Induction of the SOS DNA repair response in *Escherichia coli* by nitric oxide donating agents: dinitrosyl iron complexes with thiol-containing ligands and *S*-nitrosothiols

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Abstract The ability of nitric oxide (NO) donor compounds to induce the SOS DNA repair response in *Escherichia coli* is reported. Dinitrosyl iron complexes with glutathione and cysteine (DNIC) are the most potent SOS-inducers. *S*-Nitrosothiols (RSNO) mediate a similar response at 10–100 μM, but the response decreases sharply at concentrations above 0.5 mM. Pretreatment of the cells with the chelating agent *o*-phenanthroline (OP) prevents induction of the SOS response by all agents used. On the other hand, the toxicity of *S*-nitrosothiols is higher than that of DNIC. The EPR study shows the appearance of an EPR DNIC-type signal after incubation of the cells with *S*-nitrosoglutathione because of mutual transformation between RSNO and DNIC in the presence of accessible iron inside the cells. Pretreatment of the cells with OP leads to a decrease in this signal. Analysis of NO donor effects reveals a dual role of the iron ions in reactivity and toxicity of the compounds studied, i.e. (i) stabilization of the cytotoxic RSNO and (ii) generation of the SOS signal.

Key words: Nitric oxide; SOS DNA repair response; *S*-Nitrosothiol; Dinitrosyl iron complex; *Escherichia coli*

1. Introduction

Nitric oxide (NO) is known as a signal molecule regulating the cell cycle. It plays a crucial role in vascular relaxation, apoptosis, hypoxia, and nutrient deficiency as well as antimicrobial defense and cell injury in inflammatory diseases [1,2]. The main mechanisms of antimicrobial NO action include interference with cell division and energy production via inhibition of DNA synthesis and electron transport proteins due to nitrosylation of protein SH groups and nitrosative deamination of DNA [3,4]. Mammalian cells as well as bacteria such as *Escherichia coli* can generate a stress response providing a defense of the cells against various reactive species. Nitric oxide radicals, like superoxide anions ($O_2^{-\bullet}$), trigger the oxidative stress response by activation of the *soxRS* regulon, which is controlled by the redox-sensitive transcriptional regulator SoxR in *E. coli* [5]. The SOS DNA repair pathway plays a central role in the *E. coli* response to a wide variety of genotoxic agents. Triggering of the system can be used as a general and early sign of DNA damage. The SOS response involves induction of more than 20 genes upon blockage of ongoing DNA replication after UV or chemical exposure as well as after oxidative damage by hydrogen peroxide [6]. In this paper, we study the SOS DNA repair response as a reaction of *E. coli* cells to treatment with NO-containing agents: dinitrosyl iron complexes with thiolate ligands (DNIC) and *S*-nitrosothiols (RSNO), since various DNIC and RSNO regulatory activities have been observed earlier [7–9].

To quantify SOS response induction by the compounds tested, we used the *E. coli* strain PQ37 with an operon fusion placing *lacZ*, the structural gene for β-galactosidase, under the control of the *sfiA* gene involved in cell division inhibition. Here we provide evidence that these agents are able to induce the SOS DNA repair response in *E. coli*. It is important to take these results into account in evaluating disease states after bacterial infections in mammals.

2. Material and methods

2.1. Chemicals

Cysteine, reduced glutathione (GSH), and HEPES were purchased from Sigma (USA), ferrous sulfate from Fluka (UK). Dinitrosyl iron complexes with cysteine or glutathione were used in dimeric form and prepared by treatment of 5.4 mM $FeSO_4$ and 10.8 mM glutathione or cysteine (iron:thiol ratio 1:2) with gaseous nitric oxide in Thunberg vessels (pressure 200–300 mm Hg) in a solution (15 mM HEPES, pH 7.6) previously degassed by evacuation. Gaseous NO was synthesized by reacting $FeSO_4$ with $NaNO_2$ in 0.1 M HCl with subsequent purification in an evacuated system. *S*-Nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) were synthesized in Thunberg vessels by treatment of 50 mM glutathione or penicillamine with a mixture of gaseous NO and air for 5 min with subsequent evacuation of excess NO_2. The concentration of nitroso adducts was determined spectrophotometrically at 340 nm (molar extinction coefficient 980 M^{-1} cm^{-1}). Peroxynitrite was synthesized according the method based on mixing an acidified solution of 0.6 M $NaNO_2$ with hydrogen peroxide and subsequent stabilization by 0.9 M NaOH [10]; excess hydrogen peroxide was removed by adding manganese dioxide to the solution, with subsequent filtration. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm (molar extinction coefficient 1670 M^{-1} cm^{-1}). The compounds were synthesized just before the experiments.

2.2. *sfiA* gene expression

L medium and 63 buffer were used for growth, manipulation and storage of bacteria [11]. All studies were performed on *E. coli* PQ37 carrying a *sfiA::lacZ* operon fusion and a deletion in the normal chromosomal *lac* operon so that β-galactosidase activity was strictly dependent on *sfiA* expression. *sfiA* gene expression was monitored as described by Quillardet et al. [12]. In brief, after a 30 min treatment with NO donors, bacterial suspensions (OD_{600} = 0.36, approximately 10^8 cells/ml) were diluted in L medium according to the protocol [12], incubated at 37°C for 2 h with 4-nitrophenyl-β-D-galactopyranoside (ONPG) as chromogen, and then assayed for β-galactosidase activity colorimetrically at 420 nm. β-Galactosidase units (E) were calculated

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from the equation:

$$E = \frac{1000 \cdot D_{420}}{t}$$

where D_{420} is the absorbance at 420 nm and t is the incubation time. Buffers and reagents for β-galactosidase assays have been described elsewhere [11,12]. 2.63 nM 4-nitroquinoline-*N*-oxide (4-NQO), a well known potent SOS inducer, was used as a standard (positive control). Each experiment was performed in triplicate.

2.3. Bacterial survival

After a 90 min treatment with NO donors, samples of bacterial suspension were diluted in 63 buffer and plated on L agar plates. Colonies were scored after 24 h incubation at 37°C.

2.4. EPR study

Cells were grown aerobically in 1 l of medium to $OD_{600} \approx 0.4$. For preparation of one EPR sample, 250 ml of culture was centrifuged at $7000 \times g$ and concentrated to 5 ml prior to a 30 min incubation with small aliquots of NO donors or *o*-phenanthroline. The cells were then centrifuged, resuspended in 0.3 ml of the medium and quickly frozen in calibrated tubes for EPR analyses. X-band EPR spectra were recorded on a Radiopan spectrometer (Poland) under the following conditions: temperature 77 K, microwave power 5 mW, modulation amplitude 0.5 mT.

3. Results

We have tested the influence of NO donor compounds on induction of the SOS DNA repair response in *E. coli*. The SOS methodology allowed us to evaluate the ability of the compounds to induce expression of the *sfiA* gene. Treatment of *E. coli* cells with various NO donors resulted in induction of β-galactosidase activity. All types of NO donors examined gave a positive SOS response (Table 1).

Fig. 1 presents the dose-response relation between *sfiA* gene expression and doses of GSNO, SNAP, and glutathione-containing DNIC. DNIC appeared to be the most potent SOS inducer. The maximum *sfiA* gene expression was achieved when the DNIC concentration reached 100 μM, and remained at this level up to 2 mM. The induction of *sfiA* gene expression by GSNO was similar to that caused by DNIC at low concentrations (10–100 μM) but decreased sharply at concentrations higher than 500 μM. SNAP was the weakest SOS inducer (Fig. 1).

The toxicity of NO donors varied according to the nature of the NO-donating agent (Fig. 2). No changes in the level of surviving cells were observed after a 90 min incubation with 0.5 mM DNIC, whereas 0.5 mM GSNO or SNAP caused about 40% cell death. The fraction of *E. coli* cells which survived after treatment with 1–3 mM DNIC exceeded almost twice the number of cells which survived after treatment with the same concentrations of GSNO or SNAP. 5 mM GSNO or SNAP was 100% lethal after a 90 min incubation, whereas about 25% of the cells survived after incubation with 5 mM DNIC. This fact is all the more striking in that GSNO and SNAP contain only one NO^+-group each whereas DNIC contains two.

DNIC is a paramagnetic compound while present in a solution enriched with free thiols or after being attached to protein SH groups [13]. After incubation of *E. coli* cells with 1 mM DNIC, a typical anisotropic EPR signal was observed in frozen solution, with the axially symmetric *g*-factor with $g_\perp = 2.041$ and $g_\parallel = 2.014$ (Fig. 3a). The cell suspension, washed twice after incubation with 1 mM DNIC, displayed a decrease in the EPR signal (Fig. 3b). Incubation of the cells with 2 mM GSNO led to the appearance of a smaller EPR signal typical of DNIC (Fig. 3c), whereas incubation with 2 mM SNAP provided a significantly smaller DNIC signal. Preincubation of the cells with an iron-chelating agent, *o*-phenanthroline (OP), prevented the appearance of an EPR signal after GSNO addition (Fig. 3d) and decreased the EPR signal in cells incubated with DNIC.

Table 1 shows data on the induction of *sfiA* gene expression by NO-donating agents. *sfiA* gene expression induced by 0.5 mM DNIC or 0.5 mM GSNO was about 25% of the response to 4-NQO. *sfiA* gene induction by NO donors was similar to that induced by H_2O_2 and twice the low induction generated by 2 mM $ONOO^-$ or a 0.5 mM Fe^{2+}-citrate complex (Table 1). OP at concentrations higher than 0.1 mM inhibited the SOS response induced by all chemicals tested (Table 1). Centrifugation of the cells after preincubation with 0.1 mM OP partially reduced *sfiA* gene expression. Cell toxicity was not observed after 90 min incubation with 0.1–5 mM OP.

Table 1
Comparative study of *E. coli sfiA::lacZ* gene expression

Treatment	*sfiA::lacZ* expression, β-Gal units[a]	Induction ratio (IR)[b]	*sfiA:lacZ* expression after 0.1 mM *o*-phenanthroline	
			β-Gal units[a]	IR after centrifugation[c]
Untreated (control)	3.2	0	0.6	0
4NQO, 2.63 nM	26.6	7.3	0.9	2.1
DNIC, 0.5 mM	10.1	2.1	0.9	1
GSNO, 0.5 mM	9.1	2.0	0.7	1
Fe-citrate (1:5), 0.5 mM	5.8	0.8	–	–
H_2O_2, 0.03 mM	13.1	3.0	0.7	0.3
$ONOO^-$, 0.2 mM	3.5	0	–	–
$ONOO^-$, 2 mM	5.8	0.8	–	–

Bacteria at $\sim 10^8$ cells/ml were treated for 30 min at 37°C with chemicals where indicated and assayed for β-galactosidase activity (β-Gal) as described in Section 2.

[a]β-Gal units were measured as described in [11].

[b]Induction ratio (IR) was calculated according to:

$$IR = \frac{(\beta\text{-Gal})_s - (\beta\text{-Gal})_c}{(\beta\text{-Gal})_c}$$

where $(\beta\text{-Gal})_s$ is the β-Gal activity in cells treated with indicated agent and $(\beta\text{-Gal})_c$ is the β-Gal activity of untreated cells (control).

[c]IR is the induction ratio indicated for cells washed after incubation with 0.1 mM OP prior to β-Gal activity measurement.

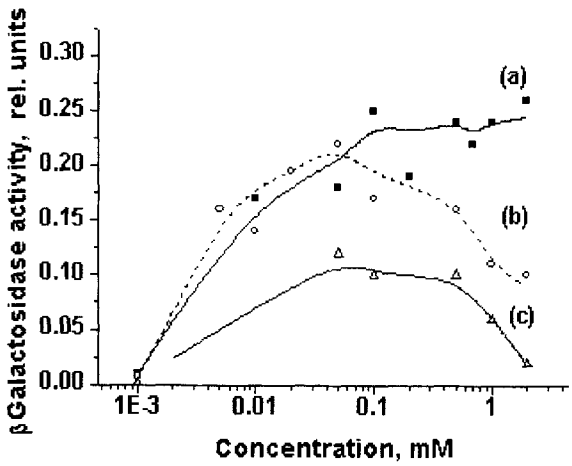


Fig. 1. Induction of the SOS DNA repair response by NO-donating agents in *E. coli* PQ37. Dependence of β-galactosidase activity on the concentration of DNIC (a), GSNO (b), and SNAP (c) was measured as described in Section 2. Relative units were calculated as the ratio of β-galactosidase activity with the NO donors used to the activity with 2.63 nM 4NQO (positive control). The results represent the average of three measurements.

4. Discussion

The data presented here show that NO donors can induce the SOS DNA repair response in *E. coli*. NO is one of the cytotoxic products liberated by activated macrophages, together with other reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\bullet}$) and $OH^\bullet$ (product of $O_2^{-\bullet}$ and H_2O_2 dismutation in the Fenton reaction catalyzed by iron). The increased susceptibility of the *E. coli recBC* mutant to NO donors suggests that DNA damage is a common result of this stress [5]. SoxR, a sensor for cell exposure to NO, stimulates the transcription of the *soxRS* regulon, including critical antioxidant defense proteins such as the oxidized DNA repair enzyme endonuclease IV and several others. Endonuclease IV deals with free radical damages such as fragmentation or oxidation of deoxyribose. Peroxynitrite, a product of a rapid reaction between NO and $O_2^{-\bullet}$, may induce oxidative DNA lesions [14]. Known DNA

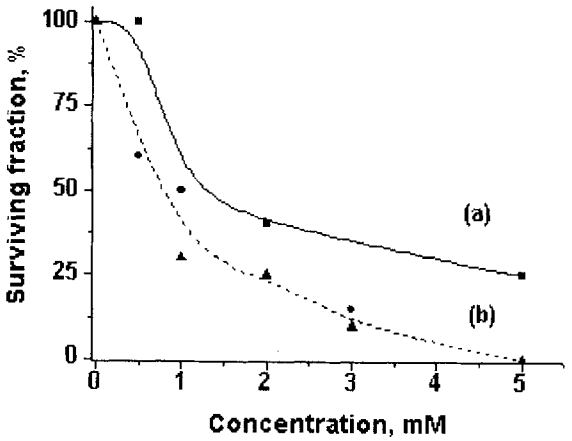


Fig. 2. Comparative toxicity of DNIC, GSNO, and SNAP. The survival of *E. coli* cells exposed to DNIC (squares, a), GSNO (triangles, b), and SNAP (circles, c) was evaluated as described in Section 2. The results represent the average of three measurements.

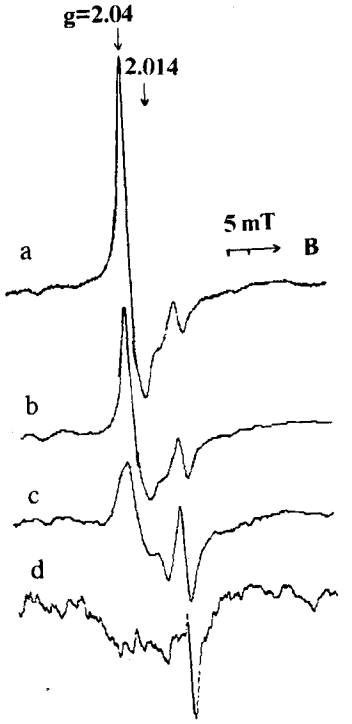
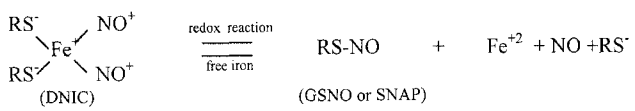


Fig. 3. EPR spectra of *E. coli* cells incubated with NO-donating agents. The samples were prepared as described in Section 2. The EPR spectra of *E. coli* cells incubated with 1 mM cysteine-containing DNIC and washed once (a) or twice (b) after incubation, incubated with 2 mM GSNO (c), and treated with 15 mM OP before the addition of GSNO (d), were recorded at 77 K, microwave power 5 mW, modulation amplitude 0.5 mT, and gains of 0.5×10^5 (a and b), 1×10^5 (c), and 2×10^5 (d).

damages that have been attributed to NO include deamination of adenine, guanine, cytosine, and 5-methylcytosine [15]. The exact nature of the lesions in SOS response induction remains to be determined. It is likely that the SOS response is induced by most types of DNA damage: single-strand breaks (SSB), double-strand breaks, and base damages [16]. It is thus not surprising that NO triggers the SOS response. Our results show that $ONOO^-$, known to cause SSB [14], contributes slightly to SOS induction. This means that the effects of DNIC and GSNO differ from those induced by peroxynitrite.

Analysis of the NO donor effects on the SOS response and cell toxicity revealed a strong influence of intracellular iron. Under physiological conditions, the NO generated may form RSNO or DNIC in reactions with thiols (such as glutathione, cysteine or protein SH groups) and iron [13]. The chemical structures of the NO donors used and the reactions which can take place inside the cells in the presence of these NO donors, iron and free thiols are given in the scheme below:



(1)

$$\text{DNIC} + \text{SH protein or Fe-S protein} \rightleftarrows \text{DNIC protein} + 2RS^- \quad (2)$$

The potent induction of the SOS response by DNIC indi-

cates that extracellular iron supplied by DNIC stabilizes the effect. The EPR study showed that DNIC enters the cells because cells treated with the agent and washed twice still exhibited a DNIC-type EPR signal (Fig. 3b). Incubation of the cells with an equivalent concentration of GSNO led to the appearance of a DNIC-type EPR signal, in agreement with the reverse reaction (1) in the scheme, which indicates the availability of iron inside the cells. The EPR signal increased until the concentration of added GSNO was about 1–2 mM. These GSNO or SNAP concentrations strikingly changed the proportion of surviving cells (Fig. 2). In accordance with the scheme, the fate of DNIC and RSNO depends on the thiol group concentration, accessible iron and stability of Fe-S proteins inside the cell. The significant increase of GSNO and SNAP toxicity in *E. coli* cells observed at concentrations which exhausted all intracellular iron for nitrosothiol binding (according to the EPR data) led us to conclude that the heterolytic thiol-to-thiol NO^+ transfer to essential SH groups of proteins was responsible for RSNO cytotoxicity. DNIC could also contribute to cytotoxicity after it has been degraded and has released RSNO (see scheme). The lower DNIC toxicity may be associated with its ability to release NO (as in the scheme), which correlates with NO antibacterial activity in *Salmonella typhimurium* [9]. Interestingly, the induction of the heat shock response, which is involved in degradation of SOS toxic products [17], was also more pronounced with DNIC than with GSNO [7].

The influence of the iron-chelating agent OP on deleterious free radical effects in biological systems can be changed from protection to sensitization due to the significant role metal ions play in $O_2^{-\bullet}$ toxicity [18]. DNA base damage can be potentiated through the intercalation of metal-OP complexes between DNA base pairs [19]. In cultured rat astrocytes, OP can mimic heat shock, inducing the heat shock protein HSP 68 [20]. In our case, however, it seems that OP decreased the effects of DNIC and GSNO by extracting iron ions from the cells. Indeed, our EPR study showed that high OP concentrations can remove all iron accessible for DNIC formation after GSNO addition (Fig. 3d). Even a low OP concentration prevented induction of the SOS response by all agents tested (Table 1). The above effect may be caused by an interaction with iron, possibly on the DNA surface, which may be responsible for NO triggering of the SOS DNA repair response, similar to that induced by $O_2^{-\bullet}$-mediated damage [21].

Intracellular iron ions are thus seen to play a dual role in the interaction of NO-donating agents with *E. coli* cells. On the one hand they can transform and stabilize GSNO in the form of less toxic DNIC; and on the other hand they play a substantial role in the formation of the SOS signal induced by NO-donating agents.

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