Selective O-Desulfation Produces Nonanticoagulant Heparin that Retains Pharmacological Activity in the Lung

ALLISON FRYER, YUH-CHIN HUANG, GOPNA RAO, DAVID JACOBY, EDWARD MANCILLA, RICHARD WHORTON, CLAUDE A. PIANTADOSI, THOMAS KENNEDY and JOHN HOIDAL

Department of Internal Medicine, Carolinas Medical Center, Charlotte, North Carolina (T.K.); Departments of Medicine (Y.-C.H., C.A.P.) and Pharmacology (R.W.), Duke University, Durham, North Carolina; Departments of Environmental Health Sciences (A.F.) and Medicine (D.J.), Johns Hopkins University, Baltimore, Maryland; Scientific Protein Laboratories, Wanaukee, Wisconsin (E.M.) and the Department of Medicine, University of Utah, Salt Lake City, Utah (J.H.).

Accepted for publication March 13, 1997

ABSTRACT

Heparin has potential use as an antiinflammatory treatment in many lung diseases but its therapeutic use is limited by inherent anticoagulant activity. The anticoagulant nature of heparin can be eliminated by a number of chemical treatments, but often not without loss of other important pharmacological activities. Lyophilization of porcine mucosal heparin under extreme alkaline conditions (pH ≥ 13) produces a nonanticoagulant heparin remarkable for the selective loss of only 2-O and 3-O sulfates, leaving 6-O and N-sulfates intact. In contrast to the commonly used nonanticoagulant analog N-desulfated, N-reacetylated heparin, selectively O-desulfated heparin retains potent activity as an inhibitor of the cationic neutrophil proteases human leukocyte elastase and cathepsin G, both in vitro and in vivo. Selectively O-desulfated heparin also inhibits complement lysis of erythrocytes, prevents ischemia-reperfusion injury of the lung, remains a potent antiproliferative treatment for cultured airway smooth muscle and normalizes altered neuronal M2 muscarinic receptor sensitivity and bronchial hyperreactivity after antigen challenge. These retained pharmacologic properties suggest possible use of this new nonanticoagulant heparin for the treatment of a variety of lung disorders.

The drug heparin presents a dazzling array of properties. In lung diseases it is used as an anticoagulant for thromboembolism, but its polyanionic nature confers a wide variety of other actions not related to anticoagulation (Jaques, 1980). Heparin is a potent antiinflammatory agent that inhibits neutrophil-derived elastase (Rao et al., 1990), complement activation (Weiler et al., 1992), platelet activating factor- and tumor necrosis factor-induced lung edema (Hocking et al., 1991, 1992), L- and P-selectins (Skinner et al., 1991), leukocyte rolling (Ley et al., 1991) and neutrophil-induced injury of pulmonary alveolar epithelium (Simon et al., 1986). Heparin can neutralize eosinophil-derived cationic airway toxins (Coyle et al., 1995; Fryer and Jacoby, 1992; Jacoby et al., 1993), reduce lymphocyte-mediated immune reactions (Lider et al., 1990) and prevent antigen-induced bronchospasm (Diamant et al., 1996). Heparin treatment blocks development and speeds resolution of hypoxic pulmonary vascular remodeling, the most common cause of right heart failure (Thompson et al., 1994), and can reduce proliferation of cultured airway smooth muscle (Kilfeather et al., 1995). More recently, heparin has even been shown to prevent injury from ischemia-reperfusion (Friedrichs et al., 1994; Black et al., 1995). With these actions, heparin might pose an ideal possible treatment for lung conditions ranging from the excess of HLE in cystic fibrosis airways (McElvaney et al., 1992) to asthma (Diamant et al., 1996; Ahmed et al., 1993) or even adult respiratory distress syndrome. However, the risk of bleeding poses an obstacle to the use of unmodified fully anticoagulant heparin for nonthrombotic indications.

Attachment of heparin to antithrombin III (Bjork et al., 1989) and some other proteins (Maccarana et al., 1993) is critically dependent on binding energies conferred by specific saccharide sequences or charged side groups, and anticoag-

ABBREVIATIONS: succinia[alanyl]2-valine-p-nitroanilide; succinia[alanyl]2-pro-phe-pNA, succinia[alanyl]2-proplyl-phenylalanine-p-nitroanilide; HLE, human leukocyte elastase; ODS heparin, heparin partially O-desulfated by alkaline lyophilization; APTT, activated partial thromboplastin time; USP, United States Pharmacopeia; Ppa, pulmonary artery pressure; Pp, tracheal pressure; W, lung weight gain from edema formation; I:E ratio, inhibitor to enzyme ratio; DOx, dextran sulfate; MAP-kinase, mitogen-activated kinase; AP-1, activator protein-1; FBS, fetal bovine serum; PBS, phosphate-buffered saline.
ultrant activity can be removed from heparin by partial chemical desulfation (Levy and Petracek, 1962; Inoue and Nagasawa, 1976). However, removal of sulfates may have variable effects on other heparin-related activities, which appear related to simple charge neutralization of cationic proteins by the anionic polysaccharide (Rao et al., 1990; Hocking et al., 1991, 1992; Simon et al., 1986; Coyle et al., 1995; Fryer and Jacoby, 1992; Jacoby et al., 1993). For these effects, sulfates are required and desulfation reduces activity (Rao et al., 1990; Weiler et al., 1992; Hocking et al., 1991, 1992; Simon et al., 1986; Diamant et al., 1996). We have tested the proposition that a portion of sulfates can be removed as long as a critical number remain to insure sufficient randomly placed electronegative charge for binding to cationic targets. We report that selective removal of some O-sulfates from heparin by lyophilization under alkaline conditions eliminates almost all anticoagulant action but preserves inhibitory activity against neutrophil proteases, complement activation, ischemia-reperfusion injury and proliferation of airway smooth muscle. Selective O-desulfated heparin, as with its parent, high performance size exclusion chromatography in conjunction with multichannel laser light scattering, using a miniDAWN detector (Wyatt Technology Corporation, Santa Barbara, CA) operating at 690 nm. Disaccharide analysis was performed by the method of Guo and Conrad (1988). In this process N-acetyl-d-glucosamine residues are deacetylated with hydrazine. The heparin is then deaminated and depolymerized by exposure to nitrous acid at pH 4 to break bonds between N-glucosamine and uronic acids, and then at pH 1.5 to break bonds between d-glucosamine N-sulfate and uronic acids. Both reactions leave O-sulfates intact, and convert glucosamine or glucosamine N-sulfate to anhydromannose, which is radiolabeled with NaB[3H4], converting anhydromannose to anhydromannitol. Radiolabeled disaccharides are then separated by reverse-phase, ion-pairing high pressure liquid chromatography.

The in vitro anticoagulant activity of heparins was studied in the APTT (Miletich, 1985), in the U.S.P. anticoagulant assay (United States Pharmacopeial Convention, 1995), and in antiXa clotting (Jesty and Nemerson, 1976) and amidoletic (United States Pharmacopeial Convention, 1995; Teien and Lie, 1977) assays.

**Effect of Heparins and Dextrans Sulfate on Neutrophil Protease Activity**

In vitro studies. The inhibitory activity of heparins or dextran sulfate against HLE and cathepsin G was monitored using the specific synthetic chromogenic substrates suc-al2-val-pNA and suc-al2-pro-phe-pNA, respectively. The method used was that described by Barrett (1981) with some modifications. The assay mixture consisted of 1 ml containing 0.3 mM substrate (100 μM, 3 mM in DMSO) in 50 mM HEPES buffer, pH 7.5. The reaction was started by addition of 100 μM HLE or cathepsin G (20 μg/ml). Activity against the substrate was determined by release of 4-nitroaniline as indicated by an increase in optical density at 405 nm over 3 min. Inhibition was assessed by preincubation of HLE or cathepsin G with various molar ratios of heparin, alkaline lyophilized heparin or dextran sulfate (17, 12, 7 and 4% sulfation by weight) for 30 min at 37°C before initiating the reaction. The substrate-dependent Ki(Ki app) was measured according to the method of Nicklin and Barrett (1984). The substrate-dependent Kapp was calculated from the slope (1/Kapp) by plotting (vi/vj) - 1 against the inhibitor concentration [I] using the relationship vi/vj = 1 + [I]Kapp where vi and vj are rates of reaction at steady state in the absence and presence of inhibitor, respectively. The true Ki was deduced by using the relationship K* = Kapp/1 + [S]/Km. The activity of HLE was also assessed with insoluble elastin as the substrate. Bovine ligament elastin was prepared by the method of Starcher and Galione (1976).
and assessed for purity by amino acid analysis. Its degradation was assayed using elastin radiolabeled with NaB[3H] after the methods described by Stone et al. (1982). The tritiated powdered elastin was homogenized and washed in PBS, pH 7.4. The reaction mixture containing the reference enzyme or sample preincubated with inhibitor was added to a 5-mg aliquot of [3H]elastin and incubated at 37°C, pH 7.4. Solubilized peptides were separated from the elastin suspension by filtration through medium-porosity filter paper. The rate of degradation was determined by quantifying the solubilized [3H]-la-
beled peptides.

In vivo studies. The ability of heparins to prevent HLE-mediated acute lung injury was assessed in female golden Syrian hamsters (Harlan Industries, Indianapolis, IN) weighing 90 to 110 g. Pentobarbital-anesthetized hamsters were injected intratracheally with 0.25 ml sterile 0.9% normal saline or 0.25 ml normal saline contain-
ing heparin or analog, followed 1 hr later by injection of HLE in 0.25 ml normal saline. Anesthetized animals were killed by exsanguina-
tion 24 hr after the treatment. The thorax was opened and lungs dissected en bloc. The trachea was cannulated with polyethylene tubing and lavaged with five sequential aliquots of 3 ml normal saline. The volume of lavage returned was similar in all groups and always >80% that instilled. Lavage fluid was centrifuged at 200 × g for 10 min. The resulting cell pellet was resuspended in 1 ml Hank's balanced salt solution for performing cell counts by hemocytometer. Differential cell counts were performed on Diff-Quik (American Scientific Products, McGaw Park, IL) stained smears. The supernatant was assayed for protein and hemoglobin, as indices of acute injury, using the Bio-Rad (Sigma) protein assay and the Sigma colorimetric hemoglobin assay.

Effect of Heparins on Complement-Mediated Red Cell Lysis
Complement-mediated red blood cell hemolysis was assessed by modification of a technique described previously (Friedrichs et al., 1994). Human blood was collected and centrifuged at 2000 × g for 10 min at room temperature. The plasma layer was discarded, and the red blood cells were washed three times with PBS. A solution of 10% erythrocytes was prepared in assay buffer (PBS containing 0.25% red blood cells were washed three times with PBS. A solution of 10% erythrocytes was prepared in assay buffer (PBS containing 0.25% }

Effect of O-Desulfated Heparin on Ischemia-Reperfusion Lung Injury
Isolation and perfusion of rabbit lungs was performed as described previously (Kennedy et al., 1989; Fisher et al., 1993). Male New Zealand White rabbits (3–3.5 kg) were given 5000 U of heparin by ear vein and anesthetized with 120 mg pentobarbital sodium. The chest was opened, and the animal was killed by rapid exsanguination from the left ventricle. Parasternal incisions were made, and the sternum and ventral portion of the ribs was removed to open the chest widely. Stainless steel cannulas were secured with umbilical tape in the trachea, left atrium and pulmonary artery. The lungs were inflated with 100 ml air and then ventilated with air containing 5% CO2, using an animal respirator. Tidal volume was adjusted to give an end-inspiratory tracheal pressure of 7 to 8 mm Hg during which a respiratory rate of 30 breaths/min at 1 mm Hg positive end-expiratory pressure was maintained. After ventilation was es-

c established, the pulmonary circulation was washed free of blood with 500 ml perfuse before recirculating flow was established at 100 ml/min. The perfusion circuit included a perfusate reservoir, roller perfusion pump and heat exchanger connected by Tygon tubing. The total volume of the perfusion system was 250 ml. The perfusion medium was Krebs-Henseleit buffer containing 3% bovine serum albumin. The buffer was prepared with deionized distilled water and maintained at 37°C and pH 7.2. Ppa and Pp were pressures were monitored by Gould pressure transducers (model P23 ID, Oxnard, CA). The perfusate reservoir was placed below the level of the lung to keep left atrial pressure at zero. Lung edema formation was monitored by loss of perfusate from the circuit as measured by reduction in weight of the perfusate reservoir, which was suspended from a force trans-
ducer (model FT10, Grass, Quincy, MA). Ppa, Pp, and W were continu-
ously recorded on a four-channel strip recorder (model 2400S, Gould).

After the lungs were isolated, instrumented and recirculating flow was established, they were perfused for 20 min to ensure integrity of the preparation. Maintenance of Ppa to less than 20 mm Hg, stable Pp, and weight of 0.1 g/min during this base-line period were the criteria used to determine if the preparation was suitable for study. In control experiments, the lungs were perfused continuously and ventilated for an additional 130 min. For ischemia experiments, perfusion was stopped for 90 min after the 20-min base-line period, but ventilation was continued. This model of ventilated ischemia was previously chosen to mimic the physiological state after pulmonary thromboembolism (Fisher et al., 1993). After the ischemic period, perfusion was re-established gradually over 45 sec. The reperfused lung was then monitored for 40 min.

Three interventions were studied. In the first, 25 mg of ODS heparin in 5 ml PBS were injected slowly into the inflow circuit before ischemia. In the second intervention 25 mg ODS heparin was injected into the inflow circuit after 90 min ischemia just before reperfusion. The third set of experiments were performed identical to the second, except that ODS heparin was charge-neutralized by addition of an equal weight of the polycation protamine sulfate to the solution before injection.

Effect of O-Desulfated Heparin on Pulmonary M2 Receptor Function in Antigen-Challenged Guinea-Pigs
The effect of O-desulfated heparin on M2 muscarinic receptor function in antigen-challenged guinea pigs was studied as reported previously (Fryer and Jacoby, 1992). Specific pathogen-free guinea pigs (Dunkin Hartley; 200–250 g) received i.p. injections with either saline (control) or 10 mg/kg ovalbumin every other day for three injections. Three weeks after the first injection, the ovalbumin-sensitized guinea pigs (but not the saline-injected) were antigen chal-
gen by exposure to an aerosol of 5% ovalbumin for 5 min on each of 4 consecutive days. On day 1 only (when acute responses to ovalbumin challenge are greatest) pyrilamine (1 mg/kg i.v.) was administered 60 min before challenge. This regime has previously been shown to increase the response to vagal stimulation in sensi-
tized animals by impairing M2 muscarinic receptor function (Elbon et al., 1995). Animals were housed in cages kept within laminar flow hoods throughout this time period.

Twenty-four hours after the last aerosol challenge, the animals were anesthetized with urethane (1.5 g/kg i.v.). None of the exper-
iments lasted for longer than 3 hr, although this dose of urethane produced a deep anesthesia lasting 8 to 10 hr. However, because paralyzing agents were used, the depth of anesthesia was monitored by observing for fluctuations in heart rate and blood pressure. Once the guinea pigs were anesthetized, both external jugular veins were cannulated for the administration of drugs. Guanethidine (10 mg/kg i.v.) was given at the start of each experiment to prevent release of norepinephrine from sympathetic nerves. Both vagi were cut and placed on shielded electrodes immersed in a pool of liquid paraffin. The electrodes were connected to a Grass SD9 stimulator (Grass
Effect of Heparins on Proliferation of Airway Smooth Muscle

Adult male Sprague-Dawley rats were killed with pentobarbital overdose and their tracheas were removed. The posterior tracheal membrane was isolated, minced and digested twice for 30 min at 37°C in Hanks’ balanced salt solution containing 0.2% type IV collagenase and 0.05% type IV elastase. Each enzyme digest was collected and centrifuged for 6 min at 500 × g at room temperature. The supernatant was removed and the pellet was resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, non-essential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin (250 ng/ml). Cells were seeded in this medium into 25-cm² flasks at 2 × 10⁵ cells per flask and incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C. Upon reaching confluence, cells were detached with 0.25% trypsin-0.002% EDTA solution for passage. Smooth muscle cell cultures demonstrated the typical “hill and valley” appearance under phase-contrast microscopy and stained specifically for α-smooth muscle actin. Immunostaining was performed using a polyclonal antibody against α-smooth muscle actin (Sigma) and visualized using an avidin-biotin-immunoperoxidase technique.

Preliminary studies demonstrated that culture of cells in the presence of 10% FBS resulted in a linear growth phase up to 120 hr. For proliferation studies, cells from passages four to eight were seeded into 24-well plates at 15,000 cells per well and cultured with Dulbecco’s modified Eagle’s medium and 10% FBS in the presence of heparin or ODS heparin at concentrations of 0, 2, 20 and 200 µg/ml. After 60 hr, cell counts were performed to study the influence of inhibitors on growth. Cells were washed twice in PBS, permeabilized by exposure to 0.5 mg/ml saponin in PBS for 5 min, fixed in absolute methanol for 10 min, stained with Giemsa-modified Wright’s stain for 3 min and washed with PBS. Cell counts were performed on 10 random fields at 40 power using a 0.01-cm² ocular grid. A total of six wells was studied at each treatment condition.

To determine whether heparins were cytotoxic, 200 µg/ml heparin or ODS heparin was added to wells of airway smooth muscle cells previously grown to confluence in DME medium and 10% FBS. After 24 hr, media were microfuged 5 min and supernatant was assayed for lactate dehydrogenase activity using a commercially available assay (Sigma). Cells were washed twice in PBS and exposed to trypan blue dye (0.04% in Hanks’ balanced salt solution). Cell counts were performed in five random fields using a 0.01-cm² ocular grid to quantitate the average number of cells that accumulated dye. A total of six wells was studied at each treatment condition.

Animal Welfare

Hamsters, rabbits and guinea pigs were handled in accordance with the standards established by the USDA Animal Welfare Acts set forth in National Institute of Health guidelines and the Policy and Procedures Manuals published by respective universities.

Statistical Analysis

Data are reported as mean ± S.E.M. The effect of heparins on HLE-induced lung injury in hamsters and airway smooth muscle proliferation were analyzed using one-way analysis of variance. Lung weight gain and Pₚₐ were compared using two-way analysis of variance on time and experimental group. In guinea pig experiments, one-way analysis of variance was used to compare the baseline bronchoconstriction and bradycardia responses to stimulation of the vagus nerves, and the initial effect of saline or ODS-heparin on vagally induced bronchoconstriction and bradycardia. The effects of saline and ODS-heparin on dose response curves to pilocarpine in antigen-challenged and control guinea pigs were compared using a two-way analysis of variance. Scheffe’s F test was used to determine whether there was a statistically significant difference between the baseline bronchoconstriction and bradycardia used to compare the baseline bronchoconstriction and bradycardia.
was used to correct for multiple comparisons. The effect of an additional 2000 U/kg heparin on the response to 100 μg/kg pilocarpine was tested using a paired t test. Significance was assumed at P < .05.

Results

Analysis of heparin structure and anticoagulant activity. Disaccharide analysis showed that alkaline lyophilization of porcine mucosal heparin produces an analog that is 2-O desulfated on α-L-iduronic acid (2-sulfate) saccharides and 3-O desulfated at N-glucosamine-N-sulfate (3,6-disulfate) (fig. 2). This partially O-desulfated heparin analog (ODS heparin) had an average molecular weight of 10,500 Da, compared to 11,500 Da for the starting material. However, ODS heparin was much more polydisperse. Whereas only 30% of the starting heparin was less than 10,000 Da and none was less than 6,000 Da, more than 60% of ODS fragments were less than 10,000 Da and 30% were less than 6,000 Da. Eight separately synthesized lots (100–1000 g) of ODS heparin showed 7.7 ± 0.9 U/mg anticoagulant activity in the USP assay and 4.9 ± 0.8 U/mg anti-Xa activity in the amidolytic assay, compared to 170 USP U/mg anticoagulant activity and 150 U/mg anti-Xa activity for the unmodified

![Disaccharide analysis of unmodified (A) and alkaline lyophilized (B) porcine mucosal heparin.](image-url)
TABLE 1

Effect of partially O-desulfated heparin on blood coagulation

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Control</th>
<th>Heparin</th>
<th>ODS Heparin</th>
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<tbody>
<tr>
<td>Time to clot formation (sec)</td>
<td>35–45</td>
<td>80</td>
<td>&gt;150</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
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<td>1.0</td>
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<td>1.0</td>
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Effect on Antithrombin 3 Activity

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Control</th>
<th>Heparin</th>
<th>ODS heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>&gt;8 min</td>
<td>42 sec</td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>&gt;7 min</td>
<td>33 sec</td>
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</tr>
<tr>
<td>1:100</td>
<td>42 sec</td>
<td>32 sec</td>
<td></td>
</tr>
<tr>
<td>1:1000</td>
<td>32 sec</td>
<td>32 sec</td>
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</tr>
</tbody>
</table>

ODS heparin was given before ischemia or just before reperfusion, or ODS heparin charge neutralized with protamine and given similarly (fig. 5A). This suggests a primary effect of nonanticoagulant heparin in preventing microvascular injury.

Effect of heparins on proliferation of airway smooth muscle. Proliferation of both vascular and airway smooth muscle is known to be reduced by heparin. Figure 6 shows that nonanticoagulant ODS heparin was equivalent to heparin in reducing FBS-stimulated proliferation of airway smooth muscle in a dose-dependent manner. The highest dose of each heparin provided an approximate 50% inhibition of cellular growth. Neither heparin nor ODS heparin was cytotoxic for rat airway smooth muscle cells, as assessed by trypan blue dye exclusion and lactate dehydrogenase activity in cell supernatants.

Effect of ODS heparin on pulmonary M₂ receptor function in antigen-challenged guinea pigs. Heparin has previously been shown to inhibit vagally induced airways hyperresponsiveness and restore altered M₂ muscarinic receptor function in antigen-challenged guinea pigs. ODS heparin also inhibits vagally induced airways hyperreactivity (fig. 7) and restores M₂ receptor function (fig. 8). In guinea pigs that were antigen challenged, saline had no effect on either vagally induced bronchoconstriction (fig. 8A) or bradycardia (fall of 62 ± 26 beats/min before saline vs 50 ± 27 beats/min 20 min after saline). In contrast, ODS-heparin decreased vagally induced bronchoconstriction, plateauing at 50% inhibition 20 min after administration (fig. 8A).
effect of ODS heparin on vagally mediated bronchoconstriction was dose related: 22.8 mg/kg reduced the response by 16%; 57 mg/kg reduced the response by 34% and 91.2 mg/kg decreased vagally induced bronchoconstriction by about 50% (figs. 7 and 8A). Vagally induced bradycardia was not altered.

Fig. 3. A, Inhibition of HLE by heparin and alkaline lyophilized partially O-desulfated heparin (ODS heparin). B, Inhibition of HLE by heparin and acid-hydrolyzed partially N-desulfated, N-reacylated heparin (NDS heparin). C, Inhibition of cathepsin G by heparin and ODS heparin. O-desulfated heparin retains inhibitory activity for both HLE and cathepsin G. In contrast, NDS heparin loses activity as an HLE inhibitor. HLE and cathepsin G were incubated with various molar ratios of enzyme to inhibitor for 30 min at 37°C before initiating the reaction. The substrate was suc-alal-val-pNA for HLE and suc-alal-pro-phe-pNA for cathepsin G. Results represent the mean of studies using nine separately synthesized lots of ODS heparin for A and three for B and C.

Fig. 4. Effect of heparin and alkaline lyophilized partially O-desulfated heparin (ODS heparin) on acute lung injury from HLE. Hamsters received 500 μg heparin or ODS heparin 1 hr before intratracheal instillation of 100 μg HLE. The lungs were excised and lavaged 24 hr later. A, Total hemoglobin in lavage (mg). Heparin and ODS heparin significantly (‡P < .01) reduced lavage hemoglobin compared to hamsters treated with HLE alone. B, Protein in lavage (μg/ml). HLE significantly (P < .05) increased lavage protein. Protein was reduced in heparin and ODS heparin-treated lungs, but values achieved statistical significance only for animals treated with heparin (‡P < .05). C, Total PMNs × 10⁶ in lavage. Heparin and ODS heparin significantly (‡P < .01) reduced lavage PMNs compared to hamsters treated with HLE alone. n = 4 in each treatment group.

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In control guinea pigs pilocarpine (1–100 μg/kg i.v.) inhibited vagally induced bronchoconstriction in a dose-dependent fashion by stimulating M2 muscarinic receptors on pulmonary parasympathetic nerves (open squares, fig. 8B). In contrast, pilocarpine had no significant effect on the response to vagal stimulation in sensitized, challenged animals (open triangles, fig. 8B). Increasing doses of ODS heparin restored the ability of pilocarpine to inhibit vagally induced bronchoconstriction in antigen challenged guinea pigs (fig. 8B). After the maximal dose of ODS heparin, the ability of pilocarpine to inhibit vagally induced bronchoconstriction in challenged guinea pigs was completely restored. There was no significant difference between the effect of pilocarpine on vagally induced bronchoconstriction in control animals (open squares, fig. 8B) and challenged guinea pigs who had received this dose of ODS heparin (closed squares, fig. 8B).

After each pilocarpine dose response curve a dose of 2000 U/kg of unmodified fully anticoagulant heparin (Elkins-Sinn) was given i.v. This dose significantly inhibits vagally induced bronchoconstriction in antigen-challenged guinea pigs (Fryer and Jacoby, 1992). In the antigen challenged animals that received 91.2 mg/kg of ODS heparin, the additional 2000 U/kg of unmodified heparin had no further effect on vagally induced bronchoconstriction (fig. 8C), suggesting that ODS heparin at this dose had completely restored M2 receptor function.

**Discussion**

As a drug, heparin has a wide array of potential pharmacologic uses. However, its activity as an anticoagulant limits its applications. Inactivating heparin as an anticoagulant, although preserving its nonanticoagulant pharmacology, would broaden the possible utility of heparin as a treatment for human diseases. This is especially true for the lung, where inflammatory and biochemical disorders ranging from asthma to ischemia-reperfusion injury are amenable to heparin therapy. Lyophilization under alkaline conditions, resulting in partial O-desulfation, appears to produce such a nonanticoagulant heparin analog.

**Figure 5.** Partially O-desulfated heparin (ODS heparin) prevents ischemia-reperfusion injury of the lung. A, Pulmonary artery pressure (PAP). B, Lung weight gain. Reperfusion caused increasing weight gain in untreated air-ventilated ischemic lungs (AI, open squares) from lung edema formation (P < .01 compared to nonischemic control lungs, closed boxes). ODS heparin (25 mg) prevented reperfusion lung edema (P < .01 compared to AI), whether given before ischemia (ODS-heparin-I, closed circles) or just before reperfusion (ODS-heparin-R, open circles). The protective effect of ODS heparin was removed by charge neutralization with protamine (P < .01 compared to AI). Weight gain in treated lungs was not significantly different than in nonischemic controls.

In control guinea pigs pilocarpine (1–100 μg/kg i.v.) inhibited vagally induced bronchoconstriction in a dose-dependent fashion by stimulating M2 muscarinic receptors on pulmo-
thrombin III (Bjork et al., 1989). Antithrombin III binding is dependent on a specific pentasaccharide sequence found in anticoagulant fractions of heparin (Marcum and Rosenberg, 1989). However, the nonanticoagulant pharmacology of heparin is in part related not to specific saccharide sequences but to its general polyanionic nature (Jaques, 1980). Inhibition of the cationic proteases HLE and cathepsin G by heparin likely occurs by electrostatic binding of heparin to the protease, covering its active site (Redini et al., 1988). This is suggested by the observation that HLE inhibition by heparin is directly related to the degree of polymer sulfation (Redini et al., 1988). The polyanionic nature of heparin likely explains its activity in binding to cationic major basic protein and restoring M₃ receptor function in sensitized, antigen-challenged guinea pigs. Other anionic compounds such as polyglutamate electrostatically bind eosinophil major basic protein and counteract its biologic activity (Coyle et al., 1995; Fryer and Jacoby, 1992; Jacoby et al., 1993).

Despite modification, partially O-desulfated heparin is a potent inhibitor of HLE and cathepsin G (figs. 3 and 4). HLE was first investigated for its potential importance in pulmonary emphysema, but HLE and cathepsin G have recently been proposed for expanded roles as mediators of inflammation. HLE and cathepsin G are potent secretagogues for serous (Sommerrhoff et al., 1990) and mucous glycoprotein (Lundgren et al., 1994) secretion and are thought important in the pathogenesis of chronic bronchitis. HLE is markedly elevated in airway secretions in cystic fibrosis (McElvaney et al., 1992) and causes both the enhanced interleukin-8 production (McElvaney et al., 1992) and the defect in neutrophil phagocytosis (Berger et al., 1989) seen in the cystic fibrosis airway. High levels of HLE are found in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome (Lee et al., 1981), and HLE inhibitors reduce lung injury in experimental models of the disease (Gossage et al., 1993; Ahn et al., 1993). HLE has also been found in human eosinophils (Lungarella et al., 1992) and lung mast cells and basophils (Meier et al., 1989), raising questions of its role in immediate hypersensitivity. Finally, HLE is found on the surface of proinflammatory monocytes (Owen et al., 1994), and HLE and cathepsin G on the monocyte surface modulate factor V procoagulant activity thought important in thrombin generation at extracellular sites of inflammation (Allen and Tracy, 1995).

ODS heparin, such as heparin, is also able to prevent ischemia-reperfusion injury (fig. 5), an important disease mechanism in the pathogenesis of myocardial infarction, stroke and pulmonary thromboembolism. The mechanism by which ODS heparin prevents ischemia-reperfusion injury is charge dependent, as shown by loss of activity when protamine is used to neutralize ODS heparin before administration (fig. 5). Prevention of ischemia-reperfusion injury by heparin is possibly mediated through inhibition of activated complement in lung interstitium, similar to the proposed protective effect of heparin in isolated refused rabbit hearts (Friedrichs et al., 1994). However, cellular injury of ischemic tissue has been attributed in part to infiltration of activated neutrophils during reperfusion (Hernandez et al., 1987). Proteolytic digestion of basement membrane has been proposed as a requirement for passage of leukocytes out of blood vessels (Delclaux et al., 1996), and HLE inhibitors reduce leukocyte extravasation into ischemic-reperfused myocardium (Nicolini et al., 1991) and bowel (Zimmerman and Granger, 1990). In addition, reperfusion of ischemic tissue results in immediate loss of as much as half of endothelial heparan sulfate (Stevens et al., 1993). Such a massive reduction in endothelial surface charge has been shown to produce endothelial permeability and lung injury that can be prevented or reduced by heparin (Chang and Voelkel, 1989) or nonanticoagulant heparin (Stevens et al., 1993).

Proliferation of smooth muscle within the airway has recently been identified as a risk factor for development of fixed airways obstruction in severe asthma (James et al., 1989). Heparin is a potent inhibitor of airway smooth muscle proliferation and has been proposed as a preventative treatment for this condition (Kilfeather et al., 1995). ODS heparin was
equally potent compared to heparin in reducing airways smooth muscle proliferation in culture (fig. 6). Although it can bind and directly inhibit growth factors (Maccarana et al., 1993; Wright et al., 1989), the antiproliferative effect of heparin on smooth muscle is thought to depend on cellular uptake and internalization (Wright et al., 1989). Subsequently, heparin indirectly reduces nuclear binding of activator protein-1 to the phorbol ester-responsive element, perhaps in part by heparin inhibition of the phosphorylation of Jun B by mitogen-activated kinases or casein kinase II (Au et al., 1993). Heparin is a known potent inhibitor of both mitogen-activated kinase (Ottlinger et al., 1993) and casein kinase II (Hathaway et al., 1980) through interaction with critically positioned positively charged amino acids.

Vagally induced airways hyperreactivity is largely mediated by inhibiting M₂ receptors on the vagus nerves. These receptors normally function to inhibit acetylcholine release from the vagus, thus limiting vagally induced bronchoconstriction. Neuronal M₂ muscarinic receptors are not functioning in animal models of asthma (Fryer and Jacoby, 1992) or in humans with asthma (Ayala and Ahmed, 1989; Minette and Barnes, 1988). Loss of M₂ receptor function is due to blockade of the receptor by endogenous eosinophil major basic protein (Fryer and Jacoby, 1992; Jacoby et al., 1993; Elbon et al., 1995). Heparin electrostatically binds major basic protein, removing it from the receptor and restoring inhibitory receptor function against vagally induced airways hyperreactivity. ODS heparin also restores M₂ muscarinic receptor function (figs. 7 and 8), and, as with heparin, may also be useful in treating clinical airways hyperreactivity in asthma (Diamant et al., 1996). Inhaled heparin has previously been shown to prevent antigen-induced bronchospasm in sheep (Ahmed et al., 1992) and exercise-induced asthma in humans (Ahmed et al., 1993) by a mechanism suggested as inositol 1,4,5-triphosphate-dependent stimulus-secretion coupling in mast cells. However, blockade of inositol triphosphate has been demonstrated only for low molecular weight heparins (average molecular weight of about 5000 Da) in cells permeabilized with saponin or digitonin so that heparin can enter the cytoplasmic space (Ghosh et al., 1988; Chopra et al., 1989). The larger unfractionated heparin (average molecular weight 12–15,000 Da) used by Ahmed et al. (1992) is not an effective inhibitor of inositol triphosphate receptors (Chopra et al., 1989), and no evidence exists to suggest that heparin is actively internalized by mast cells.

N-desulfated heparin loses its activity as an HLE inhibitor and is also reported inactive in preventing bronchospasm in sensitized antigen challenged sheep (Ahmed et al., 1992). In contrast, ODS heparin remains a potent inhibitor of neutrophil proteases (figs. 3 and 4) and complement. A clue to

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**Fig. 8.** A. Partially O-desulfated heparin (ODS heparin) inhibits vagally induced bronchoconstriction in sensitized guinea pigs challenged with ovalbumin. Electrical stimulation of the vagus nerves (15 Hz, 0.2 msec., 5–30V, 45 pulses/train) causes bronchoconstriction, measured as an increase in P ATS in mm H2O (open columns). Vagally induced bronchoconstriction 20 min after saline or ODS heparin (solid columns) is shown. Saline (0.1 ml/kg, n = 5) has no effect, but ODS heparin (91.2 mg/kg, n = 5) significantly inhibits vagally induced bronchoconstriction (*P < .05). B. ODS heparin restores the response to pilocarpine in ovalbumin challenged guinea pigs. Pilocarpine (1–100 μg/kg i.v.) significantly inhibited vagally induced bronchoconstriction in control guinea pigs (open squares □), P = .01. After antigen challenge the effect of pilocarpine on vagally induced bronchoconstriction was restored in a dose-dependent manner. In the absence of pilocarpine, vagally induced bronchoconstriction (2 Hz, 0.2 msec, 10–25 V, 45 pulses/train) was abolished (open triangles ■). Twenty min after ODS-heparin (11.4 mg/kg, closed triangles ▲). 22.8 mg/kg, closed circles ●; 57.0 mg/kg, closed diamonds ●; 91.2 mg/kg i.v., closed squares ■) the effect of pilocarpine on vagally induced bronchoconstriction was restored in a dose-dependent manner. In the absence of pilocarpine, vagally induced bronchoconstriction (2 Hz, 0.2 msec, 10–25 V, 45 pulses/train) was not significantly different between the groups of animals (controls 24.7 ± 2.0; challenged 24.0 ± 3.6; challenged + 11.4 mg/kg ODS heparin 22.9 ± 4.6; challenged + 22.8 mg/kg ODS heparin 29.9 ± 5.9; challenged + 57 mg/kg ODS heparin 25 ± 7.2; challenged + 91.2 mg/kg 22.4 ± 9.2). Results are expressed as the ratio of vagally induced bronchoconstriction after pilocarpine to vagally induced bronchoconstriction before pilocarpine. Each point is the mean of three to six animals. *P < .05 compared to control animals; †P < .01 compared to untreated antigen-challenged animals. C. The effect of pilocarpine in guinea pigs treated (solid columns) and untreated (open columns) with ODS heparin before and after additional heparin (2000 U/kg). Control guinea-pigs represented by a (−) challenged by (+). Doses of ODS heparin are listed under each set of bars. The maximum effect of pilocarpine (100 mg/kg i.v) on vagally induced bronchoconstriction before additional heparin is shown in the open columns. The response 5 min after additional heparin (2000 U/kg i.v.) is shown in the shaded columns. Results are expressed as the ratio of vagally induced bronchoconstriction after 100 μg/kg pilocarpine to vagally induced bronchoconstriction before pilocarpine. In the absence of pilocarpine, there were no significant differences between groups (see above in B). Each bar is the mean of three to six animals. *P < .05 compared to responses before additional heparin.
understanding how partially O-desulfated heparin can remain biologically active is illustrated in table 3. Dextran sulfate continues to inhibit HLE despite partial desulfation, until sulfation is reduced to less than 12%. At and above this degree of sulfation, sufficient charge is still randomly scattered along the polymer to continue effective electrostatic binding to cationic HLE. However, below a critical density of sulfates, binding with sufficient affinity is no longer likely to occur. The same principle also likely governs binding of heparin to cationic substances. ODS nonanticoagulant heparin, although of reduced sulfation, retains enough random anionic charge to effect electrostatic interaction with cationic sites of sufficient affinity to preserve many of the biologic properties of the fully anticoagulant unmodified heparin from which it was made. In contrast, N-desulfation might tend to produce a heparin with comparatively less net polyanionic activity due to formation of positive charges on desulfated nitrogens, even with N-acectylation.

Previous work with heparin fragments has suggested that not only is degree of sulfation important to HLE inhibitory activity, but that O-sulfates are more important than N-sulfates (Redini et al., 1988). The partially O-desulfated heparin produced by alkaline lyophilization remains a potent inhibitor of HLE, suggesting that structure-activity relationships for heparin fragments may not fully predict behavior of larger polysaccharides. Increasing the charge of inactive tetrasaccharide fragments by O-oversulfation is reported to increase antiproliferative effects for vascular smooth muscle, whereas desulfation of large fragments causes them to lose their activity (Wright et al., 1989). Also, 3-O sulfates have been considered a critical structural determinant of the antiproliferative activity of heparin for vascular smooth muscle (Castellot et al., 1986). The 2-O, 3-O desulfated heparin analog used in our studies remains fully antiproliferative. These results could be possibly explained by differences in the antiproliferative mechanisms of heparin for vascular versus airway smooth muscle. Heparin reduces DNA binding of AP-1 in vascular smooth muscle (Au et al., 1994). However, despite the antiproliferative effects of heparin and ODS-heparin in serum-stimulated airway smooth muscle, we have been unable to demonstrate heparin-induced reduction of AP-1 binding in electrophoretic mobility shift assays performed on nuclear protein from these cells (data not shown).

Our results suggest that nonanticoagulant ODS heparin might offer therapeutic potential in a number of lung diseases. Aerosolized, ODS heparin could inhibit elastase-mediated airway injury in cystic fibrosis (McElvaney et al., 1992) or reduce airways reactivity in asthma (Coyle et al., 1995; Fryer and Jacoby, 1992; Jacoby et al., 1993; Diamant et al., 1996). Intravenously, ODS heparin might ameliorate the lung inflammation of adult respiratory distress syndrome (Chang and Voelkel, 1989; Gossage et al., 1993) or lung ischemia-reperfusion injury after pulmonary thromboembolism (Black et al., 1995; Zimmerman and Granger, 1990; Nicolin et al., 1991). Additional studies will be needed to fully define the potential pharmacology of this new nonanticoagulant heparin in disorders of the lung and other organ systems.

Acknowledgments

The authors thank Dr. George Jakab for the use of his inhalation facilities in the Johns Hopkins School of Hygiene and Public Health, funded by National Institutes of Health Grant ES-03819. We also thank Dr. William Bell, Director of Special Hematology, Johns Hopkins Hospital, for performing anti-X, clotting studies, and Drs. Patrick Shackley and James Knobloch of Scientific Protein Laboratories for their help with disaccharide analysis and measurement of heparin molecular weights.

References


ELSON, C. L., JACOBY, D. B. AND FRYER, A. D.: Pretreatment with an antibody to interleukin-5 prevents loss of pulmonary M2 muscarinic receptor function in

TABLE 3

<table>
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<tr>
<th>I:E Ratio</th>
<th>4% DS</th>
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<td>59</td>
<td>61</td>
<td>97</td>
<td>100</td>
</tr>
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</table>

HLE activity was monitored using the specific synthetic chromogenic substrate suc-ala2-val-pNA and the method of Barrett (1981) with some modifications.

* Inhibitor to enzyme ratio.

** Dextran sulfate. Percentage sulfation of dextran sulfate represents % sulfate content by weight.