Nitric oxide inhibits DNA ligase activity: potential mechanisms for NO-mediated DNA damage

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Introduction

Several studies suggest that NO is the causative agent in a number of patho-physiological events (1–3). In deciphering potential toxicological mechanisms involving NO, a number of biological targets for NO and its derived reactive nitrogen oxide species (RNOS) or indirectly by inhibiting various repair processes. DNA ligases are enzymes which rejoin single-strand breaks and are critical for DNA integrity during processes such as gene transcription and repair. The eukaryotic and T4 DNA ligases are active in the presence of ATP and act in two steps: the formation of protein-AMP intermediates, then the ligation of DNA breaks. When T4 DNA ligase was exposed to the NO generator DEA/NO (Et$_3$N[NO(NO)]Na), a concentration- and time-dependent inhibition of these two steps, adenylylation of the protein and ligation of the substrate, was observed. This inhibition was abated by the presence of cysteine, suggesting that RNOS, rather than NO, mediated the inhibition of the ligase activity. As mammalian and T4 DNA ligases act by the same mechanism, the inhibition of DNA ligase may explain the increase in single-strand breaks reported for cells exposed to NO and provides a mechanism to increase DNA lesions without direct chemical modification of DNA by NO or RNOS.

Materials and methods

Chemicals

T4 DNA ligase was from Boehringer Mannheim. (dT)$_{16}$ and poly(dA) were from Pharmacia. (C$_5$H$_5$)$_3$Ni(NO(NO)]Na$^+$ (DEA/NO) was supplied by Dr J.Saavedra and was prepared as previously described (15). [a-$\beta$-3]$^2$P$^3$ATP (3000 Ci/mmol) was from Amersham. Dithiothreitol (DTT) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co.

Incubation of DNA ligase with NO

DEA/NO was dissolved in a basic solution (pH 11.5) at a concentration of 25 mM. The T4 DNA ligase (1 µl, 6 µg/µl, 0.45 µg protein) was diluted in a final volume of 6 µl in a buffer containing 70 mM HEPES, pH 7.5, 10 mM MgCl$_2$ and increasing amounts of DEA/NO (final pH 7.7). After 15 min at 25°C, the protein was immediately used to measure either the formation of DNA ligase-AMP intermediates or ligase activity.

Formation of DNA ligase-AMP intermediates

T4 DNA ligase (2 U, 0.150 µg protein treated or not with DEA/NO) was incubated at 25°C for 15 min in a buffer containing 66 mM HEPES, pH 8.0, 10 mM MgCl$_2$ and [a-$\beta$-3]$^2$P$^3$ATP diluted with non-radioactive ATP to a sp. act. of 250 Ci/mmol and added to a final concentration of 2 µM ATP (16). The final reaction volume was 10 µl. The reaction products were analyzed by electrophoresis through SDS-polyacrylamide gels. The DNA ligase-AMP complex was visualized by autoradiography and quantitated by densitometry.

DNA ligase activity determination

The substrate was [5',-$\beta$-3]$^2$P$^3$poligo(dT)$_{16}$poly(dA) prepared as described (10). T4 DNA ligase (0.6 U) was incubated in a buffer containing 66 mM HEPES, pH 7.5, 5 mM MgCl$_2$ and 35 µM ATP, the substrate (~40 000 c.p.m.), in a final volume of 20 µl. After 15 min at 37°C, the reaction was stopped by

\[ 4\text{NO} + O_2 \rightarrow N_2O_3 \text{[presumably N}_2\text{O}_3 \text{]} \]  

(1)

It was shown that these intermediates can damage macromolecules such as DNA on exposure to very high concentrations of NO (5–9). Further investigation of the chemistry of these intermediates revealed that RNOS could also inhibit some DNA repair proteins, in particular those that contain thiol-dependent motifs. We have reported that O$^5$-methylguani-

Abbreviations: RNOS, reactive nitrogen oxide intermediates; DEA/NO, (C$_5$H$_5$)$_3$Ni(NO(NO)]Na$^+$; DTT, dithiothreitol; BSA, bovine serum albumin; CHO, Chinese hamster ovary.

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adding 20 μl formamide dye and heating at 95°C for 10 min. The reaction products were then separated through 15% polyacrylamide–8 M urea gels. The radioactive multimers were detected by autoradiography and quantitated by densitometry.

The DNA ligase activity was measured in Chinese hamster ovary (CHO) cell extracts as described (17). Briefly, exponentially growing cells were trypsinized, suspended in a buffer containing 66 mM Tris, pH 7.6, 50 mM MgCl2 and 10% glycerol and disrupted by sonication. Cell debris was removed by centrifugation. Cell extract was incubated or not with DEA/NO (2.5 mM) for 15 min at 37°C, then with the radioactive oligo(dT)-poly(dA) substrate for different lengths of time. The reaction was stopped by heating at 68°C for 30 min, then the samples were treated with 2.5 U alkaline phosphatase for 1 h at 37°C. After addition of BSA and 10% trichloroacetic acid, the acid-precipitable material was collected on filters and the radioactivity determined.

**Results**

**Influence of NO on DNA ligase adenylylation**

The reaction mechanism of ligase involves, in a first step, the formation of a covalent ligase–AMP intermediate with a lysine–adenylate phosphoamide bond (14). To measure the influence of NO on this step of the reaction, T4 DNA ligase was treated with various DEA/NO concentrations for 15 min at 25°C prior to incubation with [α-32P]ATP. As shown in Figure 1, formation of DNA ligase–adenylate was inhibited in the presence of NO. The amount of adenylylated protein decreased with DEA/NO concentration. The amount of DEA/NO required to inhibit the activity by 50% (IC50) was 0.6 mM DEA/NO (Figure 2A). To correlate the release of NO from DEA/NO with this inhibition, a time-dependent experiment was performed. Incubating the protein with DEA/NO (1 mM) for increasing time intervals resulted in an increased inhibition of adenylylation (Figure 2B), whereas a 5 min DEA/NO treatment resulted in nearly 50% inhibition. The amount of NO released from DEA/NO was calculated using the formula:

\[ [\text{NO}] = \frac{[\text{DEA/NO}] + [\text{NO}]_0}{k + [\text{DEA/NO}]_0} \]

where \( k = 0.0005 \text{s}^{-1}, [\text{C}]_0 = 1 \text{mM} \) and \( E_{\text{NO}} = 0.89 \). The calculated total amount of NO released over 5 min was 130 ± 0.03 μmol/l. The amount of NO released from 0.6 mM DEA/NO in 15 min was 190 ± 0.05 μmol/l. There was close correlation between the amounts of NO at these IC50 values obtained in Figure 2A and B and a correlation between the rate of NO release and inhibition of the adenylylation reaction. The amount of NO required to inhibit adenylylation was similar to that for inhibition of the alkyltransferase (90 μmol/l) (10) and Fpg protein (120 μmol/l) activities (12).

**Influence of NO on the DNA ligase activity**

The enzyme was pretreated for 15 min at 25°C with various concentrations of DEA/NO, then its enzymatic activity was measured by the joining of single-strand interruptions in the double-stranded substrate [5'-32P]oligo(dT)16-poly(dA)]. Separation of the multimers in denaturing polyacrylamide gels showed that DEA/NO treatment decreased the activity of the protein (Figure 3). There was a dose-dependent inhibition with an IC50 of ~0.5 mM DEA/NO (Figure 4A). The total amount of NO released under these conditions using the above formula was 160 ± 30 μmol/l. When the protein was exposed to 1 mM DEA/NO for various time intervals (Figure 4B), there was a time-dependent decrease in its activity, with 50% inhibition observed after ~7 min. The total amount of NO released under these conditions, calculated from the above formula, was 170 ± 30 μmol/l. Comparing the amount of NO released at the IC50 values in the timing experiment (Figure 4B) versus the dose experiment (Figure 4A), there was again a correlation between the total amount of NO released and inhibition of the enzyme activity. When the DEA/NO solution was allowed to stand at 50°C at pH 7.7, which results in DEA/NO decomposition (15), no inhibition of ligase activity was observed (Table I), showing that the inhibition process is actually due to NO release. Comparison of the NO required for adenylylation and ligase inhibition was nearly identical, suggesting that a common pathway of inhibition by NO occurs for both processes.

It has been shown that NO inhibits various enzymes, not by a direct chemical reaction with the protein, but through the formation of RNOS (4). To determine whether NO or RNOS derived from the NO/O2 reaction was responsible for DNA inhibition, a time-dependent experiment (Figure 2B) was performed. Incubating the protein with DEA/NO (1 mM) for increasing time intervals resulted in an increased inhibition of adenylylation (Figure 2B), whereas a 5 min DEA/NO treatment resulted in nearly 50% inhibition. The amount of NO released from DEA/NO was calculated using the formula:

\[ [\text{NO}] = \frac{[\text{DEA/NO}] + [\text{NO}]_0}{k + [\text{DEA/NO}]_0} \]

where \( k = 0.0005 \text{s}^{-1}, [\text{C}]_0 = 1 \text{mM} \) and \( E_{\text{NO}} = 0.89 \). The calculated total amount of NO released over 5 min was 130 ± 0.03 μmol/l. The amount of NO released from 0.6 mM DEA/NO in 15 min was 190 ± 0.05 μmol/l. There was close correlation between the amounts of NO at these IC50 values obtained in Figure 2A and B and a correlation between the rate of NO release and inhibition of the adenylylation reaction. The amount of NO required to inhibit adenylylation was similar to that for inhibition of the alkyltransferase (90 μmol/l) (10) and Fpg protein (120 μmol/l) activities (12).

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Nitric oxide inhibits DNA ligase activity

Fig. 3. Inhibition of the DNA ligase activity by DEA/NO. Equal amounts of DNA ligase were treated or not with increasing DEA/NO concentrations, then incubated with the substrate \([5'-\text{32P}]\text{oligo(dT)16-poly(dA)}\) for 15 min at 37°C. The \text{oligo(dT)16} multimers were separated in polyacrylamide–urea gels and visualized by autoradiography. Lane 1, control without enzyme; lane 2, T4 DNA ligase without DEA/NO; lanes 3–5, incubated with 0.5 (lane 3), 1 (lane 4) or 1.5 mM (lane 5) DEA/NO.

Fig. 4. Influence of NO on T4 DNA ligase activity. The protein was incubated in the presence of increasing amounts of DEA/NO for 15 min before measuring its activity. The \text{oligo(dT)16} multimers were separated by electrophoresis and quantitated by densitometry (A) or the protein was incubated with DEA/NO (1 mM) for various lengths of time, then with the substrate for 15 min at 37°C (B).

ligase activity inhibition, the protein was incubated with DEA/NO in the presence of cysteine. We have previously shown that cysteine has a high affinity for RNOS species while not directly reacting with NO (18). When the T4 DNA ligase was incubated with 10 mM DEA/NO in the presence of 10 mM cysteine, significant protection of the activity was observed (Table I). A similar protection in the presence of cysteine was observed for the adenylylation reaction (data not shown). This result suggests that RNOS could be responsible for the inactivation of the protein, possibly resulting in a S-nitrosothiol protein adduct. These S-nitrosothiols decompose with time, which can result in restoration of activity in the presence of DTT (10). However, exposing the enzyme to 1 mM DEA/NO and then measuring its activity at various time intervals for 24 h revealed no recovery of the activity, even in the presence of DTT (data not shown). The effect of DEA/NO was also measured in the presence of BSA. Addition of BSA (1 mg/ml) in the DEA/NO-containing buffer showed no effect on either formation of protein–AMP intermediates (data not shown) or on ligase activity (Table I). To confirm that the presence of a large excess of protein is not sufficient to protect the enzyme against inactivation by NO, extracts from CHO cells were incubated with DEA/NO for 15 min at 37°C; ligase activity was found to be decreased by ~65%, showing that NO is able to reduce the activity even in the presence of high concentrations of proteins.

Table I. Influence of DEA/NO on T4 DNA ligase activity in the presence of cysteine or BSA.

<table>
<thead>
<tr>
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<th>DNA ligase activity(^a) (%)</th>
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<tbody>
<tr>
<td>Control protein</td>
<td>6.5 (100)</td>
</tr>
<tr>
<td>Protein + DEA/NO (1 mM)</td>
<td>0.58 (9)</td>
</tr>
<tr>
<td>Protein + expired DEA/NO (1 mM)(^b)</td>
<td>5.9 (90)</td>
</tr>
<tr>
<td>Protein + DEA/NO (1 mM) + cysteine (10 mM)</td>
<td>4.4 (68)</td>
</tr>
<tr>
<td>Protein + DEA/NO (1 mM) + BSA (1 mg/ml)</td>
<td>1.17 (18)</td>
</tr>
</tbody>
</table>

\(^a\) fmol \text{oligo(dT)poly(dA)} ligated in 15 min at 37°C by 0.1 U T4 DNA ligase.

\(^b\) DEA/NO was incubated at 50°C at pH 7.7 for 45 min prior to addition of the protein.

Discussion

The presence of NO has been shown to inhibit specific enzymes. Although DNA repair enzymes such as endonucleases III and IV or uracil DNA glycosylase are not inhibited by NO (12), proteins which contain critical thiol residues are inhibited.
This is the case for the $O^\delta$-methylguanine-DNA methyltransferase (alkyltransferase), whose active site contains a cysteine residue (10). This inhibition is expected because the RNOS formed in the NO/$O_2$ reaction has a high affinity for thiol-containing residues (18). The resulting product forms a $S$-nitrosothiol which either inactivates a thiol or can labilize zinc from the zinc finger motifs (11,12).

Our results show that T4 DNA ligase activity was inhibited in the presence of NO. Recovery of the activity was observed neither with time nor in the presence of DTT, suggesting that the reaction is different from that observed in the case of the alkyltransferase protein. A critical lysine residue is present in the active site of T4 DNA ligase protein (13). This residue initially forms an intermediate with an adenyl group from ATP, then the adenyl group is transferred to the 5'-P end of DNA. In the final step, unadenylated ligase is required for generation of a phosphodiester bond. The initial adenylation of the lysine is therefore a crucial step. In the presence of NO, nitrosation of the lysine would form a primary nitrosamine, which would rapidly rearrange to the diazonium salt, followed by hydrolysis to yield the corresponding hydroxy adduct (equation (2)).

\[
\text{Protein-NH}_2 + \text{NO}_x \rightarrow \text{protein-NH-NO} \rightarrow \text{protein-OH} + N_2
\]  
(2)

It has been reported that the lysine residue is partially in the deprotonated form in the ligase protein (14). The lone pair of electrons of the nitrogen atom that bind to the adenylate group via nucleophilic attack provides a site for electrophilic attack. In the presence of NO, nitrosation of the partially deprotonated amine could occur via NO$_x$, which then results in primary nitrosamine. Since primary nitrosamines undergo deamination (19), this nitrosamine would result in a hydroxy group (equation (2)). This chemistry represents a new mechanism by which NO (via RNOS) can interact with proteins, suggesting that the pH of the protein site of the critical lysine residue is an important determinant for interaction with RNOS.

Nitrosation of biological substances can arise through several different chemical mechanisms in vivo. In this paper, the reaction between NO and oxygen to form isomers of $N_2O_4$ (4) was used to assess the chemistry of nitrosation on ligase (equation (1)). Another potential in vivo source of nitrosating species is derived from acidic nitrite, which might be expected in areas of the gastrointestinal tract or in a phagocytic macrophage. Alternatively, $N_2O_3$ can be formed from the interaction of NO and superoxide, which results in peroxynitrite (OONO$^-$).

\[
\text{NO} + O_2^- \rightarrow \text{OONO}^-
\]  
(3)

In the presence of excess NO or superoxide, peroxynitrite is converted to nitrogen dioxide (20-22).

\[
\text{H}^+ + \text{OONO}^- + \text{NO} \rightarrow \text{NO}_2 + \text{NO}_2^-
\]  
(4)

\[
\text{H}^+ + \text{OONO}^- + \text{O}_2^- \rightarrow \text{NO}_2
\]  
(5)

Nitrogen dioxide can rapidly react with NO to form the nitrosating species, $N_2O_3$ (equation (6)).

\[
\text{NO}_2 + \text{NO} \rightarrow N_2O_3
\]  
(6)

The presence of $N_2O_3$ could then nitrosate the lysine residue of ligase, thus inactivating the enzyme.

Nitrosation of amines and thiols have been shown under various biological conditions. It has been shown that activated neutrophils and macrophages generate significant nitrosation of amines (23,24), while RNOSs such as peroxynitrite have been shown to be formed in alveolar macrophages (25). It has been suggested that nitrosation of morpholine from lipopoly saccharide-stimulated RAW macrophages occurs via equations (1) and (2) as well as equations (3)-(6) (26). In addition to cell experiments, nitrosation of amines and thiols has been shown to occur in chronically infected tissue (27-29). It would appear that amine nitrosation occurs in a variety of biological conditions which may result in inactivated ligase function. (The rate of the NO formation from stimulated macrophages and neutrophils is $-0.5-1 \text{ nmol/min/10}^6 \text{ cells}$; Calculating this value in terms of the cell volume there is $-0.5-1 \text{ nmol/l/min NO}$ produced. In these experiments, 0.02-0.4 nmol/l/min were produced during the NO exposure, suggesting that there is sufficient NO generated from these stimulated immune cells to inhibit ligase.)

Different DNA ligases have been found in mammalian cells (30). DNA ligase I is involved in the ligation of replication intermediates into high molecular weight DNA during DNA replication (31). A role in meiotic recombination has been suggested for DNA ligase II (32) and DNA ligase III activity has been related to DNA repair (33,34). Recently, a fourth DNA ligase has been identified (35). It has also been shown that an inherited molecular defect in DNA ligase I resulted in immunosuppression, lymphoma and hypersensitivity to DNA damaging agents (36). Therefore, inhibition of DNA ligase activity could play a crucial role in the cell and could explain some previous observations. Exposure of cells to NO results in an increased number of DNA single-strand breaks (6). However, when purified DNA is exposed to NO, even at doses resulting in an RNOS concentration of 1 M, there is no formation of single-strand breaks (9). This implies that direct chemical modification of DNA by NO or RNOS produces DNA breaks in vitro. Our results suggest that NO inhibits DNA ligase activity resulting in the accumulation of DNA breaks formed either during transcription or repair, thus explaining these lesion observed in cells. Another implication of NO-mediated ligase inhibition is that genotoxicity resulting from either RNOS or reactive oxygen species might be amplified. It has been shown that activated macrophages result in deamination of DNA (37). Furthermore, under conditions where superoxide and peroxide are formed, the presence of NO can induce single- and double-strand breaks, possibly by peroxynitrite or mobilization iron, which furthers Fenton-type oxidation in the nucleus (37-40). Repair of both abasic formation via deamination and direct oxidation of deoxyribose involve ligase. Thus, inhibition of ligase may increase the number of these lesions. By whichever mechanism, the increase in DNA breaks due to NO-mediated inhibition of ligase could in turn activate the tumor suppressor gene p53 (41) or activate poly(ADP-ribose) synthesis (42).

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References


