Inhibition by heparin of Fe(II)-catalysed free-radical peroxidation of linolenic acid

Marion A. ROSS, William F. LONG* and Frank B. WILLIAMSON

Polysaccharide Research Group, Department of Molecular and Cell Biology, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

Heparin, in a concentration-dependent manner, inhibited the generation of conjugated dienes and thiobarbituric acid-positive substances when incubated with Fe²⁺ and γ -linolenic acid. In the conjugated diene assay, other glycosaminoglycans, on a molar basis calculated with respect to their respective hydrated disaccharide repeat units, were less effective than heparin. Heparin which had been re-*N*-sulphonated after removal of both *N*-sulphonates and *O*-sulphates, and heparin in which iduronate residues had been reduced to idose residues, were largely unaffected in their activity. Removal of both *N*-sulphonates and *O*-sulphates greatly reduced the effectiveness of the heparin. Analysis of the effects of heparin fragments generated by heparinase I treatment of heparin showed that depolymerization decreased the effectiveness of the heparin. It is possible that heparins and related strongly acidic polysaccharides may function as endogenous antioxidants, and that sequestration by them, or harmless oxidation by them, of ions such as Fe²⁺, contributes to their effectiveness.

INTRODUCTION

The hydroxyl radical (OH[•]), generated by ionizing radiation or otherwise induced homolytic fission of water, or by a Fenton reaction involving H₂O₂ and Fe²⁺ or Cu⁺, is the most reactive of oxygen radical species. The best characterized biological damage caused by these radicals involves an autocatalytic peroxidation of polyunsaturated fatty acid residues of membrane lipids (Gutteridge & Halliwell, 1989; Halliwell & Gutteridge, 1989; Lunec, 1990). In addition to having a putative role in OH[•] generation through Fenton reactions, and therefore perhaps in first-chain initiation of lipid peroxidation, transition-metal-ion complexes (particularly of Fe²⁺ and Cu⁺, and, although they react more slowly, of Fe³⁺ and Cu²⁺) accelerate decomposition of lipid peroxides to alkoxyl and peroxy radicals. Sequestration of such metal cations by proteins that include transferrin, caeruloplasmin and albumin limits their damage potential in the plasma environment.

Heparins are strongly acidic glycosaminoglycan components of mast-cell granules, from which they are released during inflammatory reactions to tissue damage; the structurally related heparan sulphates are components of pericellular matrices. Several observations suggest a possible role for such molecules in protecting tissue from radical damage. Heparin alleviated the effects *in vivo* of radiations known to lead to free-radical production (Chaubel & Godbole, 1983), and heparin enhanced the activity *in vivo* of a superoxide dismutase (Flick *et al.*, 1983). Intravenous injection of heparin in man and some other mammals led to a prompt release of extracellular superoxide dismutase subtype C into the plasma (Karlsson & Marklund, 1987, 1988a). This may be the reason why heparin administration was effective in suppressing ischaemic paw oedema in mice (Oyanagui & Sato, 1990). Heparan sulphate proteoglycans of the vascular endothelium appear to be principal binding substances for the enzyme (Adachi & Marklund, 1989; Karlsson & Marklund, 1988b, 1989).

In addition to a role in releasing endothelium-bound superoxide dismutase, it is possible that heparins and heparan sulphates

have a more direct antioxidant function. It has been suggested that such glycosaminoglycans possess structural features which might allow them to act as free-radical sinks (Grant *et al.*, 1987a, 1990). Certainly, a relatively stable heparin radical species, generated by radiation, has been reported (Khan, 1982). Further, heparin inhibited luminol- or lucigenin-dependent chemiluminescence by neutrophils stimulated by serum-activated zymosan (Brestel & McClain, 1983; Muller-Peddinghaus & Wurl, 1987) and the 'quasi-lipoxygenase' activity of O₂ and methaemoglobin on linoleic acid (Brestel & Koes, 1989). Here, the heparin appeared to scavenge either superoxide or another potent oxidant. Cultured endothelial cells were protected by heparin from damage by oxygen radicals generated by a xanthine/xanthine oxidase system (Hiebert & Liu, 1990). A heparin-related sulphated glycosaminoglycan inhibited superoxide generation by human peripheral neutrophils (Tsuboi *et al.*, 1988), algal sulphated galactans having a chemical resemblance to heparins inhibited Cu²⁺-catalysed oxidation of linoleic acid (Allen *et al.*, 1982), saccharide-containing mucins of the respiratory and gastrointestinal tracts may scavenge oxygen radicals (Cross *et al.*, 1984; Grisham *et al.*, 1987), and heparin itself may have some superoxide dismutase activity *in vitro* (Grant *et al.*, 1988).

Heparins and heparan sulphates possess a variety of anionic groups which allow the molecules to interact with a range of biologically relevant micro- and macro-cations. Such interactions are complex and not explicable in terms of simple electrostatic theory. It is probable that they lead to changes in the biological potentials of both partners in the interaction. Copper (Grant *et al.*, 1992b) and iron (Garcia-Segura, 1977; Kojima *et al.*, 1983; Grant *et al.*, 1992a) cations are among those cations that bind strongly to heparins and similar glycosaminoglycans. E.s.r. spectroscopy shows that the iron cation binds directly to the nitrogen atom of the heparin glucosamine residues (D. Grant, W. F. Long, F. B. Williamson & M. F. Williamson, unpublished work). Molecules such as heparins might therefore influence the activity of radicals by modulating the availability or reactivity of such ions. To examine this possibility, the influence of heparin on Fe²⁺-dependent peroxidation of γ -linolenic acid was examined.

Abbreviation used: OH[•], hydroxyl radical.

* To whom correspondence should be addressed.

Similar simple 'in vitro' model systems have been used elsewhere (e.g. Aust, 1985; Knight & Voorhees, 1990).

EXPERIMENTAL

The source, preparation and properties of the heparin, the chemically modified heparins, and the heparin fragments generated by heparinase I (EC 4.2.2.7) treatment have been described previously (Grant *et al.*, 1987b, 1989, 1992b). γ -Linolenic acid (octadeca-6,9,12-trienoic acid), polyoxyethylene ether ω -1 [α -dodecyl- ω -hydroxypoly(oxyethane-1,2-diyl)], chondroitin 4-sulphate (from whale cartilage; lot no. 33F 0499), chondroitin 6-sulphate (from shark cartilage; lot no. 102F 0418), dermatan sulphate (from pig skin; lot no. 47F 0500), heparan sulphate (from bovine kidney; lot no. 60H 0932) hyaluronic acid (from human umbilical cord; lot no. 71F 0093) and α -tocopherol were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Degrees of hydration of the average dimers of heparin, chemically modified heparins, heparin fragments and other glycosaminoglycans used in the calculation of the M_r of the hydrated average disaccharide repeat unit, were taken from Atkins *et al.* (1974), Moffat (1987) and Grant *et al.* (1992a). Ferrous ammonium sulphate (Specpure grade) was from Johnson Matthey, Royston, Herts., U.K. Other materials were Analar grade reagents from BDH, Poole, Dorset, U.K.

Assay of an early stage in γ -linolenic acid peroxidation, the formation of u.v.-absorbing conjugated dienes, was essentially by the method of Knight & Voorhees (1990). Na^+ forms of the glycosaminoglycans (in water) and polyoxyethylene ether ω -1 (in water; 1%, w/v) were mixed at 37 °C in a spectrophotometer cell and allowed to equilibrate for 10 min before addition of γ -linolenic acid (in 95%, v/v, ethanol; 0.3 mM) and incubated for a further 5 min. Ferrous ammonium sulphate (in de-oxygenated water; 2.5×10^{-5} M) was then added, and the absorbance measured at 233 nm. The water used to make up reagents was de-ionized and distilled, and the reagents had the noted concentrations in a final reaction mix of 3 ml. The concentrations of heparin, of chemically modified heparins, of heparin fragments and of other glycosaminoglycans used are given in the Tables.

Assay of thiobarbituric acid-reactive substances (nominally malonaldehyde present in the sample assayed, together with that formed by peroxide degradation during the acid heating stage of the assay process) was essentially by the method of Gutteridge (1985). Heparin (in water) was incubated at 37 °C for 10 min before addition of γ -linolenic acid (0.3 mM) in polyoxyethylene ether ω -1 (in water; 1%, w/v). After a further 5 min incubation, ferrous ammonium sulphate (in de-oxygenated water; 2.5×10^{-5} M) was added. Reagents were made up in deionized distilled water and had the noted concentrations in a reaction mix of 0.5 ml. Final concentrations of the heparin used are given in Table 2 (below). After incubation for a further 10 min, thiobarbituric acid (0.5 ml of a 1%, w/v, solution in 0.5 M-NaOH) was then added, followed by 0.1 ml of 10 M-HCl. Final mixtures were heated at 100 °C for 10 min and then cooled. The absorbance of the coloured complex was measured at 532 nm.

For both assays, solutions of γ -linolenic acid were made up immediately before use, and assays reported in individual experiments were performed together. Molar absorption coefficients used to calculate the amounts of conjugated dienes and thiobarbituric acid-reactive substances produced in the assays were 2.52×10^4 and $1.56 \times 10^5 \text{ M} \cdot \text{cm}^{-1}$ respectively (Buege & Aust, 1978). Scarcity of glycosaminoglycan preparations precluded the use of multiple replicates in experiments, but the inhibitory effect of heparin (1 mM) in the conjugated diene assay reported in Table 1 (below) (an inhibition to 36% of the control value) represented the mean of 13 replicates, with an S.D. of $\pm 8\%$.

RESULTS

Results in Table 1 are derived from the increase in u.v. absorption of the reaction mix after addition of Fe^{2+} to γ -linolenic acid in polyoxyethylene ether, either alone or in the presence of various concentrations of heparin. In control experiments, the following combinations of reagents were incubated in the absence of Fe^{2+} : heparin (1 mM), polyoxyethylene ether and γ -linolenic acid; heparin and polyoxyethylene ether; heparin and γ -linolenic acid; polyoxyethylene ether and γ -linolenic acid. In no case was a change in absorption observed. Further, addition of EDTA to the reaction mix before Fe^{2+} addition inhibited the subsequent increase in absorption in an [EDTA]-dependent manner. At a final [EDTA] in the reaction mix of 1, 5, 10 and 20 μM , increases in absorption after Fe^{2+} addition (as a percentage of that increase seen in the absence of EDTA) were 99, 53, 12 and 0 respectively. This suggests that the presence of sufficient EDTA to produce an [EDTA]/ $[\text{Fe}^{2+}]$ ratio of 1 prevented the subsequent increase in absorption. Addition of Fe^{2+} to polyoxyethylene ether in the absence of γ -linolenic acid resulted in an immediate small increase in A_{233} ($\Delta A_{233} = 0.03$). This increase may represent binding of Fe^{2+} to polyoxyethylene ether; such binding might affect the reactivity of the metal ion in Fenton and other reactions (e.g. Burkitt & Gilbert, 1990; Smith *et al.*, 1990). The

Table 1. Effect of heparin on conjugate-diene production and on generation of thiobarbituric acid-reactive substances

[Heparin] values are final concentrations (in terms of hydrated disaccharide repeat unit) of heparin present in the reaction mix. Components of the reaction mixes in which conjugated dienes and thiobarbituric acid-reactive substances were measured are given in the Experimental section.

[Heparin]	Production of:			
	Conjugated dienes		Thiobarbituric acid-reactive substances	
	(nmol)	(% of control)	(nmol)	(% of control)
0	12.3	100	11.2	100
0.01	11.7	95	10.8	96
0.05	8.8	72	9.1	81
0.10	8.2	67	7.8	70
0.25	5.9	48	6.1	54
0.50	5.4	44	5.3	47
1.00	4.4	36	4.6	41

Table 2. Effect of glycosaminoglycans on conjugate-diene production

Final concentration of glycosaminoglycans (in terms of hydrated disaccharide repeat unit) in the reaction mix was 1 mM.

Glycosaminoglycan	Conjugated-diene produced	
	(nmol)	(% of control)
None	12.4	100
Hyaluronate	11.9	96
Chondroitin 6-sulphate	10.5	85
Dermatan sulphate	8.3	67
Chondroitin 4-sulphate	8.1	65
Heparan sulphate	7.1	57
Heparin	6.1	49

Table 3. Effect of chemically modified heparins on conjugate-diene production

Final concentration of modified heparins (in terms of hydrated disaccharide repeat unit) in the reaction mix was 1 mM.

Modification of heparin	Conjugated-diene produced	
	(nmol)	(% of control)
No heparin control	17.0	100
De- <i>N</i> -sulphonation, de- <i>O</i> -sulphation	15.6	92
De- <i>N</i> -sulphonation, de- <i>O</i> -sulphation, <i>N</i> -acetylation	13.6	80
De- <i>N</i> -sulphonation	9.3	55
Carboxyl reduction	8.7	51
De- <i>N</i> -sulphonation, de- <i>O</i> -sulphation, re- <i>N</i> -sulphonation	8.3	49
De- <i>N</i> -sulphonation, <i>N</i> -acetylation	6.2	36
Unmodified heparin	7.9	46

Table 4. Effect of heparin fragments on conjugate-diene production

Final concentration of heparin fragments (in terms of hydrated disaccharide repeat unit) in the reaction mix was 0.1 mM. Pools 4, 5 and 6 refer to fractions containing octa-, tetra- and di-saccharides respectively, pool 3 to a fraction containing non-sulphated *N*-acetylated fragments, and pools 2 and 1 to fractions containing fragments of progressively higher degrees of polymerization.

Pool of heparin	Conjugated-diene produced	
	(nmol)	(% of control)
No heparin control	12.0	100
Unfractionated heparin	7.3	61
Pool 1	5.1	43
Pool 2	5.8	48
Pool 3	6.0	50
Pool 4	6.6	55
Pool 5	9.9	83
Pool 6	10.7	89

results reported represent changes in A_{233} from which this small increase has been deducted.

Heparin inhibited the increase in absorption that was seen in the presence of Fe^{2+} , γ -linolenic acid and polyoxyethylene ether, and which is presumed to represent conjugated diene production, in a concentration-dependent manner (Table 1). When α -tocopherol (0.3 mM) was included in the reaction mix in place of heparin, the increase in absorption was decreased to 65% of that occurring in the absence of α -tocopherol.

Table 1 also shows that heparin, in a concentration-dependent manner, inhibited the generation of thiobarbituric acid-reactive substances in a reaction mix containing γ -linolenic acid and Fe^{2+} . Because several carbohydrates react in or interfere with this assay system, control experiments were performed, in which heparin (1 mM) was incubated with Fe^{2+} , with Fe^{2+} and polyoxyethylene ether, with Fe^{2+} and γ -linolenic acid, with polyoxyethylene ether, with polyoxyethylene ether and γ -linolenic acid, or with γ -linolenic acid before addition of thiobarbituric acid. In no case was a change in A_{532} observed.

Table 2 shows that other glycosaminoglycans were, on a molar basis estimated from the M_r of the hydrated average disaccharide repeat unit for each compound, less effective than heparin at inhibiting the increase in absorption presumed to represent conjugated diene production.

Table 3 indicates the relative inhibitory effectiveness, again on a molar basis, of a number of chemically modified heparins in the conjugated diene assay. Heparin which had been re-*N*-sulphonated after removal of *N*-sulphonates and *O*-sulphates, and heparin in which iduronate residues had been reduced to idose residues, were largely unaffected in their activity. Removal of both *N*-sulphonates and *O*-sulphates greatly decreased the effectiveness of the heparin. Replacement of *N*-sulphonates by *N*-acetyl groups slightly potentiated the action of the heparin.

Table 4 shows that depolymerization of the heparin reduced its effectiveness in the conjugated diene assay; again, in each case, concentrations of the inhibitor were expressed on a molar basis calculated from the M_r of the hydrated disaccharide unit.

DISCUSSION

Several low-molecular-mass molecules in extracellular fluids act as radical scavengers, but it is likely that at least as important an aspect of extracellular antioxidant activity depends on mechanisms that prevent metal cations from participating in radical reactions. In particular, the acute-phase plasma α_2 -glycoprotein caeruloplasmin ('ferroxidase-I', EC 1.16.3.1) catalyses the oxidation of Fe^{2+} to Fe^{3+} , together with the coupled reduction of molecular oxygen to water, a process which, unlike autoxidation of Fe^{2+} , does not result in the production of reactive oxygen intermediates. Fe^{3+} may then bind to plasma transferrin (Gutteridge, 1985, 1990; Gutteridge & Halliwell, 1989). In addition, caeruloplasmin reacts stoichiometrically with superoxide and $\text{OH}^\cdot$, and its ability also to interact non-specifically with bivalent transition-metal ions may limit further their ability to stimulate $\text{OH}^\cdot$ formation and lipid peroxidation.

Despite considerable experimental work and debate (summarized by Nieduszynski, 1989) on the mechanisms by which certain other metal cations interact with heparins, and discussion of the biological roles of these interactions, little is known about the processes of iron-cation-heparin interaction. A simple histochemical study indicated that iron bound to heparin-related glycosaminoglycans (Garcia-Segura, 1977); Fe^{2+} and Fe^{3+} were able to displace $^{45}\text{Ca}^{2+}$ from heparan sulphate (Kojima *et al.*, 1983); a pharmaceutical-grade heparin contained 1100 p.p.m. of bound iron (and many other transition metals) (Grant *et al.*, 1987b) and changes in the optical rotation of heparin in the presence of Fe^{2+} were consistent with an association constant of about 555 M^{-1} for Fe^{2+} -heparin interaction under the conditions studied (Grant *et al.*, 1992a). Binding of iron ions to the broadly related anionic polymers alginate (Seno & Yamabe, 1960) and chondroitin sulphate (Yang *et al.*, 1986) has also been noted.

The results reported here suggest that heparins and structurally related strongly anionic polysaccharides may represent part of the array of antioxidant mechanisms present in organisms. That heparin-like compounds may be effective *in vivo* is suggested by the preliminary observations that exogenous heparin inhibited lactate dehydrogenase leakage from rat primary hepatocytes treated with carbon tetrachloride, and from human secondary endothelial cells treated with hydrogen peroxide (G. Mackintosh, W. F. Long & F. B. Williamson, unpublished work). It is possible that the binding and sequestration of ions such as Fe^{3+} contributes to the effectiveness of heparin. It is not yet clear, however, that heparin-bound Fe^{2+} is unable to participate in reactions involved in lipid peroxidation, and an additional explanation for the inhibitory action of heparin is that, in its presence, Fe^{2+}

oxidation occurs. In this respect, heparin might exhibit a ferroxidase activity. Solution-phase alkaline oxidation of Fe^{2+} occurs by a mechanism that generates superoxide anion:

whereas the locally low pH around a polyanion like heparin would enable an acidic oxidation that does not involve free-radical production:

in a reaction similar to that used in proton acceptance by *Thiobacillus ferrooxidans*. The potential use of exogenous anti-oxidants based on compounds such as heparins deserves consideration.

We thank Dr. C. F. Moffat for producing the chemically modified heparins and heparin fragments, and the Scottish Home and Health Department for a grant enabling this work.

REFERENCES

- Adachi, T. & Marklund, S. L. (1989) *J. Biol. Chem.* **264**, 8537–8541
- Allen, J. C., Gardner, R. S., Parry, D. E. & Wedlock, D. J. (1982) *Prog. Ed. Nutr. Sci.* **6**, 191–198
- Atkins, E. D. T., Isaac, D. H., Nieduszynski, I. A., Phelps, C. F. & Sheehan, J. K. (1974) *Polymer* **15**, 263–271
- Aust, S. D. (1985) in *Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.), pp. 203–207, CRC Press, Boca Raton, FL
- Brestel, E. P. & Koes, M. T. (1989) *Prostaglandins Leukotrienes Essential Fatty Acids* **36**, 21–24
- Brestel, E. P. & McClain, E. J. (1983) *J. Immunol.* **131**, 2515–2519
- Buege, J. A. & Aust, S. D. (1978) *Methods Enzymol.* **52**, 302–310
- Burkitt, M. J. & Gilbert, B. C. (1990) *Free Radical Res. Commun.* **10**, 265–280
- Chaubel, K. A. & Godbole, C. S. (1983) *Radiat. Environ. Biophys.* **22**, 281–286
- Cross, C. E., Halliwell, B. & Allen, A. (1984) *Lancet* **i**, 1328–1329
- Flick, M. R., Hoeffel, J. M. & Staub, N. C. (1983) *J. Appl. Physiol. Resp. Environ. Exercise Physiol.* **55**, 1284–1291
- Garcia-Segura, L. M. (1977) *Acta Histochem.* **59**, 79–84
- Grant, D., Long, W. F. & Williamson, F. B. (1987a) *Med. Hypoth.* **23**, 67–71
- Grant, D., Long, W. F. & Williamson, F. B. (1987b) *Biochem. J.* **244**, 143–149
- Grant, D., Long, W. F. & Williamson, F. B. (1988) *Biochem. Soc. Trans.* **16**, 1030–1031
- Grant, D., Long, W. F., Moffat, C. F. & Williamson, F. B. (1989) *Biochem. J.* **261**, 1035–1038
- Grant, D., Long, W. F., Ross, M. A. & Williamson, F. B. (1990) *Proc. Harden Conf. Biochem. Soc.* **34th**, 52
- Grant, D., Long, W. F., Moffat, C. F. & Williamson, F. B. (1992a) *Biochem. J.* **282**, 601–604
- Grant, D., Long, W. F., Moffat, C. F. & Williamson, F. B. (1992b) *Biochem. J.* **283**, 243–246
- Grisham, M. B., von Ritter, C., Smith, B. F., Lamont, J. T. & Granger, D. N. (1987) *Am. J. Physiol.* **253**, G93–G96
- Gutteridge, J. M. C. (1985) in *Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.), pp. 303–307, CRC Press, Boca Raton, FL
- Gutteridge, J. M. C. (1990) in *Free Radicals, Lipoproteins and Membrane Lipids* (Crastes de Paulet, A., Douste-Blazy, L. & Paoletti, R., eds.), pp. 371–379, Plenum Press, New York
- Gutteridge, J. M. C. & Halliwell, B. (1989) *Bailliere's Clin. Haematol.* **2**, 195–256
- Halliwell, B. & Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, 2nd edn., Clarendon Press, Oxford
- Hiebert, L. M. & Liu, J. (1990) *Atherosclerosis* **83**, 47–51
- Karlsson, K. & Marklund, S. L. (1987) *Biochem. J.* **242**, 55–59
- Karlsson, K. & Marklund, S. L. (1988a) *Biochem. J.* **255**, 223–228
- Karlsson, K. & Marklund, S. L. (1988b) *Basic Life Sci.* **49**, 647–650
- Karlsson, K. & Marklund, S. L. (1989) *Lab. Invest.* **60**, 659–666
- Khan, K. A. (1982) *Proc. IUPAC Symp. Macromolecules* **28th**, 348
- Knight, J. A. & Voorhees, R. P. (1990) *Ann. Clin. Lab. Sci.* **20**, 347–352
- Kojima, S., Hama, Y., Sasaki, T. & Kubodera, A. (1983) *J. Nuclear Med.* **8**, 52–59
- Lunec, J. (1990) *Ann. Clin. Biochem.* **27**, 173–182
- Moffat, C. F. (1987) Ph.D. Thesis, University of Aberdeen
- Muller-Peddinghaus, R. & Wurl, M. (1987) *Biochem. Pharmacol.* **36**, 1125–1132
- Nieduszynski, I. (1989) in *Heparin. Chemical and Biological Properties, Clinical Applications* (Lane, D. A. & Lindahl, U., eds.), pp. 51–63, Edward Arnold, London, Melbourne and Auckland
- Oyanagui, Y. & Sato, S. (1990) *Free Radical Res. Commun.* **9**, 87–99
- Seno, M. & Yamabe, T. (1960) *Bull. Chem. Soc. Jpn.* **33**, 590–596
- Smith, J. B., Cusumano, J. C. & Babbs, C. F. (1990) *Free Radical Res. Commun.* **8**, 101–106
- Tsuboi, I., Matsuura, T., Shichijo, S. & Yokoyama, M. M. (1988) *Jpn. J. Inflammation* **8**, 131–135
- Yang, C., Bryan, A. M., Theil, E. C., Sayers, D. E. & Bowen, L. H. (1986) *J. Inorg. Biochem.* **28**, 393–405

Received 25 September 1991/24 February 1992; accepted 17 March 1992