SPECTRAL STUDIES: STRUCTURAL AND FUNCTIONAL MODIFICATIONS OF OXYHEMOGLOBIN AND DEOXYHEMOGLOBIN DURING THE INTERACTION WITH NO UNDER DIABETES MELLITUS

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S u m a r y. In vitro electronic absorption spectra of deoxyhemoglobin transition into nitrosylhemoglobin have been receiver with two characteristic absorption peaks (544.8 nm and 572.2 nm) for HbNO. The article carries the findings of the research study into the process of in vitro hemoglobin nitrosation in the norm and under experimental streptozotocin-induced diabetes mellitus (6 mg/100 g of mass). The study has revealed an increase in transition time of deoxyhemoglobin into nitrosylhemoglobin under type 1 diabetes mellitus compared with the control group. The process of in vitro deoxyhemoglobin nitrosation has been accelerated in both groups through the introduction of aminoguanidine, a selective inhibitor of inducible NO-synthase isoform, in the concentration of 1 g/1 in drinking water of test animals. Additionally the study shows that deoxyhemoglobin in rats with diabetes mellitus displays higher nitrite reductase activity compared with the control group. Aminoguanidine introduction has led to a decrease in deoxyhemoglobin nitrite reductase activity in rats with experimental diabetes mellitus.

K e y w o r d s: experimental diabetes mellitus, deoxyhemoglobin, nitrosylhemoglobin, aminoguanidine.

INTRODUCTION

The normal course of physiological and biochemical processes in humans and animals is associated in many ways with the structural and functional stability of hemoglobin, the main protein of erythrocytes, as well as with its ability to form the oxygen complex, which is supplied to the organs and tissues. Apart from O2, hemoglobin can form stable ligand forms with other compounds of both endogenic and exogenic origin, which causes the disorder of deoxyhemoglobin (RHb) dynamic equilibrium and a decrease in blood oxygen capacity. hydrogen sulfide (H2S), nitrogen monoxide (NO), carbon monoxide (CO), cyanides, aniline, phenylhydrazine, xylene, toluene etc are among compounds of exogenic origin capable of making changes to the functional activity of hemoglobin. Such a wide diversity of chemical compounds in the environment does not rule out the possibility of their combined and indirect effects on humans and animals through reduced hemoglobin oxygen capacity caused by the formation of various ligand forms [18].

NO is one of the most important mediators of the blood respiratory function. Formed in endothelial cells, NO diffuses into plasma where it quickly reacts with erythrocyte hemoglobin. NO penetrates through the cell membrane via a special protein transporter AE1 or anion exchanger. The permeability of erythrocyte membrane is relatively low for NO, which affects its bioavailability and interaction with hemoglobin [9]. In arterial blood, NO produces nitrate and methemoglobin (MetHb) during the reaction with HbO2, whereas in venous blood it produces nitrosylhemoglobin (HbNO) during the reaction with RHb, which is capable of releasing NO molecules while decomposing at high partial pressure of O2 in tissues [4, 8, 19]. In addition, there is an area in a hemoglobin globin chain where NO binds in the form of S-nitrosothiol, i.e. S-nitrosohemoglobin (SNOHb). Mass-spectrometric and crystallographic analyses have allowed us

to identify β93 – Cysteine (β93Cys) as the site of NO binding to hemoglobin [19].

Excessive NO formation is dangerous and exerts adverse impact on the gas-transport function of blood, since its bonding with β–chains considerably increases the affinity of hemoglobin for oxygen, simultaneously accumulating MetHb, which limits the supply of oxygen to tissues and leads to the development of hypoxic conditions and cell death. Under hypoxia, cells are transferred from oxygen to nitrate-nitrite respiration. Hemoglobin can reduce nitrite ions to NO, thus showing nitrite-reductase activity. The reaction is the last in the transformation chain (the so-called nitric oxide cycle): L-arginine – NO / NO_2^- – NO_3^- – NO. The property of hemoproteins to reduce nitrite ions to NO is revealed exclusively in reduced hemes [12, 17].

Therefore, hemoglobin of erythrocytes is the major place of NO deposition. Nitrogen monoxide in turn interacts with hemoglobin modifying its oxygen binding properties.

Under induced diabetes mellitus, there is a significant increase in SNOHb in the blood of test animals [14]. It is also known that the activity of NO-synthase inducible isoform (iNOS) significantly increases under the pathology [6]. Thus aminoguanidine (AG), iNOS selective inhibitor, has been used in our study [2, 3, 13].

THE AIM OF THE STUDY

The overall aim of the study was to research into structural and functional modifications of oxyhemoglobin (HbO_2) and RHb during the interaction with NO under type 1 DM.

MATERIALS AND METHODS

The study has been carried out on outbred white male rats with the weight of 160-200 g. The animals had free access to food and water in standard conditions (a 12-hour light and darkness cycle). All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the Local Ethics Committee.

The test rats were divided into four groups: 1 – control animals (C); 2 – animals treated with AG added to their drinking water (C + AG); 3 – rats with experimental diabetes mellitus (EDM); 4 – animals with induced EDM which were treated with AG added to their drinking water (EDM + AG). Experimental diabetes mellitus was induced by an intraperitoneal injection of streptozotocin (Sigma, the USA) in the dose of 6 mg/100 g of body weight dissolved in 10mM citrate buffer (pH 5.5). The development of diabetes mellitus was controlled by glucose concentrations in blood. The animals used in the experiment had glucose levels exceeding 14 mM. In the animals of the second and fourth groups, AG was administred *per os* in the concentration of 1 g/1 for 30 days. The rats of all the groups were decapitated under light ether anesthesia. Heparin was as anticoagulant (heparin: whole blood – 1:100). Hemoglobin for all the groups was isolated and identified by the described method [15]. The isolated hemoglobin used for spectrometric studies: RHb, HbO_2, and HbNO solutions were used to record absorption spectra. RHb was obtained from HbO_2 through sodium hydrosulfite ($Na_2S_2O_4$) addition (1 mg/m1 of 10mM HbO_2 solution). To obtain the nitrosyl form of hemoglobin and conduct research into RHb nitrosation, sodium nitrite ($NaNO_2$) was added to RHb solution in equimolar amounts. Electronic spectra of different ligand forms of hemoglobin were registered by Specord M-40 (Germany) spectrophotometer in the wavelength range 450–750 nm. Nitrite reductase reaction was produced as described to [10].

Statistical result processing was carried out by Microsoft Office Excel package. Discrepancies were considered reliable provided probability values were less than 5% ($P<0.05$).

RESULTS

The addition and subtraction of a ligand by a hemoglobin molecule are accompanied by its changes, which cause the corresponding changes in the electronic absorption spectrum. The absorption spectra of various ligand forms of hemoglobin (HbO_2, RHb, MetHb and NOHb) are described in detail in scientific sources [1, 5, 7, 11, 16]. As for our study, we researched into the dynamics of NO addition to hemoglobin in both healthy and diabetic rats in vitro. Such an experimental approach allowed us to characterize the rate of nitrosation/nitrosylation of that hemoprotein. RHb transition into HbNO was registered by characteristic changes in absorption in the visible electronic spectrum (Fig.1). As a result, we obtained the spectrum with two absorption peaks at 544.8 nm and 572.2 nm, which is characteristic of NOHb.

In order to study NO deposition under Type 1 DM, we conducted the research into i*n vitro* hemoglobin nitrosation against a background of AG, iNOS selective inhibitor, administration. It turned out that every test group had its own nitrosation peculiarities. The duration of RHb transition into HbNO increased by 70% under EDM compared with the control group (Table 1).

Table 1. The duration of deoxyhemoglobin transition into a nitrosoform in the norm and under EDM against a background of aminoguanidine administration (M±m, n=8-10).

Research conditions	**Duration, secs**
Control	120.10±11.30
Control + AG	75.50±6.51*
EDM	200.00±18.40*
EDM + AG	60.85±5.92*

*- probable difference compared with control, $P < 0.05$, **- probable difference compared with diabetes, $P < 0.05$

It should be noted that *in vitro* nitrosation increased both in the norm and under EDM due to AG administration (Table 1). It is highly likely that NO and peroxynitrite concentrations fell because of iNOS inhibition, thus decreasing the nitrosylation of hemoglobin molecules.

NO formation can occur during the reduction of nitrites with the help of respiratory hemoproteins. Therefore, our next task was to study the nitrite reductase link of NO formation with RHb under DM.

The kinetic curves in Figure 2 illustrate that deoxyhemoglobin in rats with EDM reveals a relatively higher level of nitrite reductase activity than RHb in the control group.

The administration of AG practically did not change the indicators studied by us in the control animals. However, RHb nitrite reductase decreased in rats with EDM when AG was administered.

DISCUSSION

Physicochemical properties and the functional state of hemoglobin seem to depend to some extent on a ligand form it takes. Interacting with different compounds, hemoglobin turns into various ligand forms, such as carboxyhemoglobin, HbNO, SNOHb, sulfhemoglobin, cyanmethemoglobin, MetHb, each performing a specific physiological function in the organism.

In vitro experiments showed that redox reactions occur when NO_2^- ions interact with deoxyhemoglobin, oxidizing RHb into MetHb and reducing NO_2^- ions to NO. Interacting with reduced hemoglobin, NO forms a stable HbNO compound. As for MetHb–NO complex, it is unstable and decomposes easily. The fact is testified by the obtained electronic absorption spectra of deoxyhemoglobin transition into HbNO (Fig. 1)

In its reactions with peripheral blood hemoglobin, NO forms MetHb, HbNO (nitrosation to Fe^{2+} in the heme group) and SNOHb (nitrosylation to β^{93}Cys) [19].

A small amount of NO avoids hemoglobin capture and reacts with plasma components producing nitrites, nitrolipids, S-nitrosothiols and other products of nitrosation/nitrosylation reactions. Each of those products is able to store, transport and isolate NO far from the place of its synthesis, thus performing the depot function [16].

While researching into the nitrite reductase activity of hemoglobin (Fig. 2), we found that deoxyhemoglobin in rats with experimental streptozotocin-induced diabetes mellitus had a relatively higher level of nitrite reductase activity compared with RHb in the control group. In all probability it happens because glycosylated aminoacid remnants of hemoglobin and later glycosylation products may serve as additional electron donors under DM.

Increased NO synthesis is observed under numerous pathological conditions, stimulating adaptation processes in a living organism. However, if NO production is excessively high, NO loses its protective properties and exerts vasodepressive and cytotoxic action. Thus NO deposition increases owing to excess NO production and decreases from its lack. It may be considered one of adaptation mechanisms, which depends on nitrogen monoxide production. On the one hand, NO deposition provides the protection against the toxicity of free NO when it is overproduced. On the other hand, it can be an additional source of nitrogen oxide when it is in deficit.

CONCLUSIONS

Consequently, the process of formation and decomposition of NO depot (which is controlled by the rate and intensity of hemoglobin nitrosation and nitrosylation) is the future direction of further research aimed at identifying the etiology of numerous pathological conditions and methods of their correction. The research into addition rates of NO and other ligands to hemoglobin, along with spec-

tral characteristics of the obtained ligand forms, provides a basis for a proper understanding of underlying mechanisms for interaction among those molecules under various pathological conditions.

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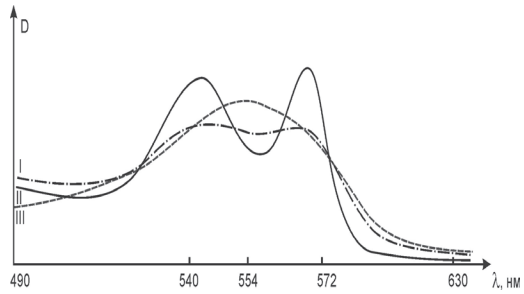

Fig. 1. Typical absorption spectra of separate ligand forms of hemoglobin in peripheral blood of healthy donors: spectrum I – HbNO; spectrum II – HbO_2; spectrum III – RHb .

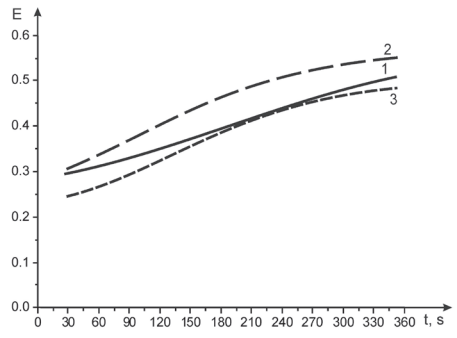

Fig. 2. Dynamics of RHb nitrite reductase activity in rats: 1 – control; 2 – EDM; 3 – EMD + AG.