METHODS

Measurement of Plasma Hemoglobin Peroxidase Activity

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We described a spectrophotometric method for measuring hemoglobin peroxidase activity in human plasma using *o*-dianisidine (*o*-DA) as the substrate and myeloperoxidase specific inhibitor 4-aminobensoic acid hydrazide (ruling out the probable contribution of myeloperoxidase to the measured parameter value). The optimal conditions (pH 5.5; 2 mM H_2O_2) have been determined, at which hemoglobin makes the main contribution to plasma oxidation of *o*-DA. A significant positive correlation between hemoglobin peroxidase activity measured by the spectrophotometric method and hemoglobin level measured by the pyridine hemochromogenic method has been detected (r=0.624; p<0.01) in plasma specimens from 16 donors. Plasma hemoglobin peroxidase activities were measured in healthy individuals and patients with type 2 diabetes mellitus and coronary heart disease. High plasma hemoglobin peroxidase activities in both groups of patients indicates disorders in the mechanisms of clearance of hemoglobin and its highly reactive derivatives and can serve as specific markers of diseases associated with oxidative stress.

Key Words: *hemoglobin; plasma peroxidase activity; myeloperoxidase; diabetes mellitus; coronary heart disease*

Measurements of the parameters characterizing the redox and inflammatory processes in the blood are essential for the diagnosis and prognosis of the course of some diseases and for monitoring the efficiency of treatment thereof. One of such parameters is plasma peroxidase activity (PA). Oxidants (free radicals, *etc.*) formed as a result of peroxidase reactions are highly reactive compounds initiating LPO, modification of proteins and nucleic acids (including oxidation, halogenation, nitration, and suture formation), and causing injuries to body tissues in inflammation.

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Plasma PA is most often measured spectrophotometrically as total PA of heme-containing proteins primarily determined by activities of myeloperoxidase (MPO) and hemoglobin (Hb) with its derivatives in the reaction of *o*-dianisidine (*o*-DA) oxidation by hydrogen peroxide [11].

Hemoglobin is the main component of erythrocyte responsible for oxygen transfer from the lungs to tissues [1,3,4,7]. However, the functional role of Hb is not confined to reversible oxygen binding. It is characterized by manifest PA [4]. Plasma contains haptoglobin whose main function is the formation of a stable complex with Hb (K_a>10^{10} M) and its elimination from circulation. In circulation, haptoglobin binds up to 1.5 g/liter Hb. Normally this complex is eliminated within 10 min. Under pathological condi-

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tions (massive intravascular hemolysis, hemorrhagic shock, hemolytic anemias, transfusion of incompatible blood, *etc.*), Hb is released in high amounts into plasma due to erythrocyte hemolysis. The toxicity of free HB in the plasma (not in erythrocytes) manifests by tissue hypoxia, excess of Hb degradation products (iron, bilirubin, porphyrins) with the development of jaundice or acute porphyria, blocking of the renal tubules by Hb-haptoglobin complexes with subsequent tubular necrosis and acute renal failure [7].

Complexes of Hb and haptoglobin exhibit PA provoking the formation of heteroaggregations with pro-oxidant activity. Detection of these heteroaggregates in the blood of patients with sepsis and the oxidative damage of cells capturing these heteroaggregates indicates the involvement of Hb in the development of oxidative stress in inflammation [10]. Hence, evaluation of extracellular plasma Hb activity is an important problem.

Several methods are used for measuring Hb concentration in the blood [3,8]. Unfortunately, measurement of plasma Hb provides no concrete information about its pro-oxidant activity and regulation of this activity in disease, and therefore we have developed a method for Hb PA evaluation in human plasma and used it for measuring Hb PA in the plasma of patients with type 2 diabetes mellitus and coronary disease.

MATERIALS AND METHODS

The following reagents were used in the study: *o*-DA, 4-aminobensoic acid hydrazide, 3% H_2O_2 (Sagmel), pyridine, and sodium dithionite (Policon Firm).

Plasma MPO PA was evaluated by *o*-DA oxidation (380 μM) after its 13.3 times dilution with phosphate-citrate buffer (60 μl plasma diluted to the sample volume of 800 μl) as described previously [2].

pH-dependent activity of Hb was evaluated in buffer solution obtained by mixing 0.1 M citric acid and 0.2 M sodium hydrophosphate after Mackilvein (further called phosphate-citrate buffer) at pH 4.0-7.0. The reaction was triggered by adding H_2O_2 (1 mM). The relationship between Hb PA and H_2O_2 concentration (0.1-4.0 mM) was studied at pH 5.5. Plasma Hb PA was measured in phosphate-citrate buffer (pH 5.5) with *o*-DA for substrate (380 μM) and MPO inhibitor 4-aminobenzoic acid hydrazide (50 mM). The reaction was triggered by H_2O_2 (2 mM). The initial plasma was diluted 13.3 times by adding buffer to the initial 60 μl of plasma to the volume of 800 μl.

Plasma Hb PA was calculated by the formula:

$$PA_{Hb}=(\Delta A_{inh}\times V)/(v\times 0.0152),$$

where ΔA_{inh} was the rate of o-DA oxidation in the presence of MPO inhibitor within 1 min, V is total volume of reaction mixture, v is plasma sample volume (in our study V/v=13.3), and 0.0152 is optical density increment corresponding to oxidation of 1 μM *o*-DA. This coefficient was determined by the calibration curve representing the direct relationship between optical density at 460 nm and *o*-DA concentration (25-125 μM) after its complete oxidation by horse radish peroxidase (0.1 μg/ml) in the presence of H_2O_2 (2 mM). Plasma Hb PA (PA_{Hb}) was expressed in arbitrary units and corresponded to oxidation of 1 μM *o*-DA by undiluted plasma in 1 min under the above conditions. The rate of *o*-DA oxidation was estimated as the slope of the initial linear part of the kinetic curve, containing a minimum of 5 experimental points, by linear extrapolation using Origin 7.0 graphic editor statistical software.

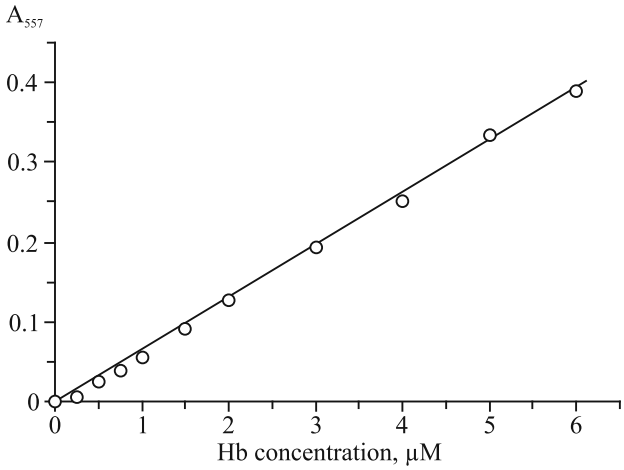

Fig. 1. Typical calibration curve representing the relationship between A_{557} optical density and Hb concentration for estimation of plasma Hb content by the pyridine hemochromogen method.

Plasma concentration of Hb was evaluated by the pyridine hemochromogenic method described previously [5] as follows. Crystalline sodium dithionite (1.5 mg) was added to the plasma diluted 8-fold with pyridine solituin and phosphate-citrate buffer (pH 7.3). Reduced pyridine hemochromogen was obtained. The absorption spectrum was then measured at 500-600 nm and 20°C on a SOLAR PV 1251s spectrophotometer. Absorption of reduced pyridine hemochromogen was estimated with consideration for diffusion and then plasma Hb concentration was evaluated using the calibration curve (successive dilutions of the initial Hb solution (40 mM) were used for measurements; the linear A_{557}-Hb concentration relationship was obtained for the concentrations of 0.25-4.00 μM; Fig. 1).

The data were statistically processed by Student's, Pirson's tests, and analysis of correlations.

RESULTS

Plasma Hb PA was studied using *o*-DA substrate. As this substrate was not Hb-specific, conditions were

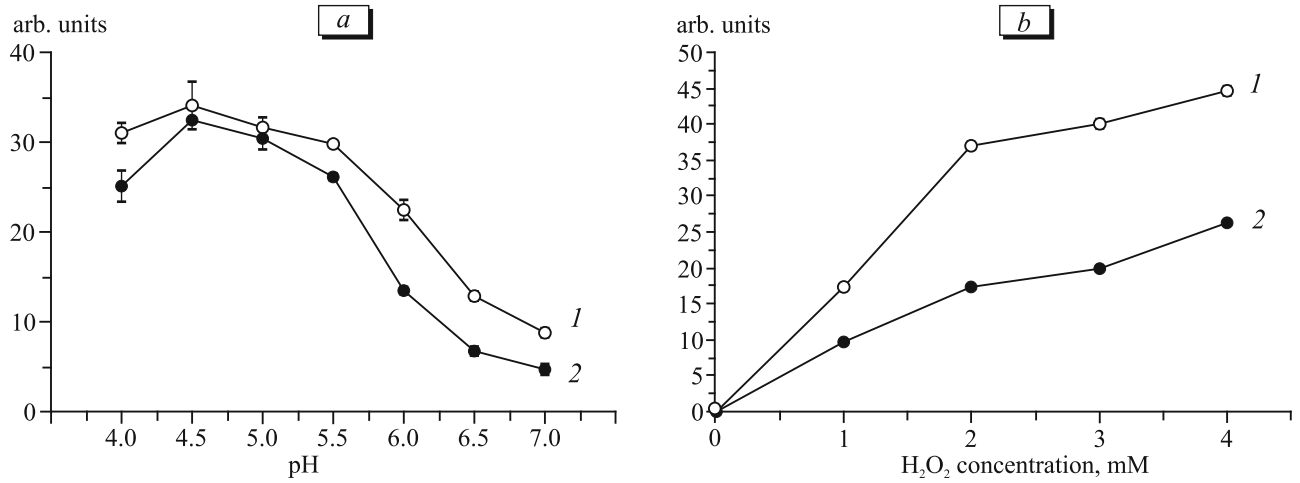

Fig. 2. Relationship between pH and Hb (1 μM) PA (phosphate-citrate buffer; pH 4.0-7.0; 380 μM *o*-DA, 1 mM H_2O_2) without (*1*) and with (*2*) blood plasma (*a*) and relationship between plasma PA and H_2O_2 concentration with 1 μM (*1*) and 0.5 μM (*2*) Hb (phosphate-citrate buffer; pH 5.5; 380 μM *o*-DA) (*b*).

selected at which Hb contribution to *o*-DA oxidation by plasma were the maximum.

As the protein PA was pH-dependent, the Hb PA (1 μM) pH-dependence was studied without blood plasma (Fig. 2, *a*, curve *1*) and in the presence of blood plasma (Fig. 2, *a*, curve *2*), minus PA of plasma proper. The Hb PA was easily recorded at pH 4.0-6.0 in solution and in the plasma. As the plasma PA was determined by Hb and MPO (which, as was shown previously [2], was active at pH 4.5-5.0), the pH of 5.5-6.0 (at which MPO activity was not high) was assumed to be the optimal for Hb detection.

A relationship between plasma PA with different Hb doses and H_2O_2 concentrations was detected (Fig. 2, *b*). Plasma Hb PA increased linearly as H_2O_2 concentration increased up to 2 mM, and hence, H_2O_2 concentration of 2 mM was selected for PA measurements.

As MPO can also contribute to the measured plasma PA value, specific MPO inhibitor (4-aminobenzoic acid hydrazide) was used for evaluation of only Hb PA. The PA of Hb-containing plasma (1 μM Hb) virtually did not change in the presence of 4-aminobenzoic acid hydrazide, while the PA of MPO-containing plasma (300 ng/ml MPO) reduced by 78% ($p<0.05$; $n=5$). Hence, the chosen substance selectively inhibited MPO PA without modulating the Hb PA and hence, using this substance, the contribution of MPO to plasma PA could be ruled out and the activity of predominantly Hb be evaluated.

In order to make sure that the chosen conditions (pH 5.5 and 2 mM H_2O_2) were optimal for measurements of plasma Hb PA, the results of PA and Hb concentration measurements in plasma samples from 16 donors were compared. A significant positive correlation ($r=0.624$; $p<0.01$) between Hb PA, measured by the spectrophotometric method with *o*-DA, and Hb concentration measured by the pyridine hemochromogenic method was detected (Fig. 3).

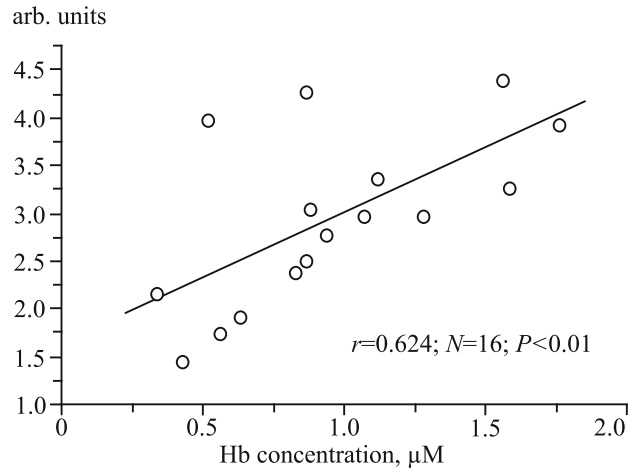

Fig. 3. Relationship between Hb PA measured by spectrophotometry and plasma Hb concentration measured by pyridine hemochromogen method.

TABLE 1. Plasma Hb PA in Donors and Patients with Type 2 Diabetes Mellitus and Coronary Heart Disease

Group	PA_{Hb}, arb. units
Donors (*N*=12)	2.45±0.28
Patients with type 2 diabetes mellitus (*N*=24)	3.55±0.33*
Coronary patients (*N*=20)	2.94±0.22*

Note. *$p<0.05$ in comparison with donors.

This method has been used to measure plasma PA in patients with type 2 diabetes mellitus and coronary heart disease. These diseases are associated with disorders in erythrocyte deformability, blood rheology, and with oxidative stress [6,12]. It is known that active oxygen species play the key role in the development of oxidative stress. These species are characterized by high reactivity and impair the molecular and cellular structures. Hemoglobin, exhibiting PA, is a source of some active oxygen species and hence, can contribute to high plasma PA [9]. Plasma Hb PA is significantly higher in diabetics and coronary patients than in donors (Table 1).

We conclude that our methodological approach can be used for measuring plasma Hb PA. Increase of this activity can serve as an extra test for prediction of the outcome and course of disease associated with oxidative stress and disorders in erythrocyte functions and blood rheology.

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