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BIOPHYSICS AND BIOCHEMISTRY

Effect of Nitrosyl Iron Complex with 3,4-Dichlorothiophenolyls on the Level of Cyclic Nucleotide *In Vitro*

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The effect of a promising NO donor, a binuclear nitrosyl iron complex (NIC) with 3,4-dichlorothiophenolyls [$Fe_2(SC_6H_3Cl_2)_2(NO)_4$], on the adenylate cyclase and soluble guanylate cyclase enzymatic systems was studied. In *in vitro* experiments, this complex increased the concentration of important secondary messengers, such as cAMP and cGMP. An increase of their level by 2.4 and 4.5 times, respectively, was detected at NIC concentration of 0.1 mM. The ligand of the complex, 3,4-dichlorothiophenol, produced a less pronounced effect on adenylate cyclase. It was shown that the effect of this complex on the activity of soluble guanylate cyclase was comparable to the effect of anionic nitrosyl complex with thiosulfate ligands that exhibits vasodilating and cardioprotective properties.

Key Words: *nitrosyl iron complexes; soluble guanylate cyclase; adenylate cyclase; cAMP; cGMP*

Nitrogen monoxide (NO) is a universal signaling molecule widely involved in the regulation of many physiological processes in the body [1,2]. Its participation in functioning of the cardiovascular, nervous, and immune systems orchestrates the functions of these important systems through complex cellular interactions.

In the physiology of the cardiovascular system, NO regulates vascular tone, BP, and cardiac function [3]. By activating soluble guanylate cyclase (sGC), NO induces the synthesis of cGMP, an important secondary messenger and a universal regulator of intracellular metabolism [4]. Elevation of cGMP concentration has various effects, such as stimulation of vasodilation, activation of ion channels, and inhibition of smooth muscle cell proliferation [5,6]. cGMP also activates cAMP-dependent protein kinase A, due to its strong homology with sGC, although with 50-fold lower selectivity [7]. In turn, activation of adenylate cyclase (AC) under the influence of some hormones (epinephrin, glucagon, *etc.*) and adrenoreceptor agonists on the G-protein, as well as NO molecules directly on the AC enzyme leads to an increase in cAMP level [8,9]. Changes in cAMP concentration affect heart rhythm, contractility, and vascular resistance [10]. The decrease cAMP level can be a result of very high content of cGMP in the cell, which leads to activation of cAMP phosphodiesterase. Thus, the coordinated interaction of cyclic nucleotides and NO is important for homeostasis in the cardiovascular system [11,12].

Low NO production in the endothelium of blood vessels leads to accelerated thrombosis, formation of atherosclerotic plaques, BP elevation, and, as a result, dysfunction of the cardiovascular system [13]. One of the main approaches aimed to solve this problem is the use of exogenous NO donors [14,15]. The binucle-

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ar neutral nitrosyl iron complex (NIC) with 3,4-dichlorothiophenolyls $[Fe_2(SC_6H_3Cl_2)_2(NO)_4]$ (complex 1) [16] is a potential source of exogenous NO that does not require additional activation for its generation. This compound can be used for targeted delivery of NO *in vivo* for the treatment of cardiovascular and other socially significant diseases [16,17].

The purpose of this work is to study the effect of complex 1 on two significant enzymatic systems sGC and AC. The effect produced by this NO donor was measured by ELISA by changes in cAMP and cGMP concentration in mouse heart homogenates.

MATERIALS AND METHODS

The experiments were approved by the Ethical Committee of the Federal Research Center of Problems of Chemical Physics and Medicinal Chemistry (Protocol No. 23/2 of February 3, 2023) and were carried out in accordance with Russian statute regulating experiments on animals.

The following reagents were used: Tris (Serva), sulfonamide (SA), HCl (Sigma-Aldrich), N-(1-naphthyl)ethylenediamine (NEDA), $Na_2HPO_4{\times}6H_2O$, and $NaH_2PO_4{\times}H_2O$ (MP Biomedicals), ELISA Kit for Cyclic Guanosine Monophosphate, ELISA kit for Cyclic Adenosine Monophosphate (Cloud-Clone Corp.).

Complex 1 and the reference complex $Na_2[Fe_2(S_2O_3)_2(NO)_4]{\times}4H_2O$ (complex 2) were synthesized as described elsewhere [16,18].

The NO-donor activity of the complexes was assessed using Griess reaction. Weighted samples of the complexes were dissolved in DMSO and 0.05 M Tris-HCl buffer (pH 7.0), respectively, which were previously purged in a flow of argon. Solutions were diluted to a final concentration of $2{\times}10^{-4}$ M with 0.05 M Tris-HCl buffer (pH 7.0). Aliquots of the resulting NIC solutions (300 μl) were incubated with 900 μl of a 0.5% SA solution in 0.25 M HCl for 5 min, then 600 μl of a 0.02% NEDA solution in 0.5 M HCl was added. After addition of SA and NEDA, the final concentration of complexes 1 and 2 in the experimental cuvette was $0.33{\times}10^{-4}$ M. After a 10-min incubation, the absorbance of the colored product was measured at 540 nm. The final concentration of NO_2^- was calculated using the calibration curve.

The concentrations of cAMP and cGMP in homogenates of mouse hearts (BDF1 mice) were assessed by ELISA. The hearts were isolated, weighed, and homogenized in an equal volume of 0.01 M sodium phosphate buffer (pH 7.03) at 10,000 rpm. The homogenates were incubated with complexes 1 and 2 in final concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M at room temperature for 20 min. The tissues were lysed with RIPA buffer (1 M Tris-HCl, 20% Triton X-100, 10% SDS, 2.5 M NaCl, DTT, sodium deoxycholate) for 2.5 h at 4°C and centrifuged for 10 min at 10,000 rpm. The supernatant was collected and frozen at -20°C for further analysis. ELISA was carried out according to the Cloud-Clone Corp. protocol. The sample solution was 10-fold diluted with 0.01 M sodium phosphate buffer (pH 7.03), applied to plate wells with adsorbed primary antibodies, and incubated at 37°C. Then, the wells were washed and incubated with secondary horseradish peroxidase-conjugated antibodies at the same temperature. At the final stage, the substrate was added. The reaction was stopped with the stop-reagent from the kit after 15-20 min of incubation as a colored product was formed. Optical density was measured using a Feyond-A400 microplate reader (Hangzhou Allsheng Instruments Co., Ltd) at 450 nm. The final concentration was calculated using previously obtained calibration curves. The experiments were repeated at least 3 times.

Statistical processing of the results was carried out using GraphPad Prism 8 software (GraphPad Software). The data from independent experiments were recalculated as a percentage of the control value and presented as *M*±*SEM*. The significance of differences between the groups was assessed using the Student's *t* test. The differences at $p<0.1$ and $p<0.05$ were considered statistically significant.

RESULTS

The NO-donor activity of complex 1 was assessed using the Griess reaction based on the kinetics of accumulation of nitrite ions, the main products of NIC decomposition in aerobic aqueous solutions [19]. Well-studied anionic nitrosyl complex 2 was used as a reference compound [18]; according to *in vivo* and

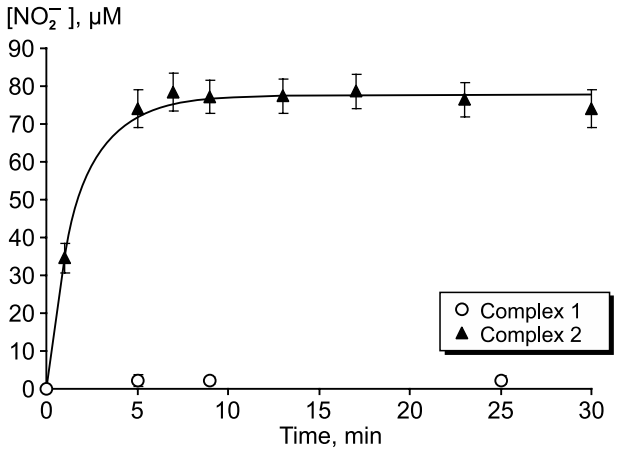

Fig. 1. Kinetics of nitrite accumulation in a buffer solution of complexes 1 and 2. Effective constant is $1.5{\times}10^{-2}$ sec^{-1} (complex 2). Conditions: the concentration of complexes 1 and 2 in the experimental cuvette is $3.3{\times}10^{-5}$ M, solvent: Tris-HCl buffer (pH 7.0), 23°C.

in vitro studies, it exhibits vasodilating, cardioprotective, and antitumor effects and is involved in the induction of gene expression of specific DNA repair pathways [20].

According to the obtained results, complex 2 in aqueous solutions is a more effective NO donor than complex 1 (Fig. 1) and generates 30-fold more NO. However, the time to the plateau for both complexes is about 5 min. Such low concentrations of released NO in case of complex 1 can be explained by the formation of insoluble binuclear nitrosyl products in aqueous buffer solutions [16]. On the other hand, in biological systems complex 1 can continuously generate 25-fold more NO than in the buffer [16], which indicates a significant influence of the environment on its NO-donor activity.

For a more detailed analysis of the influence of complex 1 on the functioning of the sGC and AC, we analyzed changes in the level of cyclic nucleotides after incubation of the complexes in three different concentrations with the heart homogenates. The concentration of both secondary messengers increased (Fig. 2). cAMP concentration increased by 2.4 times in mixtures with 10^{-4} M complex 1, and the cGMP concentration increased by 4.5 times. We also analyzed the effect of the thiolate ligand released as a result of the complex 1 decomposition on the activity of sGC and AC. After incubation of the homogenate with ligand at concentrations of 10^{-6} and 10^{-4} M, the level of cAMP increased by 1.3 and 1.7 times, which is lower than for the initial complex 1. The absence of a significant effect of 10^{-5} M 3,4-dichlorothiophenol on the cAMP content in the homogenate can be due to the intricate intracellular network of cAMP and cGMP regulatory mechanisms [8]. A detailed study is required to clarify this issue, which is beyond the scope of this work. As was expected, no effect of 3,4-dichlorothiophenol on sGC was detected.

Complex 2 at a concentration of 10^{-4} M had a slightly lower effect on sGC than complex 1 (Fig. 3). This result is especially interesting if we compare their NO-donating activity in an aqueous buffer: complex 2

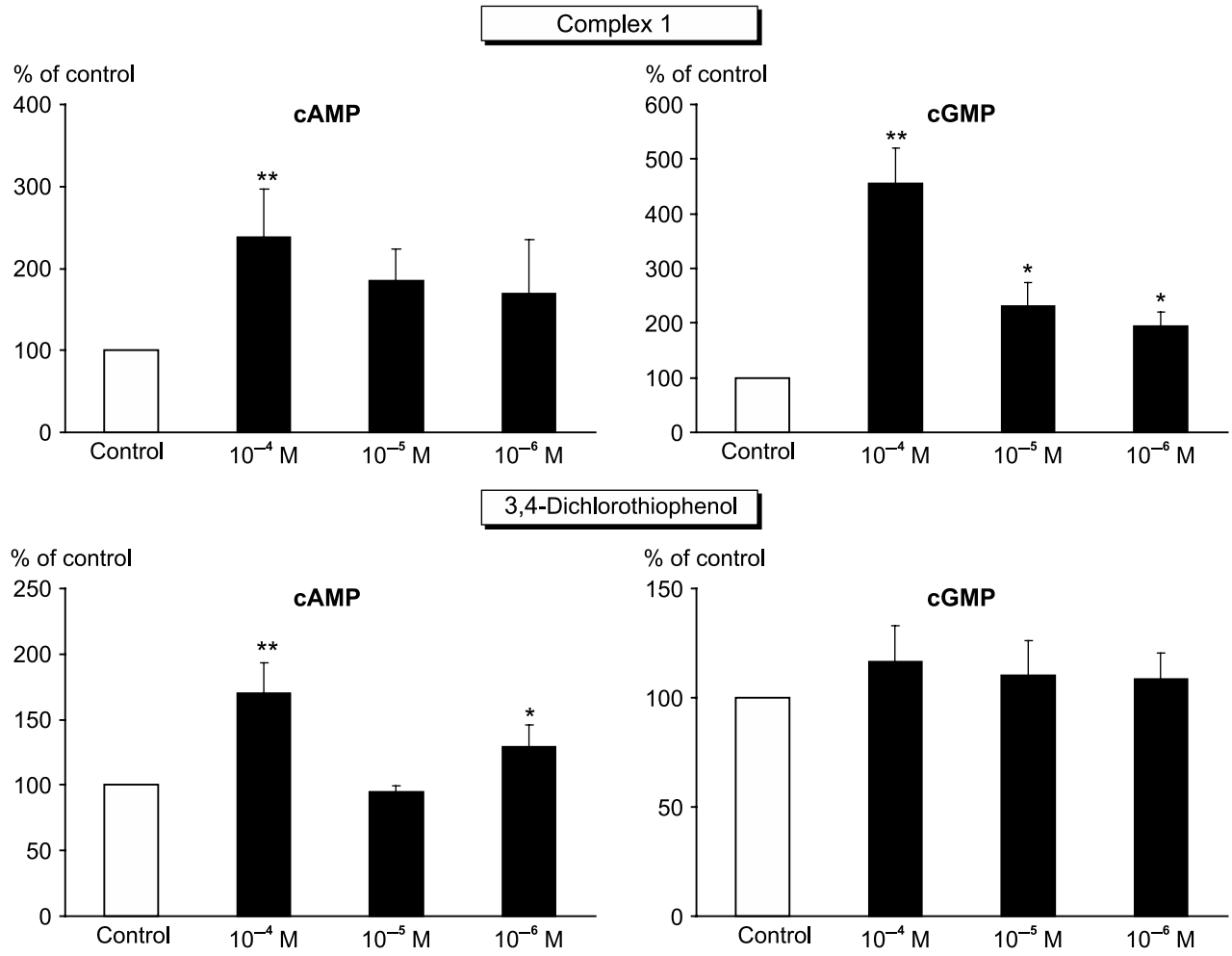

Fig. 2. Levels of cAMP and cGMP after incubation of the homogenate with complex 1 and 3,4-dichlorothiophenol at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M. Concentrations of cAMP and cGMP were measured in pg/ml. *p<0.1, **p<0.05 in comparison with the control.

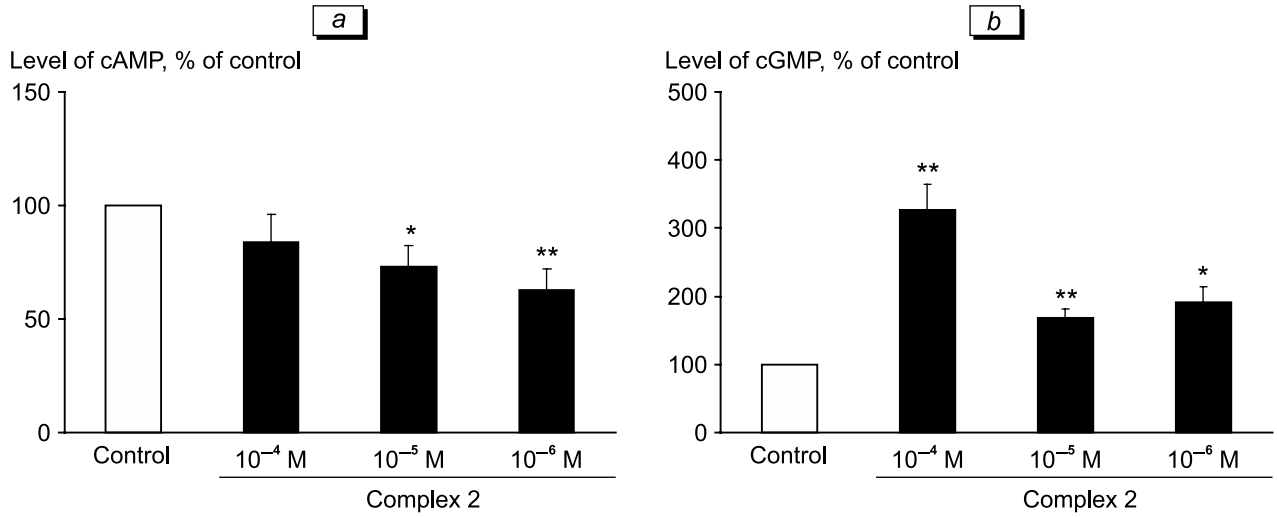

Fig. 3. Levels of cAMP and cGMP after incubation of the homogenate with complex 2 at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M. Concentrations of cAMP and cGMP were measured in pg/ml. *p<0.1, **p<0.05 in comparison with the control.

released 30-fold more NO groups than complex 1 (Fig. 1).

Thus, the obtained data confirm the fact that NO generation is significantly influenced by the environment, and, therefore, complex 1 in *in vivo* experiments can be no less effective vasodilator than complex 2.

The effect of complex 2 on AC functioning was radically different from the effect of complex 1. Thus, complex 2 at an effective concentration of 10^{-6} M reduced the level of cAMP by 1.6 times in comparison with the control. It should be noted that the effect on cAMP is consistent with the data of other studies [10,12]: a sharp release of NO into the system can cause a rapid increase in the level of cGMP, which is due to the high NO-donor activity of complex 2 (Fig. 1), and under the influence of specific phosphodiesterases in parallel, the level of cAMP may decrease. During incubation with complex 1, the release of a smaller amount of NO into the system (due to more prolonged generation of NO) has a different effect, namely both enzymatic systems work, which causes an increase in the concentrations of both secondary messengers.

Thus, despite significant differences in the NO-donating properties of the complexes in buffer solutions, complex 1 has a more pronounced effect on both enzymatic systems.

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Conflict of interest. The authors have no conflicts of interest to declare.

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