UDC: [577.127.3+57.044:546.172.6]

Вплив геміну і донорів оксиду азоту на показники метаболізму гему в печінці й сироватці крові щурів *in vivo*

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В роботі вивчено вплив *in vivo* хлориду геміну (15 мг/кг маси тіла) і донорів монооксиду азоту (NO) – нітропрусиду натрію (SNP, 1 мг/кг) і субстрату NO-синтази L-аргініну (Arg, 600 мг/кг) на активність ключових ферментів синтезу (5-амінолевулінатсинтази, АЛКС) і деградації гему (гемоксигенази, ГО), на вміст вільного гему в печінці, а також на вміст гему в сироватці крові щурів. Донори NO вводили окремо або за 30 хв до ін'єкції хлориду геміну. Рівень вільного гему в печінці оцінювали за співвідношенням активності холоферменту і загальної активності триптофан-2,3-діоксигенази (ТДО). Через 2 год після введення хлориду геміну спостерігалося значне підвищення рівня продуктів, які містять гем, і продуктів пероксидації ліпідів (ТБКРП) в сироватці крові. Ці зміни супроводжувалися зниженням активності АЛКС і збільшенням активності холоферменту і насичення гемом ТДО, що є результатом накопичення в печінці вільного гему. Через 24 год після введення хлориду геміну вміст гему в сироватці нормалізувався, а рівень ТБКРП залишався підвищеним. У печінці через 24 год дії геміну спостерігалось значне підвищення активностей ГО і АЛКС, тоді як ступінь насичення ТДО гемом знижувався, що свідчить про превалювання процесу деградації гему над його синтезом. Обидва донора NO не впливали на накопичення гему в сироватці й печінці в перші години дії геміну. Однак встановлені особливості дії SNP і L-Arg на ключовий фермент синтезу гему в печінці і рівень ТБКРП в сироватці крові. L-Arg, на відміну від SNP, запобігав накопиченню ТБКРП в сироватці, але не попереджав зниження активності АЛКС через 2 год після ін'єкції хлориду геміну. Введення самого SNP викликало підвищення рівня ТБКРП в сироватці, збільшення активності ТДО і зниження активності АЛКС в печінці через 2 год. Вміст гему в сироватці позитивно корелював з активністю холоферменту і насиченням гемом ТДО в печінці. Попередня обробка донорами NO не впливала на підвищення активності ГО, однак блокувала індукцію АЛКС, зниження активності холоферменту і ступеня насичення гемом ТДО через 24 год після введення хлориду геміну. Отже, й SNP, й Arg запобігали зниженню рівня вільного гему в печінці, що може бути пов'язано з нітрозилюванням гему в присутності донорів NO і, як наслідок, його більш повільною деградацією в гемоксигеназній реакції.

Ключові слова: *метаболізм гему, печінка, сироватка крові, гемін, донори оксиду азоту.*

In vivo effects of hemin and nitric oxide donors on parameters of heme metabolism in rat liver and serum

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In vivo effects of hemin chloride (15 mg/kg body weight) and donors of nitrogen monoxide (NO) – sodium nitroprusside (SNP, 1 mg/kg) and substrate of NO-synthase L-arginine (L-Arg, 600 mg/kg) on the activity of key enzymes of heme synthesis (5-aminolevulinate synthase, ALAS) and heme degradation (heme oxygenase, HO), on the free heme level in liver and on the content of heme in blood serum of rats were studied. NO donors were administered alone or 30 min before hemin chloride injection. The level of free heme in liver was estimated by the ratio of holoenzyme and total tryptophan 2,3-dioxygenase (TDO) activities. Two hours after hemin chloride administration a significant increase in the level of heme-containing products and lipid peroxidation products (TBARS) was found in blood serum. These changes were accompanied by decrease in ALAS activity and by increase in holoenzyme activity and heme saturation of TDO, which was the result of free heme accumulation in liver. 24 hrs after administration of hemin chloride the content of heme in serum returned to normal level, while level of TBARS remained elevated. 24 hrs after hemin action a significant increase in the activities of HO and ALAS was observed in liver, while the degree of TDO heme saturation decreased, indicating the prevalence of heme degradation over its synthesis. Both NO donors did not affect the accumulation of heme in serum and liver first hours after hemin action. However, the specific features of SNP and L-Arg effects on the key enzyme of heme synthesis in liver and the TBARS level in serum were revealed. L-Arg, unlike SNP, prevented the accumulation of TBARS in serum, but did not prevent a decrease in ALAS activity 2 hrs after hemin chloride injection. The treatment by SNP itself caused an increase in TBARS level in serum, an increase in TDO activity and a decrease in ALAS activity in liver 2 hrs after action. Heme content in serum positively correlated with holoenzyme activity and heme saturation of TDO in liver. The pretreatment with NO donors did not affect the increase in HO activity, however, it blocked the induction of ALAS, a decrease in holoenzyme activity and heme saturation of TDO 24 hrs after the administration of hemin chloride. Thus, both SNP and Arg prevented a decrease in free heme level in liver, which might be due to heme nitrosylation in the presence of NO donors and, as a result, its slower degradation in the heme oxygenase reaction.

Key words: *heme metabolism, liver, blood serum, hemin, nitric oxide donors.*

DOI: 10.26565/2075-5457-2018-31-2

Влияние гемина и доноров оксида азота на показатели метаболизма гема в печени и сыворотке крови крыс *in vivo*

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В работе изучено влияние *in vivo* хлорида гемина (15 мг/кг массы тела) и доноров монооксида азота (NO) – нитропруссида натрия (SNP, 1 мг/кг) и субстрата NO-синтазы L-аргинина (L-Arg, 600 мг/кг) на активность ключевых ферментов синтеза (5-аминолевулинатсинтазы, АЛКС) и деградации гема (гемоксигеназы, ГО), на содержание свободного гема в печени, а также на содержание гема в сыворотке крови крыс. Доноры NO вводили отдельно или за 30 мин до инъекции хлорида гемина. Уровень свободного гема в печени оценивали по соотношению активности холофермента и общей активности триптофан-2,3-диоксигеназы (ТДО). Через 2 ч после введения хлорида гемина наблюдалось значительное повышение уровня гем-содержащих продуктов и продуктов пероксидации липидов (ТБКРП) в сыворотке крови. Данные изменения сопровождались снижением активности АЛКС и увеличением активности холофермента и насыщения гемом ТДО, что является результатом накопления в печени свободного гема. Через 24 ч после введения хлорида гемина содержание гема в сыворотке нормализовалось, а уровень ТБКРП оставался повышенным. В печени через 24 ч действия гемина отмечено значительное повышение активности ГО и АЛКС, тогда как степень насыщения ТДО гемом снижалась, что свидетельствует о превалировании процесса деградации гема над его синтезом. Оба донора NO не влияли на накопление гема в сыворотке и печени в первые часы действия гемина. Однако установлены особенности действия SNP и L-Arg на ключевой фермент синтеза гема в печени и уровень ТБКРП в сыворотке крови. L-Arg, в отличие от SNP, предотвращал накопление ТБКРП в сыворотке, но не предупреждал снижения активности АЛКС через 2 ч после инъекции хлорида гемина. Введение самого SNP вызывало повышение уровня ТБКРП в сыворотке, увеличение активности ТДО и снижение активности АЛКС в печени через 2 ч. Содержание гема в сыворотке положительно коррелировало с активностью холофермента и насыщением гемом ТДО в печени. Предобработка донорами NO не влияла на повышение активности ГО, однако блокировала индукцию АЛКС, снижение активности холофермента и степени насыщения гемом ТДО через 24 ч после введения хлорида гемина. Таким образом, и SNP, и L-Arg предотвращали снижение уровня свободного гема в печени, что может быть связано с нитрозилированием гема в присутствии доноров NO и, как следствие, его более медленной деградацией в гемоксигеназной реакции.

Ключевые слова: *обмен гема, печень, сыворотка крови, гемин, доноры оксида азота.*

Introduction

Heme (Fe^{2+}-protoporphyrin) is ubiquitously used as a prosthetic group in various hemoproteins whose renewal is based on the constant heme turnover (Ponka, 1997). Each step of heme metabolism including heme synthesis, transport and degradation, is critically dependent on specific proteins and is tightly regulated (Furuyama et al., 2007). “Free” (not incorporated in hemoproteins) heme is strong lipophylic prooxidant able to damage biomolecules and biomembranes (Kumar, Bandyopadhyay, 2005). On the other hand, free heme is known as a signal molecule with multiple regulatory functions that, first of all, controls its own metabolism (Furuyama et al., 2007; Ponka, 1997; Ayer et al., 2016). Key enzyme of heme biosynthesis, 5-aminolevulinate synthase (ALAS, EC 2.3.1.37), is regulated by heme at both transcriptional and posttranscriptional levels (Ponka, 1997). Key enzyme of heme degradation, heme oxygenase (HO, EC 1.14.14.18), has two isoforms, inducible HO-1 and constitutive HO-2, both regulated by heme level but through different mechanisms (Wu, Wang, 2005; Yi, Ragsdale, 2007).

Free heme in mammals liver can originate from new heme synthesis as well as from degraded hemoproteins including heme of hemoglobin that under intravascular hemolysis is transported into hepatocytes (Smith, Morgan, 1985; Ponka, 1997). Various intracellular proteins able to bind free heme are known, and one of them is rat liver enzyme, tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11), that exists in two forms, heme-bound holoenzyme and apoenzyme, whose proportion depends on free heme level (Badawy, 2017).

Free as well as protein-bound heme can be a target for nitrogen monoxide (NO) that is synthesized from L-arginine by NO-synthases (NOS) and acts as an intracellular signal molecule (Treuer, Gonzalez, 2015). Although NO is a lipophilic radical molecule with strong affinity for heme iron (Bloodsworth et al., 2000), the formation of heme-nitrosyl complexes can limit heme participation in oxidative processes and therefore restrict heme prooxidant action (Osipov et al., 2007). NO is shown to have contradictory effect on heme oxygenase activity: it induces HO-1 at transcriptional level (Wu, Wang, 2005) but inhibits both HO-1 and HO-2 activities through heme nitrosylation in heme-binding sites (Ding et al., 1999; Kinobe et al., 2004). The regulation of ALAS and TDO activities by NO under free heme accumulation in liver has

not been deeply investigated. NO synthesis by NOS could be inhibited under stress or arginine deficiency, therefore direct NO donors are widely used to bypass this limitation (Cavicchi et al., 2000).

Taking all this into account, we studied the effects of NO donor sodium nitroprusside and NOS substrate L-arginine on the activities of the key enzymes of heme metabolism, as well as free heme level in the rat liver and heme level in blood serum under hemin action *in vivo*.

Materials and methods

Wistar male rats (160–200 g) used in the study were divided into 6 experimental groups. Hemin was dissolved in minimum volume of 1M NaOH and diluted 40 times by 0.9% NaCl. Hemin stock solution was injected intraperitoneally at final dose 15 mg/kg body weight (group 'Hemin'). Control animals (group 'Control') were injected with the corresponding volume of 0.9% NaCl. Sodium nitroprusside (SNP) was dissolved in 0,9% NaCl and administered intraperitoneally at final dose 1 mg/kg alone (group 'SNP') or 30 min before hemin chloride injection (group 'Hemin+SNP'). L-Arginine (L-Arg) was dissolved in minimal volume of 1N HCl, the solution was neutralized by 2N NaOH to pH 7.0, and then 0.9% NaCl was added to reach the required volume. L-Arg was administered intraperitoneally in dose 600 mg/kg alone (group 'L-Arg') or 30 min before hemin chloride injection (group 'Hemin+L-Arg'). The animals were decapitated under light ether anesthesia 2 or 24 hours after hemin chloride (groups 'Hemin', 'Hemin+SNP', 'Hemin+L-Arg) and 2,5 or 24,5 hrs after NO-donors administration (groups 'SNP' and 'L-Arg').

Blood was collected to obtain serum. The liver was perfused *in situ* with cooled physiological saline. Subcellular fractions were obtained by standard differential centrifugation at 2°C.

5-Aminolevulinate synthase activity was measured in liver homogenates by the amount of 5-ALA (Marver et al., 1966) and expressed in nmol 5-ALA/h per mg protein. Heme oxygenase activity was measured in post-mitochondrial fraction of liver and estimated by the amount of bilirubin using extinction coefficient 4×10^4 $M^{-1}cm^{-1}$ and was expressed as nmol bilirubin/min per mg protein (Sardana et al., 1985). Methemalbumin used as the substrate was formed *in situ* from hemin (final concentration 0.033 mM) and human serum albumin (final concentration 0.0025 mM).

Tryptophan 2,3-dioxygenase enzyme activity was measured in post-mitochondrial fraction of liver without hemin addition (holoenzyme activity) or after addition of hemin chloride in final concentration 0,002 mM (total activity). Both holoenzyme and total activity were estimated by the amount of kynurenine generated from L-tryptophan, the data expressed in nmol kynurenine/h per mg protein (Badawy, Evans, 1973). Heme saturation of TDO was calculated as the ratio of holoenzyme activity to total activity and expressed as a percentage.

The content of lipid hydroperoxides (TBARS) was measured in serum and in post-mitochondrial fraction of liver by the reaction with thiobarbituric acid (Ohkawa et al., 1979) and expressed in equivalent amounts of malonic dialdehyde (MDA) using coefficient of molar extinction $1{,}56\cdot10^5$ $M^{-1}\cdot cm^{-1}$. The level of heme-containing compounds in serum was estimated by optical density in the Soret region (390–450 nm), and expressed in ΔA/mg protein (Hrkal, Muller-Eberhard, 1971). Protein content was determined by Lowry method modified by Miller using bovine serum albumin as the standard (Miller, 1959).

Statistical processing of the data was performed using parametric methods. A threshold $p<0.05$ was considered statistically significant.

Results and discussion

Hemin chloride significantly increased serum absorbance in Soret region that reached 340% (group 'Hemin+L-Arg'), 390% (Hemin) and 430% (Hemin+SNP) of the control level two hours after hemin chloride injection (Table 1).

This makes evidence on the accumulation of heme-containing compounds that could originate from erythrocyte lysis and/or exogenous hemin presence. Pretreatment by NO donors didn't prevent and NO-donors by themselves didn't cause heme accumulation in serum at this period of time. 24 hrs after hemin chloride injection serum absorbance in Soret region was at control level except co-treatment with SNP wherein this parameter was 2.7 times higher than control (Table 1) that could be the sign of more prolonged circulation of hemolysis products.

Two hours after hemin and/or SNP treatment the content of TBARS in serum was increased to 155–160 % of control level while L-Arg prevented hemin-induced raise of TBARS level (Table 1). Free heme molecule is strong lipophilic prooxidant able to interact with cell membranes and low density

lipoproteins (Kumar, Bandyopadhyay, 2005). Moreover, iron ion released from heme could catalyze free radical processes, therefore high TBARS level can be the result of free heme accumulation.

Table 1.

The absorbance in Soret region and TBARS content of rat serum after hemin chloride and NO donors injection (M±m, n=5–6, **p*<0.05 versus control values)

Experimental group	Absorbance in Soret region, ΔA/mg protein		TBARS content, nmol MDA/mg protein	
	2 hrs	24 hrs	2 hrs	24 hrs
Control	0,035±0,005	0,034±0,006	1,00±0,09	1,00±0,09
Hemin	0,130±0,014*	0,034±0,006	1,64±0,15*	1,40±0,11*
SNP+Hemin	0,142±0,035*	0,088±0,013*	1,57±0,21*	1,54±0,11*
L-Arg+Hemin	0,112±0,018*	0,037±0,006	1,28±0,12	1,58±0,12*
SNP	0,032±0,004	0,022±0,004	1,49±0,09*	0,97±0,18
L-Arg	0,028±0,004	0,030±0,008	1,02±0,12	1,03±0,11

SNP and L-Arg revealed different effects on TBARS level in blood serum two hours after hemin chloride injection. Pretreatment by L-Arg prevented TBARS accumulation induced by hemin while SNP did not affect this parameter. The antioxidant and antiradical properties of L-Arg were shown in model systems both *in vitro* and *in vivo* wherein L-Arg was able to limit superoxide formation and lipid peroxidation (Milutina et al., 1990). On the other hand, L-Arg can be utilized for synthesis of NO that has dual antioxidant role. Firstly, it promotes the formation of heme-nitrosyl complexes (NO-Fe-protoporphyrin IX) that restricts prooxidant effects of heme (Osipov et al., 2007) and secondly, it can break free radical chain reactions through direct interaction with alkoxyl and peroxyl radicals (Chamulitrat, 1998). In contrast to L-Arg SNP is known as prooxidant (Nazari et al., 2012) and the increased TBARS level found in blood 24 hours after only SNP injection (Table 1) is consistent with this data. L-arginine by itself didn't cause the accumulation of TBARS at both periods.

The TBARS level in serum was greatly increased 24 hrs after hemin chloride injection (140–158 % of control) independently of the presence of NO donors. High level of TBARS under normal level of heme-containing compounds in serum at this time can make evidence on heme binding with blood or endothelial cells first hours after action that intensified lipid peroxidation (Balla et al., 1993).

Hemin caused significant increase in TDO holoenzyme activity in rat liver two hours after injection (Table 2), which was not prevented by NO donors. The most pronounced raise of this parameter (350% of control level) was revealed under hemin and SNP co-treatment.

It should be noted that 2 hrs after injection (Table 2) SNP by itself increased not only holoenzyme TDO activity (177% of control) similar to action of hemin but also increased total (153%) TDO activity. The raise of total TDO activity might be due to apoenzyme stabilization by heme and/or activation of apoenzyme synthesis de novo at transcription level under glucocorticoid action (Badawy, 2017).

Table 2.

The holoenzyme and total enzyme activity of TDO in rat liver after hemin chloride and NO donors injection (nmol kynurenine/h per mg protein; M±m, n=5–6, **p*<0.05 versus control values)

Experimental group	TDO holoenzyme activity		TDO total activity	
	2 hrs	24 hrs	2 hrs	24 hrs
Control	6,33±1,17	6,78±1,41	20,36±1,51	21,52±4,16
Hemin	16,95±2,62*	2,81±0,79*	21,90±2,83	15,39±3,46
SNP+Hemin	20,55±2,16*	5,17±1,16	24,34±1,79	29,62±4,85
L-Arg+Hemin	13,67±2,58*	3,88±0,35	19,80±2,70	19,15±4,41
SNP	11,02±0,84*	3,43±0,65	31,90±2,45*	19,13±4,23
L-Arg	7,06±0,71	7,80±1,62	18,74±1,99	16,06±4,68

24 hrs after hemin treatment TDO holoenzyme activity sharply decreased to 41% of control level that was prevented by NO donors. Total TDO activity was at the control level 2 hrs and 24 hrs after hemin chloride injection independently of pretreatment by NO donors (Table 2).

The free heme level in rat liver can be evaluated by the tryptophan-2,3-dioxygenase apoprotein heme saturation. This parameter was increased 2.3 times two hours after hemin action from 33% to almost 77% that made evidence on free heme accumulation in cytosolic fraction of rat liver (Table 3). The early raise of TDO heme saturation was not prevented by NO donors.

The decrease in TDO heme saturation 24 hrs after hemin action (59% of control) accompanied by the decrease in holoenzyme activity but not by apoenzyme induction or stabilization (Table 3) was the sign of heme deficiency (Badawy, 2017). Such changes could be the result of the inhibition of heme synthesis and induction of heme degradation (Kaliman et al., 1989). Both SNP and L-Arg prevented the decrease in TDO heme saturation 24 hrs after hemin treatment. Changes in free heme level were not followed by alterations of TBARS level in rat liver (Table 3). In spite of SNP effect on TDO activity, TDO heme saturation didn't change after SNP action (Table 3).

Table 3.

The heme saturation of TDO and TBARS content of rat liver after hemin chloride and NO donors injection (M±m, n=5–6, **p*<0.05 versus control values)

Experimental group	TDO heme saturation, %		TBARS content, nmol MDA/mg of protein	
	2 hrs	24 hrs	2 hrs	24 hrs
Control	33,27±4,35	30,64±2,30	0,90±0,14	0,90±0,14
Hemin	76,60±5,96*	17,95±0,93*	1,00±0,19	0,84±0,05
SNP+Hemin	89,64±2,54*	34,21±7,08	0,91±0,20	0,71±0,18
L-Arg+Hemin	66,64±9,58*	32,50±9,43	0,91±0,09	0,70±0,15
SNP	42,38±10,04	20,65±5,63	0,60±0,09	0,71±0,08
L-Arg	39,45±4,96	47,95±12,95	0,82±0,10	0,69±0,07

It is known that hepatocytes have receptor-mediated mechanism for capture of extracellular heme complexed with hemopexin with its further transport by intracellular heme-binding proteins to endoplasmic reticulum for degradation in heme oxygenase reaction (Smith, Morgan, 1985). Specific protein-mediated heme traffic into hepatocytes does not cause the activation of free radical processes (Eskew et al., 1999). Overloading of this system could result in non-specific heme transport into hepatocytes, increase in free heme pool in membranes and cytosol with promotion of lipid peroxidation (Balla et al., 1993). The accumulation of heme-containing products in serum and free heme in liver revealed first hours after hemin chloride injection (Table 1) was not accompanied by increase in TBARS content in liver (Table 3). So we can suppose the maintenance of capacity of heme-binding systems in blood plasma after this dose of hemin.

The activity of the key enzymes of heme metabolism in liver was revealed to be differently affected by NO-donors (Table 4). ALAS activity altered in two-phase manner with decrease to 50% of control level 2 hrs after hemin chloride injection and the increase to 230% 24 hrs after treatment. According to our previous data, the activity of ALAS was decreased for at least 6 hrs while the raise of ALAS activity was firstly observed 18 hrs after administration of hemin chloride in dose of 15 mg/kg b.w. (Kaliman et al., 1989). Such kind of ALAS dynamics is due to free heme level oscillations in liver cells (Braidotti et al., 1993). The accumulation of free heme is known to inhibit ALAS synthesis at transcriptional and translational levels, to destabilize ALAS mRNA and to inhibit ALAS precursor transport into mitochondria (Furuyama et al., 2007). The exhaustion of free heme pool on the contrary causes the activation of ALAS synthesis.

The decrease in ALAS activity by hemin 2 hrs after injection was prevented under co-treatment with SNP but not with L-Arg. It should be noted that SNP by itself caused the decrease in ALAS activity (to 56% of control). The similar inhibition of erythroid-specific ALAS2 in murine reticulocytes under SNP action was shown by Mickael with co-authors (Mikhael et al., 2013). Both NO donors blocked the increase in ALAS activity in the second phase of hemin action that could be due to relatively high heme concentration in liver cells according to TDO heme saturation (Table 3).

HO activity increased to 215% of control level 24 hrs after hemin chloride injection (Table 4). Liver cells have two isozymes of HO, inducible HO-1 and constitutive HO-2. Heme oxygenase-1 gene expression is known to be induced through heme responsive transcription factors under various stress and pathological conditions accompanied by free heme accumulation (Furuyama et al., 2007). The raise of inducible HO-1 activity may lead not only to degradation of excessive free heme but also to less availability of heme for synthesis of hemoproteins such as microsomal cytochrome P450 (Kaliman et al., 1989).

Table 4.

The activities of ALAS and heme oxygenase (HO) in rat liver after hemin chloride and NO donors injection (M±m, n=5–6, **p*<0.05 versus control values)

Experimental group	ALAS activity, nmol 5-ALA/h per mg protein		HO activity, nmol bilirubin/min per mg protein	
	2 hrs	24 hrs	2 hrs	24 hrs
Control	0,050±0,007	0,050±0,007	0,032±0,005	0,034±0,004
Hemin	0,025±0,007*	0,115±0,021*	0,030±0,003	0,073±0,008*
SNP+Hemin	0,034±0,011	0,057±0,013	0,028±0,001	0,073±0,004*
L-Arg+Hemin	0,025±0,006*	0,039±0,010	0,024±0,002	0,061±0,004*
SNP	0,028±0,004*	0,058±0,012	0,029±0,004	0,041±0,005
L-Arg	0,043±0,004	0,030±0,006	0,026±0,001	0,035±0,002

Hemin and NO donors, including SNP, are known to synergistically stimulate HO-1, thereby they strengthen intracellular heme accumulation (Foresti et al., 2003). Basing on this data we expected the activation of heme transfer from blood to liver and HO induction under co-treatment by hemin and NO-donors. Nevertheless, no significant difference was revealed in free heme level and the degree of HO-activity increase in liver between only hemin action and its co-action with NO-donors.

NO is known to have various effects on HO activity. NO was shown to activate HO-1 expression in rat aortic smooth muscle cells, to increase both HO-1 mRNA stability in human fibroblast cells (Wu, Wang, 2005) and HO-1 induction by heme in endothelial cells (Foresti et al., 2003). Peroxynitrite that is one of NO metabolites is the inhibitor of HO activity through the nitration of tyrosine residues in HO-1 and cysteine residues in HO-2 (Kinobe et al., 2004). The inhibitory effect of NO on heme oxygenase was also shown under action of NOS inhibitors (Mayer et al., 2003). In our experiments NO donors affected neither the basal level of HO activity nor the induction of HO by hemin (Table 4).

To sum up, hemin chloride *in vivo* (15 mg/kg b.w.) caused the significant increase in both heme-containing products and TBARS levels in blood serum two hours after injection. Heme income from blood replenished the free heme pool in liver that was confirmed by the inhibition of ALAS activity and by the increase in TDO holoenzyme activity as well as TDO heme saturation first hours after hemin chloride injection. No activation of free radical processes in liver testified that heme transport from blood to liver for degradation was performed by the help of specific transporters. 24 hrs after hemin chloride injection heme content in serum was normalized while TBARS level in serum was still higher than in control. The raise of HO activity in liver at this period could cause the fall of free heme level revealed by the decrease in TDO holoenzyme activity and heme saturation as well as the increase in ALAS activity.

Both NO donors were unable to prevent the accumulation of heme in serum and liver 2 hrs and the activation of lipid peroxidation in serum 24 hrs after hemin chloride injection. Nevertheless ALAS activity was not inhibited after co-treatment by hemin with SNP while only hemin as well as only SNP decreased its activity. SNP by itself did not cause heme accumulation in serum but increased TDO holoenzyme activity 2 hrs after injection.

Possible source for excessive heme that affected both ALAS and TDO could be the inhibition of heme degradation due to heme nitrosylation under NO release from SNP as direct NO donor (Wang et al., 2003). L-Arg but not SNP was effective in prevention of TBARS accumulation in serum 2 hrs after hemin action.

SNP delayed the normalization of heme level and did not protect from activation of lipid peroxidation in serum first hours after hemin chloride injection. Ability of SNP alone to cause TBARS accumulation in serum and to affect TDO and ALAS activities in liver makes it less safe towards cellular

metabolism than L-Arg. Thus direct NO donor SNP and L-Arg that is the substrate of NOS and has antioxidant properties (Milutina et al., 1990) revealed different effects on prooxidant indexes in blood and heme metabolism in liver.

Heme content in serum revealed strong positive correlation with TDO holoenzyme activity (r=0.83; p=0.002) or TDO heme saturation in liver (r=0.79; p=0.004) but no significant correlation with TBARS level in serum. ALAS activity negatively correlated with TDO heme saturation (r= –0.61; p=0.045). These data confirm the dependence of ALAS activity on heme level as well as the role of blood heme as potential source for free heme pool in liver. While lipid peroxidation in serum was not directly dependent on heme level under investigated dose of hemin.

Intracellular heme concentration is known to be strictly controlled at the levels of its synthesis, degradation, transport and incorporation into hemoproteins (Furuyama et al., 2007). The imbalance of these processes leads to oscillations of free heme pool whose increase under NO accumulation could cause heme nitrosylation. Heme-nitrosyl complexes formed under high NO concentration were shown to reversibly inhibit heme oxygenase that resulted in the decrease in the level of end product of heme degradation, bilirubin (Wang et al., 2003). In the experiments with glycerol model of rhabdomyolysis we also showed the decrease in bilirubin level in blood and the accumulation of heme in tissues caused by L-Arg injection (Nikitchenko et al., 2011).

Under hemin treatment both NO donors did not affect heme accumulation in liver first hours but prevented the decrease in heme level 24 hrs after injection. The absence of ALAS induction 24 hrs after co-action of hemin and NO-donors was accompanied by the maintenance of TDO holoenzyme activity and heme saturation at control level unlike after only hemin action. It should be noted that HO activity was two-fold increased at this period of time but this did not lead to the lack of heme for TDO. Thus, both SNP and L-Arg prevented a decrease in free heme level in liver, which might be due to its nitrosylation in the presence of NO donors and, as a result, slower degradation in the heme oxygenase reaction.

The ability of both NO donors to affect *in vivo* free heme level and key enzymes of heme metabolism in liver has to be taken into account under NO therapy of pathological states accompanied by increased hemolysis and hemoproteins degradation.

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Представлено: Н.І.Горбенко / Presented by: N.I.Gorbenko
Рецензент: Є.Е.Перський / Reviewer: Ye.E.Persky
Подано до редакції / Received: 10.10.2018

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