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Reaction of Peroxynitrite with Hyaluronan and Related Saccharides

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The effects of peroxynitrite on hyaluronan has been studied by using an integrated spectroscopical approach, namely electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), and mass spectrometry (MS). The reaction has been performed with the polymer, the tetrasaccharide oligomer as well as with the monosaccharides *N*-acetylglucosamine and glucuronic acid. The outcome of the presence of molecular oxygen and carbon dioxide has been also evaluated. Although ^{1}H-NMR and ESI-MS experiments did not revealed peroxynitrite-mediated modification of hyaluronan as well as of related saccharides, from spin-trapping EPR experiments it was concluded that peroxynitrite induce the formation of C-centered carbon radicals, most probably by the way of its hydroxyl radical-like reactivity. These EPR data support the oxidative pathway involved in the degradation of hyaluronan, a probable event in the development and progression of rheumatoid arthritis.

Keywords: Hyaluronan; Peroxynitrite; Electron paramagnetic resonance; Rheumatoid arthritis

Abbreviations: DBNBS, 3,5 dibromo-4-nitrosobenzenesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; D-GlcNAc, *N*-acetyl-D-glucosamine; D-GlcA, D-glucuronic acid; ESI-MS, ElectroSpray Ionisation Mass Spectrometry; GC-MS, gas chromatography mass spectrometry; TLC, thin layer chromatography

INTRODUCTION

Rheumatoid arthritis is a systemic inflammatory disorder characterized by progressive and irreversible joint destruction.[1] Although its pathogenesis remains incompletely understood, there is a substantial body of evidence which suggest that highly reactive intermediates of oxygen,[2–12] chlorine[6,8,13–16] and nitrogen,[17–23] released by activated leukocytes at the inflammation site, play a key role in the outcome of the disease. These papers suggest that, either in the cartilage and the synovial fluid, these reactive species can be involved in the degradation of macromolecules important for the maintenance of homeostasis of such tissues. Some of these studies identified hyaluronan as the major target of radical attack.[5–8,14–16,20] Hyaluronan is a high molecular weight mucopolysaccharide, presenting a linear unbranched polymeric structure consisting of a repeating disaccharides of *N*-acetyl-D-glucosamine (D-GlcNAc) and D-glucuronic acid (D-GlcA) linked by a β-(1 → 4) glycosidic bond (Fig. 1). The disaccharides are linked by β-(1 → 3) glycosidic bonds to form the hyaluronan chain.[24] Low molecular weight fragments derived from the degradation of hyaluronan have been revealed in synovial fluid of patients with rheumatoid arthritis.[25] Fragmentation, besides the ability of hyaluronan to further stimulate inflammatory cells, account almost entirely for the loss of synovial fluid viscosity.[4,14,26]

The involvement of nitric oxide ($^{\cdot}$NO) in rheumatoid arthritis has been suggested by the increased expression of mononuclear cell nitric oxide synthase,[22] as well as by the high levels

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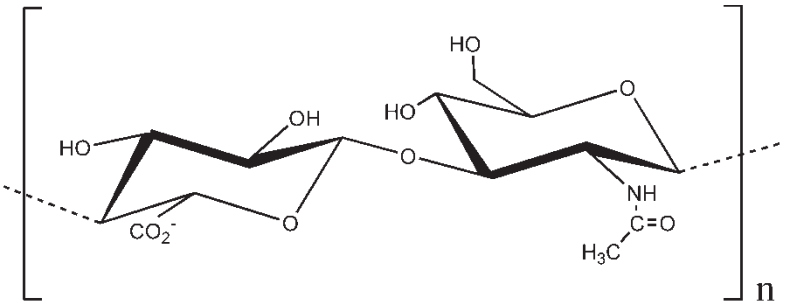
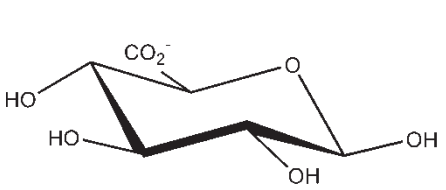
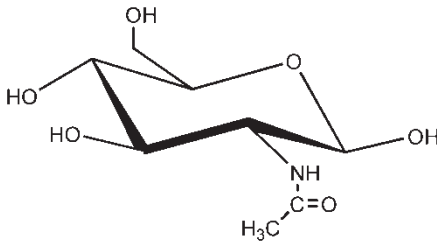


Hyaluronan



D-Glucuronic acid



N-Acetyl-D-Glucosamine

FIGURE 1 Chemical structure of hyaluronan and its monomers.

of nitrite/nitrate,[21–23] nitrosothiols[17] and nitrotyrosine[18,19] in both synovial fluid and blood serum of patients with rheumatoid arthritis.

Among the NO-derived oxidants, nitrous acid[27] and peroxynitrite[20,28] have been indicated as likely candidate for the degradation of hyaluronan. The mechanism of peroxynitrite-mediated hyaluronan depolimerization has been proposed[20,28] to be similar to that reported for other reactive oxidants, such as $^{\cdot}OH$ and hypochlorite.[5,7,16]

Peroxynitrite,[†] which is the product of the fast radical–radical reaction between $^{\cdot}NO$ and superoxide anion ($O_2^{\cdot-}$), is a reactive and short lived species that promotes oxidative tissue damage. Some oxidative reactions of biological targets, however, are not performed by peroxynitrite itself, but are probably induced by radicals ($^{\cdot}NO_2$ and $CO_3^{\cdot-}$) formed after its reaction with CO_2.[29–31] However, acidic environment such as that occurring in inflammatory conditions, in the phagolysosome, at the surface of cells, as well as in proximity of the hyaluronan (pK 2.9), can favor the CO_2-independent reactivity of peroxynitrite. The acidic environment allows $ONOO^-$ protonation with formation of peroxynitrous acid, which after homolysis (half-life of 1.9 s at pH 6.8) forms 30% $^{\cdot}OH$ and $^{\cdot}NO_2$ radicals.[32,33] This pathway of peroxynitrite decay is believed to promote hydroxylation, one-electron oxidations and nitration.[34–36] The $^{\cdot}OH$ is a more powerful oxidant than $^{\cdot}NO_2$ and $CO_3^{\cdot-}$, and is significantly less selective in the reaction with target molecules.

Interestingly, direct EPR and spin trapping studies reported that $^{\cdot}OH$ and hypochlorite can induce the formation of sugar-centered radical intermediates in the oxidative pathway leading to degradation of hyaluronan.[5,7,16] However, the integrated use of spectroscopic techniques, such as EPR, NMR and mass spectrometry (MS) may result instrumental in highlighting the oxidative pathways involved in the degradation of hyaluronan. This paper reports an investigation of the effects of the peroxynitrite with hyaluronan and its monosaccharides by using EPR, NMR and MS.

MATERIALS AND METHODS

Materials

D-GlcNAc, D-GlcA and mannitol were obtained from Aldrich. Hyaluronan was purchased from Fidia. Bovine testicular hyaluronidase, phosphate salts, diethylenetriaminepenta-acetic acid (DTPA), and 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS), were obtained from Sigma. All the solvents were of ultrapure grade from Romil.

[†]This term refers to both the anion oxoperoxynitrate (1 −), $ONOO^-$, and its conjugate acid hydrogen oxoperoxynitrate, ONOOH.

Synthesis of Peroxynitrite

Peroxynitrite was synthesized from nitrite and H_2O_2 as described by Radi *et al.*[37] and treated with MnO_2 (6 mg ml^{-1}, 30 min, 4°C) to eliminate excess H_2O_2. The peroxynitrite concentration was determined at 302 nm ($\varepsilon = 1700\,M^{-1}\,cm^{-1}$). The presence of NO_2^-, NO_3^- and H_2O_2, as contaminants of products, was ≤ 2, 49.4 and 185%, respectively, as determined by peroxynitrite decomposition at pH 4.0.[38] Decomposed peroxynitrite was obtained by neutralization with HCl before its addition to biological targets.

Synthesis of Methyl Glycosides of D-GlcNAc and D-GlcA

D-GlcNAc and D-GlcA (20 mg) were dried over P_2O_5 under vacuum overnight and separately treated with 1 M HCl/MeOH at 80°C for 20 h. The mixtures were neutralized by the addition of Ag_2CO_3, the precipitate for each sample was removed by centrifugation and the samples were separately worked up as follow. As the acid treatment removes the acetyl group from the glucosamine this last sample was treated with acetic anhydride (300 µl) overnight. After evaporation of the solvent the methyl glycosides mixture was purified as reported.[39] The glucuronic acid reaction mixture, after evaporation of methanol under a stream of N_2, was treated with 0.5 ml of 1 M NaOH for 1 h. The solution was neutralized with Dowex 50 W × 8 (H^+) cationic exchange resin (Fluka) and lyophilized. The yield was 13 and 18 mg for D-GlcNAc and D-GlcA methyl glycosides, respectively. One milligram of each sample was then acetylated and purified as reported,[39] and analyzed by GC-MS.

Enzymatic Digestion of Hyaluronan

Hyaluronan (50 mg) was dissolved in 25 ml sodium phosphate buffer (100 mM, pH 5) and treated with bovine testicular hyaluronidase (15,000 IU) at 37°C for 6 days. After this time the reaction was quenched by heating the sample for 10′ at 100°C followed by cooling in an ice bath. After lyophilization the sample was applied to a Bio-Gel P-2 column (Biorad, 1.5 × 168 cm) and eluted with Milli-Q water at a flow rate of 15 ml h^{-1} at room temperature; 3 ml fractions were collected. The fractionation yielded three fractions, namely, the exclusion volume (A, 2 mg), the tetrasaccharide (B, 22 mg) and the disaccharide (C, 20 mg).

Methyl Glycosides Peroxynitrite Reaction

Three different experiments were separately performed on methyl glycosides of glucuronic acid and *N*-acetylglucosamine with peroxynitrite. All the samples were dissolved at 2 mg ml^{-1} final concentration in 150 mM phosphate buffer. In two experiments, the samples were treated with peroxynitrite 20 mM, at final pH 4.3 and 6.9, respectively. The third sample was treated with peroxynitrite 5 mM, at final pH 4.2. To obtain the desired final pH, samples were treated with few drops of concentrated HCl or NaOH before the addition of peroxynitrite. The pH values were measured after the addition of peroxynitrite.

After 1 h the samples were deionized on Dowex H^+resin and lyophilized. The samples where then acetylated and analyzed to GC-MS. Controls without peroxynitrite were done.

Tetrasaccharide Peroxynitrite Reaction

Three samples of hyaluronan tetrasaccharide were dissolved at 4 mg ml^{-1} final concentration in 25 mM ammonium formiate pH 4.5. Two of them were treated with peroxynitrite 20 mM at final pH 6.9 and 4.3, respectively. To the last sample peroxynitrite 5 mM was added at final pH 4.2. After 1 h the samples were deionized on Dowex H^+ resin and lyophilized. The samples were then analyzed by ^{1}H NMR and ESI-MS.

NMR, GC-MS and ESI-MS

The ^{1}H spectra were obtained in D_2O at 400 MHz with a Bruker AM 400 spectrometer equipped with a dual probe, in the FT mode at 30°C. ^{1}H chemical shifts are expressed in δ relative to TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4). ESI-MS spectra were recorded with an API 100 Perkin–Elmer instrument, using a 5000 V ionization energy and 10 s as scanning time. Samples were dissolved in CH_3OH (50%) and HCOOH (20%). GC-MS spectra were obtained with a 5970 MS Hewlett–Packard instrument equipped with a 5890 Gas Chromatography and an RTX-5 capillary column (Restek, 30 m × 0.25mm i.d., flow rate, 1 ml min^{-1}, He as carrier gas). Acetylated methyl glycosides analysis was performed with the following temperature program: 150° for 5 min, 150° → 250° at 3° min^{-1}, 250° for 10 min. TLC were carried out on silica gel F254 (Merck).

EPR Measurements

To avoid metal-catalyzed nitration by peroxynitrite,[40] buffers were treated extensively with Chelex 100 (Bio-Rad, Richmond, CA). All samples contained phosphate buffer 0.15 M and 0.1 mM DTPA (referred to as phosphate/DTPA buffer). Hyaluronan tetrasaccharide and polymer were dissolved at final concentration of 100 mM and

10 mg ml^{-1}, respectively, in phosphate/DTPA buffers containing 1 mM DBNBS. Monosaccharides were tested at 300 mM final concentration in phosphate/DTPA buffers containing 10 mM DBNBS. The samples were then treated with peroxynitrite (5 mM). To obtain the desired final pH, samples were treated with few drops of concentrated HCl or NaOH before the addition of peroxynitrite. The pH values were measured before and after the addition of peroxynitrite. Peroxynitrite was added as a bolus to samples submitted to vigorous vortexing and, with the exception of decomposed peroxynitrite, was always the last addition. In some cases, a small volume of 1 M sodium bicarbonate was carefully added to the solution immediately before the addition of peroxynitrite. Where indicated, samples were bubbled with 100% oxygen or 100% argon for 30 min before the addition of peroxynitrite. The syringe used for hemogas analysis was extensively purged before sampling, and the pO_2 analysis was performed with ABL 330 (Radiometer, Copenhagen, Holland).

EPR spectra were measured at 37°C on a Bruker ECS 106 spectrometer (Bruker, Rheinstetten, Germany) equipped with a variable-temperature unit (ER4111VT). After the addition of peroxynitrite, samples were drawn up into a gas-permeable Teflon tube with 0.81 mm internal diameter and 0.05 mm wall thickness (Zeuss Industrial Products, Raritan, NJ). The Teflon tube was folded four times, inserted into a quartz tube and fixed to the ESR cavity (4108 TMH). The gas flow was air or pure N_2 as indicated. The dead time of sample preparation and ESR analysis was exactly 1 min after the last addition. All ESR spectra were corrected for baseline drift by a linear function using the software supplied by Bruker (ESP 1600 data system). Spectrometer conditions common to all spectra were: modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; microwave power, 20 mW.

RESULTS

GC-MS Analysis of Peroxynitrite-treated Methyl Glycosides of Monosaccharides

In order to evaluate the effect of peroxynitrite on both methyl glycosides of glucuronic acid and *N*-acetylglucosamine three different experiments were performed. Variations of pH values and peroxynitrite concentrations were taken into account.

From the comparison of the GC-MS chromatograms of the standards and the sample treated with peroxynitrite no modifications either in the type of signals or in the molar ratio respect to mannitol were found (results not shown).

NMR and MS Analysis of Oxidation of Hyaluronan Dimer Induced by Peroxynitrite

In order to carry out the oxidation on hyaluronan oligosaccharide, a hyaluronan enzymatic digestion was performed. The reaction products were purified by gel filtration chromatography and analyzed by ^{1}H NMR and electrospray MS. The proton NMR spectrum of the fraction B (Fig. 2) was identical with that reported in literature for a pure hyaluronan tetrasaccharide sample.[50] Positive ESI mass spectrum showed the occurrence of pseudomolecular ion peaks at m/z 799.2 $(M + Na)^+$, at m/z 777.2 $(M + H)^+$which indicated mainly the presence of a tetrasaccharide species together with a low abundant disaccharide species [m/z 398.2, $(M + H)^+$]. In this spectrum also appeared signals at m/z 759.2 $(M - H_2O + H)^+$and m/z 380.0 $(M - H_2O + H)^+$ suggesting the formation of dehydrated tetrasaccharide and disaccharide, respectively, during the enzymatic digestion. As hyaluronidase was used as purchased and no further purification steps were performed, the presence of contaminants cannot be ruled out.

The tetrasaccharide was treated with peroxynitrite in three different experiments, as described in experimental and then analyzed both by NMR and ESI-MS. In these spectra, no variations which could be assigned to a radical reaction could be detected.

The lack of evident modifications in hyaluronan and related saccharides as detected by NMR and MS analysis prompted us to study these reactions with a spectroscopic technique, EPR, specifically sensitive to radicals. It is known in fact, that a large part of peroxynitrite reactivity is due to the formation of radicals formed in the decay process.

Spin Trapping of *N*-acetylglucosamine and Glucuronic Acid Radicals Induced by Peroxynitrite

To investigate the reaction of peroxynitrite with hyaluronan and related monomers, we used DBNBS since it has been shown to be a suitable spin trap to study the radicals involved in the degradation of hyaluronan induced by Fe^{2+}/H_2O_2.[5] Figure 3 (upper panel), shows the spectra obtained after the treatment of *N*-acetylglucosamine with peroxynitrite in the presence of DBNBS. At pH 4.0 the EPR spectrum consisted of a three line isotropic signal with a nitrogen hyperfine coupling constants $a_N = 1.37 \pm 0.01$ mT (Fig. 3, Spectrum A). An adduct of DBNBS with similar spectral characteristics has been previously described in Fe^{2+}/H_2O_2-mediated oxidation of *N*-acetylglucosamine and has been assigned to the trapping of a tertiary C-centered radical of the monosaccharide.[5] In the absence of substrate, a weak isotropic signal with $a_N = 1.28$ mT was detected (Fig. 3, Spectrum B). This radical

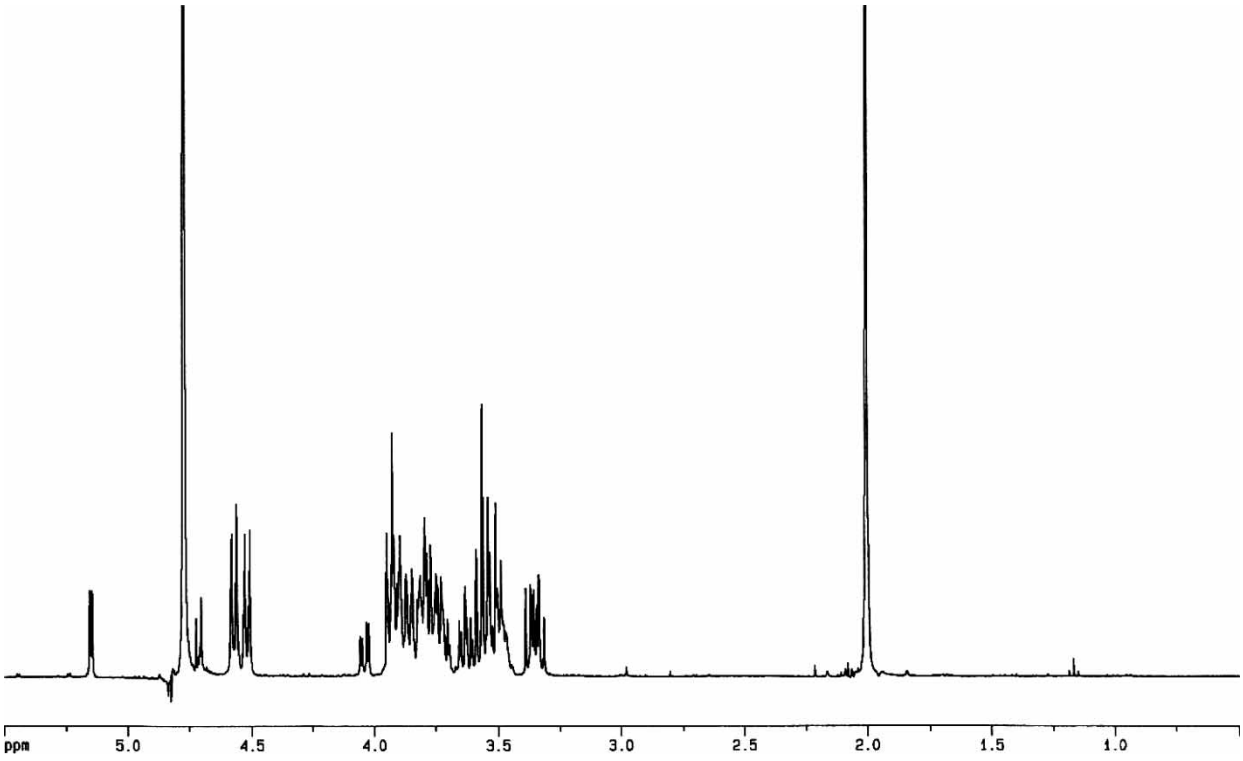


FIGURE 2 ^{1}H NMR spectrum of hyaluronan tetrasaccharide at 400 MHz ($T = 303$ K). Sample was dissolved in D_2O.

species, firstly described by Ozawa *et al.*[41] has been assigned to a DBNBS oxidation product,[42] which can be derived from peroxynitrite-dependent one-electron oxidation of DBNBS.[43]

No spectra were detected if peroxynitrite decomposed before the addition of *N*-acetylglucosamine (Fig. 3, Spectrum C), thus ruling out oxidative processes mediated by peroxynitrite decomposition products, referred in particular to nitrite at acidic pH. Indeed, Nazhat *et al.*[44] reported a three line isotropic signal ($a_N = 1.32$ mT) after the reaction of acidified solutions of nitrite with DBNBS, but this adduct was obtained at lower pH value (pH 1.5) and after significantly longer incubation times (8–16 h).

At pH 7.4, the only detected signal was that of DBNBS oxidation product and no adducts of *N*-acetylglucosamine/DBNBS were observed (Fig. 3, Spectrum D).

To further investigate the effects of pH, we studied the peroxynitrite-dependent *N*-acetylglucosamine/DBNBS adduct intensity in the pH range 4.0–8.0. As shown in Fig. 3, lower panel, the adduct intensity rapidly decreased as the pH increased above 5.5, and interestingly, the apparent pK_a of *N*-acetylglucosamine oxidation was centered at 6.3, suggesting that the oxidizing species is likely ONOOH (p$K_a = 6.5$) or a radical species derived from it.[37,46]

Further proof that DBNBS trapped a peroxynitrite-induced *N*-acetylglucosamine-centered radical was obtained by analysis of the superhyperfine structure of the central line of the DBNBS radical adduct.[45] Figure 4 shows a comparison of the superhyperfine structure of DBNBS adducts obtained after the treatment of the spin trap with peroxynitrite in the presence or absence of *N*-acetylglucosamine. The central line of peroxynitrite-treated *N*-acetylglucosamine DBNBS adduct did not show superhyperfine structure (Fig. 4, Spectrum A), while in the absence of the saccharide a triplet arising from DBNBS *meta*-protons with $a_N = 0.06$ mT was detected (Fig. 4, Spectrum B). The weakness of the signal and its faster decay (undetectable in about 3 min after the addition of peroxynitrite) did not allow to characterize this adduct further.

The addition of peroxynitrite to glucuronic acid in the presence of DBNBS did not lead to adducts formation at pH 4.4 (Fig. 5, Spectrum A), but lower pH values resulted in the detection of a faint adduct (Fig. 5, Spectrum B) and, at pH 2.2, of a triplets of doublets with hyperfine coupling constants $a_N = 1.37 \pm 0.08$ and $a_H = 0.66 \pm 0.018$ mT was clearly evident (Fig. 5, Spectrum C). Previously, a DBNBS adduct with similar hyperfine splitting constants has been detected in Fe^{2+}/H_2O_2-mediated oxidation of glucuronic acid and assigned to a C-centered radical.[5] The glucuronic acid/DBNBS adduct was undetectable in the absence of the substrate (result not shown), or if peroxynitrite decomposed before the addition of glucuronic acid

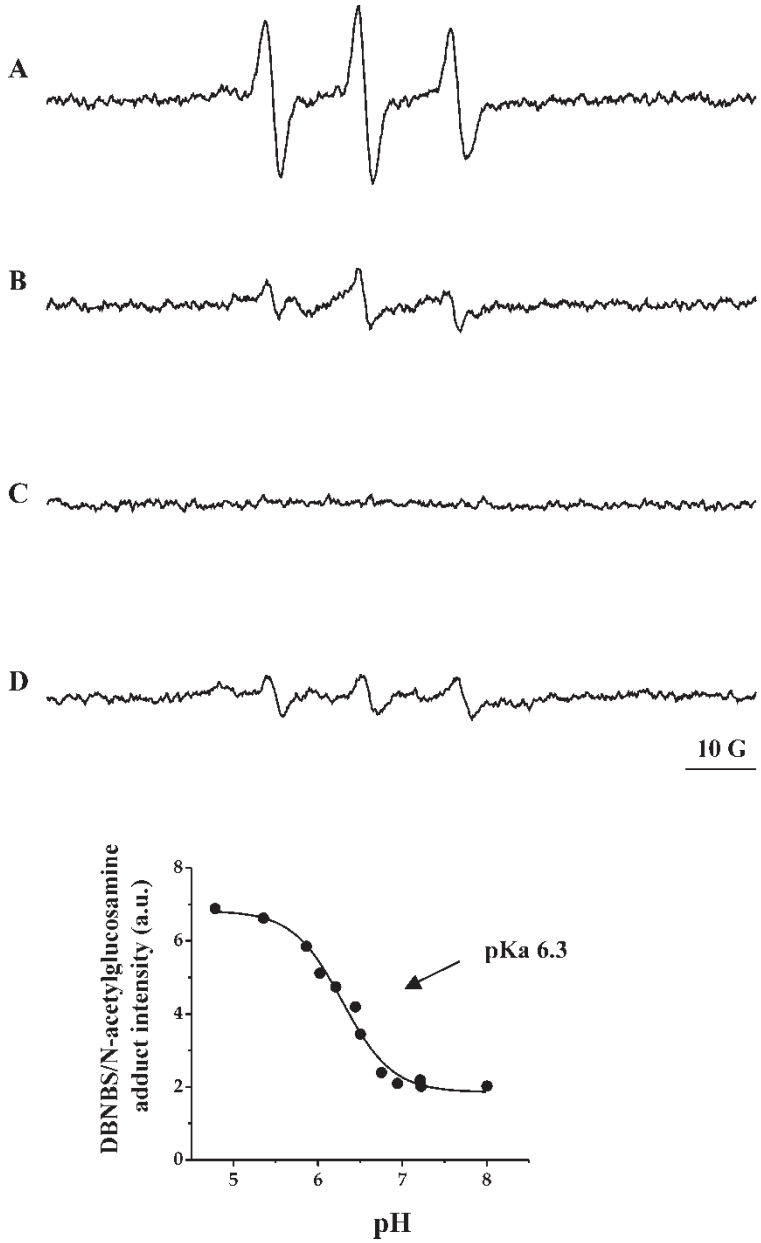


FIGURE 3 Trapping of radicals resulting from *N*-acetylglucosamine treated with peroxynitrite in the presence of DBNBS. Upper panel: EPR spectra of *N*-acetylglucosamine treated with peroxynitrite in the presence of DBNBS Spectrum A, peroxynitrite (5 mM) was added to 300 mM *N*-acetylglucosamine in phosphate/DTPA buffer, pH 4.3, containing 10 mM DBNBS; Spectrum B, sample as in spectrum A, but in the absence of *N*-acetylglucosamine; Spectrum C, sample as in spectrum A, but peroxynitrite was decomposed before the addition; Spectrum D, sample as in spectrum A, but the pH value was 7.4. Lower panel: pH dependence of DBNBS adduct yield obtained after the treatment of *N*-acetylglucosamine with peroxynitrite. Peroxynitrite (5 mM) was added to 300 mM *N*-acetylglucosamine in phosphate/DTPA buffer containing 10 mM DBNBS. To obtain the desired final pH, samples were treated with few drops of concentrated HCl or NaOH before the addition of peroxynitrite. The pH values were measured at the end of spectra acquisition. Spectra were detected 1 min after peroxynitrite addition. Spectrometer conditions were as follows: time constant, 164 ms; sweep time, 41.9 s; conversion time, 40.96 ms; gain, 4×10^3; sweep width, 100 G; modulation amplitude, 1 G; number of scans, 12.

(Fig. 5, Spectrum D). At pH 7.4 even in the presence of glucuronic acid, the EPR spectrum consisted only of the signal derived from the peroxynitrite-dependent one-electron oxidation of DBNBS (spectrum not shown).

Dose-dependent studies showed that at peroxynitrite concentrations < 5 mM the intensity of DBNBS adducts was weak compromising the definitive attribution of the adducts. At higher $ONOO^-$ concentration, the spectra were confusing due to the simultaneous presence of saccharide-centered radicals and DBNBS oxidation product that was predominant (results not shown). By the same way, the modification of the saccharide concentrations (50–500 mM) did not improve the signals (results not shown).

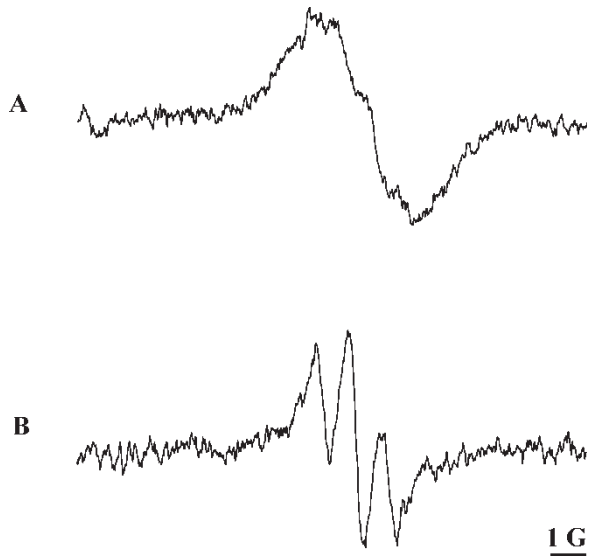


FIGURE 4 Comparison of the high resolution scans of the middle line from DBNBS adducts in the absence or in the presence of *N*-acetylglucosamine. Spectrum A, peroxynitrite (5 mM) was added to 300 mM *N*-acetylglucosamine in the presence of 10 mM DBNBS in phosphate/DTPA buffer, pH 4.5. Spectrum B, sample as in spectrum A, but in the absence of *N*-acetylglucosamine. Spectra were detected 1 min after peroxynitrite addition. Spectrometer conditions were as follows: time constant, 655 ms; sweep time, 168 s; conversion time, 163.84 ms; gain, 1×10^5; sweep width, 10 G; modulation amplitude, 0.1 G; number of scans, 1.

Effects of Oxygen on Peroxynitrite-dependent DBNBS Adducts

Molecular oxygen reacts rapidly with C-radicals[47] forming peroxyl radicals with a lifetime too short to be spin-trapped[45] and as a consequence molecular oxygen is expected to compete with DBNBS for C-radicals. In this case, the intensity of DBNBS adduct should result strongly reduced in the presence of high oxygen concentrations. We studied the effects of molecular oxygen on the formation of DBNBS adducts of peroxynitrite-treated *N*-acetylglucosamine and glucuronic acid. The oxygen tension was raised up by bubbling the samples with O_2 before the addition of the oxidant. When the pO_2 was increased by about 6 times (from 235.4 ± 6.5 up to 1432 ± 23 mmHg), the peroxynitrite-dependent DBNBS adduct intensities were decreased by about 89% for *N*-acetylglucosamine at pH 4.0, and 91% for glucuronic acid at pH 2.2.

By contrast, low oxygen tensions should allow to a greater stability of C-radicals. We performed experiments by lowering the sample oxygen tensions to 22 ± 8.1 mmHg pO_2 before the addition of peroxynitrite obtaining a 31 ± 4.6% increase of DBNBS adduct intensity. It should be considered, however,

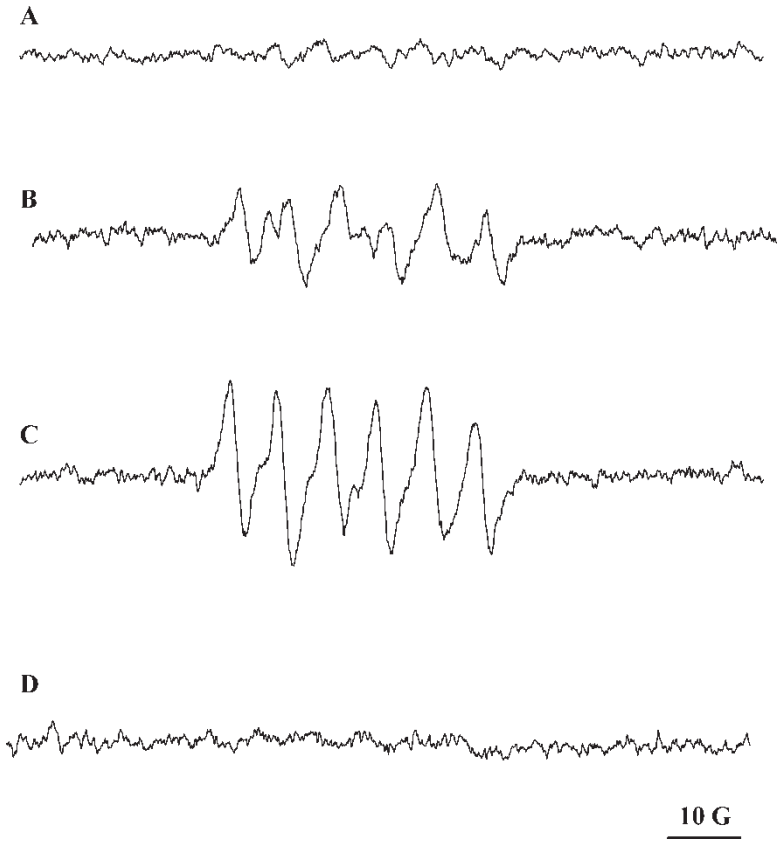


FIGURE 5 EPR spectra of glucuronic acid treated with peroxynitrite in the presence of DBNBS. Spectrum A, peroxynitrite (5 mM) was added to 300 mM glucuronic acid in phosphate/DTPA buffer, pH 4.4, containing 10 mM DBNBS; Spectrum B, sample as in spectrum A, but the pH value was 3.3; Spectrum C, sample as in spectrum A, but the pH value was 2.2; Spectrum D, sample as in spectrum C, but peroxynitrite was decomposed before the addition. Spectra were detected 1 min after peroxynitrite addition. Spectrometer conditions were as described in the legend to Fig. 1.

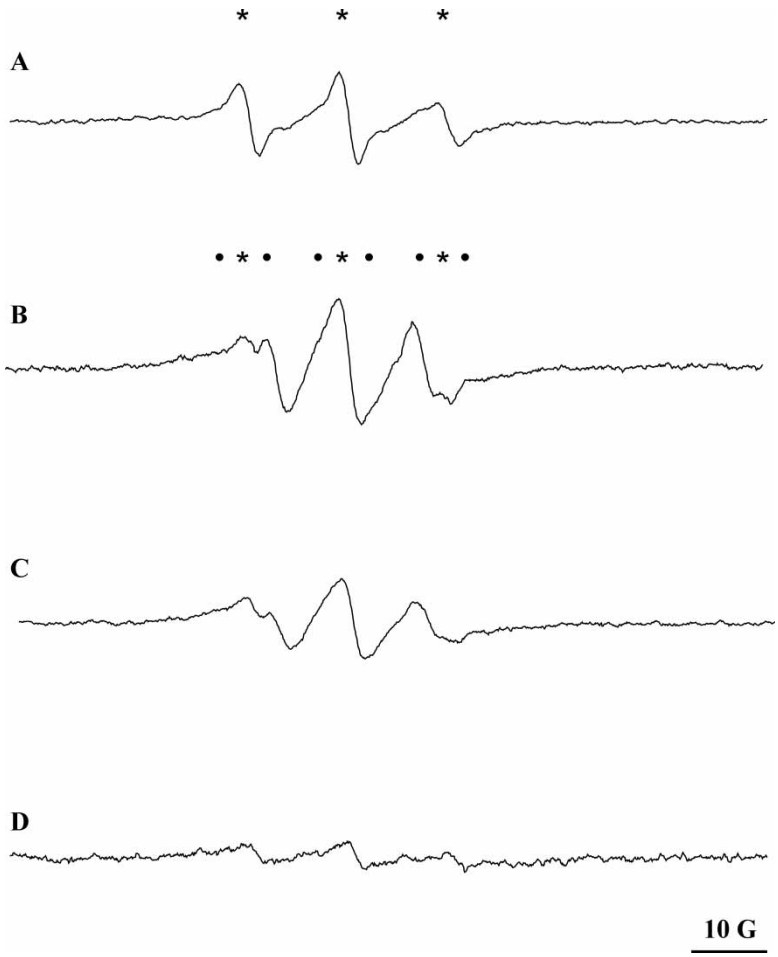


FIGURE 6 EPR spectra of hyaluronan tetrasaccharide and polymer treated with peroxynitrite in the presence of DBNBS. Spectrum A, peroxynitrite (1 mM) was added to 100 mM hyaluronan tetrasaccharide in phosphate/DTPA buffer, pH 3.2, containing 10 mM DBNBS; Spectrum B, sample as in Spectrum A, but in the presence of 10 mg ml^{-1} hyaluronan polymer; Spectrum C, sample as in Spectrum B, but the pH value was 4.5; Spectrum D, sample as in Spectrum B, but the pH value was 7.4. Adducts marked (•) are assigned to a three line isotropic signal with $a_N = 1.36$ mT, and (*) to a triplet of doublets with $a_N = 1.37$ mT and $a_H = 0.66$ mT. Spectra were detected 1 min after peroxynitrite addition. Spectrometer conditions were as described in the legend to Fig. 1.

that during peroxynitrite decay there is oxygen evolution that may limit the efficacy of sample degassing.

Spin Trapping of Radicals Induced by Peroxynitrite on Hyaluronan

Peroxynitrite was added to *N*-acetylglucosamine-glucuronic acid tetrasaccharide and hyaluronan polymer in the presence of DBNBS (Fig. 6). In the case of peroxynitrite-treated tetrasaccharide, a partially immobilized DBNBS adduct was detected (Fig. 6, Spectrum A). This signal appear to be composed by a prevailing three-line signal with hyperfine structure similar to that of *N*-acetylglucosamine ($a_N = 1.36 \pm$ mT) and an additional partially resolved adduct, whose exact identity cannot be ascertained due to its weakness.

A more anisotropic EPR spectrum was detected after the reaction of peroxynitrite with the hyaluronan polymer (Fig. 6, Spectrum B). This spectrum was substrate-dependent (undetected in the absence of polymer) and very similar to that obtained by Hawkins[5] after treatment of hyaluronan polymer with Fe^{2+}/H_2O_2. According to these authors, this spectrum can be resolved as the overlapping of a three line isotropic signal ($a_N = 1.36$ mT) and a triplet of doublets ($a_N = 1.37$ mT and $a_H = 0.66$ mT) assignable to C-centred radicals likely formed on *N*-acetylglucosamine and glucuronic acid.

At pH 4.5, the trapped species did not change, although the intensities of DBNBS adducts were reduced (Fig. 6, Spectrum C; compare with spectrum B). At pH 7.4 a weak isotropic signal assigned to DBNBS oxidation product was likely detected (Fig. 6, Spectrum D).

Effects of CO_2 on the Formation of Peroxynitrite-dependent DBNBS Adducts

As reported in the "Introduction" section, carbon dioxide/bicarbonate is the main buffering system in

tissues and is likely one of the major reactant of peroxynitrite in biological samples.[30,31,48,49] We treated monosaccharides and HA polymer with 1 or 5 mM peroxynitrite, respectively, in the presence of DBNBS and 10–100 mM bicarbonate and pH 7.4 (the related concentrations of CO_2, in equilibrium at pH 7.4, are included between 0.5 and 5 mM). Under these experimental conditions, we failed to detect any DBNBS adduct neither in the presence nor in the absence of monosaccharides and HA polymer (spectra not shown). These results suggest that at pH 7.4, the CO_2-catalyzed decomposition of peroxynitrite is faster than its reaction with DBNBS or HA related compounds.

DISCUSSION

Two previous works reported that peroxynitrite produced hyaluronan degradation as demonstrated by viscosity and agarose gel electrophoresis studies.[20,28] They proposed that the mechanism of degradation can be ascribed to the hydroxyl-like reactivity of peroxynitrite. Our EPR studies provided support to this hypothesis showing that (i) at acidic pH peroxynitrite-dependent C-centered carbon radicals are formed in monomers, in the tetrasaccharide as well as in the hyaluronan polymer (Figs. 3, 5 and 6) and (ii) the apparent pK_a of *N*-acetylglucosamine oxidation was 6.3, that is close to the pK_a value calculated for ONOOH.[37,46] The finding that DBNBS adducts induced by peroxynitrite were detectable only at mild to strong acidic conditions indicate the involvement of an oxidant species formed preferentially at acidic pH such as the hydroxyl radical formed in the decomposition process of peroxynitrous acid. At pH 7.4, we failed to detect peroxynitrite-dependent DBNBS adducts of hyaluronan either in the absence or in the presence of CO_2. This last finding can be explained by the fast reaction of peroxynitrite with CO_2 which prevents its hydroxyl-like reactivity.[30,31,33,34,51] However, it should be borne in mind that the lack of detection of spin trap adducts is not a proof that a radical species is not formed and we cannot exclude that saccharide-derived radicals are indeed formed but not trapped by DBNBS.

Previously, Hawkins *et al.*[5] used EPR to identify radical species derived by Fe^{2+}/H_2O_2-treated hyaluronan and related monosaccharides (which glucuronic acid and *N*-acetylglucosamine). These authors suggested that the $^{\cdot}OH$ reacts with sugars essentially randomly resulting in hydrogen abstraction and formation of C-centered carbon radicals. In our study, the likely involvement of carbon-centered radicals in peroxynitrite-mediated oxidation of hyaluronan monosaccharides is supported by their hyperfine structure as well as by the decrease of adduct intensities in the presence of high oxygen concentrations. Moreover, the DBNBS adducts and their hyperfine coupling constants are very close to those reported by Hawkins *et al.*[5] using the hydroxyl-radical generating system Fe^{2+}/H_2O_2, providing further evidence for the involvement of $^{\cdot}OH$ in peroxynitrite-mediated oxidations. Our GC-MS analysis, however, showed that these carbon-centered radicals do not produce evident degradations of both *N*-acetylglucosamine and glucuronic acid. This observation can be related to the instability of these radical species and to an unexpectedly higher level of selectivity in the reaction of peroxynitrite with HA. An unexpected high level of selectivity has been observed also in the reaction of HA with $^{\cdot}OH$ generated by Fe2 + $/H_2O_2$.[7]

The formation of C-centered radicals was observed not only with hyaluronan monosaccharides but also with the tetrasaccharide and the polymer, since their oxidation by peroxynitrite resulted in the trapping of adducts with similar features. As expected, the presence of glycosidic linkages between the monosaccharides in hyaluronan polymer caused a line broadening in DBNBS adducts due to a decrease of isotropy.

Hawkins *et al.*[5] used direct EPR to suggest that some of the initial sugar-derived radicals can undergo both base- and acid-catalyzed reactions (depending on the conformation of the sugar radical) to give secondary carbonyl-centered radicals through rearrangement reactions. Unfortunately, radical adducts formed by peroxynitrite are significantly less intense thus not allowing the use of direct EPR.

However, the radical species revealed by EPR did not produce evident modifications detectable by MS and NMR experiments. This result could be explained by the very low abundance of degradation products which are not displayed by these two techniques, in contrast with EPR, which is sensitive only to radicals.

Bearing in mind the likely involvement of $^{\cdot}NO$ pathway in rheumatoid arthritis,[17–19,21–23] this work suggests that peroxynitrite may be one of the involved damaging species and that depolymerization of hyaluronan is likely linked to its hydroxyl-like reactivity. Since the formation of $^{\cdot}OH$ radical from peroxynitrite is favored by the acidic environments occurring in inflamed synovial fluid near to the hyaluronan polymer (pK 2.9), peroxynitrite formation should be considered a possible pathological event in the development and progression of rheumatoid arthritis.

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