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## NITRIC OXIDE EFFECT ON THE HEMOGLOBIN-OXYGEN AFFINITY

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The biological roles of nitric oxide (NO)-hemoglobin (Hb) derivatives are obscure. It is proposed that NO can function as an allosteric regulator of hemoglobin oxygen-binding properties. We aimed to estimate the effects of NO donors and NO-synthase substrate (L-arginine) on hemoglobin-oxygen affinity (HOA) in experiments *in vitro* with the various ratios between NO formed and Hb and various oxygen pressures. HOA index (p50), blood pH, plasma and red blood cell (RBC) concentrations of nitrite/nitrate and methemoglobin amounts were measured after the experiments. In our experiments, blood incubation with NO donors (glyceryltrinitrate, molsidomine, sodium nitroprusside, S-nitrosocysteine) or NO-synthase substrate (L-arginine) did not change HOA even at NO:Hb ratio of 1:1. At the same time our results showed that oxygenated blood incubation with S-nitrosocysteine induced an oxyhemoglobin dissociation curve shift leftwards. This indicates a leading role of met-Hb in a modification of Hb oxygen-binding properties. However other NO-modified forms of hemoglobin (S-nitroso- and nitrosylhemoglobin) also may be involved in the regulation of HOA. The results obtained indicate that nitric oxide can be the allosteric effector of hemoglobin, increasing or decreasing its oxygen affinity - possibly, through the generation of different NO-Hb derivatives.

Key words: *hemoglobin oxygen affinity, nitric oxide, oxyhemoglobin dissociation curve, S-nitrosohemoglobin, nitrosylhemoglobin.*

### INTRODUCTION

Oxygen and carbon dioxide transport is the most important hemoglobin (Hb) function in body (1). In the recent time the understanding of hemoglobin gas-transporting function expanded to include nitric oxide (NO) into the respiratory cycle (2, 3, 4).

Hemoglobin interacts with NO forming the various derivatives. Under the high oxygen pressure NO oxidizes hemoglobin into the ferric species

simultaneously with nitrate generation. Nitrosylhemoglobin ( $\text{HbFe}^{2+}\text{NO}$ ) is a product of another reaction where NO adds to deoxyhemoglobin, with a resulting 6-coordinate or 5-coordinate type of the protein heme moieties (5). Cysteine residue at  $\beta$ -93 position is one additional NO-binding site of hemoglobin (6). This Hb-NO adduct is known as S-nitrosohemoglobin (SNO-Hb).

The biological role of these nitric oxide derivatives of Hb is obscure. In relation to NO, Hb has a role of depot, transporting and utilizing forms of this reactive molecule (7). The hypothesis that describes a relation between tissue oxygen pressure and Hb-NO interaction suggests that red blood cells (RBCs) can homeostatically control the blood and oxygen delivery to oxygen-deficient tissues (8, 9). Under the conditions of local hypoxia, acidosis or low blood flow NO is released from Hb creating the adequate tissue oxygen supply through the changes in regional blood flow (6, 10, 11). Some workers consider SNO-Hb as a source of bioactive NO and key determinant of cardiorespiratory cycle (8).

However, the blood amounts of NO and its derivatives are by several orders of magnitude higher than the levels required for vascular tone regulation (12). Possibly, NO can function as an allosteric regulator of hemoglobin oxygen-binding properties (13, 14).

The presence of different Hb-NO reaction products may variously influence the whole blood oxygen affinity. Higher concentrations of met-Hb and SNO-Hb shift the oxyhemoglobin dissociation curve (ODC) leftwards, and higher  $\text{HbFe}^{2+}\text{NO}$  concentration shifts it rightwards.

We aimed to estimate the effects of NO donors and NO synthase substrate (L-arginine) on hemoglobin-oxygen affinity (HOA) in experiments *in vitro* with the various ratios between NO formed and Hb.

## MATERIALS AND METHODS

*Experiments in vitro.* Heparinized blood was sampled from catheterized jugular vein of rabbits (body weight 3.5-4.5 kg; n = 21) anesthetized by sodium thiopental (50 mg/kg), and used in all the experiments. The blood samples were immediately transferred to anaerobic environment at  $+1^\circ\text{C}$  and stored until use. All investigations were performed within 5-6 hours after the blood sampling. In first series of experiments blood was anaerobically incubated for 60 min at  $37^\circ\text{C}$  with various NO donors (glyceryltrinitrate, molsidomine, L-arginine, sodium nitroprusside (SNP) or S-nitrosocysteine (Cys-S-NO)) to get the molar ratio 1:1 between tetrameric hemoglobin ( $\text{Hb}_4$ ) and NO formed. In second series of experiments blood samples were incubated with SNP or Cys-S-NO for 30 min, with final NO: $\text{Hb}_4$  molar ratio of 1:2. Initially the NO donor was added into the saturator, where the blood was saturated by oxygenating mixture (94.5%  $\text{O}_2$  + 5.5%  $\text{CO}_2$ ). Then blood was mixed with Cys-S-NO or SNP in the deoxygenating saturator, in atmosphere of 94.5%  $\text{N}_2$  + 5.5%  $\text{CO}_2$ . In the third series blood was incubated with Cys-S-NO in both saturators after the preliminary 30-min oxygenation or deoxygenation to get a final molar NO: $\text{Hb}_4$  ratio of 1:2 or 1:4. Blood incubation time with donors in 2<sup>nd</sup> and 3<sup>rd</sup> series was 30 min. In all the experiments the control blood samples were mixed with isotonic 0.9% NaCl. Experiments using the laboratory

animals were approved by of University Ethical Committee on Animal Experiments. During the experimental procedure we followed University guide for the care and use of laboratory animals.

*Determination of hemoglobin-oxygen affinity and blood pH.* P50 - i.e., oxygen pressure under that the oxygenation degree of Hb is 50% - is the HOA index. We measured p50 standard at pH=7.4,  $p\text{CO}_2=40$  mm Hg and 37°C and calculated for actual values of these parameters p50 actual. Standard p50 was determined by a mixing method using the micro gas analyzer ABL-330 ("Radiometer"). ODCs were calculated using Hill's equation. Blood pH was measured with ABL-330 ("Radiometer").

*Determination of total nitrate and nitrite ( $\text{NO}_x^-$ ) concentration.* Nitrate/nitrite amounts in plasma and erythrocytes hemolysate were determined using Griess reagent (15). Sample proteins were sedimented by 30%  $\text{ZnSO}_4$ . After the centrifugation the supernatant was incubated with metal cadmium for 12 hours thereby reducing nitrate to nitrite. Then Griess reagent was added, and total nitrite was measured spectrophotometrically.

*Methemoglobin* was measured with a spectrophotometric hemoglobin cyanide method using Drabkin reagent.

*Reagents.* The following reagents were used: L-arginine, molsidomine, sodium nitroprusside, and glyceryltrinitrate (GTN) (Sigma Chemical Co). S-nitrosocysteine (0.5 M) was prepared immediately before the use mixing 1 M  $\text{NaNO}_2$  in water with 1 M L-cysteine hydrochloride in 0.5 N HCl with 0.5 mM EDTA. Before the adding to blood, Cys-S-NO was diluted to concentration required with 0.1 M phosphate buffer (pH = 7.4) contained 0.5 mM EDTA (16).

*Statistical treatment* of results was carried out with software "Statistica". Data were expressed as means  $\pm$  standard error of mean. Differences between means were evaluated using Student's paired t-test. A value of  $p<0.05$  was considered to be statistically significant.

## RESULTS

In the 1<sup>st</sup> series of experiments blood incubation with GTN, molsidomine, L-arginine, SNP or Cys-S-NO did not change the HOA indices (both standard and actual p50) (Table 1). Total plasma  $\text{NO}_x^-$  rose only when blood was incubated with GTN (by 27.6%,  $p<0.05$ ) or SNP (by 128.6%,  $p<0.05$ ). Only Cys-S-NO significantly decreased blood pH ( $p<0.05$ ) and total  $\text{NO}_x^-$  in RBCs (by 31.1%;  $p<0.05$ ) and increased met-Hb output (by 326.1%;  $p<0.05$ ) (Tab. 1).

In the 2<sup>nd</sup> series of experiments NO donors (Cys-S-NO and SNP) were introduced into the proper saturator with a molar ratio  $\text{NO}:\text{Hb}_4 = 1:2$ . In oxygenated blood treated by Cys-S-NO the standard p50 decreased by  $3.9\pm 0.70$  mm Hg ( $p<0.05$ ), and the actual one - by  $3.4\pm 0.95$  mm Hg ( $p<0.05$ ) (Fig. 1). The met-Hb content in this sample rose by 140.3% ( $p<0.05$ ), but  $\text{NO}_x^-$  concentrations in plasma and RBCs, just as blood pH, were not significantly different from control (Table 2). Incubation of oxygenated blood with SNP did not affect the HOA indices, met-Hb content and blood pH (Tab. 2), but increased plasma  $\text{NO}_x^-$  by 114.5% ( $p<0.05$ ). During the deoxygenation, the same NO donors did not change p50 or pH, but increased the met-Hb amount (by 116.7% with SNP and by 283.3% with Cys-S-NO; both  $p<0.05$ ) and plasma  $\text{NO}_x^-$  (by 164.1% and 58.6%, respectively; both  $p < 0.05$ ). During the deoxygenation, Cys-S-NO also increased  $\text{NO}_x^-$  content in RBCs (by 23.0%).

*Table 1* NO influence on hemoglobin-oxygen affinity, methemoglobin amount and total nitrite/nitrate concentration in plasma and red blood cells (molar ratio NO:Hb = 1:1) (M±m)

Index	Control	Molsidomine	Glyceryltrinitrate	L-arginine nitroprusside	Sodium	S-nitrosocysteine
n	9	8	8	8	8	7
p50 <sub>stand</sub> , mm Hg	28,86±0,95	28,47±0,75	29,21±0,88	29,64±0,79	28,44±0,78	28,93±0,95
p50 <sub>act</sub> , mm Hg	33,5±1,27	32,9±0,66	34,26±1,22	32,55±0,98	34,77±0,99	37,81±1,71
pH, units	7,275±0,022	7,276±0,019	7,268±0,019	7,322±0,022	7,232±0,024	7,173±0,01*
met-Hb, %	0,46±0,19	0,52±0,21	0,77±0,32	0,32±0,18	0,32±0,22	1,96±0,25*
C (NO <sub>x</sub> <sup>-</sup> ) plasma, microM	305,9±15,3	288,2±16,8	390,2±25,2*	281,32±21,0	699,1±37,5*	317,1±41,59
C (NO <sub>x</sub> <sup>-</sup> ) <sub>RBC</sub> , microM	1318,4±63,3	1367,9±62,7	1393,53±62,5	1403,2±56,9	1438,3±58,9	909,0±61,7*

Note: \* - significant differences from control (p< 0.05).

Abbreviations: met-Hb - methemoglobin fraction in total hemoglobin, C (NO<sub>x</sub><sup>-</sup>)<sub>plasma</sub> - total nitrite and nitrate concentration in plasma, C (NO<sub>x</sub><sup>-</sup>)<sub>RBC</sub> - total nitrite and nitrate concentration in red blood cells.

*Table 2* NO influence on hemoglobin-oxygen affinity, methemoglobin amount and total nitrite/nitrate concentrations in plasma and red blood cells after the incubation with oxygenated and deoxygenated blood (molar ratio NO:Hb = 1:2) (M±m)

Index	Control	Sodium nitroprusside		S-nitrosocysteine	
		after oxygenation	after deoxygenation	after oxygenation	after deoxygenation
n	7	7	7	7	7
p50 <sub>stand</sub> , mm Hg	32,33±0,86	30,46±0,9	31,41±0,93	28,41±0,51*	30,15±1,05
p50 <sub>act</sub> , mm Hg	35,43±1,17	33,52±0,87	34,9±1,13	32,03±0,59*	33,8±1,22
pH, units	7,318±0,018	7,313±0,015	7,305±0,023	7,293±0,015	7,304±0,016
met-Hb, %	0,72±0,14	0,57±0,13	1,56±0,23*	1,73±0,27*	2,76±0,4*
C (NO <sub>x</sub> <sup>-</sup> ) <sub>plasma</sub> , microM	254,5±24,9	545,86±29,0*	671,9±54,54*	373,1±50,82	403,55±44,7*
C (NO <sub>x</sub> <sup>-</sup> ) <sub>RBC</sub> , microM	1791,0±82,4	2129,5±135,9	1987,0±198,7	2035,5±124,4	2203,5±163,3*

Note: \* - significant differences from control (p< 0.05).

Abbreviations: met-Hb - methemoglobin fraction in total hemoglobin, C (NO<sub>x</sub><sup>-</sup>)<sub>plasma</sub> - total nitrite and nitrate concentration in plasma, C (NO<sub>x</sub><sup>-</sup>)<sub>RBC</sub> - total nitrite and nitrate concentration in red blood cells.

In the 3<sup>rd</sup> series, the Cys-S-NO effect was studied after the previous blood oxygenation/deoxygenation, at the final NO:Hb<sub>4</sub> ratio of 1:2 or 1:4. Actual p50 in oxygenated blood did not changed at both NO:Hb<sub>4</sub> ratios (*Tab. 3*). However standard p50 decreased by 4.2±1.13 mm Hg (p<0.05) at NO:Hb<sub>4</sub> = 1:2 and by

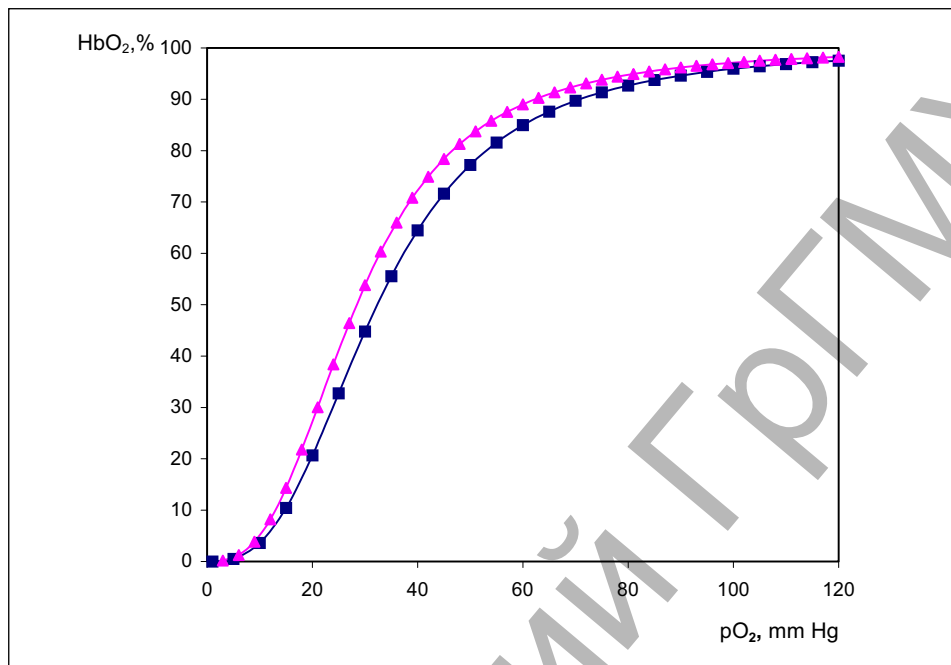


Fig.1. Position of oxyhemoglobin dissociation curve at standard conditions - pH = 7.4, pCO<sub>2</sub> = 40 mm Hg, and T = 37°C - after the incubation of oxygenated blood with S-nitrosocysteine (molar ratio of nitric oxide released to tetrameric Hb is 1:2): ▲- S-nitrosocysteine, ■- control.

Table 3 Effect of S-nitrosocysteine on hemoglobin-oxygen affinity, methemoglobin amount and total nitrite/nitrate concentrations in plasma and red blood cells after the incubation with previously oxygenated/deoxygenated blood (M±m)

Index	Control	Molar ratio of NO released to tetrameric hemoglobin = 1:2		Molar ratio of NO released to tetrameric hemoglobin = 1:4	
		after oxygenation	after deoxygenation	after oxygenation	after deoxygenation
n	6	6	6	6	6
p50 <sub>stand</sub> , mm Hg	32,44±0,75	28,22±0,54*	31,15±1,3	29,58±0,7*	28,03±0,8*
p50 <sub>act</sub> , mm Hg	37,73±0,96	34,62±1,32	37,76±1,03	35,88±0,91	34,5±1,55
pH, units	7,264±0,023	7,226±0,024	7,224±0,02	7,225±0,024	7,218±0,02
met-Hb, %	0,95±0,2	1,88±0,43	3,2±0,38*	1,7±0,16*	1,09±0,19
C (NO <sub>x</sub> <sup>-</sup> ) <sub>plasma</sub> , microM	300,3±26,9	527,57±84,6*	407,6±39,39*	447,65±37,9*	294,47±20,74
C (NO <sub>x</sub> <sup>-</sup> ) <sub>RBC</sub> , microM	740,6±85,2	1077,4±184,4	1142,8±147,3	1060,5±294,6	975,9±154,2

Note: \* - significant differences from control (p < 0.05).

Abbreviations: met-Hb - methemoglobin fraction in total hemoglobin, C (NO<sub>x</sub><sup>-</sup>)<sub>plasma</sub> - total nitrite and nitrate concentration in plasma, C (NO<sub>x</sub><sup>-</sup>)<sub>RBC</sub> - total nitrite and nitrate concentration in red blood cells.

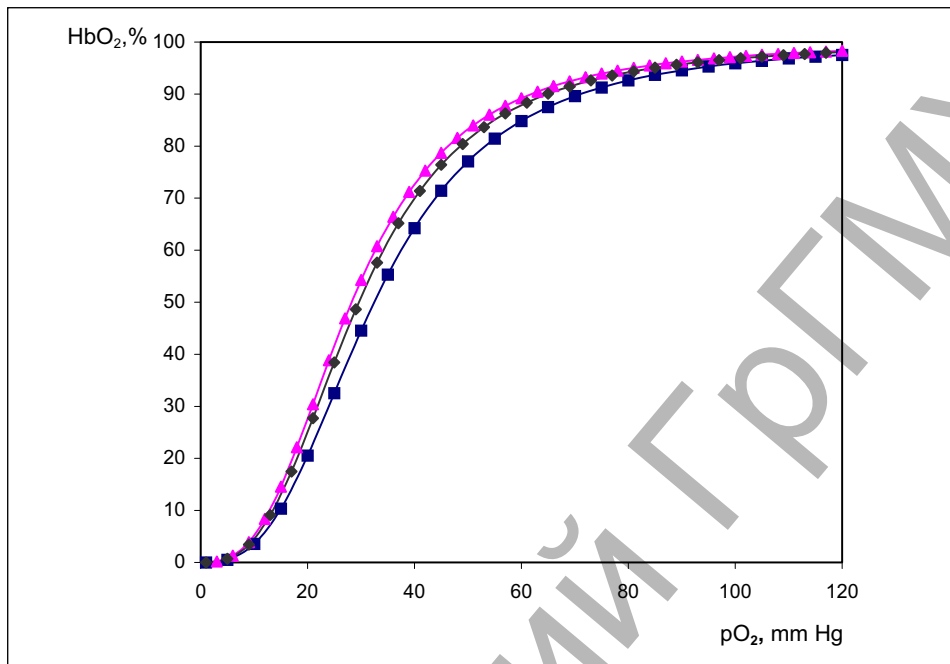


Fig. 2. Position of oxyhemoglobin dissociation curve under the standard conditions - pH = 7.4, pCO<sub>2</sub> = 40 mm Hg, and T = 37°C - after the incubation of previously oxygenated blood with S-nitrosocysteine (molar ratios of nitric oxide released to tetrameric Hb are 1:2 or 1:4): ▲ - treatment, NO:Hb = 1:2, ◆ - treatment, NO:Hb = 1:4, ■ - control.

2.9±0.89 mm Hg ( $p < 0.05$ ) at NO:Hb<sub>4</sub> = 1:4 (Fig. 2). Plasma NO<sub>x</sub><sup>-</sup> significantly rose in these cases by 75.7% and by 49.1%, respectively (both  $p < 0.05$ ), and met-Hb concentration increased only at the lowest NO level (by 78.9%,  $p < 0.05$ ). Incubation of deoxygenated blood with Cys-S-NO at NO:Hb<sub>4</sub> ratio of 1:2 did not change the standard or actual p50 values (Tab. 3). However, the met-Hb amount in this sample rose by 236.8% ( $p < 0.05$ ), and total NO<sub>x</sub><sup>-</sup> - by 35.7% ( $p < 0.05$ ). Lowering of this ratio by half decreased standard p50 (by 4.4±1.39 mm Hg;  $p < 0.05$ ) and did not change other indices (p50 actual, met-Hb and NO<sub>x</sub><sup>-</sup>). Significant changes of blood pH were not observed in this series of studies (Tab. 3).

#### DISCUSSION

We chose high NO:Hb<sub>4</sub> ratios because of the assumption that NO concentrations in arterioles and capillaries much exceed that in larger vessels. Such gradients may be explained by different endothelial NO-synthase activities in different parts of vascular system; immunohistological studies had shown that

it is the highest in arterioles and significantly lower in veins (17). Moreover, the ratio of blood volume to vessel surface is the lowest in terminal arterioles and capillaries. Therefore one can suggest that the fraction of Hb molecules interacting with NO is larger in microcirculatory bed than in other vascular segments (18).

RBCs may regulate NO bioavailability by means of oxygen partial pressure ( $pO_2$ )-dependent allosteric transition between T- and R-conformations of Hb (19, 3, 20). Under high  $pO_2$ , NO may be captured by iron of Hb in R-conformation and then be transferred to  $\beta 93$ -cysteine (21). According to the data about SNO-Hb structure, the NO binding by  $\beta 93$ -cysteine inhibits a formation of salt bridge between  $\beta 146$ -histidine and  $\beta 94$ -asparagine ultimately resulting in higher HOA (22). After the entering in poorly oxygenated tissues, SNO-Hb releases its oxygen, thereby facilitating the transition of Hb to T-conformation (strongly destabilized by the steric influence of SNO-moiety) (21). NO dissociated from Hb in T-structure can interact with heme; such notion is supported by prevalence of  $HbFe^{2+}NO$  in deoxygenated venous blood at levels, inversely related with fraction SNO-Hb (23). Spectroscopic and crystallographic investigations of  $HbFe^{2+}NO$  generation confirm the break of bond between  $Fe^{2+}$ -heme in  $\alpha$ -chain and proximal histidine (20) that must facilitate the Hb deoxygenation.

Several workers confirm the different HOAs for different NO derivatives of Hb. Thus,  $p50$  of extracellular Hb is less than 10 mm Hg (24), whereas it is  $4.3 \pm 0.27$  mm Hg for Hb containing 30% SNO-Hb (25). The presence of  $HbFe^{2+}NO$  increases a  $p50$  of Hb solution to  $39.6 \pm 1.5$  mm Hg (5). In patients with sickle cell disease that breathed by air with low NO content for 45 min HOA rose (26). Incubation with various amounts of NO or its donors (Angeli salt etc.) induced the ODC shift leftwards linearly correlated with met-Hb level, thereby suggesting a leading role of met-Hb in a modification of Hb oxygen-binding properties (27).

In our experiments, blood incubation with NO donors (GTN, molsidomine, SNP, Cys-S-NO) or NO-synthase substrate (L-arginine) did not change HOA even at NO:Hb<sub>4</sub> ratio of 1:1, despite of significant met-Hb increase and pH lowering with Cys-S-NO.

Our results showed that oxygenated blood incubation with Cys-S-NO induced an ODC shift leftwards, and the presence of met-Hb may be only one of the causes for such shift. In the previously oxygenated blood mixed with Cys-S-NO the met-Hb amount rose insignificantly, but the value of  $p50$  was even lower than in experiment without the preliminary oxygenation (when met-Hb increased significantly). The ODC shifts leftwards observed in experiment with preliminary oxygenation (NO:Hb<sub>4</sub>=1:2) may be due with presence of both met-Hb and SNO-Hb. Some experimental data (28) confirm the generation of SNO-Hb during the blood incubation with Cys-S-NO.

During the deoxygenation, blood interaction with NO donors also increases the met-Hb contents but without the expected lowering of  $p50$ . Hb oxidation results into nitrite production, with possible following NO regeneration in

reaction with deoxy-Hb after the  $pO_2$  lowering. Such reaction initially gives an intermediate  $HbFe^{3+}NO$  (29, 30). This compound is unstable and releases NO that can be captured by deoxygenated heme with formation of  $HbFe^{2+}NO$  (29, 31). Possibly, the simultaneous presence of different Hb-NO reaction products during the deoxygenation results in bidirectional effects on ODC position and ultimately does not change the  $p50$ .

Analysis of our results shows that effects of NO donors on HOA are determined by conditions of blood oxygenation and molar ratios between NO released and blood Hb; experiments with deoxygenation demonstrate this most markedly. If after the previous deoxygenation the NO:Hb ratio is 1:4, ODC is shifted leftwards despite of the constant met-Hb amount. The higher HOA observed may be due to SNO-Hb formation. Under conditions of these experiments the SNO-Hb production probably resulted from nitrosating reagent generation when NO slowly dissociated from complex with heme and reacted with oxygen (32, 31). According to Stamler, Gow (23), the oxygenation of blood containing partially iron-nitrosylated Hb promotes such  $O_2$ -dependent SNO-Hb formation.

In earlier experiments when NO donor was incubated with venous blood the value of  $p50$  lowered due to higher blood pH (33). In our investigations, the HOA changes did not correlated with the changes in blood pH thereby suggesting a leading role of NO in HOA regulation under our experimental conditions.

In patients with a congestive heart failure, Hb oxygenation in pulmonary circulation resulted in enhancing gradients of Hb-NO derivatives - with higher SNO-Hb and lower  $HbFe^{2+}NO$  levels; such gradients correlated with blood oxygen saturation (9). However the fractions of SNOHb and  $HbFe^{2+}NO$  in blood in vivo are only 0.1-1% from total Hb, seemingly making small the possible contribution of these Hb species into the whole blood oxygen-binding regulation (34). But taking in account the large microcirculatory endothelial surface, high NO synthase activity and relatively small blood volume one can suggest that at the level of microcirculatory bed the concentrations of Hb-NO reaction products may be important for gas exchange (18).

The results obtained indicate that nitric oxide can be the allosteric effector of hemoglobin, increasing or decreasing its oxygen affinity - possibly, through the generation of different NO-Hb derivatives.

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