Interaction of Nitric Oxide with Glutathione or Cysteine Generates Reactive Oxygen Species Causing DNA Single Strand Breaks

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It was found that reactive oxygen species (ROS) were generated in the interactions of nitric oxide (NO) with glutathione (GSH) or cysteine (CySH) under aerobic conditions. When supercoiled DNA was incubated with a mixture of NO/GSH, NO/CySH, NOC-7 (a NO donor)/GSH or NOC-7/CySH under aerobic conditions, DNA single-strand breaks were observed on agarose gel electrophoresis. The strand breaks were inhibited by common ROS scavengers: superoxide dismutase+catalase, the spin trapping agent 5,5-dimethyl-1-pyrroline-N oxide (DMPO), ethanol, and EDTA. The strand breaks were also caused by incubation with a mixture of S-nitrosogluthathione (GSNO) with GSH or CySH, which was inhibited by ROS scavengers. In the reaction of NO/GSH, GSNO rapidly formed and then gradually decreased, and in the reaction of GSNO/GSH, GSNO was gradually decreased. The decrease in GSNO was accelerated in the presence of superoxide+catalase. Hydroxyl radical was detected in the mixtures of NO with GSH or CySH under aerobic conditions, and thyl radicals were detected in the mixtures of GSNO with GSH or CySH under anaerobic conditions as examined in electron spin resonance studies using DMPO as a spin trap. The results indicate that the interaction of NO with thiols in the presence of O2 generates ROS that caused DNA single-strand breaks.

Key words nitric oxide; glutathione; cysteine reactive oxygen species; DNA single-strand break

Nitric oxide (NO) has many important biologic functions,1,2 but it exerts many damaging effects on lipids, proteins and DNA through conversion into reactive nitrogen oxide species including nitrogen dioxide (NO2), dinitrogen trioxide (N2O3), and peroxynitrite (ONOO−). The reactivity of NO is enhanced by oxygen (O2) through the conversion into reactive intermediates NO2 and N2O3 as in Eqs. 1 and 2, and finally into nitrite (NO2−) as in Eqs. 3 and 4.3,4)

Biologically important S-nitrosothiols such as S-nitroso-glutathione (GSNO) and S-nitroso-cysteine (CySNO) are thought to be important NO pools in biological systems.5,6 It has been generally considered that the S-nitrosothiols are produced by reactions of glutathione (GSH) or cysteine (CySH) with NO through the O2-dependent pathway as in Eq. 5, and they undergo transnitrosation as in Eq. 6 and thiolation as in Eq. 7. The chemistry of S-nitrosothiols, however, appears more complex7—10 and remains obscure. Recent investigations have suggested that glutathionyl radical (GSH·) and superoxide (O2·−) are generated in the reactions of NO/GSH10 and GSNO/GSH7 systems under aerobic conditions.

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\begin{align*}
2\text{NO} + \text{O}_2 &\rightarrow 2\text{NO}_2 \\
2\text{NO}_2 + 2\text{NO} &\rightarrow 2\text{N}_2\text{O}_3 \\
2\text{N}_2\text{O}_3 + 2\text{H}_2\text{O} &\rightarrow 4\text{NO}_2^- + 4\text{H}^+ \\
4\text{NO}_2^- + 2\text{H}_2\text{O} &\rightarrow 4\text{NO}_2^- + 4\text{H}^+ \\
\text{RSH} + \text{N}_2\text{O}_3 &\rightarrow \text{RSNO} + \text{HNO}_2 \\
\text{RSNO} + \text{R'}\text{SH} &\rightarrow \text{RSNO} + \text{R}'\text{SNO} \\
&\rightarrow \text{RSSR' + HNO}
\end{align*}
\]

The aim of the present study was to determine whether the interaction of NO/thiols in an O2-dependent generated reactive oxygen species (ROS), which would cause DNA single strand breaks. The results obtained here indicate that the interaction of NO with GSH or CySH generated ROS causing DNA single-strand breaks.

MATERIALS AND METHODS

Materials Purified air, NO gas (purity 99.9%) was obtained from Nihonsanso Ltd. (Tochigi, Japan), and highly purified nitrogen gas (purity greater than 99.9%) was obtained from Taiyo-Toyosanso Ltd. (Kanagawa, Japan). Plasmid supercoiled pBR322 DNA (in Tris buffer with EDTA) was obtained from New England Biolabs (Beverly, MA, U.S.A.). Glutathione reduced form (GSH), superoxide dismutase (SOD) [EC 1.15.1.1] (from bovine liver, 2600 units/mg), and catalase [EC 1.15.1.6] (from bovine liver, 13300 units/mg) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). 1-Hydroxy-2-oxo-3-(N-methyl-3-aminoethyl)-3-methyl-1-triazeno (NOC-7) and GSNO were obtained from Dojindo Laboratories. (Kumamoto, Japan). 5,5-Dimethyl-1-pyrroline-N oxide (DMPO) and N-tetra-butyl-α-phenyl nitrore (PBN) were obtained from Labotec (Tokyo, Japan).

All the aqueous solutions were prepared with deionized, distilled, purified water using a Milli-Q water purification system (Simpli Lab, Nihon Millipore Ltd., Tokyo, Japan), and finally by passing through a column of Chelex 100 resin (sodium form, 100—200 mesh) (Bio-Rad Laboratories, CA, U.S.A.).

NO Solution NO solution 100 ml in deaerated phosphate buffer 0.1 M (pH 7.4) was prepared by introducing pure NO gas as described elsewhere.11 Three precautions were paid in order to minimize contamination of NO2, NO gas was purified on a column of KOH pellet to remove NO2 in the NO gas tank generated by dismutation of NO before introduction into the deaerated buffer in the flask. A column of sodium hydrosulfit on glass wool was attached to the flask in order to avoid exposure of the flask contents to atmospheric O2. Nitrogen gas was purged to remove NO in the headspace of the flask. Nitrogen oxide species in the solution in the absence of O2 were determined by the modified Saltzman

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A agarose gel electrophoresis of plasmid supercoiled pBR322 DNA treated with a mixture of NO/GSH (A), NO/CySH (B), NOC-7/GSH (C), and NOC-7/CySH (D).

Plasmid DNA was incubated with NO or NOC-7 500 μM and GSH or CySH at the indicated concentrations at pH 7.4 and 37°C for 24 h under aerobic conditions. The mixture was subjected to agarose gel electrophoresis. The migration positions of form I (supercoiled) and form II (nicked open circular) DNA are indicated.

Fig. 1. Agarose Gel Electrophoresis of Plasmid Supercoiled pBR 322 DNA Treated with a Mixture of NO/GSH (A), NO/CySH (B), NOC-7/GSH (C), and NOC-7/CySH (D)

DNA single-strand breaks induced by combination of an aqueous solution of NO or the NO donor NOC-7,13,15 which gradually releases NO, with GSH or CySH were examined. Supercoiled (form I) DNA is convertible into nicked open circular form (form II) DNA by its single-strand breaks, which may be separately detected on agarose gel electrophoresis.13) When plasmid supercoiled DNA was incubated with NO 500 μM alone for 24 h under aerobic conditions, form I DNA was unchanged. In contrast, when DNA was incubated with NO 500 μM/GSH 100—2000 μM or with NO 500 μM/CySH 100—2000 μM under aerobic conditions, thiol concentration-dependent DNA single-strand breaks with formation of form II DNA occurred (Figs. 1A, B). When DNA was incubated with NOC-7 500 μM/GSH 100—2000 μM or with NOC-7 500 μM/CySH 100—2000 μM under aerobic conditions, thiol concentration-dependent DNA single-strand breaks occurred (Figs. 1C, D).

The effects of the ROS scavengers SOD+catalase, which would convert both O2· and hydrogen peroxide (H2O2) into O2 (Figs. 2A, B, lane a), spin trapping agents DMPO (lane b) and PBN (lane c), which can partially trap radical species including ROS as more stable radicals, ethanol (lane d) which can trap ·OH radical, and EDTA (lane e), which may mask metal ions to prevent generation of ·OH radical from both O2· and H2O2 via Fenton-type and/or Haber–Weiss conversion, on DNA strand-breaking induced by NO 500 μM/GSH 100 μM and NO 500 μM/CySH 100 μM were examined. All scavengers except for PBN clearly inhibited DNA single-strand breaks. DNA strand breaking induced by NOC-7 500 μM/GSH 100 μM and NOC-7 500 μM/CySH 100 μM was also inhibited by SOD+catalase and ethanol (Figs. 2C, D). The inhibition studies indicated that the species causing DNA strand-breaks were ROS with previously known DNA strand-breaking activity.16) When the effects of various amino acids on DNA strand-breaking caused by NO/GSH or NO/CySH were examined, most showed no effects at concentrations lower than 10 mM, whereas tyrosine (Tyr) and tryptophan (Trp) inhibited it in a dose-dependent manner (Figs. 3A, B). Inhibition by Tyr can be due to scavenging ONOO− formed by reaction of NO with O2·.17) which is able to cause
DNA strand-breaks. 18–20) However, ONOO− may not participate in the DNA strand breaking in the interaction of NO/thiol systems, because 3-nitrotyrosine 21) was not detected in the reaction mixture of NO/GSH with Tyr (data not shown). Tyr and Trp may affect certain processes of the interaction of NO with the thiols.

DNA strand-breaks were not observed when DNA was incubated with NO 500 μM/GSNO 100 μM under aerobic conditions (data not shown), indicating that strand-breaks did not occur during the interaction of NO and GSNO. While DNA strand-breaks were not induced by GSNO 100 μM alone (Figs. 4A, B), extensive strand-breaks were induced by interaction with GSNO 100 μM/GSH 100 or 1000 μM (Fig. 4A), and GSNO 100 μM/CySH 100 or 1000 μM under aerobic conditions (Fig. 4B). These strand-breaks were inhibited in the presence of ROS scavengers (Figs. 4A, B, lanes a—e), indicating that ROS were also generated by the interaction with S-nitrosothiols/thiols.

Under the present conditions of the reaction of NO with GSH, the production and decrease of GSNO was monitored by HPLC. NO 500 μM was added to the buffer containing GSH 10—2000 μM, and the mixture was incubated at 37°C for 24 h under aerobic conditions (Fig. 5). The time-course studies of the GSNO level in the solutions indicated that GSNO was rapidly formed depending on the initial concen-
GSH (and then decreased over 24 h. The GSNO level in the NO 500 μM/GSH 100 μM system was decreased in the presence of SOD/catalase (data not shown), suggesting that ROS decreased the GSH level in the system and thus prevented the decay of GSNO due to GSH. When GSNO 100 μM solution was incubated for 24 h, GSNO gradually decreased, and the decay was accelerated in the presence of SOD/catalase. Accelerated decay of GSNO by scavenging ROS may be caused by protection of GSH from oxidation by ROS. The results indicate that during the interaction of NO with GSH, GSNO is rapidly formed and subsequently degraded by GSH to generate ROS.

The ESR technique using DMPO as a spin trap was employed for detection of radical species. While no ESR signals were observed in the mixture of NO with DMPO, distinct ESR signals were observed in the mixtures of NO/GSH and NO/CySH under aerobic conditions. When NO 500 μM was mixed with GSH 100 or 500 μM, characteristic four line 1:2:2:1 signals due to the DMPO-OH adduct with hyperfine splitting constants of $a_N = a_H = 1.49$ mT appeared (Fig. 7A). These signals were not observed in the presence of ethanol, and instead six line ESR signals due to the DMPO-hydroxyethyl adduct with hyperfine splitting constants of $a_N = 1.59$ mT and $a_H = 2.31$ mT appeared. When NO 500 μM was mixed with CySH 100 or 500 μM, the same DMPO-OH signals were observed, which were replaced by the DMPO-hydroxyethyl signals in the presence of ethanol (Fig. 7B). These results indicate that ·OH radical was generated in the interactions of NO/GSH and NO/CySH under aerobic conditions.

While detection of ESR signals in the mixtures of GSNO with GSH or CySH under aerobic conditions was unsuccessful, distinct ESR signals were observed in the mixtures under anaerobic conditions. When GSNO 100 μM was mixed with GSH 100 μM under anaerobic conditions four line ESR signals with hyperfine splitting constants of $a_N = 1.55$ mT and $a_H = 1.53$ mT appeared (Fig. 8). Although these signals resembled those of the DMPO-OH adduct, the hyperfine splitting constants were slightly different from those of the DMPO-OH adduct and identical to those of the DMPO-SG adduct, suggesting the generation of glutathionyl (GS·) radical. When GSNO 100 μM was mixed with CySH 100 μM, six line ESR signals with hyperfine splitting constants of $a_N = 1.77$ mT and $a_H = 1.55$ mT appeared (Fig. 8). The signals and the hyperfine splitting constants were identical to those of the DMPO-CyS adduct, indicating the generation of cysteinyl (CyS·) radical. These results indicate that the interactions of GSNO with GSH or CySH generated GS· and CyS· radicals, respectively, under anaerobic conditions.
It is known that ROS generated in various systems causes DNA modification and single-strand breaks. ·OH radical reacts with the thymine base of DNA to form thymine glycol\(^{[35]}\) and with the guanine base to form 8-hydroxyguanine\(^{[35]}\) and reacts with the deoxyribose moiety by abstracting hydrogen atoms leading to sugar radicals\(^{[35]}\) which causes liberation of base moieties and strand cleavage.\(^{[18]}\) In the present study, it was found that the interactions of NO with thiols produces \(\cdot S\)-nitrosothiols and ROS under aerobic conditions, and that the interactions of \(\cdot S\)-nitrosothiols with thiols causes degradation of \(\cdot S\)-nitrosothiols accompanying concomitant generation of ROS under aerobic conditions. Generation of ROS in both the interactions of NO with thiols and the interactions of \(\cdot S\)-nitrosothiols with thiols was demonstrated by the observation of DNA single-strand breaks and the inhibiton of the breaking by ROS scavengers.

\(\cdot\text{NOO}^+\) that is produced by the reaction of NO and \(O_2^-\) \(^{[17]}\) is another possible candidate for the active species \(^{[18之外511]}\) that causes DNA single-strand breaks. However, this possibility was ruled out, because 3-nitrotyrosine \(^{[22]}\) was not produced in the reaction of Tyr with the NO/GSH system.

It has been generally thought that \(\cdot S\)-nitrosothiols are produced by reaction of NO with thiols through the \(O_2^-\)-dependent pathway, and \(\cdot S\)-nitrosothiols undergo transnitrosation and \(S\)-thiolation due to contact with thiols as shown in Eqs. 5 and 6, respectively.\(^{[5,6]}\) However generation of ROS is not involved in these pathways. Recently, more detailed chemistry of the formation and the transformation of \(\cdot S\)-nitrosothiols involving the generation of ROS has been suggested.\(^{[7–10]}\) The present results support the recently suggested mechanisms of the reactions of NO/thiols and \(\cdot S\)-nitrosothiols/thiols.

It is assumed that in the \(\cdot S\)-nitrosation of GSH by NO/\(O_2^-\), intermediary GS• radical formed by interaction of GSH with NO/\(O_2^-\) generated as in Eq. 1 plays an important role in producing GSHNO and GSSG as in Eq. 8.\(^{[9]}\) In this reaction, GS• radical may react with another GS• molecule to form GSGS•−, which in turn reduces \(O_2^-\) to produce \(O_2\).\(^{[10]}\) In the present study, the \(\cdot\text{OH}\) radical generation in the interaction of NO with thiols under aerobic conditions was confirmed using the ESR spin-trapping technique. Although GS• radical was not detected in the ESR studies, participation of GS• radical in the production of GSSMO and ROS cannot be ruled out.

\[
\text{GSH} + \text{NO}_2 \rightarrow \text{HNO}_2 + \text{GS} \\
\text{GS} + \text{GSSG} \rightarrow \text{GS} + \text{GSSG}
\]

It is also assumed that in the transformation of GSHNO in the reaction of GSHNO with GSH under aerobic conditions, GSH reacts with GSHNO to form \(N\)-hydroxysulfenamide GSN(OH)\(^{[5]}\) -SG, which would generate GS• radical and GSH \(N\)-hydroxyl radical GS•\(^{[5]}\) OH via homolytic cleavage as shown in Eq. 9. The former would react with GS• to form GSGS•−, which may reduce \(O_2^-\) to \(O_2\).\(^{[7]}\) In the present study, thyl radical generation in the interactions of GSHNO with GSH under anaerobic conditions was confirmed using the ESR spin-trapping technique. In this reaction, the generation of the thyl radicals may precede ROS generation.