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COMPLEX SYSTEMS
BIOPHYSICS

A Comparative Analysis of the Effects of Free and Bound NO on Pro- and Antioxidant Systems of the Blood

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Abstract—The dynamics of the oxidative metabolism of the blood of healthy donors (n = 30) who were exposed in vitro to nitric oxide in the form of gas or dinitrosyl iron complexes has been investigated. The intensity of lipid peroxidation and the level of malonic dialdehyde in plasma and red blood cells were determined, as well as the plasma antioxidant capacity and the level of superoxide dismutase activity in the blood samples. This study is the first to have characterized the specific features of the response of pro- and antioxidant systems of the blood to free or bound (into dinitrosyl iron complexes) nitrogen monoxide in vitro; in particular, the gas flow from the Plazon device was shown to have a pronounced prooxidant effect, which was moderately alleviated by tenfold dilution of the NO-containing mixture. The use of an experimental nitric oxide generator that was constructed at the Russian Federal Nuclear Center resulted in minimal pro-oxidant activity, while an aqueous solution of dinitrosyl iron complexes had a moderate antioxidant effect that was manifested as the limitation of lipid peroxidation in blood plasma and an increase of superoxide dismutase activity in erythrocytes.

Keywords: nitric oxide, dinitrosyl iron complexes, blood, lipid peroxidation, biochemiluminescence, superoxide dismutase activity

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The nitric oxide (NO) molecule is known to participate in a variety of reactions with organic compounds and reactive oxygen species due to its bioradical nature [1–4]. As a result, NO may exert bioregulatory activity [5, 6] or toxic effects that are mostly related to the formation of peroxynitrite ($ONOO^-$) [7, 8]; the nature of the effects depends on the actual level of NO. On the contrary, several Russian and foreign authors have reported antioxidant properties of dinitrosyl iron complexes (DNICs), the natural form of bound nitric oxide [5, 9, 10], although the mechanisms underlying the antioxidant effects are very poorly characterized. For example, the contribution of thiol-containing DNICs to the elimination of carbonyl stress substrates from a model medium was demonstrated in the doctoral thesis of S.A. Gubkina [10]; the results were confirmed by experiments on rats. K.V. Shumaev et al. used a range of model biological systems to reveal the antioxidant activity of DNICs with respect to oxidative and nitrosative stress [5, 9, 11].

On the other hand, the effects of gaseous and bound NO on the physicochemical parameters of blood homeostasis have been neither characterized nor compared by the present time. Therefore, we have analyzed the dynamics of the oxidative metabolism of blood exposed to gaseous nitric oxide or DNICs; this was the purpose of the present study.

Abbreviations: DNIC, dinitrosyl iron complexes, MDA, malonic dialdehyde.

MATERIALS AND METHODS

Preserved human blood samples that were obtained from apparently healthy donors (n = 30) were used to evaluate the effects of different forms of NO on blood. A Plazon cold plasma generator and an experimental appliance for nitric oxide synthesis that was developed at the Russian Federal Nuclear Center (RFNC) were used to generate gaseous NO. The NO-containing air flow from the latter appliance was practically devoid of ozone or other reactive oxygen species [12], unlike the flow generated by the Plazon device. DNICs with glutathione that were used as the bound form of NO were synthesized according to the procedure developed by R.R. Borodulin et al. in the research group headed by A.F. Vanin [13].

Each blood sample used in the experiments was divided into five portions of 5 mL. The first portion was left intact and used as the control; gas from the

Plazon device (moderate power, NO concentration 800 ppm; $V = 100$ mL, duration of treatment 3 min) was bubbled through the second portion; the same flow diluted tenfold with air (NO concentration 80 ppm) was bubbled through the third portion; an air and gas mixture from the experimental NO generator [12] was bubbled through the fourth portion (nitrogen oxide concentration 75 ppm, the volume and duration of exposure as above), and the fifth portion was treated with an isotonic aqueous solution of DNICs (a concentration of 3 mM and volume of 0.05 mL). DNIC concentration in solution was determined spectrophotometrically at wavelengths of 310 and 360 nm (a PowerWave XS spectrophotometer, United States). The time of incubation after NO addition equaled 5 minutes for all samples.

The intensity of lipid peroxidation, total antioxidant activity of blood plasma, and erythrocyte resistance to peroxide (as inferred from Fe-induced biochemiluminescence measured by the BKhL-06 device) were measured in the samples. The level of malonic dialdehyde (MDA) in plasma and red blood cells was estimated using the procedure developed by V.G. Sidorkin and I.A. Chuloshnikova (1993) [14]. Superoxide dismutase activity was evaluated using the procedure reported by T.V. Sirota (1999) [15].

The results were processed using the Statistica 6.0 program.

RESULTS

We assessed the state of pro- and antioxidant systems both in blood plasma and erythrocyte membranes. Analysis of biochemiluminescence parameters (Fig. 1) showed that the intensity of lipid peroxidation in blood plasma exposed to a flow of gas from the Plazon device (NO concentration 800 ppm) increased by 45% (significant change; $p < 0.05$ relative to the intact sample), in accordance with our previous results [16]. Tenfold dilution of the gas flow resulted in a decrease of the shift of the light sum of plasma biochemiluminescence; however, this value remained rather high as compared to the value for the control sample (+27%, $p < 0.05$).

The use of an experimental device for NO production that was developed at the Russian Federal Nuclear Center resulted in less-pronounced activation of lipid peroxidation as compared to the treatments that were described above, even though the concentration of NO in the mixture of air and gas (75 ppm) was close to that in the air flow from the Plazon device diluted tenfold (80 ppm). The light sum of chemiluminescence induced by the treatment was still higher than that for the control sample ($p < 0.05$ relative to the intact blood and the samples treated with the undiluted and diluted gas flow from the Plazon device).

Addition of 0.05 mL of an aqueous solution of the NO donor DNIC to the blood is comparable to the

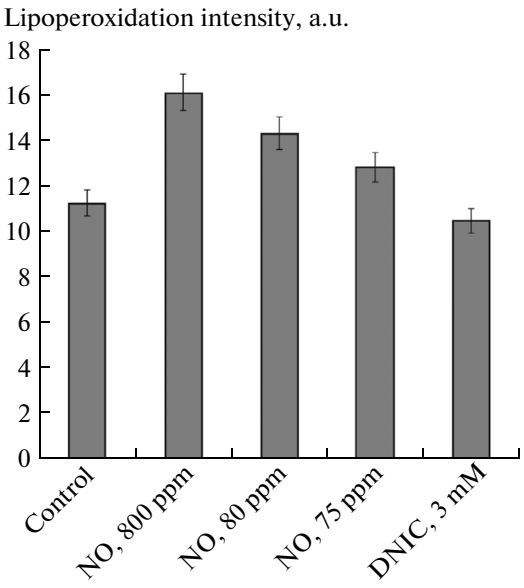

Fig. 1. The effect of nitric oxide on the intensity of lipid peroxidation in blood plasma.

treatment with the diluted gas flow from the Plazon device or the experimental NO generator with regard to the amount of NO administered to the biological fluid (9, 8, and 7.5 μg nitrogen oxide, respectively). The use of DNICs at this dose had an effect that was opposite to that of gas bubbling, causing a moderate decrease of the intensity of lipid peroxidation (by 7%, $p < 0.1$ relative to the control).

A similar pattern was detected for the total antioxidant activity of blood plasma (Fig. 2). Thus, bubbling of the undiluted gas flow from the Plazon device through the biological fluid led to a more than twofold decrease of the parameter in question as compared to the control sample ($p < 0.05$), while exposure to the flow diluted tenfold resulted in a 21% decrease in the antioxidant capacity of blood plasma ($p < 0.05$). However, neither the treatment of blood with the gas flow from an experimental NO generator nor the addition of DNIC solution changed the antioxidant potential of the biological fluid.

The results of the assessment of the content of MDA, one of the stable products of lipid peroxidation, in the blood plasma samples were in good correspondence with the trends that were detected by the biochemiluminescence analysis (Fig. 3). Bubbling of a gas flow containing 800 ppm NO through the biological fluid resulted in a 2.3-fold increase of the value of this parameter ($p < 0.05$), while an attempt to alleviate this negative effect by diluting the gas flow had a slight effect on the change (MDA concentration in the blood increased 1.9-fold relative to control, $p < 0.05$). In contrast, treatment of the blood with an almost identical amount of nitric oxide produced by the experimental NO generator (as part of the gas mixture, con-

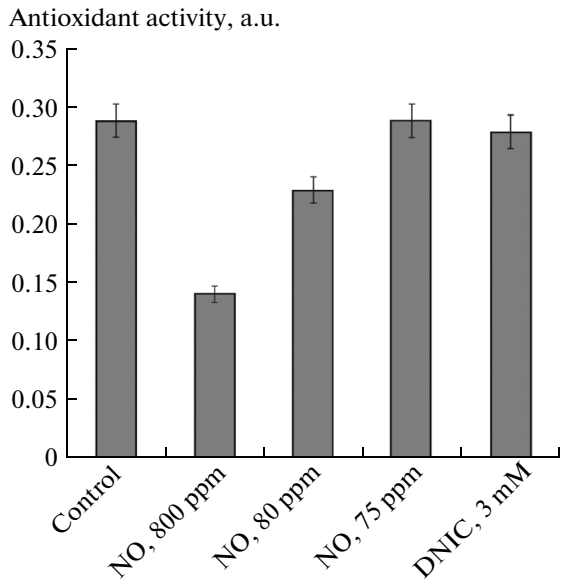

Fig. 2. The effect of nitric oxide on the total antioxidant activity of blood plasma.

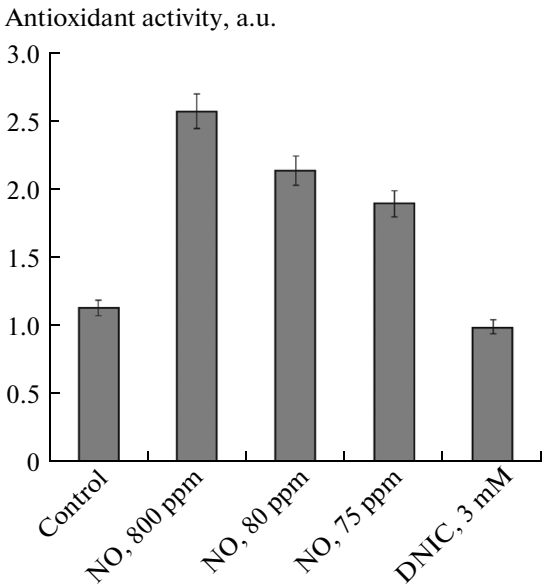

Fig. 3. Malonic dialdehyde levels in blood plasma that was exposed to free and bound nitrogen oxide.

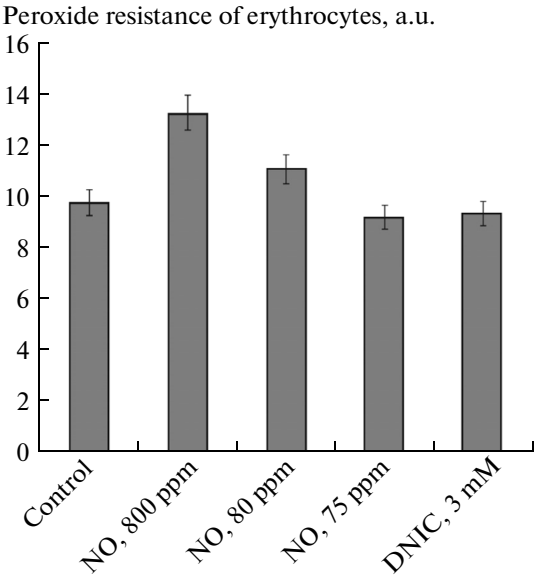

Fig. 4. The peroxide resistance of erythrocytes that were exposed to free and bound nitrogen oxide.

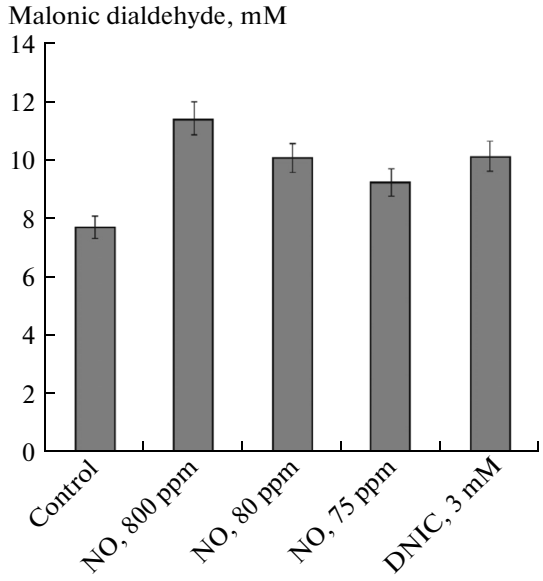

Fig. 5. The level of malonic dialdehyde in erythrocytes that were exposed to free and bound nitrogen oxide.

centration 75 ppm vs. 80 ppm in the previous case) resulted in a much smaller change of the metabolite level (1.6-fold increase, $p < 0.05$).

The pattern of MDA concentration change was more intriguing in the case of DNIC treatment: namely, the concentration decreased by 13% relative to the value for the intact sample ($p < 0.05$), which can serve as indirect proof of antioxidant properties of the compound.

The balance of the pro- and antioxidant systems in erythrocytes exhibited comparable changes (Figs. 4–6). For example, the level of peroxide resistance of erythrocytes that were exposed to the non-diluted gas flow from the Plazon device increased by 1.36 times relative to the value for the control sample ($p < 0.05$). This is indicative of a decline in the resistance of erythrocyte membranes to oxidative impact (that is, membrane disruption evoked by the treatment) and strong stimulation of lipid peroxidation in the membranes (Fig. 4).

This effect was markedly alleviated upon a reduction of nitrogen oxide concentration in the gas flow through dilution of the latter with atmospheric air (+14% relative to the value for the control sample, $p < 0.05$).

Exposure of the blood to a gas mixture produced by an experimental NO generator resulted in a minimal (7%, $p = 0.063$) decrease of the parameter relative to the intact sample, although the concentration of NO in the gas flow was similar to that in a ten times diluted gas flow from the Plazon device; this is indicative of a membrane-stabilizing effect of the factor under investigation. Notably, addition of the DNIC solution to the biological fluid had virtually no effect.

The MDA level in erythrocytes (Fig. 5) provides information on the intensity of lipid peroxidation in the membranes of those cells; notably, the use of NO at a concentration of 800 ppm evoked a sharp increase of the value in question (by 48% relative to the control value, $p < 0.05$), this being indicative of an increase of lipid peroxidation intensity evoked by the treatment. Dilution of the gas mixture with air resulted in moderate alleviation of the effect: the MDA concentration in this system increased by 31% relative to the value for the control sample that was not subjected to any manipulations ($p < 0.05$). The use of an experimental nitric oxide generator producing an air flow with NO concentration of 75 ppm had the least pronounced effect on all other parameters, but induced a significant increase of the level of the metabolite (by only 20% of the value that is characteristic of the untreated sample, $p < 0.05$). However, the increase in MDA concentration that was recorded after the exposure of blood to an aqueous solution of DNICs was comparable to that observed after treatment with a tenfold diluted gas stream from the Plazon device (32%, $p < 0.05$).

In addition to the parameters that provide a direct characteristic of lipid peroxidation intensity, we evaluated the activity of the superoxide dismutase system, since it is one of the main components of the enzymatic antioxidant defense system that mediate the inactivation of superoxide anion radicals (Fig. 6). Bubbling NO-containing gas through whole blood always resulted in inhibition of this system, but the magnitude of the effect varied considerably. Thus, the most "harsh" of the treatments that was studied, namely, exposure of blood to the gas flow from the Plazon device (nitric oxide concentration 800 ppm) caused a 1.64-fold decrease of superoxide dismutase activity relative to the control ($p < 0.05$), while decreasing the NO concentration to 80 ppm only slightly alleviated the effect (superoxide dismutase activity decreased by 29% relative to the value that is characteristic of intact blood, $p < 0.05$). The effect was the least pronounced when the gas flow from the experimental NO generator was used; in this case, superoxide dismutase activity was reduced by only 19% relative to the control ($p < 0.05$).

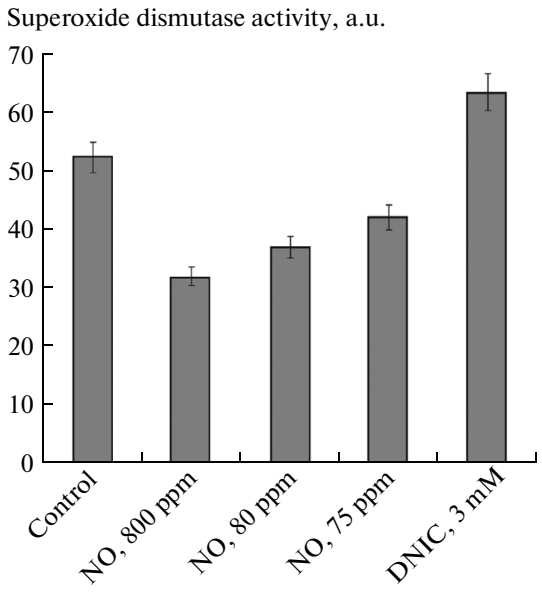

Fig. 6. The effect of nitric oxide on superoxide dismutase activity in erythrocytes.

Addition of the DNIC solution to the blood had a fundamentally different effect on the functioning of the superoxide dismutase system: namely, it induced moderate activation of the enzyme (by 21% relative to the control value, $p < 0.05$); this is indicative of almost optimal conditions for the functioning of the superoxide dismutase system. The increase in the enzyme activity could be due to antioxidant properties of the DNICs themselves. As demonstrated previously, nitric oxide bound to those complexes was capable of reacting with superoxide anion to form peroxynitrite; the latter remained bound to DNICs rather than leaking into the environment [11]. Peroxynitrite bound to DNICs could be isomerized into nitrate and subsequently released into the environment. Therefore, the emergence of free peroxynitrite capable of producing cytotoxic agents (hydroxyl radical and nitrogen dioxide) after protonation was prevented. Reduced glutathione that was used in the synthesis of DNICs could potentially contribute to the antioxidant activity of the DNIC preparation [17]; however, the description of the synthesis procedure that was used [12] clearly shows that glutathione is oxidized during the synthesis and therefore it cannot exert antioxidant action.

RESULTS AND DISCUSSION

Our previous studies addressing the parameters of energy metabolism demonstrated a differentiated response of whole human blood to nitrogen oxide administered either as gas or a component of a liquid, as well as to the gas derived from different sources (a certified Plazon device for the generation of NO-containing cold plasma and an experimental NO generator that was developed at the Russian Federal Nuclear

Center) [18]. The present study provided additional proof for our earlier findings concerning the negative effects of the gas flow from the Plazon device on the biological fluid under investigation [16, 19]. We assume that these effects were evoked by reactive oxygen species that are present in the gas stream from the Plazon device in considerable quantities that are capable of forming highly toxic peroxynitrite upon interaction with NO [7–9], as shown by additional experiments [20]. This, together with the necessity to find an optimal method to control the NO levels, which vary significantly in various pathological conditions [2, 4, 6, 21–24], justifies the validation of alternative routes of exogenous nitric oxide administration to biological systems in vivo. The three principal pathways are the following: extensive purification of the NO-containing gas stream to remove the reactive oxygen species, the use of compounds that contain bound nitric oxide that are capable of gradual release of the free gas, and the search for putative physical or chemical stimulants of endogenous NO synthesis. The latter approach is currently be performed using the Orbita device [25]; however, the reported efficacy of this device for the treatment of various diseases notwithstanding [26], the amount of additional nitric oxide that is formed under the influence of an electromagnetic field is difficult to control; therefore, the search for correlations between the amount of the gas and its biological effects is complicated.

The use of the two former approaches appears preferable; therefore they were investigated in the present study. Comparative analysis of the effects of the two NO generators for which the concentration of the active compound was nearly the same indicates that removal of reactive oxygen species from the gas mixture provides the optimal effects of the latter on lipid peroxidation processes in blood plasma and erythrocytes. Notwithstanding the stimulation of free-radical reactions (an increase of total biochemiluminescence in blood plasma and an increase of the MDA level in plasma and erythrocytes) in biological systems on any type of bolus administration of gaseous nitric oxide, the effect of the gas flow from the experimental apparatus that was developed at the Russian Federal Nuclear Center can be considered instructive, since the activation of lipid peroxidation in this case was moderate and did not result in exhaustion of the antioxidant capacity of the biological fluid. In contrast, administration of NO at high concentrations (800 ppm) combined with the presence of reactive oxygen species, including ozone [20], in the same gas flow, triggered oxidative stress in blood plasma and erythrocyte membranes and facilitated nitrosohemoglobin formation [27, 28], causing further suppression of the oxygen transport function of the erythrocytes.

The response of pro- and antioxidant systems of the blood was the most positive upon addition of an aqueous DNIC solution to the biological fluid; the amount of the active agent (in NO equivalents) was similar to that injected with a tenfold diluted gas flow from the Plazon device or the gas flow from the experimental NO generator (9, 8, and 7.5 μg, respectively). This intervention caused a moderate decrease of the intensity of plasma lipid peroxidation, with a minimal increase of MDA concentration being evidence of antioxidant properties of DNICs, rather than inhibition of peroxidation, as confirmed by the report of L.L. Gudkov et al. [5].

Thus, analysis of the dynamics of pro- and antioxidant systems of the blood demonstrated the benefits of using gaseous nitric oxide at low concentrations (less than 100 ppm) and the necessity of eliminating adventitious oxygen-containing oxidants from the gas stream. In this case, compounds containing bound NO, such as DNICs with glutathione ligands, have an optimal effect on lipid peroxidation in plasma and additionally stimulate superoxide dismutase activity in erythrocytes, in contrast to the NO-containing air stream.

CONCLUSIONS

The distinctive features of the responses of the pro- and antioxidant systems of the blood to in vitro treatment with nitrogen monoxide in a free or bound form have been characterized for the first time; the bound form was represented by DNICs, which are natural carriers of NO. A pronounced prooxidant action of the gas stream from the Plazon device on both blood plasma and erythrocyte membranes was evident; tenfold dilution of the NO-containing mixture moderately alleviated the effect. The use of the experimental nitrogen oxide generator resulted in minimal prooxidant effect on the biological sample, while an aqueous solution of DNICs had a moderate antioxidant effect that was manifested as limitation of lipid peroxidation in blood plasma and an increase of superoxide dismutase activity in the erythrocytes. The results of the experiments indicate a membrane-protective effect of the NO-containing air mixture from the experimental generator and the aqueous solution of DNICs.

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