Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide

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Abstract   Incubation of calf thymus DNA with 3-morpholino-sydnonimine (SIN-1), which simultaneously generates nitric oxide (NO) and superoxide (O_2^-), induced a significant increase of 8-hydroxydeoxyguanosine (8-OH-dG). Peroxynitrite also increased 8-OH-dG in calf thymus DNA. Addition of free hydroxyl radical (•OH) scavengers inhibited the increase of 8-OH-dG by SIN-1 or peroxynitrite. Incubation of ^{32}P-labeled DNA fragment with SIN-1 or peroxynitrite caused DNA cleavage at every nucleotide with a little dominance at guanine residues. The results suggest that NO reacts with O_2^- to form peroxynitrite and the peroxynitrite induces oxidative DNA damage through an active intermediate of which reactivity is similar to •OH.

Key words: 8-Hydroxydeoxyguanosine;
3-Morpholinosydnonimine; Nitric oxide; Superoxide;
Oxidative DNA damage; Peroxynitrite

1. Introduction

Nitric oxide (NO) and superoxide (O_2^-) produced by activated phagocytes may play an important role in the multistage carcinogenesis process, triggered by chronic infection and inflammation [1]. It was reported that NO can react with O_2 and subsequently induce DNA damage by deaminating DNA bases, resulting in mutation [2,3]. However, nitrogen dioxide formation from the reaction of NO with O_2 is relatively slow [4]. On the other hand, NO reacts with O_2^- to form peroxynitrite (ONOO$^-$) at an almost diffusion-controlled rate (3.8×10^9 M$^{-1} \cdot$s^{-1}) [5]. Peroxynitrite is a powerful oxidant and may cause damage to cells [6]. Intermediates derived from the protonated form of peroxynitrite, peroxynitrous acid, may account for much of its reactivity in biological systems. Proposed intermediates include free hydroxyl radical (•OH) [6–8], but thermodynamic calculations have been used to claim that •OH cannot be formed from peroxynitrite [9].

It has been proposed that oxidative DNA damage may be an important underlying event that leads to cancer during chronic inflammation [10]. However, to the best of our knowledge, there are no published reports investigating oxidative DNA damage by NO and O_2^-, although there are a lot of papers of in vitro studies on metal-dependent DNA damage induced by O_2^- and hydrogen peroxide (H$_2$O$_2$) [11,12]. In this study, we show that oxidative DNA damage including 8-hydroxylation

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Abbreviations: SIN-1, 3-morpholinosydnonimine-N-ethylcarbamide; 8-OH-dG, 8-hydroxydeoxyguanosine; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.

of the guanine residue is induced by concurrent generation of NO and O_2^- or by synthesized peroxynitrite. 3-Morpholinosydnonimine (SIN-1) was used as a model for the continuous release of NO and O_2^- [7].

2. Materials and methods

2.1. Materials

SIN-1 was supplied by Cassella AG, Frankfurt, Germany. Peroxynitrite was synthesized from nitrite and H$_2$O$_2$ as previously described [6]. Calf thymus DNA, superoxide dismutase (SOD) and catalase were from Sigma, St Louis, MO. [γ-[^{32}P]]ATP was purchased from Du Pont-New England Nuclear, Boston, MA. Restriction enzymes and T4 polynucleotide kinase were from New England Biolabs, Beverly, MA. Calf intestine alkaline phosphatase was from Boehringer Mannheim GmbH, Mannheim, Germany. Nuclease P1 from Yamasa shoyu Co., Chiba, Japan.

2.2. Analysis of ^{32}P-labeled DNA damage

DNA fragments were prepared from plasmid pbcNI which carries a 6.6 kb BamHI chromosomal DNA segment containing human c-Ha-ras-1 protooncogene [13]. A singly labeled 261-bp fragment (AvaI*1645–XbaI1905) and 341-bp fragment (XbaI1906–AvaI*2246) were obtained according to the method described previously [14]. The asterisk indicates ^{32}P labeling and nucleotide numbering starts with the BamHI site. The [^{32}P]DNA fragment and sonicated calf thymus DNA were incubated with SIN-1 or peroxynitrite in 10 mM sodium phosphate buffer (pH 7.2) at 37°C, heated at 90°C in 1 M piperidine for 20 min and electrophoresed as previously described [15]. Peroxynitrite was used in concentrations which did not have a significant influence on the pH of the reaction mixture. Scavengers of •OH (ethanol, sodium formate) were added where indicated. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure [14].

2.3. Analysis of 8-hydroxydeoxyguanosine (8-OH-dG)

8-OH-dG was measured by a modified method of Kasai et al. [16,17]. Calf thymus DNA (100 μM per base) was incubated with SIN-1 or peroxynitrite in 10 mM sodium phosphate buffer (pH 7.2) at 37°C. Scavengers of •OH were added when necessary. After ethanol precipitation, DNA was digested to the nucleoside by incubation with nuclease P1 and alkaline phosphatase, and analyzed by HPLC-electrochemical detection system as previously described [17].

3. Results and discussion

Fig. 1 shows the autoradiogram of double-stranded DNA fragments incubated with SIN-1. Oligonucleotides were detected as a result of SIN-1-induced DNA damage. The upper and lower bands in the control show single-stranded and double-stranded forms of intact DNA fragments, respectively. The DNA damage increased with the increase of incubation time (30 ~180 min) (data not shown). Even a low concentration (20 μM) of SIN-1 caused DNA damage. Peroxynitrite (20 μM) also induced similar DNA damage with 10 min incubation (data not shown).

The addition of ethanol and sodium formate significantly

inhibited DNA damage by SIN-1, indicating that the DNA damage is due to oxidant like •OH. SOD and catalase showed no or little inhibitory effect (Fig. 1). The addition of metal ions (Fe^{3+}, Cu^{2+}) did not increase the DNA damage. Furthermore, the metal chelating reagent DTPA had almost no effect on DNA damage by SIN-1 or peroxynitrite. These data excluded the possibility that the DNA damage was caused by active species generated from the reaction of Fe^{3+} or Cu^{2+} with O_2^-. No or little inhibitory effect of SOD can be explained by the reports that the reaction rate of O_2^- with SOD is smaller than that of O_2^- with NO [5].

To estimate the site specificity of DNA damage, the autoradiogram of treated DNA fragments electrophoresed on a longer and thinner gel was scanned with a laser densitometer (Fig. 2). Both SIN-1 and peroxynitrite caused DNA cleavage at positions of every base residue but cleavage at the positions of guanine was a little more dominant than those of the other three bases. It is considered that 8-OH-dG, a marker of oxidative DNA damage, is one of DNA base alterations induced by NO plus O_2^-. A recent study has shown that the piperidine treatment of 8-OH-dG containing DNA results in breakage of deoxyribose phosphate backbone [18]. Therefore, the backbone breakage by piperidine treatment might have occurred at NO plus O_2^--oxidized base residues including 8-OH-dG.

To confirm oxidative DNA damage, we measured the content of 8-OH-dG in calf thymus DNA incubated with SIN-1 (Fig. 3) or peroxynitrite (Fig. 4). SIN-1 induced a significant increase of 8-OH-dG with a concentration as low as 20 μM within 15 min incubation. At the higher concentration of SIN-1 (200 μM), increasing 8-OH-dG content turned to decrease with
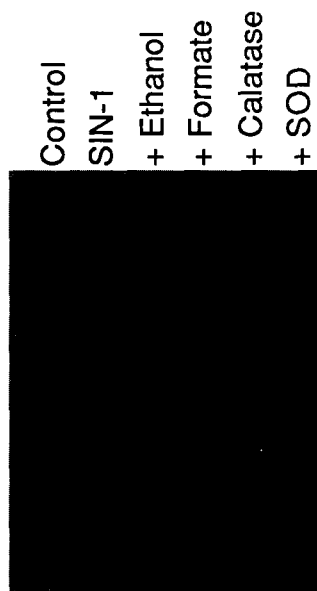

Fig. 2. Site-specificity of DNA cleavage induced by peroxynitrite or SIN-1. Singly labeled 261-base pair fragment (AvaI*1645-XbaI1905) was incubated with 1.0 mM peroxynitrite (upper) or 0.2 mM SIN-1 (lower), and treated with hot piperidine as described in Fig. 1 legend. The relative amounts of oligonucleotides produced were measured by scanning the autoradiograms with the laser densitometer. Horizontal axis, the nucleotide number of human c-Ha-ras-1 protooncogene starting with the BamHI site [13].

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragments incubated with SIN-1 in the presence of scavengers. The reaction mixture contained the ^{32}P 5' end-labeled 341-base pair fragment (XbaI1906–AvaI*2246), 1 μM per base of sonicated calf thymus DNA, 200 μM SIN-1 and 5 μM DTPA in 200 μl of 10 mM sodium phosphate buffer at pH 7.2. Where indicated, 0.8 M ethanol, 0.2 M sodium formate, 0.32 M dimethylsulfoxide, 150 units/ml catalase, or 150 units/ml SOD was added. After the incubation at 37°C for 90 min, followed by heating at 90°C for 20 min in 1 M piperidine, the DNA fragments were electrophoresed on an 8% polyacrylamide, 8 M urea gel, and the autoradiogram was obtained by exposing X-ray film to the gel.
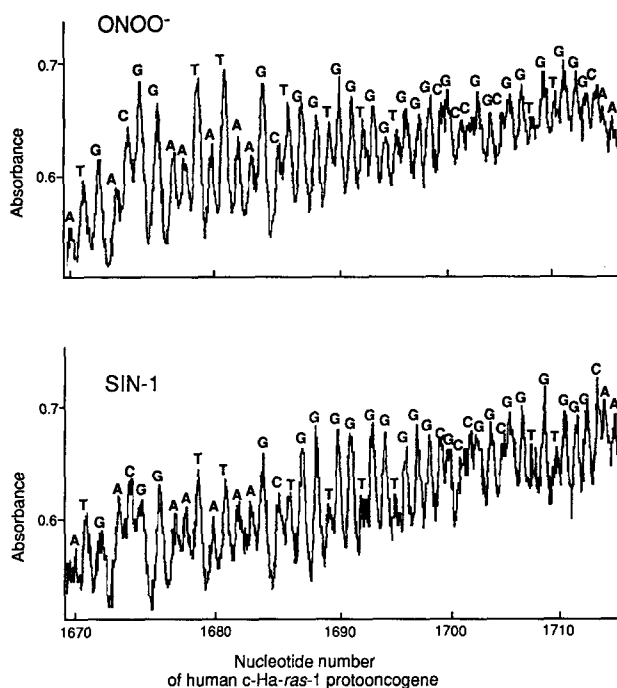
more than 30 min incubation time. This can be explained by assuming that the 8-hydroxylated guanine residue is converted into the ring cleavage residue as the reaction proceeds furthermore. Incubation of calf thymus DNA with peroxynitrite also induced a significant increase of 8-OH-dG. These results suggest that the 8-OH-dG increase by SIN-1 was induced by peroxynitrite produced from NO and O_2^-. The addition of •OH scavengers (ethanol, sodium formate) inhibited the increase of 8-OH-dG by SIN-1 or peroxynitrite (Fig. 4). In addition, DTPA did not show significant inhibitory effects on the damage to ^{32}P-labeled DNA and the 8-OH-dG formation. Therefore, it is considered that peroxynitrite generated by the reaction of NO with O_2^- induced DNA damage through a metal-independent formation of an active intermediate with high reactivity similar to •OH.

There have been two ESR spin-trapping studies concerning •OH formation by decomposition of peroxynitrite. Augusto et al. reported the formation of DMPO-OH adduct from peroxynitrite [19], whereas Shi et al. reported that DMPO does not trap •OH in peroxynitrite systems [20]. We also performed a spin-trapping experiment, resulting in the formation of DMPO-OH adduct only in the case of SIN-1, not with peroxynitrite (not shown). The conflicting results may be due to the rapid decomposition of peroxynitrite at neutral pH. It has been proposed that the very reactive intermediate, trans-peroxynitrous acid, formed from peroxynitrite would oxidize other molecules in a •OH-like manner but without releasing •OH [9]. The reactive species may be considered to be a 'bound' hydroxyl radical
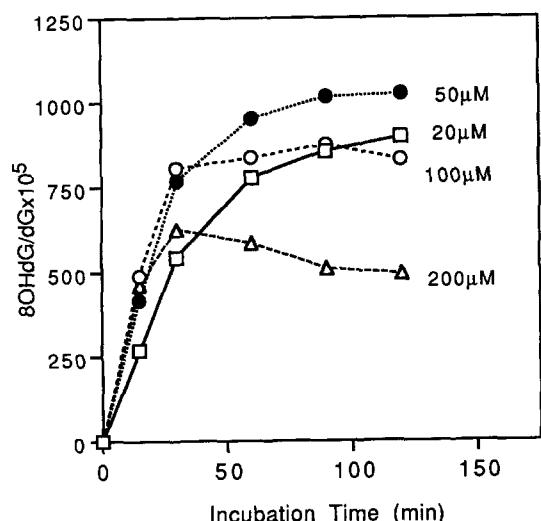
Fig. 3. Formation of 8-OH-dG in calf thymus DNA incubated with SIN-1. Calf thymus DNA (94 μM per base) was incubated with SIN-1 of the indicated concentrations in 400 μl of 4 mM phosphate buffer (pH 7.2) containing 5 μM DTPA at 37°C. After ethanol precipitation the 8-OH-dG was analyzed as described in section 2.

derived from *trans*-peroxynitrous acid. However, it is difficult to discriminate among •OH, 'bound' hydroxyl radical, and *trans*-peroxynitrous acid [9,19].

Although it was reported that NO can induce deamination of DNA, our previous results suggested that NO hardly induced DNA damage under aerobic conditions [21]. The DNA oxidation induced by NO and O_2^- generated by activated phagocytes may play a more important role in carcinogenesis than the deamination of DNA. Recently, Shibutani et al. have
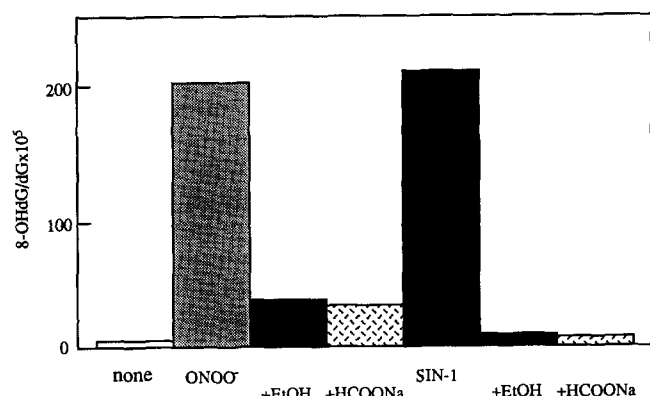

Fig. 4. Effect of •OH scavengers on 8-OH-dG formation by peroxynitrite or SIN-1. Calf thymus DNA (50 μM per base) was incubated with 450 μM peroxynitrite or 50 μM SIN-1 in 400 μl of 10 mM phosphate buffer (pH 7.2) containing 5 μM DTPA at 37°C for 15 min. Ethanol (0.5 M) or sodium formate (0.2 M) was added as indicated. After ethanol precipitation the 8-OH-dG was analyzed as described in section 2.

illustrated mutagenic replication of 8-OH-dG as a template causing G → T substitution [22]. The formation of 8-OH-dG induced by concurrent generation of NO plus O_2^- is noteworthy in relation to the observation that transversions in p53 tumor suppressor gene of G:C to T:A are most frequently seen in cancers of liver and lung [23]. The present results are expected to be valuable for the further investigations of the molecular mechanisms by which chronic inflammatory processes may initiate or enhance multistage carcinogenesis in humans.

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