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Research Article

The Gasotransmitter Mechanism of the Erythropoietin Protection Against Hepatic Reperfusion Injuries: The Role of Hydrogen Sulfide

Michail Khodosovsky*

Department of Pathophysiology, Grodno State Medical University, Belarus

***Corresponding Author:** Michail Khodosovsky, Department of Pathophysiology, Grodno State Medical University, Belarus.

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Abstract

Introduction: The erythropoietin administration can be a new method for correction of hepatic reperfusion injuries, which are an actual problem of current surgery and transplantation. The purpose of this work was to investigate the role of hydrogen sulfide in the erythropoietin protective effect during hepatic ischemia-reperfusion (HIR) in rats.

Methods: 40 male Wistar rats were divided into 4 groups: 1st (n = 10) - control; 2nd (n = 10) - HIR: hepatic ischemia (30 min, m. Pringle) and reperfusion (120 min); in 3rd group (n = 10) erythropoietin (INTAS, 1000 IU/kg) was admin 30 min before HIR; in 4th group (n = 10) rats were handled like in 3rd group, but inhibitor of cystathionine-γ-lyase (DL-propargylglycine, 50 mg/kg) was added 1hr before HIR. The parameters of pro/antioxidant balance (conjugated diens, malondialdehyde, reduced glutathione, catalase activity and ect.) were detected in blood and liver at the end of experiments.

Results: It was found that erythropoietin significantly decreased the level of lipid peroxidation products, improved parameters of antioxidant system in blood and liver at the end of reperfusion. The inhibition of endogenous hydrogen sulfide synthesis by DL-propargylglycine significantly reduced this protective effect of erythropoietin.

Conclusion: The protective effect of erythropoietin during hepatic ischemia-reperfusion is largely mediated by gasotransmitter properties of hydrogen sulfide.

Keywords: *Hydrogen Sulfide; Erythropoietin; Reperfusion; Liver; Prooxidant-Antioxidant Status; Rats*

Introduction

Elucidation of the physiological effects of endogenous hydrogen sulfide is an urgent problem of modern pharmacology and medicine. More than a dozen years have passed since the establishment of endogenous production of this compound in mammals until the point of view of it as a by-product of biochemical reactions was transformed into a biologically highly active compound participating in the mechanisms of intercellular signaling and modulating the activity of many adaptive genes [1-5]. It has been shown that H_2S is synthesized in almost all tissues, and its highest concentrations are found in the brain, heart, blood vessels, liver and kidneys [3,6]. In the liver, H_2S is synthesized from L-cysteine mainly under the influence of the enzyme cystathionine-γ-lyase. It possesses neurotransmitter properties, vasodilation, reduces platelet aggregation, easily reacts with reactive oxygen and nitrogen species, restores enzyme activity due to sulfhydration and affects cell proliferation and angiogenesis [2,5].

It has been established that during hepatic ischemia-reperfusion (HIR), exogenous H_2S improves the parameters of the oxygen transport function of the blood, increases the activity of intracellular antioxidants, induces the production of heat shock proteins (HSP-90),

suppresses apoptosis, pro-inflammatory factors, which helps to reduce reperfusion damage to the organ [7-9]. We have previously shown the protective effect of erythropoietin (EPO) in HIR [8]. Considering that EPO and H_2S are able to modulate the activity of the same adaptive genes and have a similar effect on the prooxidant-antioxidant balance in HIR [7,10-12], our goal was to clarify the role of endogenous hydrogen sulfide in the mechanism of the protective action of erythropoietin on the liver during ischemia-reperfusion syndrome in rats.

Materials and Methods

The experiments were performed on 40 male Wistar rats, weighing 280 - 360g, Animals were kept for 2 weeks in under standard vivarium conditions (temperature 22 ± 2°C, humidity 50 ± 10% and daylight cycle 8 AM - 8 PM). Animals were fed on a laboratory diet with water and food *ad libitum* until use and fasted overnight with free access to water before operation. Under combined anesthesia (sodium thiopental - 30 mg/kg, i.p., ketamine - 100 mg/kg, i.m.), liver ischemia was caused by Pringle maneuver (clamping of hepato-duodenal ligament) for 30 min, the reperfusion period lasted 120 min. At the end of the experiment, blood and liver tissues were taken to assess the parameters of prooxidant-antioxidant balance. All surgical manipulations were performed under adequate analgesia and according with the standards of ethical commission for humane behavior with experimental animals of the Grodno State Medical University.

The animals were divided into 4 groups: 1st (n = 10) - control; in the second (n = 10) the HIR was modeled; in group 3 (n = 10) - 30 min before HIR rats were injected with recombinant human erythropoietin alfa (EPO, INTAS, 1000 IU/kg, i.p.) [8], in group 4 (n = 10) the introduction of EPO was combined with an inhibitor of hydrogen sulfide producing enzyme - cystathionine γ-lyase (CSE), DL-propargylglycine (PAG, Sigma, 50 mg/kg, 60 min before HIR, i.p.) [13]. The content of hydrogen sulfide in blood plasma was determined by the spectrophotometric method [14]. ALT and AST activities in plasma were detected by kinetic method using standard "Cormay" kit.

The following indicators of the prooxidant-antioxidant state were studied: conjugated dienes (CD), malondialdehyde (MDA), Schiff bases (SB), reduced glutathione (GSH), α-tocopherol, retinol and catalase activity. The content of CD in biological material was determined by ultraviolet spectrophotometry at a wavelength of 233 nm [15]. The level of MDA was assessed by the reaction with 2'-thiobarbituric acid (TBA) [16]. The SB content was determined from the fluorescence intensity of the chloroform extract at excitation and emission wavelengths of 344 nm and 440 nm, respectively [17]. The concentration of α-tocopherol and retinol was studied by the fluorometric method in the hexane extract [18]. Sigma α-tocopherol and retinol were used as standards. The GSH content was determined according to the method of J. Sedlak, R. Lindsay (1968) [19]. Catalase activity in biological material was estimated by the spectrophotometric method based on the ability of hydrogen peroxide to form a persistently colored complex with molybdenum salts [20].

Statistical processing of the obtained data was carried out using Student's t-test, Wilcoxon or Mann-Whitney tests, depending on the normality of the distribution of samples. Differences were considered significant at $p < 0.05$.

Results and Discussion

Modeling the HIR syndrome in rats led to a decrease in the level of hydrogen sulfide in plasma of mixed venous blood at the end of reperfusion by 35.2% ($p < 0.01$) in relation to the control (See figure 1). EPO infusion promoted the restoration of the level of H_2S in the blood during HIR, whereas inhibition of cystathionine-γ-lyase under the conditions of EPO administration reduced the level of hydrogen sulfide in relation to all experimental groups and controls (Figure 1).

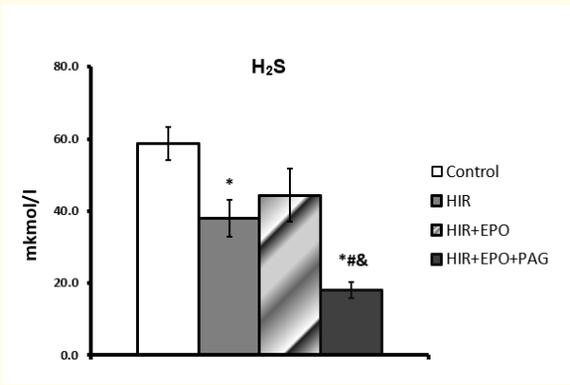

Figure 1: *The level of plasma hydrogen sulfide in experimental rats at the end of the reperfusion, where * is a significant difference in relation to the control group ($p < 0.05$), # is a significant difference in relation to the 2nd group (HIR) of animals ($p < 0.05$), & - significant difference in relation to group 3 (EPO) animals ($p < 0.05$).*

Changes in the parameters of the prooxidant-antioxidant balance are presented in table 1. It was found that the simulation of HIR in rats led to an increase in the content of lipid peroxidation (LPO) products, depletion of structural antioxidants (α-tocopherol, retinol) and a decrease in catalase activity in the blood and liver of experimental animals. In the group of animals treated with EPO (group 3), an improvement in most of the studied parameters was observed. Thus, the level of CD and SB in erythrocytes in relation to animals that did not receive the drug decreased by 62.9% ($p < 0.001$) and 39.2% ($p < 0.001$), respectively. The content of CD and SB in the liver at the end of reperfusion in relation to rats in which only HIR was modeled decreased by 78.0% ($p < 0.001$) and 73.9 ($p < 0.001$), respectively. At the same time, the studied parameters of the antioxidant system in the blood and liver tissues of rats receiving EPO improved in relation to animals of the 2nd group. Thus, in the liver at the end of reperfusion, the levels of α-tocopherol, retinol and catalase activity in animals of the 3rd group did not differ from the control ones and the content of GSH erythrocytes increased by 20.7% ($p < 0.05$) in relation to the control. The results of the study indicate that the administration of EPO to rats helps to improve the prooxidant-antioxidant balance in HIR.

The use of EPO under conditions of inhibition of hydrogen sulfide synthesis using PAG (group 4) worsened the parameters of the prooxidant-antioxidant state of the blood and liver after ischemia (Table 1). Thus, the level of LPO products in blood plasma at the end of the reperfusion period increased: CD - 2.8 times ($p < 0.001$), SB - 3.5 times ($p < 0.001$); erythrocyte catalase activity decreased 2.1 times ($p < 0.001$) in relation to the rats of the 3rd group. The content of α-tocopherol and retinol in plasma in the 4th group in relation to the control animals decreased by 14.8% ($p <0.001$) and 17.7% ($p < 0.001$), respectively. A similar dynamic of changes in the indicators of prooxidant-antioxidant balance was observed in the liver (Table 1). It should be noted that in rats of the 4th group, despite the increase in the level of CD in the liver and SB in the blood plasma in relation to the animals of the 3rd group, these LPO indices remained lower than in the 2nd group of experimental animals (Table 1).

Parameter	Control	HIR	HIR+EPO	HIR+EPO+PAG
n	10	10	10	10
CD_{plasma}, ΔE_{233}/mL	0.85 ± 0.08	4,01 ± 0,38*	1,2 ± 0,12*#	3,44 ± 0,23*&
CD_{RBC}, ΔE_{233}/mL	5.68 ± 0,48	16.73 ± 0.92*	6.14 ± 0.52#	15.47 ± 1.2*&
CD_{liver}. ΔE_{233}/g	8.52 ± 0.74	46.62 ± 2.65*	10.2 ± 0.83#	35.8 ± 2.9*#&
MDA_{plasma}. mkmol/L	2.93 ± 0.91	6.45 ± 2.99	2.38 ± 0.3*	2.95 ± 0.38&
MDA_{RBC}. mkmol/L	7.69 ± 0.96	6.07 ± 0.79	3.0 ± 0.88*#	7.44 ± 1.17&
MDA_{liver}. mkmol/g	24.94 ± 1.59	39.42 ± 2.1*	30.0 ± 1.0*#	35.88 ± 1.7*&
SB_{plasma}. U/mL	20.05 ± 1.22	184.49 ± 10.1*	46.16 ± 7.33*#	150.3 ± 9.5*#&
SB_{RBC}. U/mL	39.03 ± 2.52	65.14 ± 4.05*	42.82 ± 5.3#	57.74 ± 3.69*&
SB_{liver}. U/g	115.06 ± 5.29	469.9 ± 33.55*	126.9 ± 8.05#	388.7 ± 28.9*&
α-$tocopherol_{plasma}$. mkmol/L	20.96 ± 0.38	17.4 ± 0.34*	20.22 ± 0.37#	17.4 ± 0.34*&
α-$tocopherol_{RBC}$. mkmol/L	96.29 ± 3.74	64.48 ± 2.97*	94.91 ± 2.69#	69.92 ± 3.1*&
α-$tocopherol_{liver}$. mkmol/g	177.33 ± 4.23	141.22 ± 3.79*	165.5 ± 3.91*#	145.75 ± 1.85*&
$Retinol_{plasma}$. mkmol/L	2.32 ± 0.07	1.72 ± 0.03*	2.16 ± 0.07#	1.89 ± 0.05*#&
$Retinol_{RBC}$. mkmol/L	7.16 ± 0.3	5.67 ± 0.19*	6.94 ± 0.25#	6.47 ± 0.3#
$Retinol_{liver}$. mkmol/g	20.37 ± 0.52	16.7 ± 0.4*	19.71 ± 0.71#	17.29 ± 0.42*&
GSH_{RBC}. mkmol /g_{Hb}	50.67 ± 1.84	50.98 ± 1.97	59.71 ± 3.65*#	57.96 ± 4.99
GSH_{liver}. mmol/g	4.09 ± 0.36	1.27 ± 0.26*	5.85 ± 0.21*#	5.99 ± 0.14*#
$Catalase_{RBC}$. mmol/L*gHb*sec	1.0 ± 0.13	0.38 ± 0.06*	1.62 ± 0.17*#	0.76 ± 0.13#&
$Catalase_{liver}$. mmol/L*g*sec	3.6 ± 0.18	1.47 ± 0.16*	3.78 ± 0.12#	1.97 ± 0.23*&
ALT_{plasma}. U/L	33.5 ± 5.1	314.5 ± 36.5*	89.9 ± 13.7*#	213.7 ± 26.2*#&
AST_{plasma}. U/L	38.8 ± 5.7	351.5 ± 39.1*	88.17 ± 10.4*#	204.6 ± 29.8*#&

Table 1: *Parameters of the prooxidant-antioxidant balance in rats at the end of hepatic reperfusion during EPO administration and inhibition of hydrogen sulfide synthesis (M ± m).*

*Note: HIR: Hepatic Ischemia-Reperfusion; EPO: Erythropoietin; PAG: DL-Propargylglycine; CD: Conjugated Dienes; MDA: Malondialdehyde; SB: Schiff Bases; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; *: Significant difference in relation to the control group ($p <0.05$). #: Significant difference in relation to the 2nd group (HIR) of animals ($p < 0.05$), &: Significant difference in relation to 3 group (HIR+EPO) animals ($p < 0.05$).*

The results of the study indicate that the use of EPO before IRI significantly improves the prooxidant-antioxidant state in the blood and liver of experimental animals, while the use of an inhibitor of endogenous H_2S synthesis led to a decrease in this protective effect. It was shown that in the liver under the influence of EPO, the expression of receptors for it (EPOR) increases, which leads to inhibition of TLR2 receptors and a decrease in the level of nuclear factor - κB (NF-κB) and proinflammatory cytokines (TNF-α, IL-1, IL-6) in ischemia-reperfusion [21]. The interaction of EPO with EPOR leads to the activation of an intracellular signaling cascade involving phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt), which promotes the activation of transcription factors, incl. the nuclear factor of erythroid origin Nrf2, which is responsible for the expression of antioxidant proteins and protection against oxidative stress [11,21,22]. Thus, it was found in [11] that the activation of Nrf2 under the influence of EPO in cerebral ischemia was accompanied by a significant antioxidant effect associated with a decrease in the accumulation of hydrogen peroxide in the tissues of this organ. The results of Meng H., *et al*. [11] agree with our data on the improvement of most parameters of the prooxidant-antioxidant state, including increased catalase activity, with HIR under conditions of EPO administration. At the same time, our data on the effect of EPO during HIR in rats is difficult to explain by the activation of the nuclear apparatus of cells, because the latter requires a longer duration of experiments.

It was shown that Akt can activate CSE without participation of the genetic apparatus of cells by direct phosphorylation of the enzyme, which is the main producer of endogenous hydrogen sulfide in the liver [23]. The use of a specific inhibitor of CSE - PAG in our experiments prevented the development of most of the protective effects of EPO on the prooxidant-antioxidant state. Apparently, hydrogen sulfide was the main messenger of the protective action of EPO in HIR in rats. Endogenous hydrogen sulfide is rapidly captured by metal-containing proteins and metabolized in the mitochondria of cells [2]. It has been shown that H_2S improves mitochondrial function, maintains oxidative phosphorylation processes, prevents calcium ion-induced release of cytochrome C and apoptosis, and reduces the generation of free oxygen radicals in the respiratory chain, which prevents the development of oxidative stress in the liver [24]. It cannot be ruled out that an increase in the production of H_2S by the vascular endothelium can modify the oxygen-binding properties of hemoglobin as a result of its sulfhydrogenation [7], which can significantly affect the redox state of tissues in HIR.

The typical cellular response to hypoxia involves activation of the hypoxia-inducible factor (HIF) pathway to help cells to adapt to reduced oxygen partial pressure that occurs through stabilization of the HIF-α proteins and subsequent transcriptional upregulation of a number of genes, including EPO [25]. Using an *in vivo* murine model where mice were subjected to a 72-hour period of hypoxia (11% O_2), it was discovered that CSE knockout mice displayed lower levels of hemoglobin, EPO, and other HIF-regulated genes compared to wild-type mice, which was improved by exogenous H_2S supplementation [26]. These data are in contradiction with the data of other authors, where CSE knockout mice demonstrated an increase in the level of erythrocytes and hemoglobin, may be due to another erythropoietic factor - coproporphyrinogen oxidase [27]. However, patients suffering from chronic kidney disease, which associated with anemia and EPO deficiency, display low levels of hydrogen sulfide [26]. In our experiments HIR leads to lower level of plasma H_2S then in control rats, EPO infusion little bet improve concentration of this gasotransmitter (Figure 1). PI3K/Akt pathway can regulate HIF-α protein stability in hepatocytes [28]. At the same time HIF-1α/HIF-1β can inflow on CSE expression [29]. Taken together this data suggest that EPO protective mechanism during HIR could include activation of the H_2S production by CSE through two pathways: direct Akt/CSE activation or indirect HIF/CSE activation, which possibly potentiate each other.

Conclusion

1. Infusion of erythropoietin at a dose of 1000 IU/kg before the ischemic period reduces the activity of lipid peroxidation processes and improves the mechanisms of antioxidant defense in simulating the ischemia-reperfusion syndrome of the liver in rats.

2. The protective effect of EPO on the liver during ischemia-reperfusion is realized mainly through an increase in endogenous production of hydrogen sulfide and its gasotransmitter properties, which, in particular, is manifested in the improvement of the prooxidant-antioxidant balance in experimental animals.

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Volume 9 Issue 2 Febuary 2021