Degradation of extracellular matrix and its components by hypobromous acid

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EPO (eosinophil peroxidase) and MPO (myeloperoxidase) are highly basic haem enzymes that can catalyse the production of HOBr (hypobromous acid). They are released extracellularly by activated leucocytes and their binding to the polyanionic glycosaminoglycan components of extracellular matrix (proteoglycans and hyaluronan) may localize the production of HOBr to these materials. It is shown in the present paper that the reaction of HOBr with glycosaminoglycans (heparan sulfate, heparin, chondroitin sulfate and hyaluronan) generates polymer-derived *N*-bromo derivatives (bromamines, dibromamines, *N*-bromosulfonamides and bromamides). Decomposition of these species, which can occur spontaneously and/or via one-electron reduction by low-valent transition metal ions (Cu^+ and Fe^{2+}), results in polymer fragmentation and modification. One-electron reduction of the *N*-bromo derivatives generates radicals that have been detected by EPR spin trapping. The species detected are consistent with metal ion-dependent polymer fragmentation and modification being

initiated by the formation of nitrogen-centred (aminyl, *N*-bromoaminyl, sulfonamidyl and amidyl) radicals. Previous studies have shown that the reaction of HOBr with proteins generates *N*-bromo derivatives and results in fragmentation of the polypeptide backbone. The reaction of HOBr with extracellular matrix synthesized by smooth muscle cells *in vitro* induces the release of carbohydrate and protein components in a time-dependent manner, which is consistent with fragmentation of these materials via the formation of *N*-bromo derivatives. The degradation of extracellular matrix glycosaminoglycans and proteins by HOBr may contribute to tissue damage associated with inflammatory diseases such as asthma.

Key words: bromamine, eosinophil peroxidase, extracellular matrix, glycosaminoglycan, heparan sulfate, myeloperoxidase, protein oxidation.

INTRODUCTION

EPO (eosinophil peroxidase) and MPO (myeloperoxidase) are highly basic haem enzymes that are released extracellularly by activated eosinophils (in the case of EPO), and activated neutrophils, monocytes and some macrophages (in the case of MPO). These enzymes catalyse the reaction of H_2O_2 with Cl^- , Br^- and SCN^- (thiocyanate), to produce (pseudo)hypohalous acids [HOCl (hypochlorous acid), HOBr (hypobromous acid) and HOSCN (hypothiocyanous acid)]. These species exist as a mixture of the acid and anion forms at physiological pH values; HOCl, HOBr and HOSCN are used herein to designate these mixtures [1,2]. At physiological halide concentrations, HOBr is a significant product of EPO [1], especially when SCN^- is low, and HOBr is formed in approx. 25 % yield from H_2O_2 by MPO at physiological pH [3]. HOBr, and other brominating interhalogen species, may also be generated via the reaction of MPO-derived HOCl with Br^- [4].

The extracellular matrix is a complex material composed principally of proteoglycans (proteins bearing glycosaminoglycan chains), proteins (e.g. collagens, elastin) and the free glycosaminoglycan, hyaluronan. These species play a key role in regulating cellular adhesion, migration and proliferation, and impart important physicochemical properties to tissues. For example, basement membranes provide a scaffold for the attachment of endothelial and epithelial cells and regulate vascular permeability; these functions have been shown to depend critically on the

presence of specific components, such as heparan sulfate proteoglycans [5,6]. Binding of the highly cationic EPO and MPO proteins by the polyanionic glycosaminoglycan components of extracellular matrix (both proteoglycans and hyaluronan) is likely to localize the production of hypohalous acids to these materials *in vivo*. The degradation of extracellular matrix by hypohalous acids may have profound effects on the function and integrity of cells and tissues, and contribute to the pathophysiology of inflammatory diseases. It has been shown that co-injection of MPO and H_2O_2 into rats induces glomerular injury and proteinuria, consistent with degradation of the glomerular basement membrane by MPO-derived oxidants [7]. Heparan sulfate proteoglycans, which are critical components of the glomerular filtration barrier [6], are degraded by the $\text{MPO-H}_2\text{O}_2\text{-Cl}^-$ system *in vitro* [8] and HOCl can induce the release of protein and carbohydrate fragments from smooth muscle cell extracellular matrix *in vitro* [9]. Degradation of extracellular matrix by MPO-derived hypohalous acids may be an important process in human inflammatory lesions, such as atherosclerotic plaques and the glomerular basement membrane in human membranous glomerulonephritis, where co-localization of MPO and HOCl-modified proteins has been observed (reviewed in [10,11]). Asthma is characterized by the infiltration of eosinophils and the release of EPO and other cytotoxic granule proteins within bronchial tissue. Elevated levels of EPO, and HOBr-modified proteins, have been detected in bronchioalveolar lavage and sputum of asthmatics compared

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPO, eosinophil peroxidase; GalNAc, *N*-acetylgalactosamine acid; GlcNAc, *N*-acetylglucosamine; GlcNH₂, glucosamine; GlcNSO₃, glucosamine *N*-sulfate; HOBr, the physiological mixture of hypobromous acid and its anion present at pH 7.4; HOCl, the physiological mixture of hypochlorous acid and its anion present at pH 7.4; HOSCN, the physiological mixture of hypothiocyanous acid and its anion present at pH 7.4; MPO, myeloperoxidase; MNP, 2-methyl-2-nitrosopropane; TNB, 5-thio-2-nitrobenzoic acid; VSMC, vascular smooth muscle cell.

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with controls [12,13]. Furthermore, EPO-generated oxidants can simulate the pathological features of asthma, such as increased vascular permeability [14]. These observations implicate EPO-derived HOBr in the pathophysiology of this disease. Although there is good evidence for the extracellular matrix, and its components, being an important target for HOBr *in vivo*, the mechanisms of these reactions and their consequences are poorly understood. The reaction of HOBr with protein components is very rapid [15] and results in the modification of amino acid side-chains, unfolding, aggregation and fragmentation of the backbone [16,17]; *N*-bromo derivatives are important intermediates in these processes [16,17]. The susceptibility of glycosaminoglycans and intact extracellular matrix to degradation by HOBr has not been determined, although the related oxidant HOCl has been shown to react with glycosaminoglycans to generate polymer-bound *N*-chloro derivatives that decompose spontaneously, or via one-electron reduction by transition metal ions, to give polymer fragmentation [18–20]. Thus it was hypothesized that HOBr may degrade glycosaminoglycans, via *N*-bromo intermediates, and induce extracellular matrix fragmentation. The occurrence of these processes has been examined in the present study.

EXPERIMENTAL

Materials

Solutions and medium were prepared using water filtered through a four-stage Milli Q system. pH control was achieved using 0.1 M phosphate buffer [$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (pH 7.4)], treated with washed Chelex resin (Bio-Rad) to remove contaminating metal ions. D-Glucose was from BDH. Foetal bovine serum was from Gibco-BRL. Dulbecco's modified Eagle's medium was from JRH Biosciences. Tissue culture plates were from Falcon Becton Dickinson Labware. L-[U- ^{14}C]Proline and D-[1- ^3H]glucosamine were from Amersham Biosciences. Ultima Gold scintillant was from Packard Bioscience. L-Glutamine and sodium pyruvate were from Trace Scientific. *N*-Cyclohexylsulfamate, heparin (from porcine intestine; H-3393), NaOH, penicillin, polygalacturonic acid, SDS and streptomycin were from Sigma. Heparan sulfate (from porcine intestine) and partially de-*N*-sulfated heparin (from porcine intestine) were from Celsus Laboratories; the abundance of glucosamine residues in these samples has been reported previously [19]. Heparin octasaccharides (obtained by heparinase I cleavage of heparin) were from Dextra Laboratories. Hyaluronan (sodium salt, 120 kDa) was from Genzyme. Chondroitin sulfate A (chondroitin 4-sulfate from whale cartilage; 25–50 kDa) was from Seikagaku. All other chemicals used were of analytical grade. Stock solutions of polygalacturonic acid were stirred at approx. 95 °C for 5 min to dissolve the polymer and were filtered before use using a Nanosep MF GHP centrifugal device with a 0.45 μm pore-size filter (Pall Life Sciences). DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) from ICN was purified using activated charcoal. MNP (2-methyl-2-nitrosopropane) was dissolved in acetonitrile and diluted into reactions to give a final concentration of acetonitrile of 10% (v/v). HOBr solutions were prepared by the reaction of HOCl (40 mM) with NaBr (45 mM) in water at 22 °C for 1–60 min, with this stock diluted into 0.1 M phosphate buffer (pH 7.4) immediately prior to use. HOCl and HOBr concentrations were determined spectrophotometrically at pH 12 using molar absorption coefficients $\epsilon_{292} = 350 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [21] and $\epsilon_{329} = 332 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively [22]. O_2 was eliminated from incubations by bubbling with N_2 gas [18].

UV-visible spectroscopy

UV-visible spectra were recorded relative to 0.1 M phosphate buffer (pH 7.4) baseline using a PerkinElmer Lambda 40 UV-visible spectrometer.

Determination of *N*-bromo derivatives

N-Bromo derivatives were quantified using TNB [5-thio-2-nitrobenzoic acid; 35–45 μM in 0.1 M phosphate buffer (pH 7.4)] [18–20]. For samples that contained Cu^{2+} , EDTA (1 mM) was included to prevent oxidation of TNB by this metal ion. No other reagent interfered significantly with the assay.

EPR spectroscopy

EPR spectra were recorded, as described previously [18,20], at 22 °C, 2–30 min after addition of all the reaction components, using a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER4103TM cavity. Hyperfine couplings were measured directly from the field scan and confirmed by computer simulation using the program WINSIM (available at <http://epr.niehs.nih.gov>). Correlation coefficients between simulated and experimental data were > 0.95 for isotropic spectra. Spectra with anisotropic features gave lower correlation coefficients.

Polyacrylamide-gel electrophoresis

Polymer samples were analysed using 20 or 30 % polyacrylamide gels, as described previously [18,20]. Gels were stained with either 0.5 % Alcian Blue in 2 % acetic acid [23] or a combined Alcian Blue/silver staining method [24]. Gel images were acquired using an Umax PowerLook 1120 flatbed scanner and Silverfast Ai 6.0 software (Umax Technologies) or a Bio-Rad Gel Doc 2000 system and Quantity One software (Bio-Rad). Gel images were digitized over a linear range using Bio-Rad Quantity One software (Bio-Rad). Hyaluronidase digests of hyaluronan and chondroitin sulfate were prepared as described previously [18].

Cell culture and preparation of extracellular matrix

VSMCs (vascular smooth muscle cells; rat cell lines A7r5 and A10) were cultured in medium supplemented with 10% (v/v) foetal bovine serum, 3.5 g $\cdot \text{l}^{-1}$ glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 100 units $\cdot \text{ml}^{-1}$ penicillin and 0.1 mg $\cdot \text{ml}^{-1}$ streptomycin (herein referred to as DMEM) under an atmosphere of humidified 5% CO_2 at 37 °C. Cells were seeded at 2×10^5 cells in 12-well tissue culture plates with 1 ml of DMEM per well. Cultures were incubated for 10–12 days with the medium replaced every three days. After day one, DMEM was supplemented with 0.05 $\mu\text{Ci ml}^{-1}$ L-[U- ^{14}C]proline and 0.5 $\mu\text{Ci ml}^{-1}$ D-[1- ^3H]glucosamine. Cells were removed from the extracellular matrix using incubation for 20 min with 50 mM ammonium hydroxide, after which the extracellular matrix was washed three times with water as described previously [9]. The plates were frozen at –20 °C until required.

Treatment of the extracellular matrix with HOBr

The extracellular matrix was washed with phosphate buffer (1 ml) then incubated with 0 or 200 nmol HOBr (500 μl) at 22 °C. After 5 min incubation, aliquots (400 μl) were taken from the wells for measurement of radioactivity. The wells were then emptied of all remaining solution, refilled with phosphate buffer (500 μl) and subsequently incubated at 37 °C. Further aliquots were taken for measurement of radioactivity, in an identical manner, after 60, 180 and 300 min.

Figure 1 Structure of glycosaminoglycan *N*-bromo derivatives

(A) Hyaluronan bromamides and chondroitin sulfate bromamides (hyaluronan possesses unsulfated GlcNAc residues; chondroitin sulfate possesses 4- or 6-*O*-sulfated GalNAc residues). (B) Heparan sulfate, partially de-*N*-sulfated heparin and heparin *N*-bromo derivatives: bromamines (X = H), dibromamines (X = Br), *N*-bromosulfonamides (X = SO₃⁻) and bromamides [X = C(O)CH₃] (all polymers are variably *O*-sulfated and possess iduronic acid or glucuronic acid residues; heparin lacks GlcNH₂ residues and cannot form bromamines or dibromamines).

Measurement of radiolabelled extracellular matrix components

The total radioactivity incorporated into the VSMC extracellular matrix was quantified as described previously after solubilization using 5% SDS in 0.1 M NaOH (1 ml per well, 110°C, 10 min) [9]. A 200 µl aliquot of the solubilized matrix was added to 5 ml of scintillant and counted in a Tri-Carb 2100TR liquid-scintillant analyser (Packard Bioscience). The release of radioactivity into solution from HOBr-treated matrix was quantified by adding 400 µl aliquots to 5 ml of scintillant and subsequent counting, as above.

Statistics

Data analysis was carried out using one- and two-way ANOVA, with Dunnett's and Bonferroni post-tests respectively. Significance was assumed at the level of $P < 0.05$.

RESULTS

Formation of *N*-bromo derivatives upon reaction of HOBr with glycosaminoglycans

The formation of *N*-bromo derivatives (see Figure 1) upon reaction of HOBr with hyaluronan, chondroitin sulfate, heparin, partially de-*N*-sulfated heparin (an analogue of heparan sulfate) and heparan sulfate was investigated at 37°C and pH 7.4 by UV-visible spectroscopy.

The reaction of hyaluronan [2 mg · ml⁻¹; 5 mM in GlcNAc (*N*-acetylglucosamine) residues] with HOBr (1 mM) resulted in an increase in absorbance in the low UV region (i.e. $\lambda_{\text{max}} < 220$ nm; Figure 2A), consistent with the formation of bromamides [R-NBr-C(O)CH₃]. This increase in absorbance was complete within approx. 2 h, and spectra obtained within this period had an isosbestic point at approx. 260 nm. Similar data (results not shown) was obtained with chondroitin sulfate [2.5 mg · ml⁻¹; 5 mM in GalNAc (*N*-acetylgalactosamine) residues]. These changes were not observed upon the reaction of polygalacturonic acid (2 mg · ml⁻¹; 10 mM in galacturonic acid residues) with HOBr (1 mM), where only a slow loss of the HOBr/OBr⁻ absorbance was detected; this was complete within approx. 8 h. These data are consistent with the reaction of HOBr with hyaluronan and chondroitin sulfate occurring predominantly at their *N*-acetyl functions to generate bromamides, and not with the uronic acid residues. Elution of HOBr-treated hyaluronan and chondroitin sulfate from PD10 size-exclusion columns, and subsequent assay of the fractions, showed that the TNB-reactive material co-eluted with the parent polymer, confirming the formation of polymer-bound *N*-bromo derivatives.

The yield of these species was 20–45% (after correction for dilution during chromatography), under the conditions detailed in Table 1. These values are likely to be underestimates as a result of incomplete consumption of the HOBr, and decay of the bromamides (see below) prior to their assay.

The reaction of heparin [3.2 mg · ml⁻¹; 4.61 mM in GlcNSO₃ (glucosamine *N*-sulfate) residues, 0.59 mM in GlcNAc residues] with HOBr (1 mM) resulted in the formation of an absorbance peak with λ_{max} 307 nm (Figure 2B), assigned to *N*-bromosulfonamides (R-NBr-SO₃⁻). This conclusion is supported by the detection of a peak with λ_{max} 302 nm on reaction of the model compound *N*-cyclohexylsulfamate (R-NH-SO₃⁻, R = cyclohexyl) with HOBr (results not shown). Formation of the heparin-derived *N*-bromosulfonamides was complete within approx. 0.5 h and occurred with a yield of approx. 64% with respect to HOBr, as determined by an assay with TNB; this is likely to be an underestimate of the true yield, for the reasons outlined above. The more rapid consumption of HOBr by heparin than hyaluronan and chondroitin sulfate (at approximately equal ratios of glucosamine residues to HOBr) indicates that the rate constant for formation of *N*-bromosulfonamides (from GlcNSO₃ residues) is much higher than that for the formation of bromamides (from GlcNAc or GalNAc residues).

The reaction of heparan sulfate [3.2 mg · ml⁻¹; 0.113 mM in GlcNH₂ (glucosamine) residues, 2.96 mM in GlcNSO₃ residues and 3.74 mM in GlcNAc residues] or partially de-*N*-sulfated heparin (3.2 mg · ml⁻¹; 0.908 mM in GlcNH₂ residues, 3.79 mM in GlcNSO₃ residues and 0.60 mM in GlcNAc residues) with an equimolar concentration of oxidant with respect to GlcNH₂ residues (i.e. 0.908 mM HOBr), or less, resulted in the formation of an absorbance peak with λ_{max} approx. 291–293 nm (as determined by difference spectra relative to the parent polymer; Figures 2C and 2D). With a 2-fold excess of HOBr (with respect to GlcNH₂ residues), a pair of absorbance peaks with λ_{max} approx. 246–251 nm and approx. 379 nm were detected in difference spectra. The peak with λ_{max} 291–293 nm is characteristic of (mono)bromamines (R-NBr-H) (cf. a λ_{max} of 288 nm for taurine bromamine [25]), and the pair of peaks with λ_{max} approx. 246–251 nm (major) and approx. 379 nm (minor) are characteristic of dibromamines (R-NBr₂) (cf. absorbances at λ_{max} 241 nm and 336 nm for taurine dibromamine [25]). Formation of these chromophores, assigned to bromamine and dibromamine species, was complete within 0.5 min and the yields of *N*-bromo derivatives were 72–94% with respect to added HOBr, as quantified using TNB.

The absence of spectral features from a dibromamine when equimolar concentrations of HOBr and GlcNH₂ residues were employed is consistent with the rate constant for bromamine formation from the parent amine being significantly higher than

Figure 2 Formation of glycosaminoglycan *N*-bromo derivatives on reaction of glycosaminoglycans with HOBr

Reaction of glycosaminoglycans with HOBr at 37 °C and pH 7.4 was monitored by UV-visible spectroscopy in the region 220–500 nm. (A) Hyaluronan (2 mg · ml⁻¹; 5 mM in GlcNAc residues), 1 mM HOBr, *t* = 0.5 min, 2 h and 24 h; (B) heparin (3.2 mg · ml⁻¹; 4.61 mM in GlcNSO₃ residues and 0.59 mM in GlcNAc residues), 1 mM HOBr, *t* = 0.5 min, 0.5 h and 24 h; (C) heparan sulfate (3.2 mg · ml⁻¹; 0.113 mM in GlcNH₂ residues, 2.96 mM in GlcNSO₃ residues and 3.74 mM in GlcNAc residues), 0.113 mM and 0.225 mM HOBr (i.e. HOBr/GlcNH₂ residues = 1 and 2 respectively), *t* = 0.5 min (difference spectra obtained by subtraction of the absorbance of the unreacted parent polymer); (D) partially de-*N*-sulfated heparin (1.76 mg · ml⁻¹; 0.5 mM in GlcNH₂ residues, 2.84 mM in GlcNSO₃ residues and 0.33 mM in GlcNAc residues), 0.5 mM and 1 mM HOBr (i.e. HOBr/GlcNH₂ residues = 1 and 2 respectively), *t* = 0.5 min (difference spectra obtained by subtraction of the absorbance of the unreacted parent polymer); (E) partially de-*N*-sulfated heparin (3.2 mg · ml⁻¹; 0.908 mM in GlcNH₂ residues, 3.79 mM in GlcNSO₃ residues and 0.60 mM in GlcNAc residues), 0.25 mM HOBr (i.e. HOBr/GlcNH₂ residues < 1), *t* = 0.5 min and 24 h; (F) partially de-*N*-sulfated heparin (3.2 mg · ml⁻¹; 0.908 mM in GlcNH₂ residues, 3.79 mM in GlcNSO₃ residues and 0.60 mM in GlcNAc residues), 1.815 mM HOBr (i.e. HOBr/GlcNH₂ residues = 2), *t* = 0.5 min and 24 h.

that for dibromamine formation from the bromamine. This is consistent with the corresponding HOCl rate constants which differ by several orders of magnitude [19]. The more rapid consumption of HOBr by heparan sulfate and partially de-*N*-sulfated heparin than by the other polymers (at approx. equal ratios of glycosamine residues to HOBr), indicates that the rate constants for formation of bromamines and dibromamines are considerably higher than those for formation of *N*-bromosulfonamides or bromamides.

Glycosaminoglycan *N*-bromo derivatives were prepared for subsequent studies by reaction of the parent polymers with HOBr under the reaction conditions summarized in Table 1. Preparations of the *N*-bromo derivatives of heparin, heparan sulfate and partially de-*N*-sulfated heparin are herein identified by their most abundant *N*-bromo derivative. The absence of residual HOBr in these preparations was confirmed spectrophotometrically (for bromamines, dibromamines and *N*-bromosulfonamides) or by

passage of the HOBr-treated polymer through PD10 size-exclusion columns (for bromamides).

Stability of glycosaminoglycan *N*-bromo derivatives

All of the glycosaminoglycan *N*-bromo derivatives (bromamides, *N*-bromosulfonamides, dibromamines and bromamines) decomposed spontaneously at 37 °C and pH 7.4, as assessed by loss of their characteristic UV-visible absorbances (the spectra after 24 h are shown in Figures 2A, 2B, 2E and 2F) and assay with TNB (Figures 3A–D). Loss of all of the *N*-bromo derivatives was > 75 % within 24 h.

All of the *N*-bromo derivatives also decomposed rapidly on addition of Cu⁺ (generated *in situ* by the reduction of Cu²⁺ by Ti³⁺) and Fe²⁺ at 22 °C (Figure 4); anoxic reaction conditions were employed for these studies to prevent metal ion autoxidation. Cu²⁺

Table 1 Preparation of glycosaminoglycan *N*-bromo derivatives

Polymer	Reaction conditions				Product
	[Polymer] (mg · ml ⁻¹)	[HOBr] (mM)	Temp. (°C)	Time (min)	
Hyaluronan	4.0 (10 mM in GlcNAc)	4–4.55	37	8	Bromamides [R-NBr-C(O)CH ₃] [†]
Chondroitin sulfate	4.5 (9.1 mM in GalNAc)	4.55	37	8	Bromamides [R-NBr-C(O)CH ₃] [†]
Heparin	3.2 (4.61 mM in GlcNSO ₃ , 0.59 mM in GlcNAc)	1	37	30	<i>N</i> -Bromosulfonamides (R-NBr-SO ₃ ⁻) [†]
Heparan sulfate	3.2 (0.113 mM in GlcNH ₂ , 2.96 mM in GlcNSO ₃ , 3.74 mM in GlcNAc)*	0.113 (HOBr/GlcNH ₂ = 1) 0.225 (HOBr/GlcNH ₂ = 2)	37	0.5	Bromamines (R-NBr-H) [‡]
			37	0.5	Dibromamines (R-NBr ₂) [‡]
Partially de- <i>N</i> -sulfated heparin	3.2 (0.908 mM in GlcNH ₂ , 3.79 mM in GlcNSO ₃ , 0.60 mM in GlcNAc)*	0.908 (HOBr/GlcNH ₂ = 1) 1.815 (HOBr/GlcNH ₂ = 2)	37	0.5	Bromamines (R-NBr-H) [†]
			37	0.5	Dibromamines (R-NBr ₂) [†]

* Data for the abundance of GlcNH₂ is from [19].

[†] Only product.

[‡] The predominant product; other *N*-bromo derivatives will be generated in low yield (see text).

Figure 3 Decomposition of glycosaminoglycan *N*-bromo derivatives at 37 °C

Loss of glycosaminoglycan *N*-bromo derivatives upon incubation at 37 °C and pH 7.4 was quantified using the TNB assay. (A) Hyaluronan bromamides (326 μ M; ■) and chondroitin sulfate bromamides (512 μ M; ●); (B) heparin *N*-bromosulfonamides (726 μ M); (C) partially de-*N*-sulfated heparin bromamines (809 μ M; ■) and heparan sulfate bromamines (81 μ M; ●); (D) partially de-*N*-sulfated heparin dibromamines (1716 μ M; ■) and heparan sulfate dibromamines (162 μ M; ●). Data are means $\pm$ S.D. of three separate experiments.

at similar concentrations was an ineffective catalyst under these conditions (Figure 4).

Polymer fragmentation upon decomposition of glycosaminoglycan *N*-bromo derivatives

Changes in the molecular mass of the glycosaminoglycans upon decomposition of their *N*-bromo derivatives, with or without

added metal ions, were assessed by polyacrylamide-gel electrophoresis. In the absence of added metal ions, decomposition of hyaluronan- and chondroitin sulfate-derived bromamides at 37 °C over 24 h (cf. Figure 3A) did not result in detectable changes in molecular mass (using 20 % polyacrylamide gels; results not shown). Fragmentation occurred to varying extents upon decomposition of heparin-derived *N*-bromosulfonamides, partially de-*N*-sulfated heparin-derived bromamines and dibromamines, and

Figure 4 Decomposition of glycosaminoglycan *N*-bromo derivatives by transition metal ions

Loss of glycosaminoglycan *N*-bromo derivatives (initial concentrations as indicated below) upon reaction with Cu²⁺ (454 μM), Cu⁺ (364 μM; generated *in situ* by the sequential addition of 454 μM Cu²⁺ and 364 μM Ti³⁺) and Fe²⁺ (364 μM) under anoxic conditions at 22°C and pH 7.4 for 10 min was quantified using the TNB assay. Hyaluronan-derived bromamides (464 μM; solid bars); chondroitin sulfate-derived bromamides (380 μM; open bars); heparin-derived *N*-bromosulfonamides (646 μM; hashed bars); partially de-*N*-sulfated heparin-derived bromamines (676 μM; grey bars); partially de-*N*-sulfated heparin-derived dibromamines (1373 μM; cross-hatched bars). Data are means ± S.D. of six determinations from two separate experiments.

heparan sulfate-derived dibromamines under the same conditions (Figures 5A–5D; cf. Figures 3B–3D). The extent of fragmentation detected upon decomposition of (porcine) heparan sulfate-derived dibromamines was small, but was consistent with the fragmentation observed upon cleavage of this polymer at its GlcNH₂ residues by HOCl (via dichloramines [19]) and nitrous acid [26]. No detectable fragmentation was observed on decomposition of the heparan sulfate-derived bromamines (results not shown). This is consistent with the observation that, with partially de-*N*-sulfated heparin, bromamine-dependent fragmentation was less extensive than dibromamine-dependent fragmentation (compare Figure 5B with Figure 5C).

The reaction of Cu⁺ with hyaluronan- and chondroitin sulfate-derived bromamides, heparin-derived *N*-bromosulfonamides and partially de-*N*-sulfated heparin-derived bromamines and dibromamines resulted in significant polymer fragmentation (Figures 5E–5G). Fe²⁺ gave similar extents of fragmentation (results not shown). The fragments generated via metal ion-dependent decomposition of the hyaluronan- and chondroitin sulfate-derived bromamides gave discrete bands on high-resolution (30%) polyacrylamide gels (Figures 5H and 5I). The spacing of these bands is equidistant to those produced by hydrolytic cleavage of the parent polymers by testicular hyaluronidase, which occurs at the GlcNAc β-(1 → 4) glycosidic bonds [27] (i.e. at disaccharide intervals). This indicates that the fragmentation induced by metal ion-dependent bromamide decomposition occurs, like enzymatic cleavage, in a site-selective manner at disaccharide intervals along the polymer backbone.

Radical formation upon decomposition of glycosaminoglycan *N*-bromo derivatives

The role of radicals in the fragmentation of the HOBr-treated polymers was investigated using EPR and the spin traps MNP and DMPO (cf. studies with glycosaminoglycan *N*-chloro derivatives [18,20]). Spectral assignments were made on the basis of the observed splitting patterns and comparison of the measured hyper-

fine coupling constants with data from the literature ([18,20,28] see also: <http://epr.niehs.nih.gov/stdb>); the latter were confirmed by computer simulation. With each of the *N*-bromo derivatives, substrate-derived radicals were only detected upon addition of Cu⁺, and not with the parent compounds or with *N*-bromo derivatives that had been quenched with excess methionine. No signals were detected in the absence of the spin traps. Decomposition of the *N*-bromo derivatives in the absence of added metal ions is believed to occur via non-radical processes; the mechanism(s) of these processes are the subject of ongoing studies.

Decomposition of hyaluronan- and chondroitin sulfate-derived bromamides by Cu⁺ at 22°C in the presence of MNP (20.4 mM) or DMPO (102 mM) at pH 7.4 resulted in the detection of broad signals due to MNP and DMPO adducts of polymer-derived carbon-centred radicals [MNP, *a*(N, NO) = 1.55 mT, *a*(β-H) = 0.31 mT; DMPO, *a*(N, NO) = approx. 1.5–1.7 mT, *a*(β-H) = approx. 2.2–2.4 mT] and sharp features due to the DMPO adduct of CO₂•⁻ [*a*(N, NO) = 1.57 mT, *a*(β-H) = 1.86 mT] (Figures 6A and 6B; DMPO results not shown). The signals due to MNP adducts of carbon-centred radicals, which possessed a substrate-derived hydrogen splitting, are assigned to species with the partial structure ·CHRR'. With heparin-derived *N*-bromosulfonamides, broad signals due to adducts of polymer-derived carbon-centred radicals were detected with both MNP and DMPO [MNP, *a*(N, NO) = approx. 1.50 mT; DMPO, *a*(N, NO) = approx. 1.57 mT, *a*(β-H) = approx. 2.34 mT] as well as the MNP adduct of the SO₃•⁻ radical [*a*(N, NO) = 1.47 mT] and the DMPO adduct of the CO₂•⁻ radical (parameters as above) (Figures 6C and 6D). With bromamines derived from partially de-*N*-sulfated heparin, weak signals due to adducts of polymer-derived radicals were detected with both MNP and DMPO (Figures 6E and 6F). With the corresponding dibromamines, signals due to adducts of polymer-derived radicals were detected with both MNP and DMPO, as well as the MNP adduct of SO₃•⁻ (parameters as above) (Figures 6G and 6H). These signals were similar to those detected with the *N*-bromosulfonamides, particularly those detected with MNP (Figure 6G compared with Figure 6C), and it is likely that decomposition of low levels of *N*-bromosulfonamides present in the dibromamine preparation gave rise to these species.

Release of carbohydrate and protein components from the extracellular matrix upon reaction with HOBr

The ability of HOBr to degrade (radiolabelled) extracellular matrix (synthesized by two VSMC cell lines *in vitro*) was investigated by quantifying the release of ³H-labelled carbohydrate components and ¹⁴C-labelled protein components. The reaction of HOBr with the extracellular matrix laid down by A7r5 cells, and subsequent incubation at 37°C, resulted in the release of ³H- and ¹⁴C-labelled components in a time-dependent manner (Figures 7A and 7B). Preliminary experiments with extracellular matrix generated by A10 cells also provided evidence for an increased extent of matrix degradation as the concentration of HOBr increased. Release of these components is consistent with fragmentation of both the carbohydrate and protein components of the extracellular matrix. The time course of the release of these components is consistent with these processes occurring via the decomposition of (matrix-bound) carbohydrate and protein *N*-bromo derivatives. The release of ³H-labelled components, when expressed as a percentage of the total label incorporated, was greater than the release of ¹⁴C-labelled components, suggesting that the carbohydrate components of the extracellular matrix may be particularly susceptible to HOBr-mediated fragmentation. The background level of release detected in the absence of added oxidant is ascribed to the presence of extracellular proteases and

Figure 5 Polymer fragmentation upon decomposition of glycosaminoglycan *N*-bromo derivatives

Changes in the molecular mass of the glycosaminoglycans after decomposition of their *N*-bromo derivatives in the absence of added agents at 37 °C, or in the presence of Cu^+ at 22 °C and pH 7.4, were assessed by polyacrylamide gel electrophoresis using 20 or 30 % gels with Alcian Blue staining or with combined Alcian Blue/silver staining (hyaluronan only); densitometric analyses of the gel lanes are shown. The triangles on the horizontal scale indicate the migration positions of the Bromophenol Blue tracking dye (Δ) and the heparin octasaccharide standards ($\blacktriangle$). (A) Heparin-derived *N*-bromosulfonamides (726 μM) alone (37 °C, pH 7.4, 0.5 min and 24 h, 20 % gel); (B) partially de-*N*-sulfated heparin-derived bromamines (809 μM) alone (37 °C, pH 7.4, 0.5 min and 24 h, 20 % gel); (C) partially de-*N*-sulfated heparin-derived dibromamines (1716 μM) alone (37 °C, pH 7.4, 0.5 min, 4 h and 24 h, 20 % gel); (D) heparan sulfate-derived dibromamines (162 μM) alone (37 °C, pH 7.4, 0.5 min and 24 h, 20 % gel); (E) heparin-derived *N*-bromosulfonamides (646 μM) alone or with Cu^+ (364 μM) (22 °C, pH 7.4, 10 min, 20 % gel); (F) partially de-*N*-sulfated heparin-derived bromamines (1373 μM) alone or with Cu^+ (364 μM) (22 °C, pH 7.4, 10 min, 20 % gel); (G) partially de-*N*-sulfated heparin-derived dibromamines (1373 μM) alone or with Cu^+ (364 μM) (22 °C, pH 7.4, 10 min, 20 % gel); (H) chondroitin sulfate-derived bromamides (380 μM) alone or with Cu^+ (364 μM) (22 °C, pH 7.4, 10 min, 30 % gel), co-analysed with a partial testicular hyaluronidase (h'ase) digest of the parent polymer; (I) hyaluronan-derived bromamides (464 μM) alone or with Cu^+ (364 μM) (22 °C, pH 7.4, 10 min, 30 % gel), co-analysed with a partial testicular hyaluronidase (h'ase) digest of the parent polymer.

related matrix degrading enzymes in the matrix preparations; this is consistent with a previous study [9].

DISCUSSION

Activation of leucocytes results in the extracellular release of the highly basic haem enzymes EPO and MPO from cytoplasmic storage granules. Concurrently these cells undergo an oxidative burst with the formation of superoxide radicals, and hence H_2O_2 , via an NADPH oxidase complex. Both EPO and MPO utilize H_2O_2 , generated from this and other sources, to catalyse the oxidation of halide and pseudohalide ions to powerful oxidants. With EPO, HOBr is a major product especially in the absence of high levels of SCN^- [1]; with MPO, HOBr formation accounts for approx. 25 % of the H_2O_2 consumed at pH 7.4 [3]. As these proteins associate with components of the extracellular matrix as a result of ionic interactions, matrix components are likely to be major targets for these oxidants. Recent data have implicated excess, or inappropriate, HOBr generation in a number of human pathologies, and particularly asthma [12–14]. In fact, elevated levels of HOBr-modified proteins have been detected in bronchioalveolar lavage fluid and sputum.

In the present study it has been shown that HOBr can fragment heparan sulfate, heparin, hyaluronan and chondroitin sulfate via metal ion-dependent and independent processes, and induce the release of carbohydrate and protein fragments from extracellular matrix *in vitro*. HOBr has been shown to react selectively with the glycosamine residues (GlcNH_2 , GlcNSO_3 , GlcNAc and GalNAc) of glycosaminoglycans to generate *N*-bromo derivatives.

The reaction of HOBr with GlcNH_2 residues in these polymers generates bromamines (R-NBr-H) and dibromamines (R-NBr_2). Slower reactions occur with GlcNSO_3 residues to generate *N*-bromosulfonamides (R-NBr-SO_3^-) and with GlcNAc and GalNAc residues to generate bromamides [R-NBr-C(O)CH_3]. The reaction of HOCl with these species generates analogous *N*-chloro derivatives (chloramines, dichloramines, *N*-chlorosulfonamides and chloramides) and absolute rate constants for these reactions have been determined [18,19]. Rate constants for the reaction of HOBr with these targets have yet to be determined, however the data obtained indicate that the reaction of HOBr to generate dibromamines, *N*-bromosulfonamides and bromamides occurs at much higher rates than the corresponding reactions induced by HOCl. These data are consistent with previous studies with proteins, where the rate constants for *N*-bromination by HOBr are,

Figure 6 Radical formation upon decomposition of glycosaminoglycan *N*-bromo derivatives detected by EPR spin trapping

The formation of radicals from glycosaminoglycan *N*-bromo derivatives (approx. 340–1230 μM) upon reaction with Cu^+ (327 μM) was examined by EPR spectroscopy and spin trapping at 22 °C and pH 7.4 using the spin traps MNP (20.4 mM) and DMPO (102 mM). (A) Hyaluronan-derived bromamides, Cu^+ , MNP, after 2 min reaction. (D) Heparin-derived *N*-bromosulfonamides, Cu^+ , DMPO, after 2 min reaction. (E) Partially de-*N*-sulfated heparin-derived bromamines, Cu^+ , MNP, after 2 min reaction. (F) Partially de-*N*-sulfated heparin-derived, Cu^+ , DMPO, after 2 min reaction. (G) Partially de-*N*-sulfated heparin-derived dibromamines, Cu^+ , MNP, after 2 min reaction. (H) Partially de-*N*-sulfated heparin-derived dibromamines, Cu^+ , DMPO, after 2 min reaction. Selected spectral features and their assignment: $\circ = \cdot\text{CHRR}'\text{MNP}$ adduct [$a(\text{N}, \text{NO}) = 1.55 \text{ mT}$, $a(\beta\text{-H}) = 0.31 \text{ mT}$]; $\bullet = \text{MNP-SO}_3^-$ [$a(\text{N}, \text{NO}) = 1.47 \text{ mT}$]; $\downarrow = \text{DTBN}$ [$a(\text{N}, \text{NO}) = 1.71 \text{ mT}$]; $\blacktriangledown =$ polymer-derived carbon-centred radical adducts [$a(\text{N}, \text{NO}) = \text{approx. } 1.57 \text{ mT}$, $a(\beta\text{-H}) = \text{approx. } 2.34 \text{ mT}$]; $\nabla = \text{DMPO-CO}_2^-$ [$a(\text{N}, \text{NO}) = 1.57 \text{ mT}$, $a(\beta\text{-H}) = 1.86 \text{ mT}$]; $\blacklozenge = \text{DMPO-OH}$ [$a(\text{N}, \text{NO}) = 1.49 \text{ mT}$, $a(\beta\text{-H}) = 1.49 \text{ mT}$]. The data in panel (E) has been magnified 5-fold in the vertical scale relative to the data in panels (C) and (G). The data in panels (F) and (H) have been magnified 5-fold in the vertical scale relative to the data in panel (D).

typically, 10–100-fold higher than for *N*-chlorination by HOCl [15].

All of the glycosaminoglycan *N*-bromo derivatives decomposed readily in the absence of added agents at physiological pH and temperature and, with the exception of the bromamides, this resulted in polymer fragmentation. It has previously been shown that glycosaminoglycan dichloramines (but not other *N*-chloro derivatives) decompose readily under these conditions, resulting in polymer fragmentation [18,19]. Cleavage of heparan sulfate at its GlcNH₂ residues, via the formation of dibromamines, resulted in only small changes in molecular mass, consistent with the low abundance of GlcNH₂ residues in this polymer (0.5 per chain) and their location near the chain termini [26]. Much greater changes

Figure 7 Release of radioactivity from ³H- and ¹⁴C-radiolabelled VSMC extracellular matrix upon reaction with HOBr

³H- and ¹⁴C-radiolabelled rat VSMC extracellular matrix generated by A7r5 cells was prepared *in vitro* (using D-[1-³H]glucosamine and L-[¹⁴C]proline; see Experimental section) and release of radioactivity was measured upon reaction with 0 (■) and 200 (○) nmol HOBr as described in the Experimental section. (A) Total release of ³H-labelled extracellular matrix components, expressed as a percentage of the total label initially incorporated. (B) Total release of ¹⁴C-labelled extracellular matrix components, expressed as a percentage of the total label initially incorporated. Data are means $\pm$ S.D. of ten determinations from five separate experiments. The release of ³H material from the matrix by HOBr was significant at all the time points examined, by two-way ANOVA with Bonferroni post-test at the $P < 0.05$ level.

in mass were detected with de-*N*-sulfated heparin (an analogue of heparan sulfate) dibromamines, which have a higher abundance of GlcNH₂ residues (approx. 4 per chain, cf. data in [19]) that are located randomly along the backbone. More extensive fragmentation would also be expected with other heparan sulfates that possess GlcNH₂ residues in greater abundance, such as that from human aortae (which also has approx. 4 per chain [26]).

Cu^+ and Fe^{2+} induced rapid decomposition of all the *N*-bromo derivatives, with this resulting in polymer fragmentation. These reactions are attributed to one-electron reduction of the *N*-bromo derivatives to nitrogen-centred radicals (Reaction 1):

EPR studies using DMPO and MNP have confirmed the generation of radicals, with the species detected (polymer-derived carbon-centred radicals, $\text{SO}_3^{\bullet-}$ and $\text{CO}_2^{\bullet-}$) being of a similar nature to those detected previously from the corresponding *N*-chloro derivatives [18,20]. The *N*-chloro glycosaminoglycan reactions have been examined in detail in previous studies [18,20], with evidence obtained for selective intramolecular hydrogen atom abstraction reactions initiated by the primary

Scheme 1 Proposed reactions of glycosaminoglycan-derived nitrogen-centred radicals generated via one-electron reduction of glycosaminoglycan *N*-bromo derivatives

(A) Reactions initiated by amidyl radicals formed on hyaluronan and chondroitin sulfate. (B) Reactions initiated by aminyl, *N*-bromoaminyl, sulfonamidyl and amidyl radicals formed on heparan sulfate and heparin. Extensions of the partial structures shown are indicated by dashed bonds. Detailed evidence for the reactions of glycosaminoglycan-derived nitrogen-centred radicals has been presented previously [18,20].

nitrogen-centred radicals (1,2- and 1,5-hydrogen shifts) that generate polymer-derived carbon-centred radicals [18,20]. As the nitrogen-centred radicals generated from the *N*-bromo glycosaminoglycans are identical [R-N[•]-X, X = H, SO₃⁻, C(O)CH₃] or closely related [R-N[•]-X, X = Br, not Cl], these are proposed to undergo identical reactions (Scheme 1). Thus the radicals detected are assigned to carbon-centred radicals generated via hydrogen atom abstraction reactions of the initial nitrogen-centred radicals, and species generated via rearrangement of the resulting carbon-centred radicals (i.e. CO₂^{•-} and SO₃^{•-}; Scheme 1).

It has been shown that *N*-bromo derivatives are important intermediates in HOBr-mediated protein oxidation, with the formation of these species resulting in structural alterations and protein fragmentation [16,17]. A key route to fragmentation is believed to involve the decomposition of *N*-bromo derivatives, formed either on the backbone or on amino acid side chains, to nitrogen-centred radicals. In the case of the side-chain species, subsequent (intramolecular) hydrogen atom abstraction reactions (1,2- or 1,5-hydrogen shifts) of the initial nitrogen-centred radicals, generate peptide α-carbon-centred radicals [17]. Transition metal ions have been shown to promote damage via reduction of protein *N*-bromo derivatives to nitrogen-centred radicals [17].

Fragmentation of the glycosaminoglycans and proteins of extracellular matrix, via the reactions outlined above, can account for the observed release of these materials from extracellular matrix, synthesized by VSMCs *in vitro*, upon exposure to HOBr. The generation and subsequent decomposition of *N*-bromo intermediates appear to be crucial intermediates in matrix damage, as there is a time-dependent release of fragments that is inconsistent with direct damage by HOBr. Thus fragmentation occurs for a considerable period (hours) after the removal, or

complete consumption, of HOBr. This is consistent with the lifetime of the *N*-bromo intermediates detected on the glycosaminoglycans in the present study, and those formed on proteins reported previously [16,17].

The higher (fractional) release of carbohydrate, compared with protein, components observed in the present study needs to be interpreted with caution. These data may indicate that the glycosaminoglycans react more rapidly with HOBr than the proteins (cf. the high rate constant reported for the reaction of HOCl with GlcNH₂ residues [19]) and/or that damage to the glycosamine residues is translated more efficiently into backbone cleavage (and hence release from the matrix) than with proteins. There is considerable evidence that much of the damage induced by HOBr on proteins occurs on the side chains [15–17], rather than on the backbone, and that these modifications do not necessarily lead to protein fragmentation (and hence release of low molecular mass material). Kinetic studies with glycosaminoglycans may shed light on this point. Degradation of extracellular matrix by HOBr may be of considerable importance at inflammatory foci where EPO and MPO are released. Transition metal ions may exacerbate damage by stimulating the decomposition of matrix-bound *N*-bromo derivatives to nitrogen-centred radicals, with subsequent formation of carbon-centred radicals and polymer fragmentation. Recent studies have provided evidence for elevated levels of metal ions at some inflammatory sites, such as in atherosclerotic plaques [29], and for a correlation between metal ions levels and the extent of protein oxidation at such sites [30]. The degradation of extracellular matrix components by HOBr might be expected to have profound effects on both the structural integrity of tissues, and cellular and tissue function. Further investigation of the consequences of these reactions may provide further insights

into the role of HOBr-mediated damage in the pathogenesis of inflammatory diseases.

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REFERENCES

- 1 Van Dalen, C. and Kettle, A. J. (2001) Substrates and products of eosinophil peroxidase. *Biochem. J.* **358**, 233–239
- 2 Kettle, A. J. and Winterbourn, C. C. (1997) Myeloperoxidase: a key regulator of neutrophil oxidant production. *Redox Rep.* **3**, 3–15
- 3 Senthilmohan, R. and Kettle, A. J. (2006) Bromination and chlorination reactions of myeloperoxidase at physiological concentrations of bromide and chloride. *Arch. Biochem. Biophys.* **445**, 235–244
- 4 Henderson, J. P., Bryn, J., Williams, M. V., Mueller, D. M., McCormick, M. L. and Heinecke, J. W. (2001) Production of brominating intermediates by myeloperoxidase: a transhalogenation pathway for generating mutagenic nucleobases during inflammation. *J. Biol. Chem.* **276**, 7867–7875
- 5 Iozzo, R. V. (2005) Basement membrane proteoglycans: from cellar to ceiling. *Nat. Rev. Mol. Cell Biol.* **6**, 646–656
- 6 Morita, H., Yoshimura, A., Inui, K., Lodeura, T., Watanabe, H., Wang, L., Soininen, R. and Tryggvason, K. (2005) Heparan sulfate of perlecan is involved in glomerular filtration. *J. Am. Soc. Nephrol.* **16**, 1703–1710
- 7 Johnson, R. J., Couser, W. G., Chi, E. Y., Adler, S. and Klebanoff, S. J. (1987) New mechanism for glomerular injury: myeloperoxidase–hydrogen peroxide–halide system. *J. Clin. Invest.* **79**, 1379–1387
- 8 Klebanoff, S. J., Kinsella, M. G. and Wight, T. N. (1993) Degradation of endothelial cell matrix heparan sulfate proteoglycan by elastase and the myeloperoxidase–H₂O₂–chloride system. *Am. J. Pathol.* **143**, 907–917
- 9 Woods, A. A. and Davies, M. J. (2003) Fragmentation of extracellular matrix by hypochlorous acid. *Biochem. J.* **376**, 219–227
- 10 Nicholls, S. J. and Hazen, S. L. (2005) Myeloperoxidase and cardiovascular disease. *Arterioscler., Thromb., Vasc. Biol.* **25**, 1102–1111
- 11 Malle, E., Buch, T. and Grone, H. J. (2003) Myeloperoxidase in kidney disease. *Kidney Int.* **64**, 1956–1967
- 12 Aldridge, R., Chan, T., van Dalen, C., Senthilmohan, R., Winn, M., Venge, P., Town, G. and Kettle, A. (2002) Eosinophil peroxidase produces hypobromous acid in the airways of stable asthmatics. *Free Radical Biol. Med.* **33**, 847–856
- 13 Andreadis, A. A., Hazen, S. L., Comhair, S. A. A. and Erzurum, S. C. (2003) Oxidative and nitrosative events in asthma. *Free Radical Biol. Med.* **35**, 213–225
- 14 Mitra, S. N., Slungaard, A. and Hazen, S. L. (2000) Role of eosinophil peroxidase in the origins of protein oxidation in asthma. *Redox Rep.* **5**, 215–224
- 15 Pattison, D. I. and Davies, M. J. (2004) Kinetic analysis of the reactions of hypobromous acid with protein components: implications for cellular damage and the use of 3-bromotyrosine as a marker of oxidative stress. *Biochemistry* **43**, 4799–4809
- 16 Hawkins, C. L. and Davies, M. J. (2005) The role of aromatic amino acid oxidation, protein unfolding, and aggregation in the hypobromous acid-induced inactivation of trypsin inhibitor and lysozyme. *Chem. Res. Toxicol.* **18**, 1669–1677
- 17 Hawkins, C. L. and Davies, M. J. (2005) The role of reactive *N*-bromo species and radical intermediates in hypobromous acid-induced protein oxidation. *Free Radical Biol. Med.* **39**, 900–912
- 18 Rees, M. D., Hawkins, C. L. and Davies, M. J. (2003) Hypochlorite-mediated fragmentation of glycosaminoglycans and related *N*-acetyl glucosamines: evidence for chloramide formation, free radical transfer reactions and site-specific fragmentation. *J. Am. Chem. Soc.* **125**, 13719–13733
- 19 Rees, M. D., Pattison, D. I. and Davies, M. J. (2005) Oxidation of heparan sulphate by hypochlorite: role of *N*-chloro derivatives and dichloramine-dependent fragmentation. *Biochem. J.* **391**, 125–134
- 20 Rees, M. D. and Davies, M. J. (2006) Heparan sulfate degradation via reductive homolysis of its *N*-chloro derivatives. *J. Am. Chem. Soc.* **128**, 3085–3097
- 21 Morris, J. C. (1966) The acid ionization constant of HOCl from 5 °C to 35 °C. *J. Phys. Chem.* **70**, 3798–3805
- 22 Kumar, K. and Margerum, D. W. (1987) Kinetics and mechanism of general acid-assisted oxidation of bromide by hypochlorite and hypochlorous acid. *Inorg. Chem.* **26**, 2706–2711
- 23 Cowman, M. K., Slahetka, M. F., Hittner, D. M., Kim, J., Forino, M. and Gadelrab, G. (1984) Polyacrylamide-gel electrophoresis and Alcian Blue staining of sulfated glycosaminoglycan oligosaccharides. *Biochem. J.* **221**, 707–716
- 24 Min, H. and Cowman, M. K. (1986) Combined Alcian Blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution. *Anal. Biochem.* **155**, 275–285
- 25 Thomas, E. L., Bozeman, P. M., Jefferson, M. M. and King, C. C. (1995) Oxidation of bromide by the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase: formation of bromamines. *J. Biol. Chem.* **270**, 2906–2913
- 26 Westling, C. and Lindahl, U. (2002) Location of *N*-unsubstituted glucosamine residues in heparan sulfate. *J. Biol. Chem.* **277**, 49247–49255
- 27 Meyer, K. (1971) Hyaluronidases. In *The Enzymes*, vol. 5 (Boyer, P. D., ed.), pp. 307–320, Academic Press, New York
- 28 Buettner, G. R. (1987) Spin trapping: ESR parameters of spin adducts. *Free Radical Biol. Med.* **3**, 259–303
- 29 Stadler, N., Lindner, R. A. and Davies, M. J. (2004) Direct detection and quantification of transition metal ions in human atherosclerotic plaques: evidence for the presence of elevated levels of iron and copper. *Arterioscler., Thromb., Vasc. Biol.* **24**, 949–954
- 30 Stanley, N., Stadler, N., Woods, A. A., Bannon, P. G. and Davies, M. J. (2006) Concentrations of iron correlate with the extent of protein, but not lipid, oxidation in advanced human atherosclerotic lesions. *Free Radical Biol. Med.* **40**, 1636–1643

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