Nitric oxide degradation of heparin and heparan sulphate

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NO is a bioactive free radical produced by NO synthase in various tissues including vascular endothelium. One of the degradation products of NO is HNO₂, an agent known to degrade heparin and heparan sulphate. This report documents degradation of heparin by cultured endothelial-cell-derived as well as exogenous NO. An exogenous narrow molecular-mass preparation of heparin was recovered from the medium of cultured endothelial cells using strong-anion exchange. In addition, another narrow molecular-mass preparation of heparin was gassed with exogenous NO under argon. Degradation was evaluated by gel-filtration chromatography. Since HNO₂ degrades heparin under acidic conditions, the reaction with NO gas was studied under various pH conditions. The results show that the degradation of exogenous heparin by endothelial cells is inhibited by NO synthase inhibitors. Exogenous NO gas at

concentrations as low as 400 p.p.m. degrades heparin and heparan sulphate. Exogenous NO degrades heparin at neutral as well as acidic pH. Endothelial-cell-derived NO, as well as exogenous NO gas, did not degrade hyaluronan, an unrelated glycosaminoglycan that resists HNO₂ degradation. Peroxynitrite, a metabolic product of the reaction of NO with superoxide, is an agent that degrades hyaluronan; however, peroxynitrite did not degrade heparin. Thus endothelial-cell-derived NO is capable of degrading heparin and heparan sulphate via HNO₂ rather than peroxynitrite. These observations may be relevant to various pathophysiological processes in which extracellular matrix is degraded, such as bone development, apoptosis, tissue damage from inflammatory responses and possible release of growth factors and cytokines.

INTRODUCTION

A vascular smooth-muscle relaxing factor produced by endothelium was discovered by Furchgott and Zawadzki [1], and its chemical identity was shown to be NO [2,3]. NO is produced from L-arginine by the enzyme NO synthase, which exists in constitutive and inducible forms [4]. The enzyme is inhibited by derivatives of L-arginine, including N^ω-nitro-L-arginine (L-NNA) and N^G-monomethyl-L-arginine (L-NMMA). Human umbilical-vein endothelial cells (HUVECs) have been shown to produce NO, either constitutively [5] or stimulated by bradykinin, α-thrombin, L-arginine and ionomycin [6,7]. NO is also produced by neuronal cells, and is involved in neural transmission [8]. Large quantities of NO, as well as superoxide, are produced by neutrophils and monocytes on inflammatory stimulation, such as by lipopolysaccharide [2]. Superoxide combines with NO to make peroxynitrite [9], a very powerful non-specific degradative agent with hydroxyl-radical-like properties [10].

Heparin is an anticoagulant–antithrombotic glycosaminoglycan, that is a co-polymer of uronic acid (iduronic or glucuronic acid) and glucosamine. It is heterogeneous in size, charge and anticoagulant activity. It is highly sulphated, with about 2.5 sulphate groups per disaccharide moiety [11–13]. In addition, heparin stimulates the growth of endothelial cells, yet inhibits growth of vascular smooth-muscle cells [14–16]. Heparan sulphate is the main proteoglycan of the extracellular matrix of HUVECs [17]. Its glycosaminoglycan structure is similar to that of heparin, except that it has fewer N-sulphate moieties and more glucuronic acid residues [18]. Hyaluronic acid, or hyaluronan, is an unsulphated glycosaminoglycan consisting of alternating glucuronic acid and N-acetylglucosamine residues.

HNO₂ is one of many decomposition products of NO [10,19]. A predominant rapid chemical method to depolymerize heparin

and heparan sulphate involves the use of HNO₂ [20–23]. HNO₂ cleaves N-sulphated or free glucosamine moieties. This deaminative reaction results in elimination at the glycosidic bond and yields anhydromannose moieties on the new reducing ends of the products. The breakdown products of heparin after HNO₂ treatment have been structurally characterized by Bienkowski and Conrad [20]. Since the amino groups of the glucosamine moieties of hyaluronan are blocked with N-acetyl groups, this glycosaminoglycan is resistant to HNO₂ degradation. We have recently reported that hyaluronan is susceptible to degradation by peroxynitrite, through a hydroxyl-radical-like mechanism [24]. Thus we tested hyaluronan for susceptibility to NO degradation, and heparin for susceptibility to peroxynitrite, to determine the pathway of heparin degradation.

This report documents and characterizes the degradation of heparin by both endogenous and exogenous NO. These results suggest that NO and its products can degrade biologically important glycosaminoglycans; thus NO may have additional physiological and pathological roles.

MATERIALS AND METHODS

Materials

Heparin (porcine), hyaluronan (bacterial), L-NNA and L-NMMA were from Sigma (St. Louis, MO, U.S.A.). Bio-Gel P-100 and Bio-Gel P-4 were from Bio-Rad (Richmond, CA, U.S.A.). QAE-Sephadex A-25 was from Pharmacia/LKB Biotechnology (Piscataway, NJ, U.S.A.). Collagenase was from Gibco (Grand Island, NY, U.S.A.) or Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Tissue culture media and reagents were from Gibco or Sigma. Fetal bovine serum was from Flow/ICN (McLean, VA, U.S.A.) or Sigma.

Abbreviations used: L-NNA, N^ω-nitro-L-arginine; L-NMMA, N^G-monomethyl-L-arginine; HUVEC, human umbilical-vein endothelial cell; V₀, void volume (gel-filtration column); V_T, total volume (gel-filtration column).

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Figure 1 Gel-filtration chromatography of porcine heparin on a Bio-Gel P-100 column (2.6 cm $\times$ 96 cm), eluted with 0.5 M LiCl/0.05 M imidazole, pH 7.45

Heparin concentration was measured by the carbazole assay [25]. The heparin was divided into six fractions, as indicated by the dashed lines. Fraction 4 was used for the NO gas studies and fraction 3 was the one incubated with the cultured endothelial cells.

Heparin preparations

Narrow molecular-mass fractions of heparin (porcine; Sigma) were prepared by gel filtration on a column (2.6 cm $\times$ 96 cm) of Bio-Gel P-100, eluted with 0.5 M LiCl/0.05 M imidazole, pH 7.45. Heparin was assayed by the carbazole method [25]. The separation is shown in Figure 1. The heparin was divided into six fractions, as indicated by the dashed lines on the Figure. Fraction 2 was used for NO gas studies and fraction 3 was incubated with cultured endothelial cells.

Degradation of heparin by endothelial cells

HUVECs were grown as previously described [26]. Experiments were performed on confluent cells between the second and fourth passage in T-75 flasks containing about 3.4×10^6 cells/plate.

Complete growth medium was made substituting heparin fraction 3 (90 μ g/ml) for unfractionated heparin. Confluent HUVECs were incubated in T-75 flasks for 48 h at 37 °C with 5% CO₂ in 15 ml of this complete medium. The pH did not change significantly in any of the incubation mixtures. Medium not exposed to cells (15 ml) served as a control. Some incubations with heparin fraction 3 were also supplemented with L-NNA (0.5 mM) or L-NMMA (0.5 mM). At the end of the incubation period, the media were collected and frozen at -20 °C until the next step.

Heparin was recovered from the cell culture medium by strong-anion-exchange chromatography, concentrated, then analysed for molecular-mass distribution on a gel-filtration column, as follows. LiCl was added to both fresh and conditioned media to a final concentration of 1.0 M, and the samples were brought to pH 5.0 with acetic acid. Heparin was recovered from fresh or incubated media (15 ml for each) using matched 3.5 ml QAE-Sephadex A-25 columns previously equilibrated with 1.0 M LiCl/0.1 M acetate, pH 5.0. After sample application, the column was eluted with the equilibrium buffer, which removed more than 99% of the protein in the medium (A_{280}). After the medium pH indicator dye had been eluted with 1.0 M LiCl, heparin was eluted from the column with 3.0 M LiCl/0.1 M acetate, pH 5.0. Heparin-positive fractions, as determined by Alcian Blue stain-

ing, were combined, then concentrated by precipitation with sodium-acetate-saturated ethanol. After being redissolved in water, the samples were desalted by ultrafiltration on a YM-2 membrane (Amicon, Beverly, MA, U.S.A.) and lyophilized (SpeedVac; Savant). Alternatively, the heparin fractions were dialysed, then lyophilized. Both procedures retain the original fraction-3 heparin but lose the lowest-molecular-mass species that would be generated by degradation.

Recovered heparin samples were redissolved in water (0.5 ml) and applied to a Bio-Gel P-100 column, equilibrated with 0.5 M LiCl/0.05 M imidazole, pH 7.4. V_o was 25 ml and V_T 92 ml. Heparin was analysed by Alcian Blue and by the carbazole reaction. Since most low-molecular-mass species are lost, comparisons were made between the original fraction-3 heparin peak recovered from the medium incubated with cells (conditioned medium) and the recovery of the same peak from unincubated (fresh) medium. Thus data are expressed as percentage recovery of fraction-3 heparin peak relative to recovery of the same peak from fresh medium.

Incubation of hyaluronan with HUVECs

Hyaluronan (0.1 mg/ml) was dissolved in culture medium, and 5 ml was incubated with HUVECs in T-25 flasks (approx. 1×10^6 cells) for 48 h, as above. After incubation, samples were treated with Pronase, then concentrated by evaporation to 1 ml. Degradation was evaluated by the electrophoretic method of Lee and Cowman [27] and compared with unincubated hyaluronan.

Chemical treatment of glycosaminoglycans

Treatment of heparin with NO gas

Solutions of heparin fraction 2 were prepared at a final concentration of 10 mg/ml in buffers at pH 1.5 (0.6 M KCl/HCl, pH 1.5), pH 4.0 (0.12 M sodium citrate, pH 4.0) [21,22] and pH 7.4 (0.5 M LiCl/0.05 M imidazole or 0.2 M phosphate). One ml of each, in a Teflon-coated screw-capped glass tube (16 mm $\times$ 100 mm), was carefully pre-gassed with argon for over 1 min, then bubbled for 30 s with a slow stream of NO gas (> 98.5%; Linde) at room temperature in a fume hood. No yellow colour, characteristic of NO₂ formation, was observed in the test tube. Assuming that the NO gas was saturated, the concentration of NO was estimated to be approx. 2 mM (based on a solubility of 5.7 cm³/100 ml at 20 °C). Another solution of heparin was adjusted to pH 1.5 and treated with 1 M HNO₂ by following the standard procedure [21]. After 10 min at room temperature, the solutions at pH 1.5 and 4.0 were neutralized with five or six drops of saturated NaHCO₃. The samples were left at room temperature overnight and then refrigerated until run on a Bio-Gel P-100 gel-filtration column (1 cm $\times$ 113 cm), in 0.5 M LiCl/0.05 M imidazole, pH 7.4, as above. Identical heparin solutions at each pH, not treated with NO gas, were run as controls.

Treatment of heparin and heparan sulphate with 400 p.p.m. of NO

Heparin fraction 2 was dissolved at 5 mg/ml in the same pH 1.5 and 4.0 buffers as above. Heparan sulphate (bovine; Upjohn) was purified by chondroitinase ABC treatment, followed by ion-exchange chromatography on DE-52 (prepared by Dr. B. Lahiri, New York Medical College). A 2 mg/ml solution was prepared in the pH 1.5 and 4.0 buffers, as above. These solutions were carefully bubbled with argon, followed by 400 p.p.m. of NO in N₂ for 30 s. Again, no yellow colour was observed. The samples were left overnight at room temperature before gel-filtration

analysis. For comparison, untreated controls were also analysed by gel filtration.

Treatment of heparin with peroxynitrite

Fraction-2 heparin (5 mg/ml) was treated with 5 mM synthetic peroxynitrite [9] in 0.2 M phosphate buffer, pH 7.4. These conditions were sufficient for degradation of hyaluronan [24]. After overnight incubation, analysis by gel filtration was carried out as above.

Treatment of hyaluronan with NO gas

Hyaluronan (5 mg) in 5 ml of 0.2 M phosphate buffer at pH 7.4 was pre-equilibrated with argon gas for 1–2 min and then bubbled with NO gas for 1 min. No yellow colour was observed. After 10 min at room temperature, this sample was refrigerated overnight, then analysed for depolymerization, as compared with an untreated control, by electrophoresis [27].

RESULTS

Heparin fractions

In order to observe more clearly subtle changes in heparin molecular mass caused by partial degradation, narrow molecular-mass fractions were employed for this study. This strategy allows observation of small shifts in molecular mass that would be difficult to observe with unfractionated heparin. Thus fraction 3 from Figure 1 was used for cell incubations, and fraction 2 was used for chemical treatment experiments.

Degradation of glycosaminoglycans by endothelial cells

In the first set of experiments, heparin fraction 3 was recovered from growth medium to which this fraction had been added. Recovery from incubated medium was compared with recovery from unincubated medium (control). Figure 2(A) shows the elution patterns for fraction-3 heparin recovered from

Figure 2 Recovery of fraction-3 heparin from fresh (----) or incubated (—) fraction-3 heparin-containing medium in the absence (A) or presence (B) of L-NNA (0.5 mM)

Incubation with cells was for 48 h at 37 °C.

Table 1 Relative recovery of heparin from medium

Cells	Addition	Recovery (% of control)
No	None	100* (control)
Yes	None	45
Yes	L-NNA or L-NMMA (0.5 mM)	105

* Absolute recovery of fraction-3 heparin from medium in the absence of cells was 48.4%.

Figure 3 Separation of intact from degraded fraction-2 heparin after NO treatment in acid pH conditions

One ml of each heparin solution (10 mg/ml) was pre-gassed with argon, then bubbled for 30 s with a slow stream of NO gas (> 98.5%; Linde) at room temperature in a fume hood. Another identical solution of heparin was adjusted to pH 1.5 treated with 1 M HNO₂ by following the standard procedure [21] (A). After 10 min at room temperature, the solutions were neutralized with five or six drops of saturated NaHCO₃. The samples were left at room temperature overnight (in the fume hood) and then run on a Bio-Gel P-100 gel-filtration column (1 cm × 113 cm) as described in the Materials and methods section. —, Untreated controls; —, digested samples. In the heparin colorimetric assay, interfering blue and green colours, formed from the carbazole reagents reacting with inorganic substances, occurred after fraction 43. Fractions in the low-molecular-mass region before that volume produced the characteristic magenta colour of the uronic acid reaction with the carbazole reagents. (B) and (C) Gel-filtration patterns of the reaction products at pH 1.5 and at pH 4.0 respectively. The volume of each fraction was 2 ml.

unincubated medium and incubated medium. There was considerable loss of the main heparin peak in those samples incubated with HUVECs, as shown by the smaller peak, compared with the control. The NO synthase inhibitor L-NNA completely protected heparin from degradation (Figure 2B), as shown by a peak that had the same area as the control. Mean relative recoveries of fraction-3 heparin from three experiments are presented in Table 1. Both L-NNA and L-NMMA inhibited degradation of heparin, resulting in complete recovery. These results suggest that the primary degradative process of extracellular exogenous heparin by HUVECs may involve NO. In contrast with heparin, hyaluronan was not degraded by incubation with cultured endothelial cells (results not shown). This suggests that neither

Figure 4 Separation of intact from degraded fraction-2 heparin after NO treatment at pH 7.4 using two different buffers: phosphate (A) and imidazole (B)

Samples were treated as described for Figure 3 except that they were not neutralized. Interfering blue colours started at fraction number 39 for these analyses. The volume of each fraction was 2 ml. —, Control, pH 7.4; —, NO gas, pH 7.4.

Figure 5 Further comparison of the low-molecular-mass oligosaccharides obtained from Figure 3 using Bio-Gel P-4 gel filtration

(A) Oligosaccharides from HNO₂ treatment; (B) oligosaccharides from NO gas treatment at pH 1.5. Similar patterns were obtained for all pH conditions at which there was degradation. The volume of each fraction was 2 ml.

superoxide nor peroxynitrite were present in the medium in sufficient quantity to depolymerize hyaluronan.

Chemical treatment of heparin

These experiments were designed to show that NO degradation of heparin was by a reaction similar to the known degradation of heparin by HNO₂, a reaction that occurs under acidic conditions. Reactions were carried out under an argon atmosphere, to limit the formation of higher nitrogen oxidation species.

The gel-filtration pattern of the products after treatment of fraction-2 heparin with 1 M HNO₂ at pH 1.5 by the published method [21] (Figure 3A) was compared with the patterns of the products after treatment with NO gas (> 98.5%) at pH 1.5 (Figure 3B) and at pH 4.0 (Figure 3C). Complete degradation of fraction-2 heparin was demonstrated by the complete loss of the high-molecular-mass peak of the control (on the left) and the

Figure 6 Treatment of heparin with 400 p.p.m. of NO

The Bio-Gel P-100 gel-filtration pattern of control fraction-2 heparin (untreated; ○) was compared with the patterns from 400 p.p.m. NO treatment at pH 1.5 (△) and pH 4.0 (□). The volume of each fraction was 2 ml.

appearance of a new peak at low molecular mass. (The reaction only destroys the amino sugar and not the uronic acid, which is the moiety measured by the carbazole reaction [25].) For heparin treated with NO gas at pH 7.4, the type of buffer made a difference (Figure 4). In phosphate buffer, NO gas caused complete degradation of heparin (Figure 4A), but in imidazole, a nitrogen-containing buffer, degradation did not occur (Figure 4B). These data suggest that total degradation can be achieved by NO under both acidic and neutral conditions. Possibly, nitrogen-containing compounds, such as imidazole, may inhibit this reaction.

Further fractionation of degradation products

The low-molecular-mass breakdown products from Figures 3 and 4 were concentrated and rechromatographed on Bio-Gel P-4. Figure 5 shows the elution pattern of the products from treatment with NO at pH 1.5 (Figure 5B), compared with the pattern from treatment with 1 M HNO₂ (Figure 5A). The Bio-Gel P-4 elution patterns from NO gas using other pH conditions were similar, thus demonstrating that HNO₂ and NO gas yield similar heparin-breakdown products.

Treatment of heparin and heparan sulphate with 400 p.p.m. of NO

To show that this reaction can occur at a physiological concentration of NO, heparin and heparan sulphate were bubbled with 400 p.p.m. of NO (Figure 6 and 7 respectively). At pH 1.5 (△), both heparin and heparan sulphate were substantially degraded. At pH 4.0 only slight degradation of heparin was observed, as shown by the slight widening of the bottom of the peak. Heparan sulphate was not degraded at pH 4.0 under these conditions.

Treatment of heparin with peroxynitrite

We have reported that hyaluronan is susceptible to degradation by peroxynitrite at pH 7.4 [24]. To determine the effect of peroxynitrite on heparin, we treated heparin (5 mg/ml) with 5 mM peroxynitrite at pH 7.4, and found it to be resistant to this reagent (Figure 8).

Treatment of hyaluronan with NO

Figure 9 shows the results of treating hyaluronan with NO gas. Compared with the control (Figure 9A), depolymerization of

Figure 7 Treatment of heparin sulphate with 400 p.p.m. of NO

The Bio-Gel P-100 gel-filtration pattern of control heparan sulphate (untreated; ○) was compared with the patterns at pH 1.5 (△) and pH 4.0 (□). The volume of each fraction was 2 ml.

Figure 8 Treatment of heparin with peroxynitrite

The gel-filtration pattern of control fraction-2 heparin (○) was compared with the pattern resulting from treatment with 5 mM peroxynitrite (□). Interfering green colour was observed after fraction 40 in the peroxynitrite-treated sample. The volume of each fraction was 2 ml.

Figure 9 Treatment of hyaluronan with NO gas

(A) Untreated control; (B) 1 mg of hyaluronan/ml of 0.2 M phosphate buffer, pH 7.4, was pre-equilibrated with argon gas for 1–2 min and then bubbled with NO gas for 1 min. After 10 min at room temperature, the sample was refrigerated overnight, then analysed for depolymerization by electrophoresis [27].

hyaluronan was not achieved with NO gas (Figure 9B), showing that this glycosaminoglycan is not susceptible to degradation by NO. Hyaluronan is also not susceptible to degradation by HNO_2 because of its *N*-acetyl groups [20–22].

DISCUSSION

Heparin interacts with and binds to endothelial cells [28,29] resulting in changes, such as suppression of endothelin-1 production by HUVECs [26,30]. On binding to HUVECs and smooth-muscle cells, heparin is internalized, degraded and re-secreted [31–34]. Metabolism and degradation of heparin and heparan sulphate are lysosomal processes [35,36]. Whether these enzymic processes involve NO is unknown; however, the data in this study support such an involvement.

Cultured HUVECs produce and secrete NO [5–7], causing relaxation of adjacent vascular smooth-muscle cells. One of the degradation products of NO is HNO_2 [10,19]. HNO_2 cleaves heparin and heparan sulphate at *N*-sulphate (pH 1.5) or free amino (pH 4) glucosamine residues by a deaminative mechanism [20–23]. The active species, the nitrosonium cation (NO^+), may also form directly from NO gas in the presence of an appropriate (but unknown) electron acceptor (Scheme 1). Along with cleavage of the glycosidic bond, the amino group is eliminated as N_2 (the sulphate group is also eliminated), yielding an anhydromannose at the new reducing end of each oligosaccharide [20]. These results indicate that NO gas cleaves heparin by a similar mechanism.

HNO_2 originates from the reaction of NO with higher oxidation species of nitrogen such as NO_2 (forming N_2O_3) or from N_2O_4 [10,19]. In experiments with exogenous NO gas incubation argon was used to produce microanaerobic conditions, which may mimic physiological or lower oxygen concentrations. NO_2 and N_2O_3 were undoubtedly present (and producing HNO_2), as they would be *in vivo*; thus this study cannot distinguish between NO (forming NO^+ directly) or HNO_2 as the nitrosating species (Scheme 1). Further studies are necessary to clarify this point. The present work does eliminate peroxynitrite as a degradative reagent for heparin and heparan sulphate (see below).

Scheme 1 Proposed mechanism for depolymerization of heparin or heparan sulphate by NO or HNO_2

In aqueous solutions, NO in the presence of O_2 forms higher nitrogen-oxidation species intermediates (N_2O_3 or NO_2) which convert into HNO_2 [10,19]. We propose that NO and HNO_2 may share a common intermediate, the nitrosonium cation (NO^+), which attacks *N*-sulphated or free amino groups of the glucosamine residues of heparin. The amino group leaves as nitrogen, with elimination of the glycosidic bond, forming the anhydromannose derivative. R' and R'' represent the remainder of the heparin or heparan sulphate molecule, particularly the intact uronic acid moieties.

Scheme 2 Summary of NO degradation of heparin and heparan sulphate

In the presence of O_2 , NO is converted into HNO_2 in water, and, in the presence of superoxide anion ($O_2^{\cdot-}$), it is converted into peroxynitrite. Each of these products has a different glycosaminoglycan specificity: HNO_2 cleaves heparin and heparan sulphate (solid arrow), but does not degrade hyaluronan (dashed arrow). Peroxynitrite degrades hyaluronan (solid arrow), but does not cleave heparin or heparan sulphate (dashed arrow).

The results reported here show that heparin is degraded by exogenous NO, even at neutral pH, and that degradation of heparin by HUVECs is inhibited by L-NNA and L-NMMA. Although L-NNA and L-NMMA scavenge radical species, such as hydroxyl radical, free-radical concentrations in HUVEC medium were too low to depolymerize hyaluronan, which is susceptible to such degradation [24]. Heparin is resistant to degradation by peroxynitrite (Figure 8), possibly because of the highly negatively charged sulphate groups. At neutral pH, degradation was observed when phosphate buffer was used, but not with 0.05 M imidazole buffer; the nitrogen of imidazole may react preferentially with NO or HNO_2 by a concentration effect.

The control experiments with hyaluronan, a glycosaminoglycan that is resistant to HNO_2 but susceptible to peroxynitrite degradation [24], help substantiate the proposed mechanism. Hyaluronan was found to be resistant to degradation by NO gas and by HUVECs, but is susceptible to peroxynitrite degradation [24], showing that heparin degradation by NO is through NO or HNO_2 , rather than through peroxynitrite (Scheme 2). Thus, under normal growth conditions, peroxynitrite production by HUVECs is not high enough to cause hyaluronan degradation. As additional evidence for a HNO_2 pathway, the gel-filtration elution pattern of NO-degradation products matched the pattern of authentic HNO_2 -derived products (Figure 5).

Cellular degradation of heparin and heparan sulphate involves lysosomes, in which there would be an appropriate acidic environment for degradation of these compounds by NO. Inflammatory response cells also produce acidic conditions, which may be suitable for heparan sulphate degradation. This reaction may be sensitive to nitrogen-containing molecules that can attenuate degradation. Despite the presence of molecules capable of inactivating NO *in vivo* (such as iron, proteins and other sources of free amino groups), degradation of heparin by cultured endothelial cells was found to occur in complete growth medium (Figure 2).

The major proteoglycan of endothelial cell extracellular matrix is heparan sulphate [37,38]. Since heparan sulphate is also susceptible to breakdown by HNO_2 , degradation by NO (Figure 7) would be expected. The discovery that NO gas degrades heparin and heparan sulphate (of the extracellular matrix) may explain certain observations. For example, patients receiving nitroglycerine, an NO donor *in vivo*, have been reported to become refractory to heparin [39], suggesting that the released NO degrades heparin. Animals chronically exposed to NO gas were shown to develop pulmonary lesions [40]; this could also be due to degradation of heparan sulphate of the extracellular matrix. This latter observation may have relevance in therapeutic applications of NO for pulmonary hypertension [41].

Treatment of cultured endothelial cells with endotoxin

(resulting in stimulation of NO) significantly alters the apparent biosynthesis of heparan sulphate of the extracellular matrix [42]. Heparan sulphate of the extracellular matrix binds growth factors [43], chemokines [44] and enzymes such as extracellular superoxide dismutase [45]; thus NO, by degrading extracellular matrix heparan sulphate, may control the release of these regulatory factors [43]. Macrophages and neutrophils release large amounts of NO [2] and hypochlorous acid [46] on inflammatory stimulation, which may result in increased degradation of extracellular-matrix glycosaminoglycans. Inflammatory processes may also release excess NO and superoxide anion, forming peroxynitrite, which may degrade hyaluronan [24], but not heparan sulphate. Degradation or altered synthesis of hyaluronan of joint synovial fluid has been linked to rheumatoid arthritis. Thus the balance between NO and superoxide anion determines which glycosaminoglycan component of the extracellular matrix is destroyed and may be important in regulating disease processes [47]. The relative concentrations of NO and superoxide have also been shown to regulate lipid peroxidation [48]. In this study, unstimulated cultured endothelial cells did not degrade hyaluronan, suggesting insufficient superoxide (and therefore peroxynitrite) formation.

Since NO is constitutively produced by endothelial cells, this degradation of heparan sulphate may be part of its normal turnover. Heparan sulphate degradation by NO may be important in processes such as bone development and apoptosis. Thus NO may be involved in the regulation and pathology of extracellular-matrix metabolism.

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