂S content increased twice in cardiac mitochondria in PAG and PAG+L-cysteine groups (Table 1), whereas there was only 25% increase in H₂S content in the L-cysteine group.

It is known that NO is metabolized to NO₂⁻ and then to NO₃⁻ as a stable metabolite of NO. The nitrites content was significantly increased in cardiac mitochondria of PAG and PAG+L-cysteine groups in 2.8 and 1.3 times respectively that is in agreement with significant induction of cNOS activity in 3.7 and 3.8 times and iNOS activity in 5.8 and 4.7 times in these groups. NADPH-dependent nitrate reductase restores NO₃⁻ to NO₂⁻. In L-cysteine group iNOS activity and reductase activity was increased by 2.8 and 2.1 times. Similar results observed in PAG and PAG+L-cysteine groups, the nitrate reductase activity was increased by 2.8 and 2.5 times respectively that may contribute to increased nitrite content. Although the nitrate content was not changed significantly in any of the groups. That indicates the stable level of peroxynitrite generation in normoxic conditions of our experiment. Additionally, O₂⁻ generation rate, MDA and DK levels were decreased in mitochondria of PAG+L-cysteine group (Table 2) indicating no membrane damage and oxidative stress under PAG+L-cysteine pretreatment.

Glutathion levels in heart tissue under CSE inhibition

259 Biochemical analysis of the heart homogenates showed that PAG+L-cysteine increased GSH and GSSG
260 levels in cardiac tissue in 2.24 and 1.86 times respectively ($P<0.05$ vs. control for both) (Figure 2). The
261 inhibitor of glutathione BSO prevented PAG+L-cysteine mediated increase of GSH and GSSG levels in
262 cardiac tissue.

263

264 **Impaired Frank-Starling response of the heart under glutathione inhibition**

265 To evaluate the role of glutathione in Frank-Starling response, rats were administrated with BSO, an
266 inhibitor of the first enzyme of glutathione synthesis. In BSO pretreated group, LVDP did not differ
267 significantly from the control values and averaged 142 ± 10.0 mmHg at 135 μ L of stretching (Figure 4).
268 However, EDP curve was sharply increased during volume load procedure: at 135 μ L of balloon volume,
269 EDP averaged 85.4 ± 6.0 mmHg (42.8 ± 4.9 mmHg in control, $P<0.01$) suggesting an impaired myocardial
270 capacity for relaxation.

271 Administration of PAG+L-cysteine on the background of BSO showed significant improvement of heart
272 function comparing to BSO group and remained the reaction in control group. These data support the idea
273 about improved elasticity of myocardium under PAG+L-cysteine treatment.

274

275 **PAG+L-cysteine improved Frank-Starling response of myocardium via NO-mediated pathway**

276 To evaluate the role of NO in PAG+L-cysteine effect, we used L-NAME as an inhibitor of NO synthesis.
277 L-NAME pretreatment considerably reduced PAG+L-cysteine effect that was observed at the first step of
278 volume load procedure (Figure 3). The maximal value of LVDP was detected at 135 μ L and averaged
279 127.6 ± 4.8 mmHg, however, it was significantly lower comparing to PAG+L-cysteine group ($P<0.01$).
280 Additionally, L-NAME increased myocardial stiffness: at the balloon volume of 235 μ L, EDP was
281 significantly higher in L-NAME+PAG+L-cysteine group (97 ± 0.5 mm Hg) than in the control (76 ± 4.5
282 mm Hg, $P < 0.05$) or in PAG + L-cysteine group (65 ± 3.1 mm Hg, $P < 0.01$). These data clearly indicate
283 participation of NO in PAG + L-cysteine induced positive inotropy.

284

DISCUSSION

The Frank-Starling law of the heart describes the dependence of the strength of myocardial contraction on the volume of blood flow into the heart. Increased volume of blood flow induces greater myocardium contraction during next systole. This physiological mechanism provides the necessary speed of blood circulation in the body under physical activity and stress. Growing evidence has demonstrated that gas transmitters NO and H₂S are inherent components of cardiovascular system regulatory cascades. Whereas the role of NO has been thoroughly studied for the last decades, the role of endogenous H₂S in heterometric regulation of the heart function needs to be investigated further. Decreased H₂S level in aortic tissue and plasma was found in aging, hypertension and coronary disease (Jiang et al., 2005; Mys et al., 2020; Yan et al., 2004). These states are characterized with decreased Frank-Starling response of the heart. Although exogenous H₂S donors like NaHS demonstrate beneficial effects on cardiovascular system including improvement of Frank-Starling response (Shymans'ka et al., 2012) the role of endogenous H₂S in regulation of Frank-Starling response of the heart remains unclear.

We found that, on the Langendorff perfused isolated rat heart, PAG+L-cysteine significantly improved functional reserves of myocardium and increased H₂S level in cardiac mitochondrial and increased glutathione level in cardiac tissue. We assumed that PAG inhibited CSE enzyme that made L-cysteine more available as a substrate for 3-MPST and GCL. Increased H₂S, in turn, could increase cNOS activity and NO content that greatly improved Frank-Starling response of the isolated rat heart. The importance of de novo glutathione and NO synthesis in PAG+L-cysteine induced positive inotropy was confirmed in experiments with BSO, as an inhibitor of glutathione, and L-NAME, as an inhibitor of NO production. BSO and L-NAME abrogated effect of PAG+L-cysteine pretreatment. Thus, our data suggest that up-regulated glutathione and mitochondrial H₂S synthesis improves Frank-Starling response of the heart at least in part via cNOS-dependent mechanism.

In this study, we used L-cysteine as a substrate for H₂S synthesis and PAG as an inhibitor of H₂S producing enzyme CSE. L-cysteine alone had no significant effect on P-V dependence of isolated rat heart (Figure 1). Pretreatment with both, PAG and L-cysteine, greatly improved Frank-Starling response

of the isolated heart as increased increment of LVDP in volume load procedure (Figure 1). This result was unexpected since PAG is widely used as an inhibitor of H₂S synthesis. On the other hand, PAG is an inhibitor of CSE which is a cytoplasmic source of H₂S in the cell. Another H₂S producing enzyme is 3-MPST which is mostly located in mitochondria. MPST produces H₂S from 3-mercaptopyruvate, which is produced by CAT from cysteine and alfa-ketoglutarate. In our experiments, PAG inhibited the CSE pathway of L-cysteine utilization and increased the bioavailability of L-cysteine for CAT that, in turn, activated H₂S production by 3-MPST. Indeed, testing of the cardiac mitochondria for H₂S content showed that H₂S levels were elevated twice in PAG+L-cysteine group comparing to untreated control (Table 1). Thus, our results support the idea that 3-MPST but not CSE is the primary enzyme responsible for H₂S synthesis in the myocardium (Kuo et al., 2016), which participates in heterometric regulation of the heart function.

Another finding of this study is that inhibition of CSE with PAG may redirect L-cysteine to glutathione production by GCL enzyme which synthesized gamma-glutamylcysteine from L-glutamate and L-cysteine. Previously we have shown that PAG+L-cysteine provided cardioprotective effects against ischaemia-reperfusion revealing antioxidative effect of the used combination of compounds (Sahach et al., 2013). Here, we have shown that PAG+L-cysteine increased both reduced and oxidized glutathione levels in the heart homogenates (Figure 2). That finding is supported by ^{*}O₂⁻ generation and products of lipid oxidation data. Our results suggest that PAG+L-cysteine pretreatment decreased ^{*}O₂⁻ generation in cardiac mitochondria. As result, decreased MDA and DK concentration. It maintained the mitochondrial membrane integrity and function of mitochondrial protein complexes. That is basis for improved Frank-Starling response.

Considering that PAG+L-cysteine combination induces H₂S production by 3-MPST, our data confirm the results of others who showed the possibility of glutathione upregulation by H₂S (Kimura et al., 2010). It was also reported that exogenous H₂S upregulated expression of GCL catalytic and modifier subunits, and formation of GSH in a monocytes cell model (Jain et al., 2014). Thus, there are two possible ways of

glutathione induction under PAG+L-cysteine administration: the direct glutathione synthesis from L-cysteine and the induction of glutathione synthesis by newly synthesized H₂S by 3-MPST.

The glutathione inhibitor BSO caused sharp increase in EDP curve (Figure3) that indicated robust disturbances in calcium management in cardiomyocytes under BSO administration. Moreover, BSO prevented PAG+L-cysteine induced increase of the GSH and GSSG levels. Considerably, depletion of glutathione under BSO administration led to reduced SERCA glutathionylation and, thus, decreased activity of these channels which, in turn, reduced calcium transport from the cytoplasm into the sarcoplasmic reticulum. As a result, calcium overload of cardiomyocytes led to the impaired relaxation of the myocardium and to the rapid growth of EDP curve which we observed in the BSO+PAG+L-cysteine group. Thus, our data indicate that heterometric regulation of the heart function requires adequate glutathione levels that might be upregulated by PAG+L-cysteine pretreatment.

It should be noticed that improved Frank-Starling response induced by PAG+L-cysteine combination was associated with increased cNOS activity and nitrite content in cardiac mitochondria (Table 1). It seems that NO produced by cNOS made significant contribution to increased P-V dependence of the heart since the application of the cNOS inhibitor, L-NAME, abolished positive inotropy induced by PAG+L-cysteine (Figure 4). Except *de novo* NO synthesis, it is likely that PAG+L-cysteine pretreatment increased reutilization of NO from nitrates/nitrites and nitrosothiols. Here, we have found that PAG+L-cysteine administration was accompanied by increased nitrate reductase activity (Table 1). It was shown that exogenous H₂S enhanced NO production involving both endothelial NO synthase and nitrite reduction mechanisms (Bir et al., 2012). The sulfide donor, GYY4137, promoted the release of NO from S-nitrosothiols, thus, modulating soluble nitrosothiol-mediated guanylyl cyclase (Cortese-Krott et al., 2014) which produces cyclic GMP and mediates relaxation of the smooth muscles. Previously we have shown that stimulation of endogenous H₂S synthesis increased cNOS activity, reduced the levels of nitrates and led to vasorelaxation of aortic rings in old rats (Mys et al., 2020). Thus, PAG+L-cysteine pretreatment regulates NO production in the heart by *de novo* and reduction mechanisms that, in turn, enhances the increase in LVDP and potentiated the diastolic response of the isolated rat heart.

CONCLUSIONS

In the present work we showed that PAG+L-cysteine administration positively influenced the Frank-Starling response of the heart involving NO and GSH. PAG+L-cysteine administration induced mitochondrial H₂S synthesis, increased glutathione levels, cNOS and nitrate reductase activity in the heart. To our knowledge, this is the first work that demonstrates the importance of endogenous H₂S produced by mitochondrial pathway in heterometric regulation of the heart function. We assume that in the case of PAG+L-cysteine administration, H₂S is the key component that regulates downstream processes including glutathione synthesis, NO production by *de novo* and/or by reduction mechanisms, intracellular Ca²⁺ cycling etc. Also, we showed that the presence of glutathione in sufficient quantities in cardiac tissues is important for adequate realization of the Frank-Starling law. It increases contractility in response to mechanical stretch as well as for dilatation. Inhibition of glutathione with BSO significantly reduced Frank-Starling response and increased diastolic stiffness of myocardium. Thus, the Frank-Starling response of the heart depends on a tight interaction between H₂S, glutathione and NO. Our results contribute to better understanding of the basic physiological mechanism underlying the heart function in conditions of stress or increased volume load regime. Improved myocardial response to mechanical stretch due to increased H₂S, NO and glutathione synthesis might be promising approach in treatment of myocardial dysfunction in some pathological and age-related conditions in patients with heart failure, arterial hypertension or diabetes.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

388 **DECLARATIONS**

389 **Ethics Approval**

390 All experiments were carried out in compliance with the Directive of the Parliament of Ukraine №3447-
391 IV “On the protection of Animals from Cruel Treatment” (2006) and Directive 2010/63/EU of the
392 European Parliament and of the Council on the protection of animals used for scientific purposes
393 (22.09.2010). The study was approved by the Institutional Committee for Biomedical Research Ethics
394 (protocol 4/20 from 22.05.20). The manuscript does not contain clinical studies or patient data.

395 **Conflicts of Interest**

396 On behalf of all authors, the corresponding author states that there is no conflict of interest.

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400 **Author contribution**

401 All authors contributed to the study conception and design. Material preparation, data collection and
402 analysis were performed by Yulia V.Goshovska, Raisa A. Fedichkina and Yulia P. Korkach. The first
403 draft of the manuscript was written by Yulia V. Goshovska and all authors commented on previous
404 versions of the manuscript. All authors read and approved the final manuscript.

406 **Data Availability Statement**

407 The data that support the findings of this study are available from the corresponding author upon
408 reasonable request.

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Figure legends

Figure 1 Effect of volume load on the left ventricular developed pressure (LVDP) and end diastolic pressure (EDP) of isolated heart of rats treated with (A) PAG, L-cysteine or (B) PAG+L-cysteine. Values are expressed as means $\pm$ SEM. Comparison between groups was made using Kruskal-Wallis ANOVA test ($n \geq 5$ per group, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ the difference versus control, # $P < 0.05$, ## $P < 0.01$, *** $P < 0.001$ the difference between L-cysteine and control group)

Figure 2 GSH (A) and GSSG (B) levels in heart homogenates in control, PAG+L-cysteine, BSO+PAG+L-cysteine groups. Values are expressed as means $\pm$ SEM. Comparison between groups was made using Kruskal-Wallis ANOVA test ($n=8$ per group, * $P < 0.05$, ** $P < 0.01$, versus control, # $P < 0.05$, ## $P < 0.01$ versus PAG+L-cysteine group)

Figure 3 The effect of volume load on the left ventricular developed pressure (LVDP) and end diastolic pressure (EDP) in isolated rat heart in control, BSO and BSO+PAG+L-cysteine group. Values are expressed as means $\pm$ SEM. Comparison between groups was made using Kruskal-Wallis ANOVA test ($n \geq 5$ per group, ** $P < 0.01$ versus control, ## $P < 0.01$ versus BSO+PAG + L-cysteine group)

Figure 4 Effect of volume load on the left ventricular developed pressure (LVDP) and end diastolic pressure (EDP) in isolated rat heart after treatment with NOS and CSE inhibitors. 1 – control; 2 – PAG+L-cysteine; 3 – L-NAME+PAG+L-cysteine. Values are expressed as means $\pm$ SEM. Comparison between groups was made using Kruskal-Wallis ANOVA test ($n \geq 5$ per group, *the difference is significant versus control, # the difference is significant versus PAG + L-cysteine group)

Table 1

H₂S content and indexes of NO metabolism in cardiac mitochondria

Group	H ₂ S, <i>nmol mg⁻¹ of protein</i>	NO ₂ ⁻ , <i>pmol mg⁻¹ of protein</i>	NO ₃ ⁻ , <i>nmol mg⁻¹ of protein</i>	cNOS activity, <i>pmol min⁻¹ mg⁻¹ of protein</i>	iNOS activity, <i>pmol min⁻¹ mg⁻¹ of protein</i>	Nitrate reductase activity, <i>nmol min⁻¹ mg⁻¹ of protein</i>
Control	4,48 ±0,44	172±18	68,8 ±15,5	5,85 ±1,12	1,61±0,12	0,59±0,04
L-cysteine	5,62±1,25	159±17	48,6 ±10,4	6,95 ±1,22	4,61±0,68*	1,26±0,41*
PAG	10,0±1,95*	488±60*	51,4 ±10,5	21,7±2,97*	9,48±1,07*	1,70±0,22*
PAG+L- cysteine	9,0±1,25*	226±18*	73,0 ±13,5	22,45±2,51*	7,6±1,25*	1,48±0,23*

Values are expressed as means ± SEM. Comparison between groups was made using Kruskal-Wallis ANOVA test (n≥5 per group, * P<0.05 versus control)

Table 2

Markers of oxidative stress in cardiac mitochondria

Group	*O ₂ ⁻ generation, <i>rel. un.</i>	MDA, <i>nmol/mg of protein</i>	DK, <i>ng/mg of protein</i>
Control	22,1 ±2,81	2,22 ±0,45	2,12 ±0,25
PAG+L- cysteine	5,03± 0,78*	0,96 ±0,10*	1,41 ±0,26

Values are expressed as means ± SEM. Comparison between groups was made using Kruskal-Wallis ANOVA test (n≥5 per group, * P<0.05 versus control)