The Cytostatic Action of Dinitrosyl Iron Complexes with Glutathione on Escherichia coli Cells Is Mediated by Nitrosonium Cations Released from These Complexes

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Abstract—This study demonstrates a bacteriostatic effect of binuclear dinitrosyl iron complexes with glutathione on Escherichia coli TN300 cells. It has been quantified by the colony formation assay. The bacteriostatic effect exerted by these complexes increases considerably in the presence of diethyldithiocarbamate. Our results suggest that this effect is caused by the intense release of nitrosonium cations, NO+, from the complexes, which decompose under the action of diethyldithiocarbamate. A similar effect is observed when E. coli cells are treated with diethyldithiocarbamate 40 min after the addition of sodium nitrite or S-nitrosothiol. Notably, the level of dinitrosyl iron complexes observed in the bacterial cells due to the effects of sodium nitrite or S-nitrosothiol is almost the same as that obtained after treatment with thiol-containing complexes. The bacteriostatic effects of the NO molecules released from nitrite or S-nitrosothiol during their brief interaction with bacteria were significantly smaller than the bacteriostatic effect of NO+. We deduce therefrom that the nitrosation cations released from DNICs are responsible for the observed bacteriostatic effect of these complexes in E. coli cells.

Keywords: dinitrosyl iron complex, nitric oxide, nitrosonium cations, S-nitrosoglutathione, bacteriostatic effect, Escherichia coli cells

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Presently, it is thought that endogenous nitric oxide NO, enzymatically produced from L-arginine, is the main effector of immune defense against bacterial infections in humans and animals [1–4]. However, it is still unknown whether NO itself or the product of its one-electron reduction, the nitrosonium cation NO+, is responsible for the inhibition of bacterium proliferation in the body. On the one hand, NO molecules can be converted to peroxynitrite, a highly cytotoxic agent [3–6]; on the other hand, NO+ can S-nitrosylate various vital proteins [3, 4, 7–9] to suppress their activity.

Here we attempt to shed light on this issue in experiments with Escherichia coli cells and dinitrosyl iron complexes (DNICs) as donors of NO and NO+. As shown in [10–12], their formation from DNICs involves the disproportionation of NO molecules bound to the iron(II) with the presence of thiol-containing ligands (RS−), as shown in Scheme 1. According to this scheme, the nitroxyl anion NO− forming during NO disproportionation undergoes hydrolysis. It is protonated and released from the ligand environment of iron as a nitrous acid or nitrite. Two HNO molecules are transformed to nitrous acid N2O and water. The vacant site at the iron atom is filled by a third NO molecule to form a mononuclear DNIC molecule (M-DNIC). It can be characterized by the resonance structure [(RS−)2Fe(ONO)2] or [(RS−)2Fe(NO)(NO)]. As for the hypothetical hydrolysis of the nitrosonium cation that arises in DNIC owing to NO disproportionation, its interaction with the hydroxyl anion, which would convert NO+ to nitrous acid or nitrite, is prevented by the neutralization of its positive charge by the electron density from sulfur atoms of the thiol-containing ligands, which are strong π donors.

As shown in [10–13], the resonance structures presented for M-DNICs are also present in the binuclear DNIC form (B-DNIC): [(RS−)2Fe2+(NO−)2(NO)2] or [(RS−)2Fe2+(NO+)4].
According to the data reported in [11, 12], nitrosyl cations, like NO molecules, can be released from DNIC in equal amounts in the establishment of equilibrium between the complex and its components and in the irreversible degradation of the latter, caused by the removal of iron or thiol-containing ligands. Dithiocarbamate derivatives, $R_2=N–CS_2$, are the most prominent agents inducing DNIC degradation. These compounds can tear the nitrosyl iron group $[Fe^{2+–NO}]$ off dinitrosyl iron fragments $[Fe^{2+–(NO)(NO^+)]}$ of DNICs to form a stable EPR-active mononitrosyl complex (MNIC) with dithiocarbamate derivatives, as illustrated in Scheme 2 [14–16].

Formerly, our team demonstrated such a transformation of DNICs by dithiocarbamate derivatives diethyl dithiocarbamate (DETC) or $N$-methyl-D,L-glucamine dithiocarbamate (MGD) in living organisms [15, 16].

It has been shown that the MNIC–DETC or MNIC–MGD formed in this transformation tightly bind the neutral NO molecules originally present in the DNIC and prevent their action on biological targets. Thus, the NO molecules are removed from the process. Meanwhile, nitrosonium cations are released from the degrading DNIC according to Scheme 2 and bound to various thiols. As shown in [17, 18], the interaction of these cations with thiols to form $S$-nitrosothiols rather than with hydroxyl anions resulting in NO$^+$ hydrolysis is determined by the significantly greater affinity of the cations to thiols.

It is supposed that the accompanying $S$-nitrosylation of various thiol-containing proteins is responsible for DNIC cytotoxicity [19–24]. This action of B-DNIC with mercaptosuccinate was observed in our experiments with MCF-7 tumor cells. It was greatly enhanced by the presence of MGD, which increased the number of cations released from the B-DNIC [25].

The goal of this work was to investigate in detail the contribution of DETC to the antibacterial (bacteriostatic) action of B-DNIC with glutathione (B-DNIC-GSH) on $Escherichia coli$ bacteria and to find out what component of this DNIC, nitric oxide molecules or nitrosonium cations, suppresses bacterium propagation (colony-forming activity).

MATERIALS AND METHODS

Materials. Iron sulfate was purchased from Fluka (Switzerland). Reduced glutathione, sodium nitrite,
and sodium diethyldithiocarbamate were purchased from Sigma (United States). Gaseous NO was obtained by the reaction of iron sulfate with sodium nitrite NaNO₂ in 0.1 M HCl and purified by cryosublimation in vacuo [13].

**Synthesis of the binuclear dinitrosyl iron complex with glutathione and S-nitrosoglutathione.** The binuclear dinitrosyl iron complex with glutathione (B-DNIC-GSH) was prepared by the treatment of iron sulfate + GSH solution with gaseous NO at the molecular ratio Fe²⁺ : GSH = 1 : 2 and at NO pressure 100–150 mmHg. Iron sulfate dissolved in 0.5 mL of distilled water (pH 5.5) was loaded into the upper container of a Thunberg vial, and 4.5 mL of GSH solution in 15 mM HEPES buffer pH 8.0 were loaded into the lower container. Air was evacuated from the vial, and NO was injected. The vial was shaken for 5 min, the solutions were mixed in the NO atmosphere, and shaken for further 5–10 min, which was sufficient for B-DNIC-GSH formation. Then nitric oxide was evacuated from the vial. The complex concentration was deduced from the optical absorbances at 310 and 360 nm with the molar attenuation coefficients ε = 4600 and 3700 M⁻¹ cm⁻¹, respectively (corrected for one iron atom in B-DNIC) [13].

S-Nitrosoglutathione (GS-NO) synthesis was based on the ability of nitrous acid HNO₂, arising in nitrite protonation in acidic medium, to S-nitrosylate thiols. Glutathione solution (11 mM) was mixed in the air with 10 mM NaNO₂, and the neutral mixture was acidified to pH 2–3. The solution immediately turned pink. After 1.5 h, the solution was decacified to neutral pH. The GS-NO concentration was assessed from its light absorbance at 334 nm, ε = 980 M⁻¹ cm⁻¹.

**Experiments with bacterial cells** were carried out with *Escherichia coli* TN530 cells. Their survival was assessed from their colony-forming activity (CFA) expressed as percentage of the same index in untreated control cells. Cell suspension was grown in LB broth at 37°C for 18 h. A sample volume was diluted fiftyfold with the same medium, and the cells were grown for 3 h within the exponential stage to the titer 3–5 × 10⁸ cells/mL, which corresponded to the reference sample.

The experiments on the effect of B-DNIC-GSH, NaNO₂, and GS-NO on bacterial cells were conducted as follows: (1) The dose-dependences of the bacteriostatic activities of the agents were tested separately. (2) and (3) The effects of DETC on the bacteriostatic activities were recorded with the synchronous addition of DETC and each of the agents into the culture and with the addition of DETC after 40-min exposure to B-DNIC-GSH, NaNO₂, or GS-NO. In the first step, 1 mL of increasing concentrations of B-DNIC-GSH, NaNO₂, or GS-NO was added to a cuvette with 1 mL of bacterial cells. The mixture was incubated for 30 min and inoculated onto agar at tenfold dilution. The agar was incubated at 37°C for 24 min, and colonies were counted to assess the colony-forming activities and, correspondingly, the bacteriostatic activities of B-DNIC-GSH, NaNO₂, and GS-NO. One colony was taken to correspond to one cell. The effect of DETC (2.5 mM) on the bacteriostatic action of B-DNIC-GSH, NaNO₂, and GS-NO was studied in the same way, with addition at the same time as the agents or 40 min later.

**Optical and EPR measurements.** Optical measurements of B-DNIC-GSH solutions were carried out with a UV-2501PC spectrometer (Shimadzu Europa GmbH, Germany) in flat quartz cuvettes with a 10 mm optical path at room temperature in the air. EPR spectra of M-DNICs with thiol-containing ligands (GSH and thiol-containing cell proteins) and of MNIC-DETC arising in bacterial cultures were recorded in a quartz Dewar vessel with liquid nitrogen using a modified RadioPan radiospectrometer (Poland) at the microwave power 5 mW and high-frequency amplitude mod-
Concentrations of EPR-active paramagnetic centers were assessed by double integration of their EPR signals with an M-DNIC-GSH solution of known concentration as a reference. Some EPR measurements were conducted at room temperature in quartz capillary tubes 1 mm in diameter.

Statistical evaluation. The results are presented as means of three independent replications ± standard deviation ($M \pm SD$).

RESULTS AND DISCUSSION

Physicochemical properties of B-DNIC-GSH and MNIC-DETC. In contrast to M-DNICs with thiol-containing ligands, showing EPR signals with $g_\perp = 2.04$ and $g_\parallel = 2.014$ ($g_{\text{mean}} = 2.03$), referred to as signal 2.03 [26], (Fig. 1a, spectrum 1), B-DNIC-GSH is diamagnetic and EPR-silent. As mentioned above, its concentration can be determined from light absorbance at 310 and 360 nm (see the spectrum in Fig. 1b).

As reported in [15, 16], solutions of MNIC-DETC in hydrophobic media, e.g., in cell membranes, are characterized by the EPR spectrum 2 in Fig. 1a with the mean $g$ factor value 2.04 and a clearly resolved triplet hyperfine structure (HS). The complex is poorly soluble in aqueous media, where its triplet structure is blurred; therefore, the signal looks like an unstructured symmetrical singlet at $g = 2.04$ (Fig. 1a, spectrum 3).

Antibacterial (bacteriostatic) action of B-DNIC-GSH on E. coli cells. The data in Fig. 2 show that E. coli cells are relatively resistant to the bacteriostatic action of B-DNIC-GSH. The 50% CFA was achieved only at about 5 mM concentration of the complex. Moreover, lower concentrations (0.03–0.1 mM) favored cell proliferation, increasing CFA to 120% of the control value. Cell treatment with DETC produced a similar effect.

As indicated above, the effect of DETC on the bacteriostatic action of B-DNIC-GSH was studied at the concentrations 2.5 and 0.5 mM, respectively. According to the dose-response curves presented in Fig. 2, the CFA values were 50 ± 5 and 88 ± 5%. In the independent stochastic process model, where B-DNIC-GSH and DETC are assumed to act simultaneously not affecting each other’s state, their combination is expected to produce a CFA value of 44% (Fig. 3, bar 3). In fact, the simultaneous exposure of cells to DETC and B-DNIC-GSH reduced this index to 7 ± 5% (Fig. 3, bar 4). When DETC was added to cells 40 min after the B-DNIC-GSH addition, cell propagation ceased completely, and CFA went down to zero (Fig. 3, bar 5).

In agreement with data quoted in INTRODUCTION, this dramatic enhancement of the bacteriostatic action of B-DNIC-GSH by DETC may be caused by B-DNIC-GSH decomposition, which produces EPR-active MNIC-DETC and releases nitronium cations from B-DNIC-GSH. These cations may adversely affect bacterial cells. This hypothesis was convincingly proven by EPR. In experiments with simultaneous addition of B-DNIC-GSH and DETC, the unstructured singlet signal shown in Fig. 1a (spectrum 3), characterizing MNIC-DETC located, presumably, in the intercellular fluid, was recorded instead of the EPR signal with $g_\perp = 2.04$ and $g_\parallel = 2.014$ ($g_{\text{mean}} = 2.03$), characteristic of MNICs with thiol-containing ligands, which was recorded at 77 K in cells treated with B-DNIC-GSH alone (Fig. 1a, spectrum 1).
When DETC was added 40 min after B-DNIC-GSH, the EPR signal with triplet HS shown in Fig. 1a, spectrum 2, was recorded at 77 K. This signal is characteristic of MNIC-GSH in hydrophobic medium, presumably, cell membranes. It replaced the EPR signal 2.03 (Fig. 1a, spectrum 1), recorded in the cells prior to DETC addition.

Thus, there is every reason to state that the dramatic enhancement of the bacteriostatic action of B-DNIC-GSH added together with DETC is mediated by nitrosonium cations released from B-DNIC-GSH. The NO molecules originally present in the complex are incorporated into stable MNIC-DETC and were removed from the process, as stated above; that is, they cannot affect bacterial cells.

The 2.03 signal, characteristic of M-DNICs with thiol-containing ligands, was absent from cells not treated with B-DNIC-GSH. The EPR signal characteristic of MNIC-DETC found in the cells after treatment with DETC was also absent.

Antibacterial action of sodium nitrite and S-nitrosoglutathione on E. coli cells. The dose dependence of E. coli resistance to the bacteriostatic action of NaNO2 and GS-NO was close to that to B-DNIC-GSH (Fig. 4). The 50% CFA value of all these compounds was achieved at concentrations about 5 mM.

This similarity might be due to the emergence of M-DNICs with thiol-containing ligands in cells incubated in 0.5 mM NaNO2 or GS-NO. Judging from the amplitude of signal 2.03, the M-DNIC concentration was approximately equal to the concentration in bacterial cells exposed to 0.5 mM B-DNIC-GSH. This observation agrees with our earlier results [12, 16] and data obtained by other authors [27–29], which report that both M-DNICs and B-DNICs with thiol-containing ligands arise in microbial and animal cells exposed to nitrite or S-nitrosothiols. These complexes are probably formed by gaseous NO released from the said compounds. At shorter durations of exposure of bacterial cells to 0.5 mM NaNO2 or GS-NO, 3–5 min, no significant amounts of DNICs with thiol-containing ligands were noticed.

In contrast to experiments with B-DNIC-GSH, the stimulating effect of DETC on the bacteriostatic action of 0.5 mM NaNO2 or GS-NO was observed only when DETC was added 40 min after the agent but not with simultaneous addition (Fig. 5, bars 8, 9 and 6, 7, respectively).

Judging from the EPR data presented below, MNIC-DETC was produced in the former case by the interaction of DETC with M-DNIC and B-DNIC that had formed before with the presence of NaNO2 or GS-NO. In the latter case, MNIC-DETCs also arose in the culture, but not with DNICs; rather, they were formed mostly by NO released from NaNO2 or by GS-NO with iron present in the incubation medium and in bacterial cells.

The formation of MNIC-DETC in the reaction of DETC with NO and iron admixture rather than from M-DNIC and B-DNIC could not result in the emergence of nitrosonium cations in the cell culture. Obviously, this is why no notable effect of DETC on the bacteriostatic action of nitrite and GS-NO was observed for its addition together with the agents.

Consider the EPR results that brought us to this inference. The EPR signal of MNIC-DETC with the triplet HS shown in Fig. 1a was recorded at 77 K in bacterial cells after 40-min exposure to NaNO2 or GS-NO.
NO. This implies that the complex was formed by the interaction of the agents with the DNIC having arisen in the cells, whose hydrophobic compartments housed the dissolved MNIC-DETC. This process generated nitrosonium cations, which suppressed cell propagation. In experiments with simultaneous treatment with DETC and NaNO2 or GS-NO, the unstructured symmetrical signal of MNIC-DETC shown in Fig. 1a (spectrum 3) was recorded in the cells at 77 K. This is indicative of the location of the complex in the aqueous intercellular medium, where MNIC-DETC is poorly soluble, rather than inside cells. Hence, the complex was not formed in the reaction of DETC and DNIC, which might occur inside cells, but in the intercellular medium in the reaction of DETC with NO released from NaNO2 or GS-NO and with iron, present in the incubation medium or in cells themselves. Obviously, this process could not result in the appearance of nitrosonium cations in the intercellular medium.

This inference is in full agreement with measurements of the CFA of cells simultaneously treated with DETC and either NaNO2 or GS-NO (Fig. 5, bars 6 and 7). The CFA values were 40 ± 5 and 35 ± 5%, respectively, comparable with the values calculated for the additive action of DETC and NaNO2 or GS-NO on bacterial cells (Fig. 5, bars 4 and 5).

Concentrations of DNICs with thiol-containing ligands and MNIC-DETC in cell suspension. We recorded the EPR signal of M-DNICs with thiol-containing ligands (signal 2.03), giving way to the more intense signal of MNIC-DETC with triplet HS in E. coli cells treated first with gaseous NO at 100 mmHg for 15 min, as in [13], and then with DETC. Judging from the amplitude of the EPR signal of M-DNICs appearing in cells after treatment with NO, their concentration was 2.0 ± 0.5 μM, almost the same as the concentration of the complexes arising in the same suspension after 40-min exposure to 0.5 mM B-DNIC-GSH, NaNO2, or GS-NO at 1.5 ± 0.5, 1.5 ± 0.5, and 2.5 ± 0.5 μM, respectively. After the subsequent addition of 2.5 mM DETC, the concentrations of the resulting MNIC-DETC were 4.0 ± 0.5, 5.0 ± 0.5, and 5.0 ± 0.5 μM, respectively, whereas in cells first treated with gaseous NO this concentration was 6.5 ± 0.5 μM. Our reports [15, 16] explain the higher MNIC-DETC level than M-DNIC by the fact that binuclear DNICs with thiol-containing ligands react with DETC, and such DNICs appear in cells in higher amounts than their mononuclear counterparts.

The formation of M-DNICs and B-DNICs in bacterial cells treated with gaseous NO or its donors at approximately equal concentrations may be caused by the limitation of the process by one of the DNIC components. As in this synthesis neither gaseous NO level nor iron excess (in experiments with cell treatment with gaseous NO or B-DNIC-GSH) affect the concentration of the forming DNICs, we infer that cellular thiol-containing compounds (mainly thiol-containing proteins) are the limiting factor in DNIC formation.

The MNIC-DETC level in cells simultaneously treated with DETC (2.5 mM) and 0.5 mM NaNO2 or GS-NO was 50 μM. This indicates that the concentration of NO rapidly released from nitrite or GS-NO and incorporated into MNIC-DETC was no lower. It was much higher that the levels of M-DNIC and B-DNIC with endogenous thiol-containing ligands arising in cells 40 min after incubation with NaNO2 or GS-NO (about 1.5 and 5 μM). Correspondingly, the level of the released nitrosonium cations was low as well. Nevertheless, the bacteriostatic effect of NO released from NaNO2 or GS-NO (Fig. 5, bars 2 and 3) was much lower than the action of NO+ cations (1.5–5 μM) released in the reactions of DETC with M- and B-DNICs arising in cells after 40-min incubation with NaNO2 or GS-NO (Fig. 5, bars 8 and 9). This fact unambiguously indicates that the bacteriostatic effect of these cations is higher than that of NO molecules.

CONCLUSIONS

The principal result of our study is the detection of the dramatic enhancement of the bacteriostatic action of binuclear DNIC-GSH on Escherichia coli TN530 bacteria by DETC. Similar results were previously obtained in [30] and in our work [25] in experiments with Jurkat and MCF-7 tumor cells, respectively. Instead of DETC, use was made of the water-soluble dithiocarbamate derivative MGD, and instead of B-DNIC-GSH, M-DNIC-thiosulfate [30] or B-DNIC-mercaptopussuccinate [25]. These agents were added simultaneously with MGD. Their use drastically increased cell death in comparison to the individual action of MGD, thiosulfate, or mercaptosuccinate.

Exogenous synthetic DNICs were used in the mentioned studies [25, 30] and in this work. It is worth mentioning that the formation of endogenous M- and B-DNICs with thiol-containing ligands in cells with the presence of NO from exogenous or endogenous sources was demonstrated in our early studies of 1960s, later summarized in Vanin’s monographs [31, 32], and in later experiments. It is likely that these endogenous complexes act as the key effectors of T-cell-mediated immunity. This conjecture was evidenced for in 1990s by the detection of M-DNICs with thiol-containing ligands in activated macrophages, in which NO was intensely produced by inducible NO synthase [33–36]. These complexes could pass from macrophages to co-cultivated tumor cells and cause their death [34].

As DNICs with thiol-containing ligands can supply nitrosonium cations [10–12], and these cations can inhibit E. coli propagation [this work] and cause tumor cell death [25, 30], it is conceivable that the ability of immunocompetent cells to suppress the
reproduction of bacterial pathogens involves the formation of DNICs with thiol-containing ligands, which act as nitrosonium cation donors. The cytotoxic action of NO$^+$ ions as the key effectors of T cell-mediated immunity may be based on their ability to S-nitrosylate vital thiol-containing proteins and enzymes [30].

These enzymes may include proteases of various viruses, among them, coronaviruses SARS-CoV-2. Therefore, DNICs with thiol-containing ligands may be helpful in coronavirus disease treatment. This suggestion was put forward in [37] and confirmed by testing the B-DNIC-GSH + DETC mixture on Syrian hamsters infected with SARS-CoV-2 [38].

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interests. The authors declare that they have no conflicts of interest.

Statement on the Welfare of Animals. This article does not contain any studies involving humans or animals performed by any of the authors.

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