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Original Contribution

IMPORTANCE OF GUANINE NITRATION AND HYDROXYLATION IN DNA IN VITRO AND IN VIVO

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Abstract—Guanine (Gua) modification by nitrating and hydroxylating systems was investigated in DNA. In isolated calf thymus DNA, 8-NO_2-Gua and 8-oxo-Gua were dose-dependently formed with peroxynitrite, and 8-NO_2-Gua was released in substantial amounts. Myeloperoxidase (MPO) with H_2O_2 and NO_2^- reacted with calf thymus DNA to form 8-NO_2-Gua dose dependently without release of 8-NO_2-Gua. The frequency of strand breaks was higher than the sum of 8-NO_2-Gua and 8-oxo-Gua, particularly in the MPO-treated DNA, indicating the importance of other types of damage. The activation of human neutrophils and lymphocytes with phorbol ester did not induce 8-NO_2-Gua and 8-oxo-Gua in their nuclear DNA. However, 8-NO_2-Gua was found in calf thymus DNA co-incubated with activated neutrophils in the presence of NO_2^-. No significant formation of 8-NO_2-Gua was found in liver DNA from mice treated with *Escherichia coli* lipopolysaccharide. The incubation of peroxynitrite or MPO-H_2O_2-NO_2^--treated DNA with formamidopyrimidine glycosylase (Fpg) released 8-oxo-Gua, but not 8-NO_2-Gua, indicating that 8-NO_2-Gua is not a substrate for Fpg. Although 8-NO_2-Gua was generated in isolated DNA by different nitrating systems, other types of damage were formed in abundance, and the lesion could not be found reliably in nuclear DNA, suggesting that the biological importance is limited. © 2000 Elsevier Science Inc.

Keywords—Nitrating agents, Oxidizing agents, Peroxynitrite, Myeloperoxidase, Inflammation, DNA damage, 8-oxo-Guanine, 8-NO_2-Guanine, Formamidopyrimidine glycosylase (Fpg), DNA repair, Free radicals

INTRODUCTION

DNA damage induced by endogenous reactive intermediates, in particular reactive oxygen species (ROS), has attracted considerable interest and generated extensive discussion on the relevance of endogenous DNA damage to aging and cancer [1]. 7-Hydro-8-oxo-2′-deoxyguanosine (8-oxodG) as one of the most abundant and mutagenic residues generated selectively by ROS, has been extensively used as a biomarker for ROS attacks on DNA [2,3].

Recently, the importance of reactive nitrogen species (RNS) and related DNA damage has been proposed [4]. The endogenous free radical nitric oxide ($NO^\bullet$), from the L-arginine pathway catalyzed by nitric oxide synthase, is the precursor of RNS. $NO^\bullet$ reacts rapidly with the superoxide anion ($O_2^{\bullet -}$) to form peroxynitrite [5]. Another pathway for RNS generation during inflammation involves myeloperoxidase (MPO). Activated immune cells secrete MPO, which uses hydrogen peroxide and nitrite (NO_2^-), a major end product of $NO^\bullet$ metabolism, to generate RNS-related effects [6–8].

The basal endogenous formation of $NO^\bullet$ is approximately 1.2 mmol/d [9]. Inflammatory stimulation in an animal model caused a 9-fold elevation of $NO^\bullet$ generation [10]. It has been proposed that the modification of biomacromolecules by RNS could be a mechanism of increased risk of certain cancers related to the chronic infection and inflammation [11]. RNS can induce various DNA lesions, including nitration, deamination, and oxidation of bases, as well as formation of abasic sites and strand breaks [4,12–15]. The nitration of DNA bases by

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peroxynitrite, particularly at the C-8 position of guanine, has been reported by pioneer studies. The results from in vitro studies with isolated DNA showed that 8-nitroguanine (8-NO_2-Gua) is the main type and isoform of the nitrated bases from the reaction with exogenous nitrating agents [12,16–18]. However, formation of 8-NO_2-Gua has not been investigated in biological systems, and the relevance of this lesion is thus unknown. Indeed, inflammatory reactions with activation of cells producing ROS, RNS, and MPO could give ample opportunities for nitration of guanine in cellular DNA. Moreover, information on the possible enzymatic repair of 8-NO_2-Gua is lacking.

In the present study, we investigated the formation of 8-NO_2-Gua and 8-oxo-Gua in isolated and nuclear DNA in vitro and in vivo by various nitrating and hydroxylating systems. Formamidopyrimidine glycosylase (Fpg) was also applied to investigate whether this base excision repair enzyme can recognize 8-NO_2-Gua in DNA.

MATERIALS AND METHODS

Chemicals

Guanine, 8-aminoguanosine, 2-amino-6,8-dihydroxypurine (8-oxo-Gua), 2-deoxyguanosine (dG), DNA from calf thymus, nitrite (NO_2^-), manganese dioxide, butylated hydroxytoluene (BHT), phorbol 12-myristate 13-acetate (PMA), MPO, lipopolysaccharide (LPS) from *E. coli* serotype 26:B6, and agarose (Sigma A-9539) were purchased from Sigma (St. Louis, MO, USA). Lymphoprep-1.077 (Nycomed Pharma AS, Oslo, Norway), dextran weight: 500,000 (Pharmacia Fine Chemical Lot 11648), sodium dithionite (Merck KGaA, Darmstadt, Germany), and DNA molecular weight marker XIII (50 bp ladder; Boeheringer Mannheim, Mannheim, Germany; catalog number 1721-925) were purchased from the sources indicated. Fpg was received as a kind gift from Dr. Serge Boiteux.

Peroxynitrite synthesis

Peroxynitrite was synthesized with a quench-flow technique [19,20]. The concentration of peroxynitrite was quantified before use by measuring the absorbance at 302 nm, using a molar extinction coefficient of 1700 $M^{-1} \cdot cm^{-1}$ [21]. Residue hydrogen peroxide was excluded by filtering the solution through a manganese dioxide column.

8-NO2-Gua synthesis and purification

In order to synthesize 8-NO_2-Gua as reference compound, guanine (0.01 mM) was dissolved in 0.01 M hydrochloric acid (HCl) by 4 h of stirring at 80°C, and peroxynitrite was added as described by Yermilov et al. [18]. The reaction mixture was separated by high-performance liquid chromatography (HPLC; Merck-Hitachi: L-7200 autosampler, L-7100 pump, and L-7300 UV detector, Darmstadt, Germany). Four hundred microliters of the reaction mixtures was injected directly onto a preparative reverse-phase Nucleoside C18 column (16.0 mm × 25 cm, 5 μM, Saülentechnik Knauer, Berlin, Germany). 8-NO_2-Gua was eluted at 11 min (maximal UV absorbance at 387 nm) by 20% methanol in water (flow rate of 4 ml/min) and was collected by a Gilson fraction collection (model 202, Villier le Bel, France). The collection was loaded onto C18 Cartridges (Sep-Pak Plus, Waters, Milford, MA, USA) for further desalting and was first washed with 0.01 M HCl and then eluted with 0.01% ammonia. The purified yield was dried in a speed vacuum centrifuge and weighed. The product identity was confirmed by HPLC-EC (reducing it with sodium hydrosulfite to 8-NH_2-Gua detected electrochemically [EC]) and by HPLC with tandem mass spectrometry (MS-MS). Without reduction, no 8-NH_2-Gua peak appeared upon injection of the yield. The MS-MS spectrum was recorded on an API 365 triple quadrupole mass spectrometer equipped with a turbo-ionspray source (SCIEX, Thornhill, CA, USA). The sample was continuously infused from a Harward Model '11' syringe pump (Harvard Apparatus, South Natick, MA, USA) at a methanol flow rate of 0.6 ml/h. Electrospray was performed in the negative ion mode using nitrogen as the nebulizing and curtain gas. The spray needle potential was −3.6 kV. A collision energy of 10.7 eV was applied with nitrogen as the collision gas. The precursor ion at m/z 195 was collisionally activated, indicating a molecular weight of 196 (mW of nitroguanine = 196).

Incubation of DNA

Calf thymus DNA (0.20 mg/ml) was prepared in 10 mM phosphate buffer (pH 6.5) and incubated in a 1 ml aliquot. Different concentrations of peroxynitrite (0–500 μM) obtained by spontaneous decomposition with variable storage time were added directly into the DNA solution and stirred for 5 min. HCl 66 μl (1 M) was added later to keep consistency with the components in the control incubations. The controls were designed by decomposing 100 μl of peroxynitrite with 66 μl of 1 M HCl before adding it to the DNA solution. Calf thymus DNA was also incubated with 100 μM EDTA, 150 μM H_2O_2, 60 nM NO_2^-, and 30–90 nM MPO in 50 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 min. After the reactions, the DNA was precipitated by two volumes of ice-cold 96% ethanol. The supernatant was first concentrated in a speed vacuum centrifuge and then

filtered by 30 kDa molecular weight cut-off filters (Whatman, Maidstone, UK). The filtrate was further dried under speed vacuum and resuspended in 200 μl of mobile phase for the quantification of 8-NO_2-Gua and 8-oxo-Gua by HPLC-EC. The recovered DNA was divided into two aliquots for the further treatments without or with Fpg.

An aliquot of DNA was resuspended in 0.1 ml of the buffer (40 mM HEPES, 0.1 M potassium chloride, 0.5 mM EDTA, 200 μg/ml bovine serum albumin, pH 8.0) for Fpg incubation (4 μg Fpg protein/sample) at 37°C for 20 min. After incubations, DNA and the reaction medium were analyzed for modified guanine bases by HPLC-EC.

DNA modification by PMA-stimulated human neutrophils and lymphocytes

The human neutrophils and lymphocytes were separated from heparinized blood of healthy donors with the Ficoll-Hypaque method [22]. The cells were washed twice and diluted to approximately 2.5×10^7 cells/ml in phosphate-buffered saline (10 mM sodium phosphate, 10 mM potassium chloride, 140 mM sodium chloride, and 5 mM glucose, pH 7.3). One milliliter of neutrophils or lymphocytes was incubated with 200 nM PMA, 60 nM NO_2^-, and 0.20 mg of calf thymus DNA as the target at 37°C for 60 min. The reaction was terminated by centrifugation at 800 × *g* and 4°C for 10 min. The cells were collected for the extraction of nuclear DNA as described elsewhere [23], whereas the target calf thymus DNA in the incubation medium was precipitated by ethanol as described above.

Animal experiments

Male NMRI mice (20–22 g body weight) from the animal center at the Panum Institute, Copenhagen, were housed in an environmentally controlled facility operating on a 12 h dark/light cycle at 22–24°C with free access to a standard diet and tap water. Twenty-four mice were randomly divided into two groups. One group received LPS (5 mg/kg in saline intraperitoneally [ip]), and the other group received saline ip as the control. Six hours after the administration, the mice were sacrificed, and DNA was extracted from 500 mg liver tissue as described elsewhere [23,24].

HPLC analysis of 8-NO2-Gua and 8-oxo-Gua

All solutions for this assay were saturated with argon gas. 8-Aminoguanine (8-NH_2-Gua, the product of 8-NO_2-Gua reduction) standard was prepared from

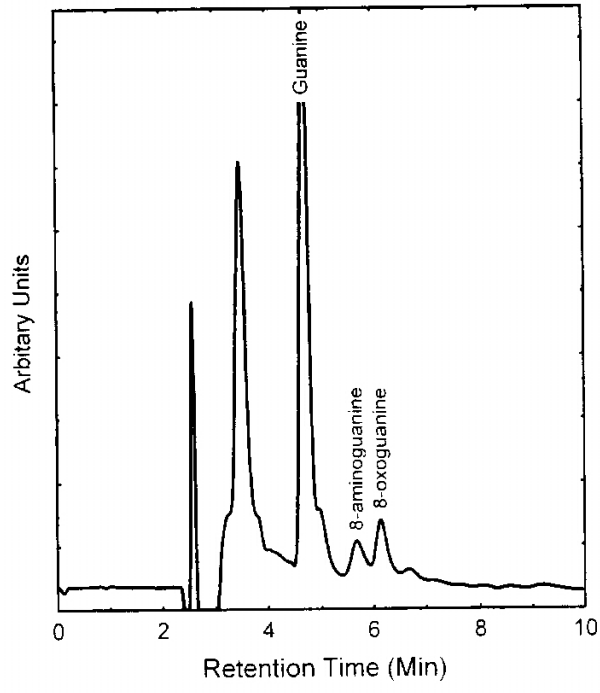

Fig. 1. Typical HPLC chromatograms from the electrochemical detector for quantification of 8-NO_2-Gua and 8-oxo-Gua.

8-aminoguanosine with 300 μl of formic acid and 10 μl of 5% BHT (in methanol) at 130°C for 30 min. After hydrolysis, the liquid part was removed in a speed vac, and the residue was dissolved in 200 μl of mobile phase. 8-Oxo-Gua and guanine stock solution were prepared freshly in 0.05 M HCl at 95°C by 4 h of continuous stirring. The dried DNA samples or reaction medium was hydrolyzed by formic acid as above, with argon gas filled in the containers. After hydrolysis, the liquid part was removed in a speed vac, and the residue was dissolved in 200 μl of mobile phase. Five μl of sodium hydrosulfite (40 mM in water, prepared fresh) was added to reduce 8-NO_2-Gua to 8-NH_2-Gua. The samples were analyzed with a Merck-Hitachi HPLC system with an electrochemical detector (ESA Coulochem, Bedford, MA, USA) with a 5011 analytical cell with electrode 1 at 0 mV and electrode 2 at 295 mV [18]. The samples were separated by a reverse-phase Nucleoside C18 column (4.6 mm × 25 cm, 5 μM, Beckman). Guanine was detected by UV at 254 nm. The mobile phase consisted of 12.5 mM citric acid, 25 mM sodium acetate, and 25 μM EDTA, pH 2.9, at a flow rate of 1 ml/min. The retention times of guanine, 8-NH_2-Gua and 8-oxo-Gua were 4.61, 5.85, and 6.20 min (typical chromatogram shown in Fig. 1). A series of purified 8-NO_2-Gua (2.5, 5, 10, 25, 50, and 100 nM, four samples each) was prepared to evaluate the molar conversion rate from 8-NO_2-Gua to 8-NH_2-Gua in comparison with an 8-NH_2-Gua series. The conversion efficiency was 61.5 ± 6.0% (mean ±

SD) and was linear in the selected range. Accordingly, the 8-NO_2-Gua concentrations measured by calibration with 8-NH_2-Gua were corrected for this.

Alkaline agarose gel electrophoresis

Agarose (1.5%) was prepared in 50 mM NaCl with 4 mM EDTA and allowed to set completely. The gel was presoaked in alkaline electrophoresis buffer (30 mM NaOH and 2 mM EDTA) overnight at room temperature. DNA samples were mixed and loaded in the gel with denaturing loading buffer (1 M NaOH, 50% glycerol, and 0.05% bromocresol green). The electrophoresis was run at 50 V until the dye came to three quarters of the height of the whole gel apparatus. The gel was then neutralized in 500 ml of 0.2 M Tris-HCl (pH 8.0) for 30 min at room temperature and stained with ethidium bromide for the visualization under UV. The frequency of DNA strand breaks was recorded as 2/Mn, where Mn is the number of bases in a molecular marker, coinciding with the maximum fluorescence intensity in the lane [25]. In order to make it comparable to the frequencies of modified guanine determined by HPLC, the scale was adjusted to 10^5 Gua.

Statistics

Multiple groups were compared by means of a one-way ANOVA test. Duncan's multiple range test was used for post hoc comparison of means. Two groups were compared by the *t*-test for independent samples. Differences were considered significant when $p < .05$.

RESULTS

8-NO_2-Gua and 8-oxo-Gua were generated dose dependently with peroxynitrite in isolated calf thymus DNA. The molar level of 8-oxo-Gua in DNA was several folds higher than 8-NO_2-Gua (Fig. 2, top). However, 8-NO_2-Gua was released in great amounts in the medium during the reaction, whereas no 8-oxo-Gua was released (Fig. 2, bottom). The incubation of peroxynitrite-treated DNA with Fpg did not change the level of 8-NO_2-Gua but led to a considerable decrease in the 8-oxo-Gua level in the DNA (Fig. 2, top). In addition, Fpg incubation did not result in the release of 8-NO_2-Gua but in a striking release of 8-oxo-Gua into the reaction medium (Fig. 2, bottom).

As shown in Table 1, the formation of 8-NO_2-Gua and 8-oxo-Gua was detected in calf thymus DNA treated with the MPO-H_2O_2-NO_2 system. 8-NO_2-Gua was generated only in the complete system and was dependent on the concentration of MPO, for example, 1.9 per 10^5 Gua at 90 nM MPO, whereas this base could not be detected in the reaction medium. In contrast, treatment of DNA with 16 μM of peroxynitrite generated an 8-NO_2-Gua frequency of 1.8 per 10^5 Gua in the strand and gave off 3.1 nM of 8-NO_2-Gua into the reaction medium (Fig. 2). Formation of 8-oxo-Gua was dependent on H_2O_2 but not on MPO or NO_2^-. Fpg treatment did not change the level of 8-NO_2-Gua in MPO-H_2O_2-NO_2^--treated DNA or release this base into the medium. However, 8-oxo-Gua was decreased in the MPO-H_2O_2-NO_2^--treated DNA by Fpg treatment and was detectable in the medium of the samples with the highest concentration of MPO in the system.

Alkaline agarose electrophoresis indicated the abundant formation of strand breaks in the calf thymus DNA (Fig. 3). After the peroxynitrite and MPO-H_2O_2-NO_2 treatment, the frequencies of strand breaks were 2–6 and 30–120 times higher than the sum of 8-NO_2-Gua and 8-oxo-Gua in DNA and in the reaction medium, respectively (Table 2).

Exposure of human neutrophils and lymphocytes to PMA in the absence or presence of NO_2^- did not induce detectable formation of 8-NO_2-Gua or raise the levels of 8-oxo-Gua in the nuclear DNA of the activated cells (data not shown). However, 8-NO_2-Gua was found in calf thymus DNA co-incubated with PMA-activated neutrophils in the presence of NO_2^- (Table 1). On the other hand, no differences were found with respect to 8-oxo-Gua in calf thymus DNA among all designed groups in the cell incubation experiment (Table 1).

The administration of LPS to mice induced a minor trace of 8-NO_2-Gua in the nuclear DNA from the liver of 2 out of 12 animals. The amount of 8-NO_2-Gua was 0.62 and 0.55 nM (corresponding to 0.5 and 0.4 8-NO_2-Gua per 10^5 Gua) in these two samples, respectively. This was close to the limit of quantification, which was set to 0.5 nM of 8-NO_2-Gua added to control DNA. There was no significant difference in 8-oxo-Gua in the DNA between control (1.95 $\pm$ 0.46 per 10^5 Gua) and treated animals (2.18 $\pm$ 0.64 per 10^5 Gua).

DISCUSSION

The present study shows that 8-NO_2-Gua can be formed in DNA by several nitrating systems, and we could for the first time demonstrate that these include biological systems involving MPO or activated neutrophils. However, other types of DNA damage were generated simultaneously in abundance, and 8-NO2-Gua was not reliably detected in nuclear DNA in vitro and in vivo, suggesting that the biological relevance may be limited. Moreover, 8-NO_2-Gua was not repaired by Fpg.

Peroxynitrite has been presumed to be a mediator of cellular and tissue injury in various pathological situa-

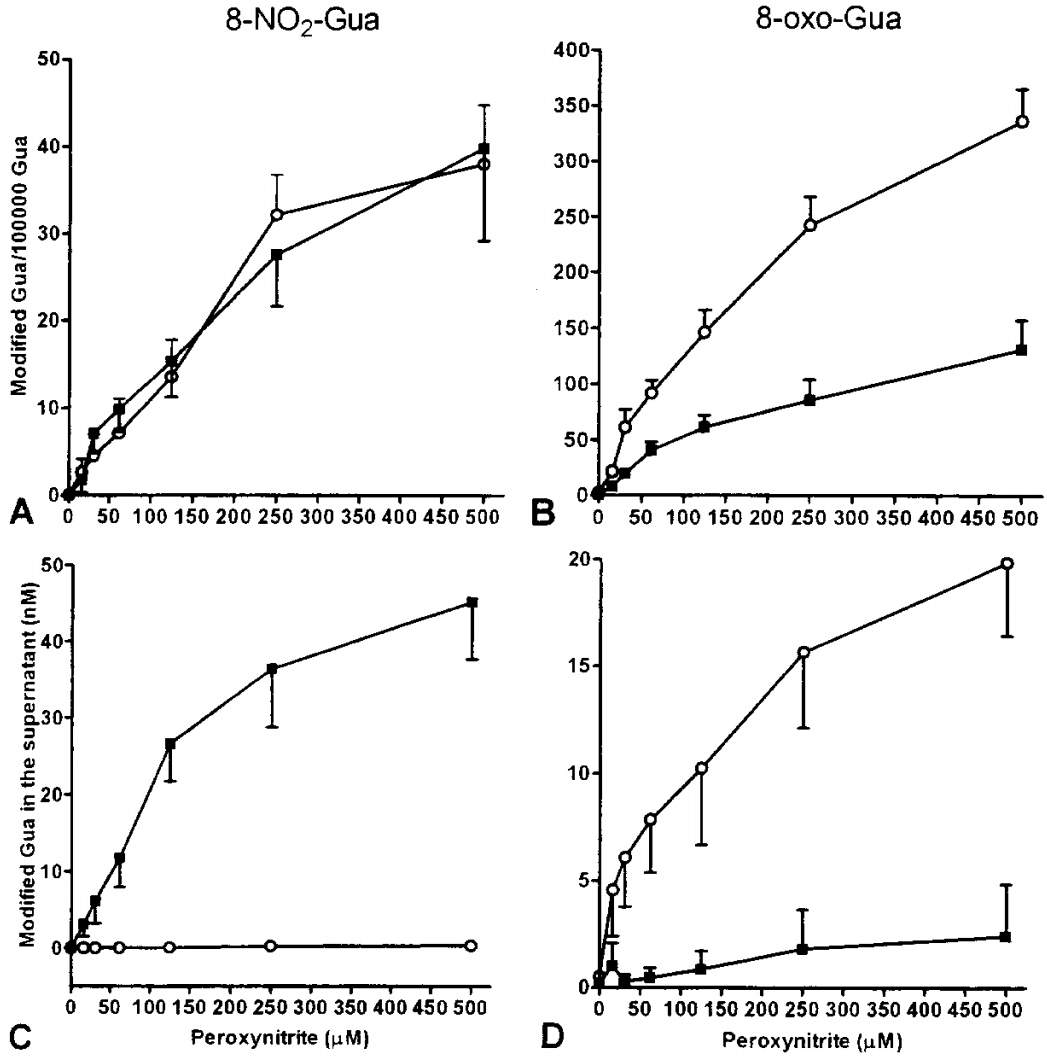

Fig. 2. Level of 8-NO_2-Gua (left panel) and 8-oxo-Gua (right) in DNA (top) after treatment of calf thymus DNA with increasing concentrations of peroxynitrite, and subsequent incubation with (■) or without (○) fapyguanine glycosylase (Fpg). The levels of the modified bases in the initial peroxynitrite medium (■) and in the Fpg incubation medium (○) are shown in the bottom panel. Data points are means ± SD of five experiments.

tions and can induce DNA damage, including nitration and hydroxylation of guanine at the C-8 position [4,26–30]. In agreement with previous studies [12,16–18,31], the present data showed that peroxynitrite induced formation of 8-NO_2-Gua and 8-oxo-Gua dose dependently in calf thymus DNA. Other RNS, such as free radical nitrogen dioxide ($NO_2^{\bullet}$) and nitryl chloride (NO_2Cl), are also capable of nitrating surrounding molecules [32,33]. Free radical nitrogen dioxide can be derived from NO_2^- through a one-electron oxidation via composing a complex with H_2O_2 and MPO, an abundant protein in neutrophils and one that is induced in inflammation [34]. Nitryl chloride is also produced by the reaction of NO_2^- with hypochlorous acid (HOCl) formed via MPO-catalyzed reaction [35]. Nitration of macromolecules occurs in a wide range of inflammatory diseases involving neutrophil and macrophage activation [36]. Indeed, the present data show that an MPO-H_2O_2-NO_2^- system can induce formation of 8-NO_2-Gua in calf thymus DNA, although the exact responsible RNS and the possible involvement of chloride remain to be investigated. In agreement, it was recently reported that MPO-H_2O_2-NO_2^- could nitrate monodeoxyguanosine [37]. Similarly, activated neutrophils were capable of inducing 8-NO_2-Gua in calf thymus DNA in the present study. Thus, RNS capable of nitrating guanine in DNA may be formed during inflammation. In contrast, the formation of 8-oxo-Gua in calf thymus DNA was not dependent on MPO or activated neutrophils but rather on the presence of H_2O_2, which, together with trace amounts of transition metals, could oxidize DNA in a Fenton-like reaction. In addition, hypochlorite also generated by MPO along with

Table 1. The Formation of 8-NO_2-Gua and 8-oxo-Gua in Calf Thymus DNA (Target DNA) Incubated with MPO-H_2O_2-NO_2^- System and Activated Human Neutrophils

Condition	8-NO_2-Gua in target DNA without Fpg (8-NO_2-Gua/10^5Gua)	8-NO_2-Gua in target DNa with Fpg (8-NO_2-Gua/10^5Gua)	8-oxo-Gua in target DNA without Fpg (8-oxo-Gua/10^5Gua)	8-oxo-Gua in target DNA with Fpg (8-oxo-Gua/10^5Gua)
Myeloperoxidase-H_2O_2-NO_2^- system				
Complete system with 30 nM MPO	0.74 ± 0.22	0.63 ± 0.28	4.16 ± 0.30	2.82 ± 0.30**
Complete system with 60 nM MPO	1.35 ± 0.25	1.28 ± 0.18	4.04 ± 0.47	3.01 ± 0.25**
Complete system with 90 nM MPO	1.91 ± 0.43	1.87 ± 0.42	4.35 ± 1.20	2.75 ± 0.35**
Complete system minus				
MPO	0	NA	4.13 ± 0.53	3.03 ± 0.22**
H_2O_2	0	NA	2.59 ± 0.57*	2.58 ± 0.29
NO_2^-	0	NA	3.70 ± 0.48	3.08 ± 0.67
Activated neutrophils-NO_2^- system				
Complete system	0.91 ± 0.19	1.08 ± 0.21	2.66 ± 0.27	2.63 ± 0.39
Complete system minus				
Cells	0	NA	2.33 ± 0.30	2.94 ± 0.31
NO_2^-	0	NA	2.65 ± 0.21	3.21 ± 0.48
PMA (200 nM)	0	NA	2.58 ± 0.36	2.88 ± 0.14

* $p < 0.05$ in comparison with 8-oxo-Gua in calf thymus DNA treated with the complete MPO-H_2O_2-NO_2^- systems and the complete MPO-H_2O_2-NO_2^- system minus NO_2 without Fpg.

** $P < 0.05$ in comparison with the corresponding group without Fpg treatment.

NA = not analyzed.

nitrite can degrade 8-oxoGua and generate other base modifications in DNA [38,39]. Such multiple reactions could also explain the very high frequency of strand breaks compared with 8-NO_2-Gua and 8-oxo-Gua found by means of alkaline agarose gel electrophoresis of DNA incubated with the MPO system. In contrast, the frequency of strand breaks was about four times higher than the combined level of base damage after incubation with

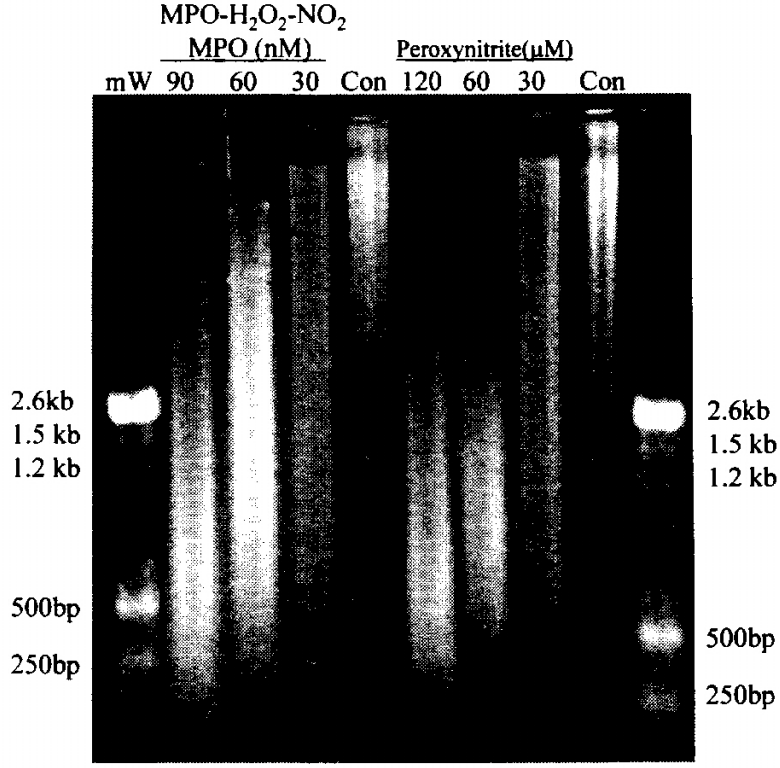

Fig. 3. Alkaline agarose gel display of the DNA strand breaks induced by peroxynitrite or myeloperoxidase (MPO) with H_2O_2 and NO_2^-.

Table 2. Formation of Strand Breaks, Estimated by Agarose Gel Electrophoresis, and of Base Modifications (8-NO_2-Gua and 8-oxo-Gua), Estimated by HPLC-EC, in Calf Thymus DNA Treated with Increasing Concentrations of Peroxynitrite or Myeloperoxidase (MPO) Together with H_2O_2 and NO_2^-

	Peroxynitrite (μM)			MPO (nM) with H_2O_2 and NO_2^-		
Parameter	30	60	120	30	60	90
Strand breaks in DNA	160	720	880	140	360	760
(8-NO_2+8-oxo)-Gua in DNA	69	104	160	4.8	5.4	6.2
8-NO_2-Gua in medium	7	14	30	0	0	0

Values are average number of lesions per 10^5 Gua.

peroxynitrite, although other types of base modifications may certainly occur, and 8-oxoGua may be partly degraded [31,40,41].

A crucial issue is whether in vivo–generated nitrating agents are capable of modifying nuclear DNA, for example, during inflammation [28–30]. Although administration of PMA to immune cells stimulates the release of nitrating and hydroxylating agents [42–44], as shown by formation of 8-NO_2-Gua in calf thymus DNA in the present study, this lesion was not detectable in the nuclear DNA from the activated neutrophils. Thus, the reactive species formed outside the cells do not appear to reach the nucleus that is also protected by efficient antioxidants [45].

To further test a similar situation in vivo, mice were pretreated with LPS, known to result in an increased oxidative burst in neutrophils and other immune cells as well as in iNOS expression [46–48]. The treatment protocol has previously induced substantial iNOS activity in mice, demonstrated by excessive NO_2^- levels in plasma [22]. However, the formation of trace amounts of 8-NO_2-Gua in nuclear DNA from the liver in only two samples was not reproducible in 10 more mice. The detection limit of the assay did not exclude the formation of this lesion under these conditions, but the levels should at least be below 0.5 per 10^5 Gua. Moreover, an optimum time window that takes formation and depurination or repair into account will be difficult to determine.

During peroxynitrite treatment of DNA, substantial amounts of 8-NO_2-Gua, but not 8-oxodG, were released into the reaction medium, indicating the rapid depurination of the former base. The spontaneous rate of 8-NO_2-Gua depurination has been measured with a half-life of 4 h in a similar study [17]. In contrast, 8-NO_2-Gua could not be found in the medium of the MPO-H_2O_2-NO_2^- and DNA incubation, even though the frequency of 8-NO_2-Gua was higher than that from peroxynitrite-treated (16 μM) DNA releasing 8-NO_2-Gua. This result suggested that the rapid depurination of 8-NO_2-Gua from peroxynitrite-treated DNA is not spontaneous per se but is rather an effect of peroxynitrite. The violent treatment of DNA with bolus peroxynitrite was accompanied by sharp changes in pH and ionic strength, which could lead to the protonation of the base, followed by direct cleavage of the glycosyl bond [49,50]. This process might lack selectivity and could result in the falloff of both modified and nonmodified bases and result in depurination as well as depyrimidination in spite of the more stable glycosylic linkage of pyrimidine to deoxyribose [6]. The 3′-phosphodiester bonds associated with the deoxyribose residues left at sites of base loss in DNA are labile and can be hydrolyzed by a β-elimination reaction [51–54]. On the other hand, the formation of 8-NO_2-Gua from MPO-H_2O_2-NO_2^- treatment might be more firmly attached to the strand because of the mild reaction environment.

In general, the level of 8-oxo-Gua in DNA was higher than that of 8-NO_2-Gua. Part of that difference after peroxynitrite treatment could be due to depurination as described above. Moreover, a higher relative rate of nitration would probably have been found if carbon dioxide/bicarbonate had been used in the peroxynitrite-based system, as previously demonstrated by Yermilov et al. [16]. In the biological systems, the levels of 8-oxoGua was relatively high, possibly due to artefactual oxidation of Gua despite the use of argon during sample work-up, especially during the process of hydrolysis at high temperature (130°C). Thus, the level of 8-oxoGua measured in cells and mouse liver by enzymatic hydrolysis of the extracted DNA to deoxynucleosides is usually below 0.5 8-oxodG per 10^5 dG in our laboratory [24].

Although the present data may question the significant formation of 8-NO_2-Gua in nuclear DNA, repair may be required if it is formed because spontaneous depurination appears to act rather slowly. The Fpg enzyme repairs other similar modifications of Gua, for example, 8-oxoGua and FapyGua, by base excision and would accordingly be the first candidate for potential repair of 8-NO_2-Gua [55]. However, incubation of the peroxynitrite-treated or MPO-H_2O_2-NO_2^--treated DNA with Fpg did not release 8-NO_2-Gua, although 8-oxo-Gua was released, and the level in the DNA was reduced by more than half in accordance with other studies [56]. These results indicate that Fpg was capable of recognizing 8-oxo-Gua but not 8-NO_2-Gua, despite the similarity in chemical structure of the two forms of oxidized guanine. Nevertheless, it cannot be excluded that other DNA repair enzymes may deal with 8-NO_2-Gua in DNA.

In summary, 8-NO_2-Gua can be generated in isolated calf thymus DNA by peroxynitrite, MPO-H_2O_2-NO_2^-, and activated neutrophils in the presence of NO_2^-. However, simultaneous formation of 8-oxoGua and a large number of strand breaks, in particular in the MPO sys-

tem, suggest that nitration of guanine may have less quantitative important than other forms of DNA damage, even during inflammation. Moreover, it was not possible to demonstrate the nitration of guanine in nuclear DNA reliably in vivo or in vitro. The rapid depurination of 8-NO_2-Gua from peroxynitrite-treated DNA appears not to be spontaneous per se but rather an effect of peroxynitrite, whereas Fpg was incapable of releasing this lesion, suggesting that it is not involved in the potential repair. Taken collectively, these data question the pathophysiological importance of nitrated guanine in DNA.

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ABBREVIATIONS

RNS—reactive nitrogen species
ROS—reactive oxygen species
MPO—myeloperoxidase
8-NO_2-Gua—8-nitroguanine; 8-oxo-Gua, 8-oxoguanine
LPS—lipopolysaccharide
PMA—phorbol myristate acetate