Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination

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Abstract Peroxynitrite is a strong oxidant formed by reaction of nitric oxide with superoxide in inflamed tissues. We have demonstrated that 8-nitroguanine is formed dose-dependently in calf thymus DNA incubated with low concentrations of peroxynitrite in vitro. 8-Nitroguanine in acid-hydrolyzed DNA was chemically reduced into 8-aminoguanine, which was analyzed using high performance liquid chromatography with electrochemical detection. Only peroxynitrite, but not nitrite, tetranitromethane nor NO-releasing compounds, formed 8-nitroguanine. Antioxidants and desferrioxamine inhibited the reaction. 8-Nitroguanine was depurinated from DNA incubated at pH 7.4, 37°C (t1/2 = ~4 h). Peroxynitrite did not increase 8-oxoguanine levels in DNA.

Key words: Peroxynitrite; 8-Nitroguanine; DNA damage; 8-Oxoguanine; Nitric oxide; Superoxide

1. Introduction

Chronic infection and inflammation have been associated with an increased risk of a variety of human cancers. Reactive oxygen and nitrogen species generated by inflammatory cells have been proposed to induce DNA and tissue damage, chromosomal aberrations and mutations, which contribute to the multistage process of carcinogenesis [1–3].

Recent studies have shown that NO reacts rapidly with superoxide anion (O2−) to form peroxynitrite, which is a strong oxidant and can initiate reactions characteristic of hydroxyl radical (HO•), nitronium ion (NO2+), and nitric oxide radical (NO) [4–6]. It has been shown that essentially all of the NO produced by macrophages activated with phorbol 12-myristate-13-acetate is converted to peroxynitrite [7]. Increased levels of deamination and oxidation products of DNA bases have been detected in macrophages activated with lipopolysaccharides and interferon-γ [8]. Peroxynitrite formed by macrophages has been implicated as one possible mechanism for oxidative DNA damage [8].

Increasing evidence now suggests that peroxynitrite is a major agent causing tissue damage induced by inflammation in vivo [9–11]. Peroxynitrite oxidizes sulphhydryl groups and induces membrane lipid peroxidation [12–14]. It also nitrates tyrosine residues in proteins to form 3-nitrotyrosine, which is now measured as a marker of peroxynitrite-induced protein damage [15–17]. Since peroxynitrite is a relatively stable compound (the half-life is about one second at physiological pH [5]), it could penetrate the nucleus, where it might induce damage in DNA. It has been reported that peroxynitrite induces strand breaks in plasmid DNA [18] and oxidative damage in isolated DNA in vitro [19]. We recently found that guanine reacts rapidly with peroxynitrite under physiological conditions to form several substances, two of which are yellow [20]. On the basis of chromatographic and spectral evidence we identified the major compound (which accounts for about 80% of all compounds formed) as a novel adduct, 8-nitroguanine (nitroGua) [20]. In order to study the biological significance of this new adduct, it is essential to determine whether nitroGua is formed in DNA.

For this study, we have developed a new sensitive and specific method to analyze nitroGua in DNA, which we have used to measure the formation and stability of nitroGua in DNA. Various factors known to influence oxidation and nitrination reactions by peroxynitrite have also been studied in relation to their effects on the formation of nitroGua in DNA.

2. Materials and methods

2.1. Chemicals

NitroGua was synthesized by reaction of guanine with peroxynitrite as described previously [20]. 8-Aminoguanine was prepared by acid hydrolysis of 8-aminoguanosine (Sigma Chemical Co., St Louis, MO) [21]. Peroxynitrite was synthesized in a quenched flow reactor and excess hydrogen peroxide was destroyed by granular manganese dioxide [4,22]. 3-(4-Morpholinyl)-sydnone imine (SIN-1) was a gift from Hoechst Laboratories (Paris). All other chemicals were commercially available.

2.2. Confirmation of nitroGua in DNA hydrolysates

In order to confirm the formation of nitroGua in DNA, we carried out the reaction on a large scale (50 mg calf thymus DNA, 0.1 mM peroxynitrite in 50 ml 0.1 M phosphate buffer, pH 7.0). DNA was precipitated with ice-cold ethanol (2 volumes), washed twice with 100 ml of 75% ethanol and dried in a Savant Speed-Vac. DNA was hydrolyzed in 50 ml of 0.1 N HCl for 30 min at 100°C and dried under vacuum. The bases were separated by preparative high performance liquid chromatography (HPLC) (1.0 × 25-cm, Nucleosil C 18, 5 μm, Société Française Chromato Colonne, Neuilly Plaisance, France) under isocratic conditions with 20 mM ammonium formate buffer, pH 6.0 at a flow rate of 2 ml/min. Fractions containing nitroGua were collected and concentrated. The concentrated fractions were analyzed in a Spectrophysics SP 8800 HPLC system with a 0.46 × 15 cm ultrahigh ODS column (5 μm, Beckman, Berkeley, CA) under isocratic conditions with the same ammonium formate buffer as above at a flow rate of 0.8 ml/min. A Waters Lambda-Max model 481 UV/vis LC spectrophotometer was used to detect nitroGua at 396 nm and 8-aminoguanine at 254 nm.
2.3. Reaction of calf thymus DNA with peroxynitrite

The reactions were carried out at room temperature (~20°C). Peroxynitrite prepared in 1 N NaOH at various concentrations (100 μM) was added to a reaction mixture (final volume, 1 ml) containing 0.1 M sodium phosphate buffer, pH 7.0, calf thymus DNA (0.2 mg), 100 μM diethylaminediacetic acid (DTPA), a metal chelator and an appropriate amount of HCl to neutralize the NaOH present in the peroxynitrite solution (final pH was 7.2). Control experiments were performed using the same concentrations of decomposed peroxynitrite.

2.4. Reactions of calf thymus DNA with NO-releasing compounds, sodium nitrate and tetramethylmethane

The NO-releasing compounds (10 or 100 mM) were dissolved freshly in water, except for 5-nitro-N-acetyl-lysine which was prepared in 50% DMSO. The solution (100 μl) was added to a reaction mixture containing 0.1 M sodium phosphate buffer, pH 7.0, calf thymus DNA (1 mg/ml) and 100 μM DTPA (final volume, 1 ml). Tetranitromethane was added directly to the reaction mixture to give a final concentration of 1 or 10 mM. The reaction mixtures were incubated at 37°C for 1–18 h.

2.5. Effects of ferric and ferrous ions, hydroxyl radical scavengers, glucose, anti-oxidants and metal chelators on nitroGua formation in DNA

The effects of ferric ion- or ferrous ion-ethylenediaminetetraacetic acid (EDTA) on the formation of nitroGua in DNA were studied by adding FeCl3·6H2O or FeCl2·4H2O (final concentrations of 1, 10 and 100 μM) and a 1.5-fold excess EDTA to the reaction mixture (1 ml) containing 0.1 M sodium phosphate buffer, pH 7.0, calf thymus DNA (1 mg), 100 μM DTPA and 0.1 mM peroxynitrite. Similarly, a reaction mixture containing 0.1 M sodium phosphate buffer, pH 7.0, calf thymus DNA (1 mg/ml), 100 μM DTPA and 0.1 mM concentrations of scavengers and antioxidants, except for uric acid (0.1 mM), was incubated with 0.1 mM peroxynitrite. Effects of metal chelators (DTPA, EDTA and desferrioxamine) were studied in the presence of 0.1 M sodium phosphate buffer, pH 7.0, calf thymus DNA (1 mg), 0.1 mM of a chelator and 0.1 mM peroxynitrite.

2.6. Depurination of nitroGua formed in DNA

NitroGua-containing calf thymus DNA (100 mg) was prepared as described in 2.2. Isolated DNA was dissolved in phosphate buffered saline, pH 7.4 at a concentration of 500 μg/ml. The solution was incubated at 37°C for up to 6 h.

2.7. Determination of nitroGua in DNA

After the reaction, DNA was precipitated with cold ethanol (2 volumes), washed twice with 2.5 ml of 75% ethanol and 1 ml ethanol and dried in a Savant Speed-Vac. The dried DNA samples were hydrolyzed in 0.1 N HCl (~1 ml/mg DNA) at 100°C for 30 min. HCl was removed in a Speed-Vac and the residue was dissolved in 100 μl of 0.1 M Tris-HCl buffer, pH 8.5. To a 50 μl aliquot, a small amount of sodium hydrosulfite was added in order to reduce nitroGua to 8-aminoguanine [20]. We have therefore developed a new method to analyze nitroGua in DNA sensitively and specifically, that involves acid-hydrolysis of DNA, and chemical conversion of nitroGua into 8-aminoguanine, which is then detected by HPLC-EC. Fig. 1 shows typical chromatograms of acid-hydrolysates of calf thymus DNA after incubation in vitro with 0.1 mM peroxynitrite (Fig. 1A, b) or with decomposed peroxynitrite (Fig. 1A, a). Both samples showed a peak corresponding to oxoGua before acid-hydrolysis, excluding the possibility that nitroGua is present as a depurinated compound in the sample. Similarly, DNA treated with decomposed peroxynitrite did not contain nitroGua. These results indicate that DNA hydrolysates contained nitroGua, which was converted to 8-aminoguanine by chemical reduction. The same DNA sample did not show this yellow peak before acid-hydrolysis, excluding the possibility that nitroGua is present as a depurinated compound in the sample.

Although nitroGua is not detectable by EC, 8-aminoguanine has been reported to be electrochemically active [21]. As shown above, nitroGua is easily reduced by sodium hydrosulfite to 8-aminoguanine [20]. We have therefore developed a new method to analyze nitroGua in DNA sensitively and specifically, that involves acid-hydrolysis of DNA, and chemical conversion of nitroGua into 8-aminoguanine, which is then detected by HPLC-EC. Fig. 1 shows typical chromatograms of acid-hydrolysates of calf thymus DNA after incubation in vitro with 0.1 mM peroxynitrite (Fig. 1A, b) or with decomposed peroxynitrite (Fig. 1A, a). Both samples showed a peak corresponding to oxoGua before reduction with sodium hydrosulfite (Fig. 1B, b). After the reduction, bases from peroxynitrite-treated DNA showed a new peak at a retention time corresponding to 8-aminoguanine (Fig. 1A, a), but such a peak was not observed in samples from DNA treated with decomposed peroxynitrite (Fig. 1A, b). The method is sensitive and specific, the limit of detection for nitroGua being approximately 1 pmol injected. The presence of nitroGua can be confirmed by injecting the sample before and after reduction with sodium hydrosulfite, nitroGua being specifically detected by EC only after the reduction.
Nitration of guanine in DNA, but not peroxynitrite itself. Peroxynitrite has been reported to generate both OH\(^-\) and nitrogen dioxide radical (NO\(^2\)) by homolytic cleavage [4,6]. Our results suggest that peroxynitrite generates a nitrating agent (or several), which effectively reacts with guanine moieties in DNA to form nitroGua, whereas an HO\(^-\)-like compound may not be generated from peroxynitrite, or if generated, it reacts with other components in the reaction mixture more rapidly than with guanine.

NitroGua was formed in DNA treated with peroxynitrite, but was not detected in DNA incubated with NO-releasing compounds (S-nitroso-N-acetyl-D,L-penicillamine, sodium nitroprusside, SIN-1), sodium nitrite or the strong nitrating agent, tetranitromethane during incubation for up to 18 h. We studied the reaction of DNA with SIN-1 more extensively, because it has been reported that SIN-1 releases both NO and O\(^2\)-, which may react to form peroxynitrite [23,24]. SIN-1 increased dose-dependently the level of oxoGua, but not that of nitroGua in DNA. Incubation of DNA (1 mg/ml) with 10 mM SIN-1 for 6 h resulted in the formation of 1.19 ± 0.15 mmol oxoGua/mol guanine, whereas a control DNA sample incubated without SIN-1 contained 0.08 ± 0.01 mmol oxoGua/mol guanine. The formation of oxoGua was inhibited by the addition of either superoxide dismutase (500 U/ml) alone, catalase (500 U/ml) alone or the two in combination, by 65, 60 or 70%, respectively. Thus, the formation of oxoGua by SIN-1 could be mediated by O\(^2\)- or hydrogen peroxide generated from SIN-1. No nitroGua was detected in any DNA sample incubated with SIN-1 for a short (30 min) or long period (18 h), although in each case oxoGua was formed. One possible reason for this observation could be that peroxynitrite is not formed from SIN-1 under the present conditions. Further studies are needed to elucidate the mechanism by which oxoGua, but no nitroGua, is formed in DNA treated with SIN-1. We are currently studying formation of nitroGua and oxoGua in DNA in reaction systems where both NO and O\(^2\)- are generated simultaneously.

Nitrated tyrosine and phenolics by peroxynitrite has been reported to be catalyzed by ferric ion [4,25]. We have examined the effects of ferric and ferrous ion on nitroGua formation in DNA. As we previously observed for nitration of guanine in vitro [20], Fe\(^3+\)-EDTA at concentrations 1, 10, 100 and 1000 \(\mu\)M did not affect the formation of nitroGua in DNA (DNA and peroxynitrite concentrations were 1 mg/ml and 0.1 mM, respectively). Under the same conditions, Fe\(^2+\)-EDTA at concentrations of 1, 10 and 100 \(\mu\)M did not affect the reaction, but a high concentration (1000 \(\mu\)M) inhibited it by ~45%. Fe\(^3+\)-EDTA, but not Fe\(^2+\)-EDTA, dose-dependently increased the level of oxoGua in DNA treated with either peroxynitrite or decomposed peroxynitrite, possibly by oxidation with HO\(^-\) generated from Fenton-type reactions.

Fig. 3 shows the effects of various scavengers of hydroxyl radical, antioxidants and glucose on the formation of nitroGua in DNA incubated with peroxynitrite. N-Acetylcysteine as well as antioxidants such as ascorbic acid and uric acid significantly inhibited the reaction. Hydroxyl radical scavengers such as DMSO, D-mannitol and ethanol did not affect it. Metal ion chelators such as EDTA and DTPA also did not affect the

Fig. 2. Effect of peroxynitrite concentration on formation of nitroGua and oxoGua in DNA. One millilitre of 100 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM DTPA and 0.2 mg calf thymus DNA was incubated with various concentrations of peroxynitrite or decomposed peroxynitrite at room temperature (~20°C). o----o, nitroGua formed with peroxynitrite; o---o, oxoGua formed with peroxynitrite; o----•, nitroGua formed with decomposed peroxynitrite; •----•, oxoGua formed with decomposed peroxynitrite.

Fig. 3. Effects of hydroxyl radical scavengers, glucose, anti-oxidants and desferrioxamine on the formation of nitroGua in DNA incubated with peroxynitrite. One millilitre of 100 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM DTPA, 1 mg calf thymus DNA and a test compound was incubated with 0.1 mM peroxynitrite at room temperature (20°C). The concentrations of test compounds were 1 mM, except for uric acid and desferrioxamine, whose concentrations were 0.1 mM. The results are expressed as % of control without a test compound which formed 2.92 ± 0.15 mmol nitroGua/mol guanine in DNA (mean ± S.D., \(n = 3\)). *, significantly different from controls at \(P < 0.005\).
peroxynitrite in inflamed tissues, like several other oxidized compounds, serve as a specific marker of DNA damage induced by NO and peroxynitrite. Peroxynitrite induces damage in DNA in cells and tissues. NitroSGua could be a novel adduct that forms uniquely by peroxynitrite and no other NO-releasing compound or nitrating agent forms the same adduct. NitroSGua is rapidly depurinated from DNA, yielding apurinic sites which are potentially mutagenic. Peroxynitrite could therefore play an important role in carcinogenesis by inducing DNA and tissue damage. Our method to analyze nitroSGua in DNA treated with peroxynitrite in vitro will be useful to study whether peroxynitrite induces damage in DNA in cells and tissues. NitroSGua could serve as a specific marker of DNA damage induced by NO and peroxynitrite in inflamed tissues, like several other oxidized DNA bases, such as oxxoGua, which have been measured as markers of oxidative damage.

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References


![Fig. 4. Removal of nitroguanine from DNA by depurination. Calf thymus DNA containing nitroguanine was dissolved in phosphate buffer saline, pH 7.4 (500 μg/ml) and incubated at 37°C. At every 1 h incubation, the reaction mixture was removed and DNA was precipitated with ethanol and washed twice with 75% ethanol. The washings were combined and dried in a Speed-Vac for the analysis of free nitroguanine liberated from the DNA into the medium. ––, nitroguanine in DNA; •–•, free nitroguanine liberated in the medium.](image-url)