The antioxidant activity of heparins.

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The production of species of oxygen free radicals is a normal consequence of aerobic metabolism, and is also associated with activated macrophages and polymorphonuclear leucocytes. The presence of abnormally high concentrations of such radicals is associated with chronic inflammatory conditions, particularly neoplasia, where radical-generating cells are continuously activated. Sites of chronic inflammation can be environments of low pH probably resulting from lactic acid metabolism. This decrease in pH causes the release of potentially dangerous metal ions, in particular Fe(II) and Cu(I), from proteins such as caeruloplasmin and transferrin [1]. These transition metal ions are implicated in areas of biological damage due to their involvement in Fenton reaction with H₂O₂, leading to the generation of reactive hydroxyl radicals [2] and autocatalytic peroxidation of polyunsaturated fatty acid residues of membrane lipids [3]. 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 free radical damage [4]. In reaction conditions relevant to cells, heparin enhances the activity in vivo of superoxide dismutase and, in addition to a role in releasing endothelium-bound superoxide dismutase, heparin itself may have some superoxide dismutase activity in vitro [5]. Cultured endothelial cells were protected by heparin from damage by oxygen radicals generated by a xanthine/xanthine oxidase system [6]. It has been suggested that such glycosaminoglycans possess structural features which might allow them to act as free-radical sinks [7]. Heparin and heparan sulphates possess a variety of anionic groups which allow the molecules to interact with a range of biologically relevant micro- and macromolecules. Such interactions are complex and not explicable in terms of simple electrostatic theory. It is probable that they lead to changes in the structure of both partners in the interaction. Free iron and iron cations are among those that bind strongly to heparins. Despite considerable experimental work and debate on the mechanisms by which certain other metal cations interact with heparins and the biological roles of these interactions, little is known about the processes of iron-cation-heparin interaction.

In this transaction, we demonstrate the antioxidant activity of heparin over a range of pH values and compare this activity to that of the acute-phase protein caeruloplasmin, by measurement of the formation of u.v.-absorbing conjugated dienes, an early stage of lipid peroxidation. Heparin (octadeca-6,9,12-trienoic acid), polyoxyethylene ether (ω-1-[y-dodecyl-o-hydroxypoly(oxyethylene)-1,2-diyl]), heparin (porcine intestinal mucosa; lot no. 29F 0314) and caeruloplasmin (lot no. 19F 9457) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Degrees of hydration of the average dimer of heparin used in the calculation of the molecular mass of the hydrated average disaccharide repeat unit was discussed in an earlier communication [8]. 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.

The method was essentially that of Knight and Voorhees [9]. The antioxidant (in water; heparin at 1 mmol dm⁻³; caeruloplasmin at 0.26 μmol dm⁻³) and polyoxyethylene ether (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 mmol dm⁻³) and incubated for a further 5 min. Ferrous ammonium sulphate (in de-oxygenated water; 25 μmol dm⁻³) was then added, and the change in absorbance measured at 233 nm. A control was included which involved replacing the antioxidant with water. De-ionised and distilled water was used to make up the reagents, which had the notated concentrations in a final reaction volume of 3 cm³. The pH was varied using dilute hydrochloric acid and dilute sodium hydroxide.

Results in Table 1 show the increase in u.v. absorption of the reaction mix after addition of Fe(II) for oxyradical acid in polyoxyethylene ether, in the presence of either heparin or caeruloplasmin, at a range of pH values from 3.5 to 7.4. At all pH values studied, heparin inhibited the increase in absorption that was seen in the presence of Fe(II), γ-linolenic acid and polyoxyethylene ether, and which is presumed to represent conjugate diene production. In contrast, caeruloplasmin, although very effective in inhibiting the increase in absorption at physiological pH, was much less so at low pH.

Several low-molecular-mass molecules in extracellular fluids act as free scavengers, but it is likely that at least as important an aspect of extracellular antioxidant activity depends on mechanisms that prevent redox-active transition metal cations from participating in radical reactions. In particular, the acute-phase plasma α2-glycoprotein caeruloplasmin ('ferroxidase-1', EC 1.16.3.1) catalyses the oxidation of Fe(II) to Fe(III), together with the coupled reduction of molecular oxygen to water, a process which, unlike the autoxidation of Fe(III), does not result in the production of reactive oxygen intermediates. Fe(III) may then bind to plasma transferrin at physiological pH. In addition, caeruloplasmin reacts stoichiometrically with superoxide and hydroxy radicals, and its ability to interact non-specifically with bivalent transition-metal ions may limit further their ability to stimulate hydroxyl radical formation and lipid peroxidation. Caeruloplasmin may, however, be susceptible to free radical damage. Chronic inflammation can be associated with lower than normal pH. The results presented here indicate that caeruloplasmin may become inactive under these conditions. Heparin, however, remains active even at low pH, suggesting that heparin may be a particularly effective antioxidant in situations of chronic inflammation, that is, at low pH and in the presence of protein-damaging oxygen free radicals. Also, at low pH, Fe(III), the most stable oxidation state of iron at low pH, will precipitate and nucleate the formation of iron (III) hydroxide, which may continue to activate macrophages. Heparin is an effective anti-crystallization agent [10].

It is likely that heparins exert much of their antioxidant activity by binding and altering the redox chemistry of ions such as Fe(II). Potentiometric titration and polarimetry have provided an insight into interactions that occurs between heparin and Fe(II) [11]. In conclusion, heparins possess newly discovered antioxidant activities which may be of critical importance under biological conditions of reduced pH.

Table 1. Antioxidant activity of heparin and caeruloplasmin

<table>
<thead>
<tr>
<th>pH</th>
<th>Lipid peroxidation as % of control</th>
<th>Heparin</th>
<th>Caeruloplasmin</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>30.9</td>
<td>269.9</td>
<td></td>
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<tr>
<td>4.5</td>
<td>43.9</td>
<td>119.1</td>
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<td>5.5</td>
<td>41.3</td>
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<tr>
<td>7.3</td>
<td>31.5</td>
<td>85.3</td>
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</table>

Fe(II)-induced conjugate diene formation was measured at 233 nm and the protective ability of heparin (1 mmol dm⁻³) and caeruloplasmin (0.26 μmol dm⁻³) was studied. Results shown are expressed as a percentage of the control values at 10 min (AA₂₃₃ of 0.131, 0.121, 0.115 and 0.140 at pH 3.5, 4.5, 5.5 and 7.3, respectively).

6 Hiebert, L.M. & Liu, J. (1990) Atherosclerosis 83, 47-51