

**CARDIOPROTECTIVE EFFECT OF H₂S AND GLUTATHIONE SYNTHESIS
MODULATION IS MEDIATED BY INHIBITION MITOCHONDRIAL
PERMEABILITY TRANSITION PORE OPENING**

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Hydrogen sulfide (H₂S) was recently classified as the third gaseous transmitter produced by two cytosolic cystathionine γ -lyase (CSE) and cystathionine β -synthase, and one mitochondrial enzyme – 3-mercaptopyruvate sulfurtransferase. It was clearly shown that H₂S protects against cardiac ischemia/reperfusion (I/R) injury in a wide range of exogenously applied doses of H₂S donors. Cell damage within I/R injury is caused by extensive reactive oxygen species (ROS) mainly produced by mitochondria. ROS flashes are associated with massive opening of mitochondrial permeability transition (MPT) pores and contribute to deterioration of heart function. However, it may be prevented in case of pharmacological inhibition of MPT pores opening. Mixture of mitochondrial metabolites released through the opened MPT pores can be detected in situ as increased optical density of outflow solutions at a wavelength of 245-250 nm and was called mitochondrial factor (MF). One of the most powerful antioxidant agents that preserve redox status in tissues is tripeptide glutathione. It forms in two ATP-dependent reactions and exists in two forms reduced and oxidized one. Glutathione and H₂S have a common precursor – amino acid L-cysteine. In this study, we used Langendorff isolated rat heart model to investigate the effect of H₂S and glutathione synthesis modulation on MPT pores opening in I/R injury. Rats were pretreated intraperitoneally with D,L-propargylglycine (11,3 mg/kg), an inhibitor of H₂S-producing enzyme CSE, L-cysteine (121 mg/kg) and buthionine sulfoximine (BSO, 22,2 mg/kg) an inhibitor of first step of glutathione synthesis. Cardiac function, oxygen metabolism and MPT pores opening in situ were measured. We clearly showed that treatment with PAG and L-cysteine provided pharmacological precondition and exerted cardioprotective effect inhibiting MPT pores opening as greatly decreased MF release from isolated heart. Pretreatment with BSO abolished cardioprotective effect of PAG+L-cysteine combination. Absorbance spectra in L-cysteine pretreated group did not differ from the control. Thus, we demonstrate that PAG+L-cysteine induced cardioprotection mediated via inhibition of MPT pores opening.

Keywords: L-cysteine, glutathione, ischemia/reperfusion, mitochondrial permeability transition pore, cardioprotection

Hydrogen sulfide (H₂S) was recently classified as the third gaseous transmitter and is considered to play a critical role in varieties of physiological and pathological conditions. The unique properties of H₂S in cardiovascular system were demonstrated in regulation of the endothelium-dependent vasorelaxation [4, 31, 35], prevention of inflammation [14], stimulation of angiogenesis [4, 20]. It is becoming increasingly clear that H₂S protects against cardiac ischemia/reperfusion(I/R) injury in a wide range of exogenously applied doses of H₂S donors [3, 11, 12, 27, 28]. It was further shown that the myocardial cell shape and viability are significantly increased [2], while the myocardial infarct size is decreased after a prolonged I/R when treated with donors of H₂S, like NaHS or Na₂S [6, 11, 16, 30, 36]. However, little is known about physiological

effects of endogenously produced H₂S. Several H₂S-producing enzymes were identified, namely cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). CBS and CSE are pyridoxal-5'-phosphate-dependent enzymes those localized in cell cytoplasm. CBS is responsible for H₂S production mostly in brain [34] while CSE is more abundant in heart and vascular smooth muscle cells, particularly in aorta [35]. A major substrate for CBS and CSE is L-cysteine that is metabolized with production of ammonium, pyruvate and H₂S [32]. The third pathway of H₂S biosynthesis was identified in mitochondrial matrix as 3-mercaptopyruvate sulfurtransferase (3MST) enzyme producing H₂S from 3-mercaptopyruvate, which, in turn, is produced from L-cysteine and α-ketoglutarate by cysteine aminotransferase [25].

L-cysteine is a precursor not only for H₂S but for glutathione synthesis as well. Glutathione belongs to the first line of antioxidant defense and exists in reduced (GSH) and oxidized (GSSG) state. GSH forms in two ATP-dependes steps by γ-glutamylcysteine synthetase and GSH synthetase. The first reaction of GSH synthesis is the rate-limiting reaction that can be inhibited by GSH or some chemical agents, like buthionine sulfoximine (BSO). GSH synthesis is localized in cytoplasm but then it is transported to cell organelles. Particularly, mitochondria contain about 10 % of cell's glutathione [15]. It was shown that GSH level was depleted in I/R injury [4, 7, 19]. In brain, injections of glutathione ethyl ester increased glutathione content in mitochondria [1]. In cardiac tissue, GSH was reduced by pretreatment with BSO that aggravated I/R injury of myocardium in pigs [29]. Intravenous infusion of glutathione increased GSH content, reduced infarct size and improved recovery of contractile function of the ischemized myocardium [29]. Thus, sufficient level of GSH may predispose the resistance of myocardium to ischemia. Stimulation of endogenous GSH production instead of exogenous application could be beneficial for the heart tissue in oxidative stress conditions like I/R.

Mitochondria are the major source of ROS production in the cell. In normal conditions, electron leaking 2–4 % from electron-transport chain (ETC) forms superoxide anion that, however, greatly increased in I/R. Recent data suggests that superoxide flashes originating from the mitochondrial permeability transition (MPT) pore [33]. ROS flashes occur because of functional coupling between ETC proteome and MPT pores opening. In I/R MPT pores opening leads to mitochondria swelling, ETC uncoupling, dissipation of proton gradient, drastic decrease in ATP synthesis and release of cytochrome C from the mitochondrial matrix initiate apoptosis [5, 8, 9]. These lead to cardiac disfunction. Previously we have shown that the mixture of metabolites released from mitochondria via MPT pores into outflow solution from the isolated heart might be detected by UV spectroscopy [21]. The outflow solution collected during 1st minute of reperfusion are characterized with extremely increased optical density at a wavelength of 245-250 nm and were called mitochondrial factor (MF) [17]. We have also shown that various agents (vitamin E, coenzyme Q, ischemic preconditioning etc) can significantly reduce reperfusion disturbances of myocardial function by keeping MPTP in a closed state on reperfusion that correlates with the significantly decreased levels of MF [10, 23, 24]. Thus, the determination of MF can serve as a valuable tool for evaluation of the closed/open state of the MPTP and new candidates for cardioprotective agents.

Previously we have showed that stimulation and blockade of H₂S synthesis improved recovery of isolated rat heart function after I/R [22]. In this study, we would like to show that such modulation occurs via induction of endogenous glutathione production and inhibition of MPT pores opening.

Methods

All experiments were carried out in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental Purposes (Strasbourg, 1986) and pro-

protocols approved by the Institutional Committee for Biomedical Research Ethics. We used male Wistar rats aged 6 months, weighing 300–320 g kept on a standard diet in vivarium of Bogomolets Institute of Physiology (Kyiv, Ukraine) with free access to water. Animals were divided into 4 groups: 1 – control group, 2 – L-cysteine pre-treated group (121 mg/kg, 30 min before decapitation), 3 – D,L-propargylglycine pre-treated group (PAG, 11,3 mg/kg, 40 min before decapitation), 4 – PAG (10 min) + L-cysteine (30 min) pre-treated, 5 – buthionine sulfoximine (BSO, 22,2 mg/kg) +PAG (10 min)+L-cysteine (30 min) pre-treated group.

Isolated hearts by Langendorff preparation

The aorta of isolated heart was cannulated and the perfusion of the coronary vessels of the isolate heart was performed retrogradely under stable perfusion pressure of 75–80 mmHg with non-recirculating Krebs-Henseleit solution (in mmol/L): 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 24 NaHCO₃, 1.2 KH₂PO₄, 10.0 glucose, 2.5 CaCl₂, pH 7.4. The perfusing solution was aerated with gas mixture (95 % O₂, 5 % CO₂). Temperature of perfusion solution was held constant at 37 °C throughout the whole experiment. The pulmonary artery was catheterised with a tube of a proper size for effluent collection. Thus, a coronary flow was measured as perfusion solution volume passed through the heart per minute. The following parameters of cardiac function were assessed: left ventricular developed pressure (LVDP), calculated as the difference between systolic and diastolic pressure in the left ventricle; end-diastolic pressure (EDP); the maximal (dP/dt_{max}) and minimal (dP/dt_{min}) value of the first derivative of left ventricular pressure, and heart rate. LVDP was monitored with a water-filled latex balloon inserted into the left ventricle, inflated to give initial EDP of 2.5–5 mmHg. The balloon was connected to the strain gauge 746 ('Mingograph-82', 'Elema', Sweden) and PC with Global Lab software. This allowed continuous monitoring and registration of pressure changes throughout ischemia (20 min) and reperfusion (40 min). During the ischemia the hearts' temperature was kept stable by immersion of hearts into the bath with Krebs-Henseleit solution (37 °C).

Oxygen consumption by myocardium

'Arterial' samples of perfusion solution were collected from the tap place close to the cannulated aorta. Samples of the 'venous' perfusate were collected without exposure to air by inserting the needle of testing syringe into the pulmonary catheter. Oxygen tension of those perfusate samples was measured with an oxygen electrode of gas analyzer (BMS 3 Mk-2 Radiometer, Copenhagen). Arterial-venous difference was calculated and used for oxygen consumption calculation by formula proposed by Neely [18]:

$$\frac{\frac{O_2 \text{ consumption (mmole/hr per g)}}{\text{arterio - venous } O_2 \text{ tension (mm Hg)}}}{\frac{760 \text{ (mmHg)}}{\text{solubility of } O_2 \text{ at } 37^\circ \text{C (ml/ml H}_2\text{O)}}} \cdot \frac{\text{coronary flow (ml/hr)}}{\text{dry weight of heart (g)}}$$

Oxygen cost of myocardial work (OCMW) was expressed as the ratio of oxygen consumption to the heart work (the product of LVDP and the heart rate).

Evaluation of mitochondrial permeability transition (MPT) pore opening in situ

Effluents probes (2,0–2,5 mL) were collected from the pulmonary catheter before and immediately after ischemia. The coronary solutes were tested in UV spectra (230–260 nm). Data were presented as graphs of dependence of the samples absorbance from the wave length. The

appearance of “peek” of absorbance at 250 nm of solutions taken at reperfusion indicated MPT pore induction.

Statistics

The data are expressed as the mean \pm standard error. Data were calculated by Kruskal-Wallis with Mann-Whitney *post hoc* analysis. P values less than 0.05 were considered significant.

Results and Discussion

Hearts were perfused by Langendorff technique at stable perfusion pressure. After 15-20 min of stabilization period hearts reached the functional ‘plateau’ with repetitive meaning of LVDP and coronary flow. At a start of each experiment the EDP value was settled in range of 2.5–5 mmHg. The difference in initial values of cardiodynamic parameters between experimental groups might be considered as the effect of chemicals injected before Langendorff preparation.

L-cysteine increased the rate of pressure development (dP/dt) of isolated heart (see Table), however, no significant changes were observed in LVDP and heart rate values. OCMW was decreased by 20 % in L-cysteine group comparing to non-treated control. The same dynamic was observed in PAG treated group but in LVDP and coronary flow changes were significant. OCMW was decreased by 30 % in PAG group comparing to control. The administration of PAG and L-cysteine significantly increased LVDP and dP/dt. Despite of two-times decrease in coronary flow, the value of OCMW was decreased by 40 % indicating more effective oxygen utilization by myocardium in PAG+L-cysteine group. However, this improving effect was abolished when animals were pretreated with glutathione depletor BSO.

The initial values of cardiodynamics of isolated rat heart under H₂S and glutathione modulators

Group/Parameter	Control (n=8)	L-cysteine (n=6)	PAG (n=4)	PAG+L-cysteine (n=5)	BSO+PAG+L-cysteine (n=8)
LVDP, mmHg	100 \pm 3.7	117.2 \pm 4.1	122 \pm 10.4*	123.8 \pm 7.7*	85.3 \pm 10.2#
dP/dt _{max} , mmHg s ⁻¹	1455 \pm 93	1829 \pm 73*	1795 \pm 112*	1875 \pm 85**	1226 \pm 150##
dP/dt _{min} , mmHg s ⁻¹	1157 \pm 75	1475 \pm 64*	1384 \pm 25	1405 \pm 20*	927 \pm 78###
Coronary flow, mL min ⁻¹	13.3 \pm 0.63	10.4 \pm 0.96*	8.8 \pm 0.63**	6.8 \pm 0.84**	7.8 \pm 0.97
Heart rate, beats min ⁻¹	188 \pm 17	189 \pm 13	168 \pm 30	153 \pm 15	178 \pm 26
Oxygen consumption, mmol hr ⁻¹ g ⁻¹	1.19 \pm 0.13	1.17 \pm 0.11	0.87 \pm 0.09	0.72 \pm 0.09*	0.94 \pm 0.13
Oxygen cost of myocardial work, $\times 10^{-6}$, mmol of O ₂ beat ⁻¹ g ⁻¹	1.09 \pm 0.12	0.86 \pm 0.03	0.76 \pm 0.06	0.65 \pm 0.07*	1.15 \pm 0.14#

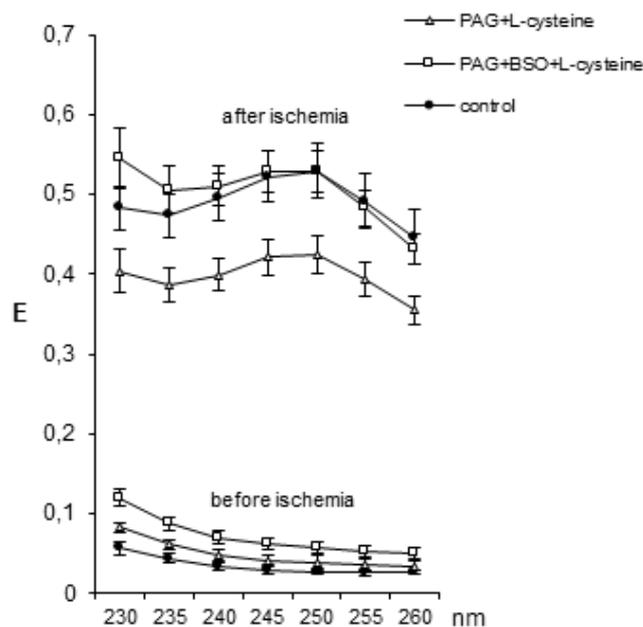
Values are means \pm SEM Oxygen tension of buffer entering the heart averaged 450 mmHg. The average dry heart weight was 0.2 g. *P<0.05, **P<0.01 vs control, # P<0.05, ## P<0.01, ### P<0.001 vs PAG+L-cysteine as calculated by Kruskal-Wallis with Mann-Whitney *post hoc* analysis.

These data show the improving effect of PAG+L-cysteine combination at heart contractile function and oxygen consumption of non-ischemized myocardium. This effect seems to be glutathione dependent since inhibitor of glutathione BSO abolished the effect PAG+L-cysteine as decreased heart rate, LVDP, dP/dt and increased oxygen cost of myocardial work.

PAG+L-cysteine pretreatment caused restoration of cardiac function during reperfusion period without EDP deterioration. LVDP restoration was 106 % comparing with 42.7 % in con-

control group ($p < 0.005$). dP/dt_{\max} averaged 104.0 % and dP/dt_{\min} – 93.5 % comparing with 42.8 % and 38.8 % respectively in control group ($p < 0.005$). Oxygen metabolism in myocardium was preserved as OCMW was significantly lower in reperfusion period and increased only by 34 % ($p < 0.03$) comparing to the control group. These data clearly indicate cardioprotective effect PAG+L-cysteine pretreatment due to more effective oxygen utilization by ischemized myocardium comparing with non-treated control.

We studied the optical density of the effluent probes to evaluate the release of mitochondrial factor as marker of MPT pores opening and mitochondrial damage. A figure shows that absorbance spectra of coronary effluents were maximal at 250 nm in all groups. It should be noticed that absorbance spectra in L-cysteine pretreated group did not differ from the control (0.586 ± 0.069 rel. un.) (not shown). Pretreatment with PAG+L-cysteine significantly decreased the peak of absorbance of effluents (0.423 ± 0.023 rel. un. vs 0.529 ± 0.034 in I/R group, $p < 0.05$). PAG pretreated group repeated the dynamic of PAG+L-cysteine group (0.448 ± 0.056 rel. un.) (not shown). However, this effect was absent in BSO pretreated group (0.528 ± 0.026 rel. un.). Thus, PAG+L-cysteine provide pharmacological precondition and exert cardioprotective effect inhibiting MPT pore opening.



Absorbance spectra of coronary effluents collected before and during 1st min of reperfusion of isolated rat heart. I/R induced absorbance of outflow solutions was significantly decreased by PAG+L-cysteine. Inhibition of glutathione synthesis with BSO abolished membrane protective effect of PAG+L-cysteine.

Burst ROS production and mitochondrial Ca^{2+} overload are precipitating factors in cardiac I/R injury, with subsequent triggering of the MPT pores opening, mitochondria swelling, cytochrome *c* release, apoptotic and necrotic cardiomyocyte death [8]. This mixture of substances has mitochondrial origin since the release of UV-absorbing solutes is inhibited by MPT pore inhibitor cyclosporine A or by ischemic preconditioning [10, 17, 26]. It is suggested that this mixture contain ATP decomposition products, i.e. adenosine and inosine etc. Thus, I/R is accompanied

with loss of the phosphate carriers and energetic units by the cell. Prevention of this process and preservation of mitochondrial dysfunction could provide fast recovery of myocardial contractile function after I/R. We used PAG as an inhibitor of CSE for directing L-cysteine to glutathione production. We have found that such manipulation with L-cysteine significantly decreased release of mitochondrial factor from ischemized heart indicating lower ROS production, inhibited MPT pores opening and preserved ATP-producing function of mitochondria under PAG+L-cysteine pretreatment.

Thus, inhibition of endogenously produced H₂S with PAG in combination with L-cysteine provided cardioprotection against ischemia/reperfusion injury that was vastly dependent on *de novo* glutathione formation since *glutathione depressor* BSO abolished cardioprotective effect of PAG+L-cysteine. The changes in cardiodynamic data were supported by relevant changes in oxygen cost of myocardial work and shows inhibitory effect of PAG+L-cysteine on I/R-induced MPT pores opening in myocardium.

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КАРДИОПРОТЕКТОРНИЙ ЕФЕКТ МОДУЛЯЦІЇ СИНТЕЗУ H_2S І ГЛУТАТІОНУ ПРИ ІШЕМІЇ-РЕПЕРFUЗІЇ ОБУМОВЛЕНІЙ ПРИГНІЧЕННЯМ ВІДКРИТТЯ МІТОХОНДРІАЛЬНИХ ПОР ТРАНЗИТОРНОЇ ПРОНИКНОСТІ

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Сірководень (H_2S) був класифікований як третій газоподібний посередник, що виробляється ферментами: двома цитозольними – цистеїн- γ -ліазою і цистатіон- β -синтазою та одним мітохондріальним – меркаптопіруват-сульфур-трансферазою. Було показано, що H_2S захищає від ішемічно-реперфузійного (I/R) пошкодження серця в широкому діапазоні застосовуваних доз екзогенних донорів H_2S . Пошкодження клітин за умов ішемії-реперфузії спричиняється великими дозами АФК, що в основному виробляються мітохондріями. Вибухоподібне збільшення концентрації АФК в цитозолі асоційоване з масовим відкриттям мітохондріальних пор транзиторної проникності (МРТ) та з виділенням із мітохондрій суміші речовин, названої мітохондріальним фактором. Усе це сприяє розвитку I/R порушення функції серця. Такому розвитку подій можна запобігти за допомогою фармакологічного гальмування відкриття МРТ пор. Нами показано, що мітохондріальний фактор можна визначити у відтікаючих від тканин розчинах спектрофотометричним методом на довжині хвиль 245-250 нм. Одним із найпотужніших антиоксидантів у тканинах, який бере участь у забезпеченні окисно-відновного балансу, є трипептид глутатіон. Він синтезується в клітинах у результаті двох АТФ-залежних реакцій. Глутатіон і H_2S мають спільного попередника – амінокислоту L-цистеїн. У цьому дослідженні ми використовували модель ретроградної перфузії коронарних судин сердець щурів, ізольованих за методом Лангендорфа, щоб дослідити вплив модуляції синтезу H_2S і глутатіону на відкривання МРТ пор в умовах ішемії-реперфузії. Вимірювали скоротливу активність міокарда, оцінювали метаболізм кисню та відкривання МРТ пор *in situ*. Щурам внутрішньоочеревинно вводили D,L-пропаргілгліцин (11,3 мг/кг), інгібітор цистатіонін γ -ліази, L-цистеїн (121 мг/кг) та бутіонін-сульфоксимін (BSO, 22,2 мг/кг) – інгібітор синтезу глутатіону. Ми показали, що сумісне введення D,L-пропаргілгліцину та L-цистеїну здійснювало кардіопротекторний ефект і зменшувало оптичну щільність розчинів, що відтікали від ішемізованого серця. Попереднє введення BSO скасовувало кардіопротекторну дію комбінації PAG+L-цистеїну. Таким чином, ми показали, що PAG+L-цистеїн має кардіопротекторний ефект, який опосередковується інгібуванням відкриття МРТ пор.

Ключові слова: L-цистеїн, глутатіон, ішемія-реперфузія, мітохондріальні пори транзиторної проникності, кардіопротекція