EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Plasma NO-Binding Activity in Patients with Hematological Malignancies: Suppressive Effect of Glucocorticoids

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Plasma NO-binding activity was studied in patients with various forms of hematological malignancies. The method used in the study quantitatively evaluated the plasma capacity to bind NO, which reflects the intensity of NO production and the degree of patient's stress resistance. Plasma NO-binding activity significantly decreases in patients with hematological malignancies. Glucocorticoid treatment promotes the decrease in plasma NO-binding activity, which was dose-dependent.

Key Words: *nitrogen oxide; plasma NO binding activity; glucocorticoids*

NO is an unstable molecule with half-life period from several milliseconds to 5-6 seconds (according to different sources). The involvement of NO in physiological and pathophysiological processes in the body and in antistress defense is hardly possible without special NO deponents/transporters prolonging the life-span and extending the zone of its action and performing an important stress-limiting function by neutralizing nitrogen oxide excess in hyperexpression of inducible NO synthase, caused, for example, by inflammation and oxidative stress. Among these deponents are high-molecular-weight S-nitrosothiols (RS-NO), products of thiol reaction with nitrozonium cation (NO^+) [4] and dinitrosyl complexes of non-heme iron (DCNI) with protein thiol groups [3]. It is assumed that RS-NO and DCNI are balanced due to capacity to mutual transformation. This system of dynamic equilibrium includes NO, iron ions, and free thiols. This suggests a relationship between the number of nitroxide deponents and total level of NO production; that is, factors preventing NO production in the body should also lead to suppression of its NO-depositing capacity. In order to verify this hypothesis, we studied NO-binding activity (NO-PBA) of the plasma in healthy subjects and patients with hematological malignancies and changes in this parameter during glucocorticoids treatment (inhibitors of endogenous NO synthesis).

MATERIALS AND METHODS

For evaluation of plasma NO content by the level of stable metabolites (NO_2^-/NO_3^-), protein-free plasma was prepared from 5 ml heparin-treated venous blood using the method [1], frozen, and stored at -20°C (so that NO-PBA were analyzed simultaneously in patients with hematological malignancies and controls). In order to detect NO_2^- ions in protein-free plasma (after its defrosting), 0.04 ml Greiss reagent (1% sulfanilamide, 0.1% N-[1-naphthyl]ethylenediamine, 7.5% H_3PO_4 in equal volumes) was added to 0.16 ml plasma. Spectrophotometry of the samples was carried out on an

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Anthos htII multichannel spectrophotometer at λ=540 nm. Quantitative evaluation of nitrite ions (in μM) was carried out in standard $NaNO_2$ solution. Protein-free plasma (0.05 ml) was mixed with 50 mM sodium nitroprusside (0.15 ml) and saline (0.1 ml). The samples were incubated for 60 min at room temperature, exposed for 5 min after addition of 0.01 ml 30% $ZnSO_4$, and then centrifuged for 15 min at 10,000 rpm. The content of NO_2^- (in μM) in the supernatant was measured after 5-min preincubation of 0.08 ml supernatant with 0.02 ml Greiss reagent at 20°C. Plasma-free solution of sodium nitroprusside served as the control. ΔNO_2^- was determined by the difference in NO_2^- content (μM) between the plasma-free sample with sodium nitroprusside and the sample with plasma. Summary NO production included NO_2^- content in protein-free plasma without sodium nitroprusside and ΔNO_2^-. NO-PBA was estimated as [ΔNO_2^-/sodium nitroprusside (control)]×100%.

The data were statistically processed using Microsoft Excel software.

NO-PBA was studied in 58 patients (30 men, 28 women) with various hematological malignancies (14 cases with acute leukemia, 7 with chronic myeloleukemia, 3 with idiopathic myelofibrosis, 8 with chronic lymphocytic leukemia, 9 with malignant lymphoma, 11 with multiple myeloma, and 6 with Hodgkin's disease). Patients' ages varied from 17 to 80 years (mean age 51 years). NO-PBA was analyzed before therapy and during antitumor and/or symptomatic therapy.

Control group consisted of 25 normal subjects (13 men and 12 women) aged 14-78 years (mean age 44 years).

Diets were virtually inessential for the results of NO-PBA analysis, and hence, the patients received their common ration on the day of blood collection (no limitation of nitrate-containing foodstuffs).

RESULTS

The mean NO-PBA in patients with hematological malignancies (without consideration for therapy) differed significantly from the normal (Table 1).

Cytostatics used as monotherapy or in various combinations without glucocorticoids virtually did not modify NO-PBA. The only exclusion was a female patient aged 32 years with chronic myeloleukemia (disease duration ~1 month) receiving hydroxyurea monotherapy (daily dose 3 g). The level of NO-PBA measured at the end of 1-month chemotherapy was extremely high in this patient: 99.9%. This elevation cannot be attributed to the effect of hydroxyurea (NO donor drug), because this drug in the same or lower dose (2 g/day) had virtually no effect on NO-PBA in other patients with chronic myeloleukemia and idiopathic myelofibrosis. Later a hereditary form of hemolytic anemia (Minkowski—Chauffard disease), characterized by increased plasma level of free hemoglobin (active acceptor of NO) was diagnosed in this patient, which seemed to be responsible for so high level of NO-PBA.

TABLE 1. NO-PBA in Patients with Hematological Malignancies and Normal Subjects

Group	NO-PBA, % *M*±*m*	Coefficient of variations, %
Control	31.3±1.2	19.2
Main, all hematological malignancies	22.1±1.7*	58.4

Note. *p<0.05 compared to the control.

Oral and parenteral glucocorticoids (prednisolone, dexamethasone) in combination with other cytostatics and/or as monotherapy, exhibited a clear-cut inhibitory effect on NO-PBA. The mean level of NO-PBA in patients receiving no glucocorticoids was significantly higher than in those treated with glucocorticoids. Moreover, the mean levels of NO-PBA differed significantly in patients receiving different doses of the drugs. NO-PBA in patients treated with glucocorticoids (n=30) was 17.9±1.2%, in those receiving no glucocorticoids (n=28) 26.4±1.0%, in patients receiving prednisolone in a dose of 60 mg/m²/day and more (n=17) 14.9±1.4%, while in those receiving this drug in a dose of 30 mg/m²/

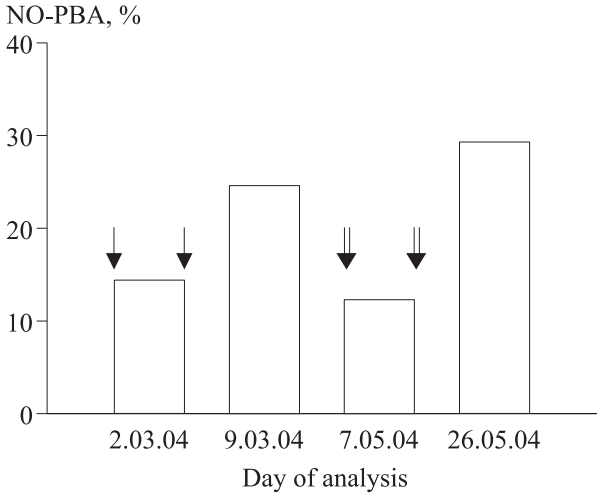

Fig. 1. Individual fluctuations in NO-PBA in a female patient with acute undifferentiated M_0 leukemia during remission receiving intravenous dexamethasone (4 mg/daily; two courses: 01.03.04-05.03.04 and 17.04.04-07.05.04). Thin arrows: course 1 of drug therapy; double arrows: course 2 of drug therapy.

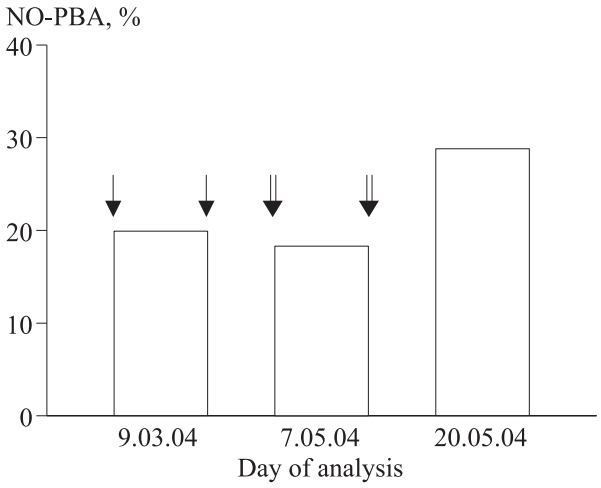

Fig. 2. Individual fluctuations in NO-PBA in a female patient with acute lymphoblastic leukemia B (remission induction) receiving prednisolone (60 mg/m^2/day) by VRP and POMP protocols and during symptomatic therapy. Thin arrows: VRP; double arrows: POMP.

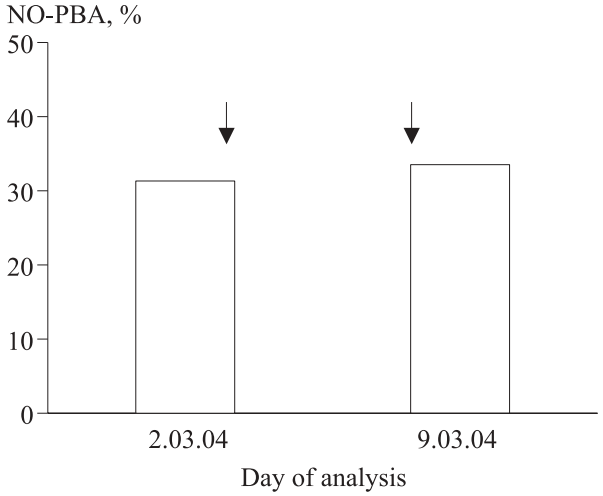

Fig. 3. Individual fluctuations in NO-PBA in a female patient with acute myeloblastic M_4 leukemia during remission, treated with cytosinarabinoside (20 mg/day). Arrows show the beginning and end of drug therapy.

day and lower (n=13) 24.0±1.6%. The suppressive effect of glucocorticoids was significant and depended on the daily dose of the drugs. The mean NO-PBA level in patients with hematological malignancies receiving no glucocorticoids (26.4±1.0%) was lower than in controls (31.3±1.2%), though the difference between these groups was negligible.

Repeated analysis of NO-PBA was made in 8 patients with acute leukemia at different stages of the disease (remission induction, first remission, relapse, second remission, antirelapse treatment; Figs. 1-3). Two short courses of intravenous dexamethasone monotherapy (4 mg daily) during remission in a female patient with acute undifferentiated M_0 leukemia were paralleled by an almost 2-fold drop of NO-PBA level in comparison with the values after drug discontinuation (Fig. 1). A similar effect was observed in a female patient with acute lymphoblastic leukemia receiving oral prednisolone (60 mg/m^2/day) by the VRP and POMP protocols (Fig. 2). Cytosine arabinoside in low doses (20 mg/day) during remission in a female patient with acute M_4 myeloblastic leukemia did not change the NO-PBA level (Fig. 3).

Instead of summary (two-component) NO-PBA value we estimated mainly one-component value in our studies, because the initial concentration of NO_2^- ions in the plasma (before sodium nitroprusside) was detected in only 12 (20.7%) of 58 patients with hematological malignancies and in 5 (20%) of 25 normal subjects and this concentration was low (2.70±0.85 and 2.90±0.88 μM, respectively). The level of plasma nitrite ions was zero in the absolute (about 80%) majority of cases. It seems that NO-PBA value is largely determined by the plasma content of NO-binding molecules, NO deponents (primarily RS-NO and DCNI), the synthesis of which directly depends on the intensity of endogenous NO generation.

On the other hand, it seems that along with plasma proteins containing SH group, hemoproteins also can bind NO. For this reason we tried to clear out the contribution of these proteins to NO-PBA and estimated r coefficient of linear correlation between protein content (remaining in the sample after its precipitation with $ZnSO_4$) and NO-PBA. The coefficient r was equal to -0.05, and we therefore neglected NO-binding capacity of plasma proteins in our study.

Hence, our preliminary data indicate that the method for evaluation of NO-PBA is a perspective method which deserves clinical trials. Presumably, due to this method it will be possible to evaluate (at least indirectly) the intensity of NO production and the effects of therapy on this production. This is confirmed by the data on the inhibitory effect of glucocorticoids on NO-PBA, which coincide with the data on the suppressive effect of glucocorticoids on inducible NO-synthase [2]. Presumably, the NO-PBA value is an indicator of stress resistance and can be used for predicting the course of hematological malignancies and complications of therapy, because the plasma level of NO deponents ("physiological equivalents" can reflect not only the level of NO production, but also patient's antistress potential. An important advantage of this method in comparison with other indirect methods for NO evaluation (by the level of NO_2^-/NO_3^- stable metabolites [1]) is that there is no need in reduction of NO_3^- ions (mainly eliminated from the body) into

NO_2^- ions by metal cadmium or nitrate reductase. The only justification of this procedure is an appreciable (by almost an order of magnitude) improvement of the sensitivity of the method. However, it may be fraught with risk of erroneously high results of analysis because of exogenous nitrates consumed with food, which limits the possibility of studies under conditions of common nutrition.

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