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Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite

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Abstract **Carbon dioxide has been reported to react with peroxynitrite ($ONOO^-$), a strong oxidant and nitrating agent, to form an $ONO_2CO_2^-$ adduct, altering the reactivity characteristic of peroxynitrite. We found that bicarbonate (0–10 mM) caused a dose-dependent increase of up to 6-fold in the formation of 8-nitroguanine in calf-thymus DNA incubated with 0.1 mM peroxynitrite, whereas it produced no apparent effect on 8-oxoguanine formation. In contrast, bicarbonate inhibited peroxynitrite-induced strand breakage in plasmid pBR322 DNA and thymine-propenal formation from thymidine. We conclude that CO_2/HCO_3^- reacts with peroxynitrite to form a potent nitrating agent, but also to inactivate hydroxyl-radical-like activity of peroxynitrous acid.**

Key words: Carbon dioxide; Peroxynitrite; 8-Nitroguanine; 8-Oxoguanine; DNA strand break; Thymine-propenal

1. Introduction

Nitric oxide ($^{\bullet}NO$) reacts rapidly with superoxide anion ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$), which is a strong oxidant and nitrating agent, that can initiate reactions characteristic of the hydroxyl radical ($HO^{\bullet}$), nitronium ion (NO_2^+) and nitrogen dioxide radical ($NO_2^{\bullet}$) [1–4]. Increasing evidence suggests that peroxynitrite is a major agent responsible for tissue damage induced by inflammation in vivo [5–8]. Peroxynitrite oxidizes sulfhydryl groups and induces membrane lipid peroxidation [9–11]. It also nitrates tyrosine residues in proteins to form 3-nitrotyrosine, which can be measured as a marker of peroxynitrite-induced protein damage [12–14]. Since peroxynitrite is a relatively stable compound (its half-life is about 1 s at physiological pH [3]), it could penetrate the nucleus, where it might cause damage in DNA. It has been reported that peroxynitrite induces strand breaks in plasmid DNA [15–18] and oxidative damage in isolated DNA in vitro [19]. Peroxynitrite reacts with nucleobases and nucleosides to form adducts such as 8-nitroguanine (nitro^{8}Gua) [20], 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine [21] and base-propenals [18]. We recently reported that nitro^{8}Gua is formed dose-dependently in DNA incubated with low concentrations of peroxynitrite under physiological conditions [22].

Carbon dioxide has been reported to react rapidly with peroxynitrite (3×10^4 M^{-1} s^{-1}) forming an $ONO_2CO_2^-$ adduct [23,24], and alters the reactivity of peroxynitrite [11,25–28]. Peroxynitrite-induced luminol chemiluminescence is significantly enhanced in the presence of bicarbonate [27]. High concentrations of bicarbonate protect bacteria (*Escherichia coli*) and parasites (*Trypanosoma cruzi*) from the cytotoxic effects of peroxynitrite [28,29]. Bicarbonate has also been reported to inhibit aromatic hydroxylation and nitration of phenylalanine, and to increase the yield of 3-nitrotyrosine and dityrosine from the reaction of peroxynitrite with tyrosine [25]. Recent studies show similar enhancing effects of carbon dioxide or bicarbonate on nitration by peroxynitrite of tyrosine [25,30], *p*-hydroxyphenyl acetate [31,32] and albumin [33]. Because carbonate is abundant (≥25 mM) in physiological fluids [23,24], for this study we have determined the effects of bicarbonate-derived carbon dioxide on the DNA damage mediated by peroxynitrite. We analysed single-strand breaks in plasmid pBR322 DNA, and formation of nitro^{8}Gua and oxo^{8}Gua in calf-thymus DNA, as well as thymine-propenal formation from thymidine, as markers of DNA damage induced by peroxynitrite.

2. Materials and methods

2.1. Chemicals

Peroxynitrite was synthesized in a quenched-flow reactor and excess hydrogen peroxide was destroyed by granular manganese dioxide [3,34]. Nitro^{8}Gua was synthesized by reaction of guanine with peroxynitrite as described [19]. 8-Aminoguanine (amino^{8}Gua) was prepared by acid hydrolysis of 8-aminoguanosine (Sigma Chemical Co., St Louis, MO). All other chemicals including 8-oxoguanine (oxo^{8}Gua) (Aldrich, Milwaukee, WI) were commercially available.

2.2. Reaction of calf-thymus DNA with peroxynitrite in the presence of $NaHCO_3$ and determination of nitro^{8}Gua and oxo^{8}Gua in DNA

The reactions were carried out at room temperature (~20°C). Peroxynitrite prepared in 1 N NaOH (1 mM, 100 μl) was added to a reaction mixture (final volume, 1 ml) containing 0.1 M sodium phosphate buffer, pH 7.0, calf thymus DNA (1 mg), 100 μM diethylenetriaminepentaacetic acid (DTPA), and an appropriate amount of HCl to neutralize the NaOH present in the peroxynitrite solution (final pH was 7.5). Control experiments were performed using the same concentrations of decomposed peroxynitrite.

Nitro^{8}Gua and oxo^{8}Gua in DNA were analysed according to the method of Yermilov et al. [22]. Briefly, after the reaction, DNA was precipitated with cold ethanol (2 vols.), washed twice with 2.5 ml of

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Abbreviations: nitro^{8}Gua, 8-nitroguanine; oxo^{8}Gua, 8-oxoguanine; amino^{8}Gua, 8-aminoguanine; EC, electrochemical detector; DTPA, diethylenetriaminepentaacetic acid; DMSO, dimethylsulfoxide.

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75% ethanol and once with 1 ml ethanol and dried in a Savant Speed-Vac. The dried DNA samples were hydrolysed 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 nitro^{8}Gua to amino^{8}Gua. The samples before and after reduction were analyzed using a Spectraphysics HPLC (model SP 8810) equipped with two reverse-phase columns in series (15×0.46 cm Ultrasphere ODS column, 5 μm Beckman) under isocratic conditions with a 12.5 mM citric acid, 25 mM sodium acetate buffer containing 25 μM EDTA (pH 5.2) at a flow rate of 1 ml/min. The reduced nitro^{8}Gua (i.e. amino^{8}Gua) and oxo^{8}Gua were detected using an electrochemical detector (EC) (Waters model M460) at a potential of +600 mV. Guanine was detected by a UV spectrophotometer (Spectra Series UV 100, thermoseparation) at 254 nm. Under these conditions, retention times of amino^{8}Gua, oxo^{8}Gua and guanine were 12.7, 14.6 and 12.9 min, respectively. Experiments were carried out in duplicate.

2.3. Analysis of DNA single-strand breaks

Conversion of the covalently closed circular double-stranded supercoiled plasmid DNA (form I) to a relaxed open circle (form II) and a linear form (form III) was used to investigate DNA strand breaks induced by peroxynitrite. The experiments were carried out by incubating plasmid pBR322 DNA (100 ng) in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DTPA, 0.5 mM peroxynitrite, an appropriate amount of HCl to neutralize the NaOH present in the peroxynitrite solution and 0–50 mM $NaHCO_3$ at ~20°C (final volume 10 μl). Similarly, the reactions were carried out using 100 mM sodium phosphate-0.1 mM DTPA buffer at pH 4.5 and pH 8.5. After the reaction, 2 μl of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol) was added to the reaction mixture and an aliquot (8 μl) was loaded onto a 0.7% agarose gel prepared with 45 mM Tris-borate/1 mM EDTA (pH 8.0) containing 0.5 μg/ml ethidium bromide. The gel was run at 8.5 V/cm for 90 min. Percentages of supercoiled (form I), relaxed (form II) and linear (form III) forms were calculated using an Imaging Densitometer Model GS-670, Bio-Rad (Hercules, CA). All experiments were carried out in triplicate.

2.4. Formation and analysis of thymine-propenal

The experiments were carried out by incubating 10 mM thymidine in 100 mM sodium phosphate buffer, pH 4.5, containing 0.1 mM DTPA, 1.0 mM peroxynitrite, an appropriate amount of HCl to neutralize the NaOH present in the peroxynitrite solution and 0–10 mM $NaHCO_3$ at ~20°C (final volume 1 ml). After the reaction, the mixture was evaporated to dryness and the residue was dissolved in 100 μl distilled water. The reaction products in a 20 μl aliquot were separated by thin-layer chromatography (TLC) with silica gel 60 F_{254}, using the solvent system ethyl acetate:isopropyl alcohol:water (74:17:9). Products were first detected under UV light. Base-propenals were then visualized by spraying the TLC plate with 0.6% 2-thiobarbituric acid and heating for 10 min at 100°C [35]. Spot areas were calculated by an Imaging Densitometer, using an authentic thymine-propenal as a standard [35]. All experiments were carried out in triplicate.

Table 1
Effect of pH and bicarbonate on DNA single-strand breaks induced by peroxynitrite

pH	$NaHCO_3$ (mM)	Single-strand breaks (% of open circular form)[a]	
		Peroxynitrite	Decomposed peroxynitrite
8.6	0	24±14	7±10
7.5	0	66±7	10±6
4.2	0	88±1	10±4
4.2	0.05	83±2	8±2
4.2	0.5	80±2	13±9
4.2	5	59±4	4±5
4.2	50	8±3	1±6

[a]Conversion of the covalently closed circular double-stranded supercoiled plasmid DNA (form I) to a relaxed open circle (form II) was analysed to investigate DNA strand breaks induced by peroxynitrite. The experiments were carried out in 100 mM sodium phosphate buffer (final pH values 4.2, 7.5 and 8.6), containing plasmid pBR322 DNA (100 ng), 0.1 mM DTPA and 0.5 mM peroxynitrite. Effects of 0–50 mM $NaHCO_3$ on the strand breaks induced by peroxynitrite at pH 4.2 were similarly studied.

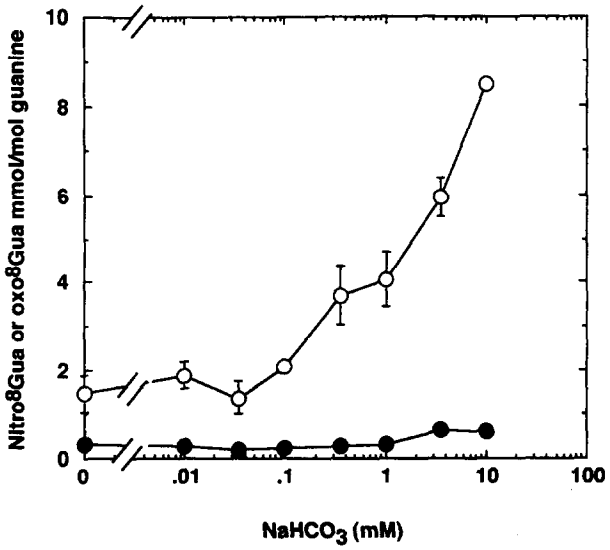

Fig. 1. Effects of $NaHCO_3$ on the formation of nitro^{8}Gua and oxo^{8}Gua in DNA incubated with peroxynitrite. 1 ml of 100 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM DTPA and 1 mg calf thymus DNA, was incubated with 0.1 mM peroxynitrite at room temperature (~20°C) (final pH 7.5). Nitro^{8}Gua (○—○) and oxo^{8}Gua (●—●) in DNA (mean±S.D., $n=2$) were analysed by HPLC-EC according to the method of Yermilov et al. [22].

3. Results and discussion

We have used a newly developed method to analyze nitro^{8}Gua in DNA [22]. The method involves acid hydrolysis of DNA and chemical conversion by sodium hydrosulfite of nitro^{8}Gua into amino^{8}Gua, which is then detected by HPLC-EC. Oxo^{8}Gua and amino^{8}Gua can be detected by HPLC-EC without reduction. The levels of nitro^{8}Gua and oxo^{8}Gua in DNA formed with 0.1 mM peroxynitrite were 1.47±0.41 and 0.34±0.08 mmol/mol guanine, respectively. Decomposed peroxynitrite did not generate nitro^{8}Gua, but formed oxo^{8}Gua at the level of 0.08±0.01 mmol/mol guanine. Non-treated calf-thymus DNA contained oxo^{8}Gua at the level of 0.05±0.01 mmol/mol guanine. The DNA samples treated with either peroxynitrite or decomposed peroxynitrite contained no detectable level of amino^{8}Gua.

We have examined the effect of exogenously added $NaHCO_3$ on peroxynitrite-mediated nitration and oxidation of guanine in DNA (Fig. 1). The level of nitro^{8}Gua increased gradually with increasing concentrations of $NaHCO_3$ in the reaction mixture up to 10 mM. The yield of nitro^{8}Gua increased by about 6-fold when the $NaHCO_3$ concentration was 10 mM (8.46±0.05 mmol/mol guanine), compared with the level without $NaHCO_3$ (1.47±0.41 mmol/mol guanine). The level of oxo^{8}Gua was not affected by the presence of low concentrations of $NaHCO_3$ (0.01–1 mM) in the reaction mixture, but at higher concentrations of $NaHCO_3$ was increased 2-fold (from 0.34±0.08 mmol/mol guanine at 0 mM $NaHCO_3$ to 0.59±0.08 mmol/mol guanine at 10 mM), as shown in Fig. 1. However, higher concentrations (10 mM)

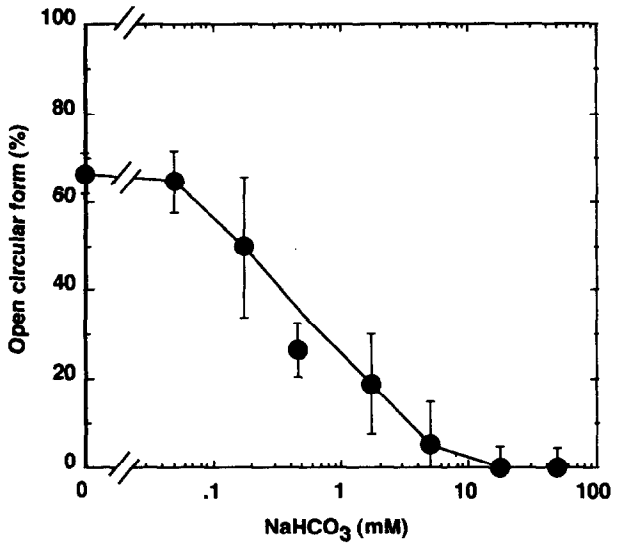

Fig. 2. Effect of $NaHCO_3$ on DNA single-strand breaks induced in plasmid pBR322 by peroxynitrite. The experiments were carried out by incubating plasmid pBR322 DNA (100 ng) in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DTPA and 0.5 mM peroxynitrite at room temperature (~20°C) (final volume 10 μl). Percentages of open-ring relaxed form (form II) (mean ± S.D., $n = 3$) are plotted against $NaHCO_3$ concentration.

of $NaHCO_3$ also increased 2-fold the levels of oxo^{8}Gua in DNA formed with decomposed peroxynitrite (from 0.08 ± 0.01 to 0.15 ± 0.03 mmol/mol guanine).

Peroxynitrite at 0.5 mM induced a conversion of 66 ± 7% of open-ring form II from a supercoil form (form I). As shown in Fig. 2, the addition of $NaHCO_3$ decreased this peroxynitrite-mediated formation of single-strand breaks dose-dependently. In order to attain 50% inhibition of the strand breakage caused by 0.5 mM peroxynitrite, at least an equimolar concentration of $NaHCO_3$ (0.5 mM) was needed. As the mechanism for peroxynitrite-mediated DNA strand breakage has not been elucidated extensively, we examined the effect of pH (Table 1). We found that peroxynitrite induced significantly more single-strand breaks at acidic pH than at neutral or alkaline pH: conversion of the supercoiled form I to form II was 88 ± 1, 66 ± 7 and 24 ± 14% at pH 4.2, 7.5 and 8.6, respectively. Decomposed peroxynitrite did not cause single-strand breaks significantly under the above pH conditions.

We previously reported that treatment of various deoxynucleosides with peroxynitrite leads to dose-dependent formation of 2-thiobarbituric acid-reactive substances, which showed the same R_F values on TLC as authentic base-propenals (base-CH = CH-CHO), which are highly cytotoxic compounds [18]. The yield of base-propenals was greater under acidic conditions (pH < 6) than at neutral or alkaline pH (pH 7.0) [18]. In this study, we examined the effect of $NaHCO_3$ (0–10 mM) on thymine-propenal formation by the reaction of thymidine (10 mM) with 1 mM peroxynitrite at pH 4.5. Under these conditions, about 2 μM thymine-propenal was formed in the absence of $NaHCO_3$, whereas the reaction was inhibited by 41 and 88% with 1 and 10 mM concentrations of $NaHCO_3$, respectively (Table 2).

Low concentrations of peroxynitrite induce strand breaks in plasmid DNA [15–18], although the mechanism and reactive species are not known. Salgo et al. [17] recently reported that induction of single-strand breaks by peroxynitrite is not only unaffected by the free radical scavenger, mannitol, but is also amplified by two other scavengers, benzoate and dimethylsulfoxide (DMSO). These authors suggest that hydroxyl radical is not involved in peroxynitrite-mediated DNA damage, but rather that an activated form of peroxynitrite (a hydroxyl radical-like intermediate, ONOOH*) formed during the decomposition of peroxynitrous acid (ONOOH) is responsible for the damage. Benzoate and DMSO intercept this intermediate and convert it to NO_2, which causes the DNA damage, rather than allowing it to decompose to an unreactive nitrate [17]. We have demonstrated that more DNA single-strand breaks are induced by peroxynitrite under acidic conditions than at neutral or alkaline pH. It is therefore likely that reactive species derived from ONOOH, rather than $ONOO^-$, cause the strand breaks. Base-propenals are also more easily formed under acidic conditions [18]. It has been reported that base-propenals are formed by cleavage of the deoxyribose ring with hydroxyl radical generated during the treatment of DNA with bleomycin in the presence of Fe^{2+} and oxygen or with γ-irradiation [35,36]. Therefore, hydroxyl-radical-like intermediate(s) (ONOOH*) generated from peroxynitrous acid (ONOOH) could be involved in the base-propenal formation and possibly in the induction of DNA single-strand breaks.

It has recently been reported that carbon dioxide reacts rapidly with peroxynitrite (3×10^4 M^{-1} s^{-1}) to form an $ONO_2CO_2^-$ adduct [23,24]. Although the exact molecular mechanism involved in CO_2-catalysed nitration by peroxynitrite is not known, Pryor and co-workers [31] have suggested that this $ONO_2CO_2^-$ adduct rearranges to give a nitrocarbonate anion, $O_2N\text{-}OCO_2^-$, which may serve as the proximal oxidant and nitrating agent in biological systems. It has also been proposed that the $ONO_2CO_2^-$ adduct could undergo either a heterolytic or a homolytic cleavage at the weak peroxo O-O bond, yielding $CO_3^{2-}+NO_2^+$ or $CO_3^{\bullet-}+{}^\bullet NO_2$, respectively [32].

Bicarbonate exerted different effects on the DNA damage induced by peroxynitrite. As has been shown for nitration of tyrosine [25,30], *p*-hydroxyphenyl acetate [31,32] and albumin [33], we have found that $NaHCO_3$ enhanced peroxynitrite-mediated nitration of guanine in DNA up to about 6-fold (Fig. 1). On the other hand, there was no apparent effect of bicarbonate on oxo^{8}Gua formation in DNA treated with peroxynitrite. Similarly, it has recently been reported that oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) induced by peroxynitrite is only marginally increased by $NaHCO_3$ [31]. On the other hand, $NaHCO_3$ inhibited peroxynitrite-mediated strand breakage and thymine-propenal formation (Fig. 2 and Table 2). Denicola et al. [32] recently

Table 2
Inhibitory effect of bicarbonate on thymine-propenal formation from reaction of thymidine with peroxynitrite at pH 4.3

$NaHCO_3$ (mM)	% of control
0	100 ± 3
1	59 ± 13
10	12 ± 6

The experiments were carried out in 100 mM sodium phosphate buffer, pH 4.5, containing 10 mM thymidine, 0.1 mM DTPA, 1.0 mM peroxynitrite and 0–10 mM $NaHCO_3$ at ~20°C (final volume was 1 ml). After the reaction, thymine-propenal was analysed by TLC [35] as described in the text.

reported that bicarbonate partially inhibited the oxidation of thiols, DMSO, oxyhemoglobin and cytochrome c^{2+} and totally inhibited the hydroxylation of benzoate [32]. These results indicate that the reactive species involved in strand breakage and base-propenal formation are different from those involved in nitration of guanine. For the former reactions, most probably ONOOH* or ONOOH are responsible and the reaction of peroxynitrite with CO_2 leads to the formation of the ONO_2CO_2H adduct, resulting in decreased amounts of the reactive intermediate(s). The ONO_2CO_2H adduct is a strong nitrating agent, but has low hydroxyl-radical-like activity.

In conclusion, we have demonstrated that bicarbonate enhances peroxynitrite-mediated formation of nitro^{8}Gua in DNA, whereas it inhibits strand breakage and base-propenal formation. These in vitro data suggest that an $ONO_2CO_2^-$ adduct, formed from the reaction of CO_2/HCO_3^- with peroxynitrite, is a potent nitrating agent, but has less hydroxyl-radical-like activity. Further in vivo studies on the role of CO_2/HCO_3^- in peroxynitrite-mediated tissue/DNA damage and cytotoxicity are needed.

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