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Interaction of Secretory Leukocyte Protease Inhibitor with Heparin Inhibits Proteases Involved in Asthma*

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Melissa A. Fath‡, Xiaojun Wu‡, Ronald E. Hileman‡, Robert J. Linhardt‡§¶, Mohammed A. Kashem‖, Richard M. Nelson‖, Clifford D. Wright‖, and William M. Abraham**

*From the ‡Division of Medicinal and Natural Products Chemistry, College of Pharmacy, and §Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, Iowa 52242, ‖Amgen Inc., Boulder, Colorado 80301, and the **University of Miami School of Medicine, Miami Beach, Florida 33140*

Protease inhibition by secretory leukocyte protease inhibitor (SLPI) is accelerated by the sulfated polysaccharides. The nature of the SLPI-polysaccharide interaction, explored with affinity chromatography, indicated that this interaction was sensitive to the charge and type of polysaccharide. Dextran and chondroitin had the lowest affinity for SLPI, followed by dermatan, heparan, and dextran sulfates. While heparin bound SLPI tightly, the highest affinity heparin chains unexpectedly contained a lower level of sulfation than more weakly interacting chains. Heparin oligosaccharides, prepared using heparin lyase I were SLPI-affinity fractionated. Surprisingly, undersulfated heparin oligosaccharides bound SLPI with the highest affinity, suggesting the importance of free hydroxyl groups for high affinity interaction. Isothermal titration calorimetry was used to determine the thermodynamics of SLPI interaction with a low molecular weight heparin, an undersulfated decasaccharide and a tetrasaccharide. The studies showed 12–14 saccharide units, corresponding to molecular weight of ~4,800, were required for a 1:1 (SLPI:heparin) binding stoichiometry. Furthermore, an undersulfated decasaccharide was able to bind SLPI tightly (K_d ~13 nM), resulting in its activation and the inhibition of neutrophil elastase and pancreatic chymotrypsin. The *in vitro* assessment of heparin and the decasaccharide and tetrasaccharide using stopped-flow kinetics suggested that heparin was the optimal choice to study SLPI-based *in vivo* protease inhibition. SLPI and heparin were co-administered by inhalation in therapy against antigen-induced airway hyperresponsiveness in a sheep bronchoprovocation model. Heparin, in combination with SLPI demonstrated *in vivo* efficacy reducing early and late phase bronchoconstriction. Heparin also increased the therapeutic activity of SLPI against antigen-induced airway hyperresponsiveness.

Secretory leukocyte protease inhibitor (SLPI)[1] is a nonglycosylated serine antiprotease with a molecular weight of 11,700 (29). It has been isolated from human bronchial secretions, seminal fluid, cervix uteri secretions, parotid saliva, articular cartilage chondrocytes, and intervertebral disc fibrochondrocyte (2, 3). The biological function of SLPI is believed to be the inhibition of elastase, cathepsin G, and other proteases, thereby protecting tissue from self-degradation by these enzymes (2, 4). SLPI is composed of two homologous domains and contains many positively charged amino acid residues (15 lysine and 5 arginine residues) (2). It is within clusters of these positively charged amino acid residues that the heparin polyanion probably interacts (5, 14).

Heparin is biosynthesized in mast cells as a proteoglycan consisting of a central core protein from which multiple glycosaminoglycan (GAG) chains extend (6–8). On isolation and purification from tissue, such as lung, heparin is released from its protein core and isolated as a GAG. GAG heparin is a polydisperse, highly sulfated, linear polysaccharide comprised of repeating 1→4 linked uronic acid and glucosamine residues, with a molecular weight range of 5,000–40,000 and an average molecular weight (M_r(avg)) of 14,000 (9, 10). While it has as many as 10 different saccharide residues that comprise its sequence, not all sequences are biosynthetically favored. The major sequence in heparin is →4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S(1→ (where IdoAp is idopyranosyluronic acid, GlcNp is 2-amino-2-deoxyglucopyranose and S is sulfate) comprising from 75 to 90% of its structure (Fig. 1) (9, 10). Low molecular weight (LMW) heparin has an M_r(avg) ~5,000 and is prepared from heparin by chemical or enzymatic treatment as a clinical antithrombotic agent (11). Homogeneous heparin oligosaccharides, ranging in molecular weight from 665 to 4655, have also been prepared from heparin using heparin lyase I (12, 13). These oligosaccharides have been purified to homogeneity and structurally characterized using high-field multidimensional NMR spectroscopy (13).

SLPI binds heparin and LMW heparin tightly (K_d of 6 ± 2 nM and 50 ± 9 nM, respectively) resulting in an increase in SLPI's rate of elastase inhibition (1). Heparin also increases inhibition of chymotrypsin by SLPI (14). Recent studies by Ying *et al*. (15) showed that heparin could restore the elastase inhibition by SLPI after the inactivation of SLPI through the oxidation of a critical methionine residue. Faller *et al*. (16) have shown that SLPI interacts with heparin through seven ion pair interactions and have inferred that 85% of the binding energy is electrostatic in nature.

We report the further characterization of the interaction between SLPI and heparin by using affinity chromatography. Heparin-derived oligosaccharides with high affinity for SLPI were isolated and their structure identified. The interaction of heparin and heparin-derived oligosaccharides with SLPI was assessed using isothermal titration calorimetry (ITC) (17) as was their activation of SLPI's inhibition of various proteases,

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¶ To whom correspondence should be addressed: University of Iowa, PHAR-S328, Iowa City, IA 52242. Tel.: 319-335-8834; Fax: 319-335-6634; E-mail: robert-linhardt@uiowa.edu.

[1] The abbreviations used are: SLPI, secretory leukocyte protease inhibitor; GAG, glycosaminoglycan; LMW, low molecular weight; ITC, isothermal titration calorimetry; PAGE, polyacrylamide gel electrophoresis; pNA, *p*-nitroanaline; PBS, phosphate-buffered saline; dp, degree of polymerization.

using stopped-flow kinetics (18). Finally, the *in vivo* activation of SLPI by heparin was studied using a sheep bronchoprovocation model for late asthmatic response (19).

EXPERIMENTAL PROCEDURES

Materials

Porcine heparin (M_r(avg) 14,000), low molecular weight heparin (M_r(avg) 4,800), and heparan sulfate (M_r(avg) 11,000) were obtained from Celsus Laboratories (Cincinnati, OH). Chondroitin (M_r(avg) 25,000) and dermatan sulfate (M_r(avg) 11,000–25,000), sodium salts, and heparin/heparan sulfate disaccharide standards for capillary electrophoresis were purchased from Seikagaku America, Inc. (Rockville, MD). Dextran (M_r(avg) 1,000), dextran sulfate (M_r(avg) 5,000), heparin-Sepharose 4B prepacked columns, CNBr-activated Sepharose 4B *N*-Suc-Ala-Ala-Pro-Phe-pNA and MeO-Suc-Ala-Ala-Pro-Val-pNA were purchased from Sigma. Heparin lyase I (heparinase I, EC 4.2.2.7), heparin lyase II, (heparinase II, no EC number), and heparin lyase III (heparinase III, EC 4.2.2.8) were a gift from IBEX (Montreal, Canada). Recombinant human SLPI was expressed and purified as described previously (20). Recombinant protein was >99% pure as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high performance liquid chromatography and contained <0.72 enzyme unit lipopolysaccharide/mg of protein. Bovine pancreatic chymotrypsin was purchased from Boehringer Mannheim. Human neutrophil elastase and human neutrophil cathepsin G were from Calbiochem-Novabiochem, San Diego, CA. Dialysis tubing was purchased from Spectrum Laboratory Products (Houston, TX).

Methods

Affinity Chromatography Studies

Heparin-Sepharose Affinity Chromatography of SLPI—SLPI (100 μg in 10 μl buffer) was loaded onto a 2.5-ml heparin-Sepharose column (1 mg/ml) was first washed with 20 mM sodium phosphate, pH 7.2, buffer and a 50-ml linear salt gradient of 0–1.5 M NaCl in 5 mM sodium phosphate buffer, pH 7.4, was used to elute the column. The eluent was monitored at 280 nm, and the salt concentration was determined using a conductivity meter and fractions were collected.

Preparation of SLPI-Sepharose—CNBr-activated Sepharose (300 mg) was washed with 75 ml of 1 mM hydrochloric acid and swollen for 15 min at room temperature. The swollen beads were suspended in coupling buffer (100 mM sodium bicarbonate, 100 mM NaCl, pH 8.4) containing 10 mg of *N*-acetylated heparin (21) (to protect SLPI's interacting lysine residues), SLPI (1 mg) was added slowly to avoid precipitation, and the slurry was mixed at 4 °C for 12 h. The gel was then washed with coupling buffer, 100 mM sodium acetate buffer, pH 4.5, resuspended in 1 M ethanolamine, pH 8.9, to block remaining reactive sites on the beads, and the slurry was again mixed at 4 °C for 12 h. The gel was subsequently poured into a column (0.5 × 2 cm) and thoroughly washed with 5 mM sodium phosphate buffer, pH 7.4, containing 2 M NaCl and stored at 4 °C until used. A control column was also prepared using the same method but without adding the SLPI-*N*-acetylated heparin mixture. All affinity chromatography was performed at 4 °C.

SLPI-Sepharose Affinity Chromatography of Polysaccharides—The SLPI-Sepharose column was equilibrated in 5 mM, pH 7.4, sodium phosphate buffer. In separate experiments, heparin (500 μg), heparan sulfate (500 μg), dermatan sulfate (500 μg), chondroitin (600 μg), dextran (600 μg), or dextran sulfate (1.2 mg) were affinity fractionated on this column. After loading the polysaccharide, the column was washed with 5 mM sodium phosphate buffer, pH 7.4, and a 20-ml linear salt gradient (from 0 to 1.75 M NaC1) in the same buffer was used to elute the column and fractions were collected. GAGs were detected by carbazole assay (22), dextran and dextran sulfate by phenol-sulfuric acid assay (23), and salt concentrations were determined by measuring the conductivity.

Individual fractions from the SLPI-Sepharose affinity-fractionated heparin were exhaustively dialyzed against distilled water (500 molecular weight cutoff membranes) and freeze-dried. Each fraction (10–50 μg) was enzymatically depolymerized for 10 h at 30 °C using 1 milliunit of heparin lyase I, II, and III in 20 μl of 50 mM sodium phosphate buffer containing 100 mM NaCl and 2 mg/ml bovine serum albumin. The samples were heated to 100 °C for 5 min to inactivate the heparin lyases and analyzed immediately using reverse polarity capillary electrophoresis as described previously (24). Briefly, a normal fused silica capillary (75 μm inner diameter, 70 cm long) was used with 20 mM phosphoric acid adjusted to pH 3.5 by 1 M dibasic sodium phosphate as the running buffer. Each sample (~10 nl) was applied by vacuum injection and separated using 20 kV. Samples were detected by absorbance at 232 nm and the peaks identified by co-migration of disaccharide standards.

SLPI-Sepharose Affinity Chromatography of Partially Depolymerized Oligosaccharides—Heparin was digested to 30% reaction completion using heparin lyase I and fractionated by Sephadex G50 gel exclusion chromatography into sized oligosaccharide mixtures as previously reported (13). Two size-fractionated oligosaccharide mixtures were chosen for these studies, one in which the average oligosaccharide size was a hexasaccharide and the other in which the average oligosaccharide size was a hexadecasaccharide. The hexasaccharide mixture (50 and 500 μg) was loaded onto the SLPI-Sepharose column equilibrated with 5 mM sodium phosphate buffer, pH 7.4, the column was washed with the same buffer and then eluted in the same buffer with 20 ml of linear 0–1.5 M NaCl. The eluent was monitored by conductivity and at 232 nm. Fractions corresponding to the interacting peak were collected, exhaustively dialyzed (500 molecular weight cutoff membranes) and lyophilized. The hexadecasaccharide mixture was similarly fractionated. Fractions from the affinity chromatography experiments were subjected to gel electrophoresis (PAGE) (25). Sample (5–10 μg) were dissolved in 10–15 μl of distilled water and 15 μl of a 50% (w/v) sucrose solution and subjected to electrophoresis on a 20-cm-long linear gradient (12–22% total acrylamide) gel for 5 h at 400 V or through a 32-cm-long gradient gel for 18 h at 400 V. The gels were stained with Alcian blue (0.5% in 2% acetic acid) for 30 min, destained by washing with distilled water for 24 h, and silver-strained as described previously (25).

Binding, Inhibition, and Rate Studies

ITC—ITC of SLPI with LMW heparin and the homogeneous heparin-derived tetrasaccharide and decasaccharide (13) were performed as described previously (17). Titrations of SLPI (1 ml at 100–200 μM) with tetrasaccharide (10 10-μl injections at 8–9 mM) were in 50 mM sodium phosphate buffer, pH 7.4, containing 10–150 mM NaC1 to vary ionic strength. Titrations of LMW heparin (1 ml at 100 μM) or decasaccharide (1 ml at 142 μM) with SLPI (20 5-μl injections at 853 μM) were in 50 mM sodium phosphate buffer, pH 7.1, containing 100 mM NaC1.

Effect of Polysaccharides on Proteinase Inhibition by SLPI—The effect of heparin and other polysaccharides on the inhibition of three proteases by SLPI was determined by reacting a fixed concentration of protease and substrate with a series of SLPI concentrations in the presence or absence of 10 μg/ml polysaccharide. After 15 min of incubation at 37 °C, residual enzymatic activity was measured as the rate of change in absorbance at 405 nm caused by formation of pNA on a SpectraMAX 340 plate reader (Molecular Devices, Sunnyvale, CA). Human neutrophil elastase was assayed in a reaction mixture with 7.5 nM enzyme and 0.3 mM MeO-Suc-Ala-Ala-Pro-Val-pNA (26) in 100 mM Tris-HCl, pH 8.3, 0.1 M NaCl, 1% bovine serum albumin. Human cathepsin G was assayed in a reaction mixture with 16 nM enzyme and 0.4 mM *N*-Suc-Ala-Ala-Pro-Phe-pNA (27) in 625 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 0.125% Brij 35. Bovine pancreatic chymotrypsin was assayed using 13 nM enzyme and 100 μM *N*-Suc-Ala-Ala-Pro-Phe-pNA (28) in 100 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$. The K_m for each substrate was determined by iteratively fitting the initial velocities to the Michaelis-Menten equation using Sigma Plot (Jandel Scientific, San Rafael, CA). Data were visualized by plotting according to the Lineweaver-Burk transformation. The concentration of elastase, cathepsin G, and chymotrypsin were determined by active site titration with human SLPI (29). The inhibition constant (K_i) of recombinant human SLPI for each protease was determined as described previously (30).

Determination of Rates of Inhibition—The association rate constant for inhibition of proteases by SLPI was determined using the progress curve method (31). Enzyme containing heparin or heparin-derived oligosaccharide (120 μl) was added to a mixture (120 μl) of SLPI and substrate, and the concentration of product (absorbance at 402 nm) was measured as a function of time at 25 °C using stopped-flow apparatus (Biologic SFM-3, Molecular Kinetics, Pullman, WA). Human neutrophil elastase (10 nM) containing heparin or heparin-derived oligosaccharide (10 μg/ml) was added to a mixture of SLPI (50 to 100 nM) and substrate MeO-Suc-Ala-Ala-Pro-Val-pNA (1.0 mM) in 50 mM Hepes, pH 7.4, 100 mM NaCl. Human neutrophil cathepsin G (30 nM) containing heparin or heparin-derived oligosaccharide (10 μg/ml) was added to a mixture of SLPI (150–350 nM) and substrate *N*-Suc-Ala-Ala-Pro-Phe-pNA (1.0 mM) in 50 mM Hepes, pH 7.4, 100 mM NaCl. Bovine pancreatic chymotrypsin (40 nM) containing heparin or heparin-derived oligosaccharide (10 μg/ml) was added to a mixture of SLPI (250 to 500 nM) and substrate *N*-Suc-Ala-Ala-Pro-Phe-pNA (500 μM) in 50 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$. The observed pseudo-first order rate constant (k_{obs})

TABLE I
SLPI-Sepharose affinity chromatography of polysaccharides

Polysaccharide	Charge per disaccharide	Sodium chloride concentration	
		peak	Highest affinity chains
		M	
Dextran	0	0.00	0.01
Chondroitin	−1.15	0.04	0.20
Dermatan sulfate	−2	0.24	0.46
Heparan sulfate	−2	0.12	0.59
Dextran sulfate	−4.5	0.26	0.64
Heparin	−3.7	0.26	0.74

for the approach to the steady state was obtained by nonlinear regression analysis of the progress curves using Biokine software (Biologic Instruments de Laboratories). The apparent second-order association rate constant (k_a) for proteinase-SLPI complex formation was calculated from k_{obs} using the following relationship (14),

$$k_a = k_{obs}(1 - v_s/v_o)\frac{(1 + [S]/K_m)}{[I_0]} \quad \text{(Eq. 1)}$$

where v_s is the steady state velocity, v_o is the initial velocity, $[I_0]$ is the SLPI concentration, K_m is the Michaelis constant for the specific substrate, and [S] is the substrate concentration. We have calculated k_a using the following relationship.

$$k_a = k_{obs}\frac{(1 + [S]/K_m)}{[I_0]} \quad \text{(Eq. 2)}$$

To account for the effect of the factor v_s/v_o on the k_a in Eq. 1, we have estimated the values of v_s and v_o from the progress curve using the Biokine software. In most cases the corrected values were within the experimental error of the method (*i.e.* v_s/v_o = 0.1–0.2).

Antigen-induced Airway Responses in Sheep

Airway Mechanics—Adult ewes (median weight ≈ 30 kg) were instrumented as described previously (32). Mean pulmonary flow resistance (R_L) was calculated from an analysis of 5–10 breaths by dividing the change in transpulmonary pressure by the change in flow at midtidal volume. Immediately after R_L determination, thoracic gas volume (V_{tg}) was measured in a constant volume body plethysmograph to calculate specific lung resistance (SR_L) by the equation $SR_L = R_L \times V_{tg}$).

A Raindrop jet nebulizer (Puritan-Benett, Lenexa, KS), operated at a flow rate of 6 liters/min, was used to generate droplets with a mass median aerodynamic diameter of 3.6 ± 1.9 μm. Aerosol delivery was controlled using a dosimetry system (32), which was activated for one second at the onset of the inspiratory cycle of a piston respirator (Harvard Apparatus Co., South Natick, MA). Aerosols were delivered at a tidal volume of 500 ml and a respiratory rate of 20 breaths/min.

Sheep with airway hypersensitivity to *Ascaris suum* antigen which exhibited both early and late phase bronchoconstriction were used for these studies. Sheep were challenged with *Ascaris suum* extract (82,000 protein nitrogen units/ml in phosphate-buffered saline (PBS)) (Greer Diagnostics, Lenoir, NC) delivered as an aerosol at a rate of 20 breaths/min for 20 min. Changes in SR_L were monitored for 8 h after antigen challenge. A paired *t* test (two-tailed) was used to compare peak early and late phase bronchoconstriction responses.

Airway Hyperresponsiveness—Base-line airway responsiveness was determined by measuring the SR_L immediately after saline inhalation and consecutive administration of 10 breaths of increasing concentrations of carbachol (0.25, 0.5, 1.0, 2.0, and 4.0%, w/v). Airway responsiveness was estimated by determining the cumulative carbachol breath units required to increase SR_L by 400% over the post-saline value (PC_{400}). One breath unit was defined as 1 breath of an aerosol containing 1% w/v carbachol (32). Antigen-induced airway hyperresponsiveness was determined by repeating the carbachol dose response 24 h after antigen challenge. A paired *t* test (one-tailed) was used to evaluate hyperresponsiveness studies.

RESULTS AND DISCUSSION

Affinity chromatography experiments were performed on SLPI-Sepharose using various polysaccharides (Table I). Affinity for SLPI increased with the increasing negative charge of the polysaccharides studied. Dextran, an uncharged 1→6-linked polymer of α-D-glucopyranose, failed to bind. Chondroitin, containing one carboxylate group and ~0.15 sulfate groups/disaccharide unit, bound weakly to the column, eluting as a sharp peak at 0.04 M NaCl. Heparan sulfate and dermatan sulfate, both containing one carboxylate group and ~1 sulfate per disaccharide unit, bound with moderate affinity eluting with 0.12 and 0.24 M NaCl, respectively. Heparin and dextran sulfate (Fig. 1) bound with high affinity to SLPI both eluting from the SLPI-Sepharose column with peaks at 0.26 M NaCl. Heparin contained chains having a higher affinity for SLPI (eluting at 0.74 M NaC1) than the highest affinity chains in dextran sulfate (eluting at 0.65 M NaC1). This is surprising as heparin has a lower negative charge density (−3.7/disaccharide, one carboxylate and ~2.7 sulfate groups) than dextran sulfate (−4.5/ disaccharide). These results suggest that while SLPI-polysaccharide interaction increases with increased anionic character of the polysaccharide, this interaction is not strictly dependent on charge density. The glycosidic linkage, type of saccharide present, and molecular weight may also be important in displaying the interacting groups of the polysaccharide in a productive manner.

Heparin eluting from SLPI-Sepharose gives a broad peak with significant trailing (up to 0.74 M) (Table I). However, when SLPI is fractionated on heparin-Sepharose, it gives a sharp and symmetric peak at 0.26 M (not shown). These data suggest that affinity fractionation of heparin is taking place on SLPI-Sepharose and that tightly binding chains might contain structurally unique SLPI-binding sites. In contrast, dextran sulfate fractionated on a SLPI-Sepharose column gave a much sharper symmetrical peak, suggesting that fractionation was the result of ion-exchange with the most highly charged chains binding with the highest avidity. Previous studies report that SLPI elutes from heparin-Sepharose at 0.7 M (16) and 0.5 M (14) NaC1, while the current study shows a lower affinity (0.26 M NaC1) between heparin and SLPI. These previous studies used Hitrap columns (Amersham Pharmacia Biotech) having a significantly higher concentration of heparin (10 mg/ml of beads) compared with a 0.75–1 mg/ml ligand density in the column used in the current study. The increased ligand density may cause an increase in both binding strength and nonspecific interactions (33).

To study the structural differences of heparin affinity-fractionated on SLPI-Sepharose, the disaccharide composition of selected heparin fractions was depolymerized using a mixture of heparin lyases I, II, and III, and analyzed by capillary electrophoresis. The disaccharide composition of heparin eluting at 0.26 M NaC1 (the peak fraction) was compared with heparin eluting at 0.58 M NaC1 (the highest affinity fraction). The disaccharide analysis of both fractions afforded a predominant peak at 7 min assigned to the trisulfated disaccharide (Fig. 2, *peak 1*), ΔUA2S(1→4)-α-D-GlcNpS6S (where ΔUA is 4-deoxy-α-L-*threo*-hexa-4-enopyranosyluronic acid) that arises from heparin's major sequence (Fig. 1). The heparin fraction with highest affinity for SLPI contained an increased amount of undersulfated disaccharides of the structure ΔUAp(1→4)-α-D-GlcNpS, ΔUAp(1→4)-α-D-GlcNpAc6S, and ΔUAp(1→4)-α-D-GlcNpAc (Fig. 2, *peaks 2–4*, respectively) with the ΔUAp arising from unsulfated iduronic or glucuronic acid residues present in heparin. The enrichment of undersulfated sequences in the heparin fraction with the highest affinity toward SLPI-Sepharose is surprising, since highly sulfated chains in heparin most often exhibit the greatest affinity for heparin binding proteins (5). The enrichment of chains containing undersulfated sequences suggests some degree of specificity in the SLPI-

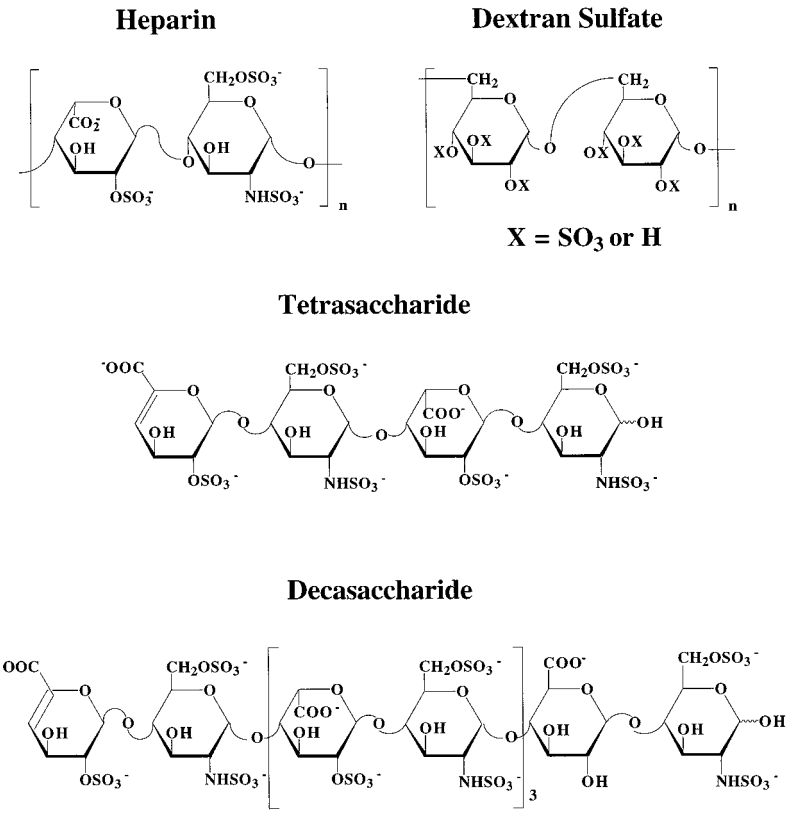

FIG. 1. **Structure of the major repeat units of the polysaccharides and oligosaccharides used in this study.**

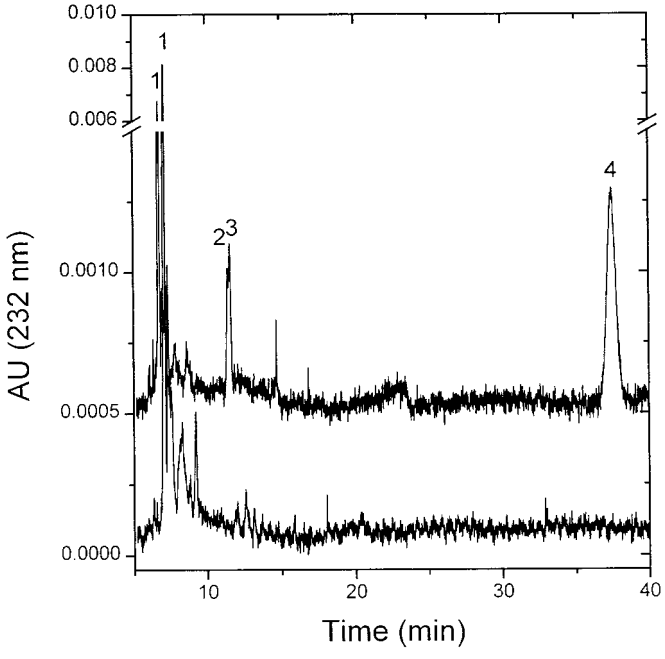

FIG. 2. **Disaccharide analysis of affinity-fractionated heparin following heparin lyase digestion using capillary electrophoresis.** The lower electropherogram corresponds to the fraction having average affinity (peak fraction; and the top electropherogram corresponds to the highest affinity fraction. Peaks are labeled: *1*, ΔUAp2S(1→4)-α-D-GlcNpS6S;*2,* ΔUAp(1→4)-α-D-GlcNpS; *3*, ΔUAp(1→4)-α-D-GlcNpAc6S; and *4*, ΔUAp(1→4)-D-GlcNpAcα,β.

heparin interaction. It is also possible that the less highly sulfated glycosaminoglycan heparan sulfate may be an endogenously important ligand for SLPI. Furthermore, the additional hydroxyl groups present in undersulfated iduronic or glucuronic acid residues enriched in the heparin, having the highest affinity for SLPI, suggest that the SLPI-heparin interaction may involve a hydrogen-bonding component. This is supported by the observation of Faller *et al.* (16). that the SLPI-heparin interaction is not purely ionic.

Heparin-derived oligosaccharide mixtures, prepared through the partial digestion of heparin with heparin lyase I, were next used to better understand the structural requirements for SLPI binding to heparin. An oligosaccharide mixture, comprised primarily of hexasaccharides (~80%) but containing small amounts of di-, tetra-, octa-, and decasaccharides, was initially used for these experiments. This mixture was fractionated on SLPI-Sepharose (Fig. 3, *A* and *B*) and analyzed by gradient polyacrylamide gel electrophoresis (PAGE) (Fig. 3*C*), a method that fractionates oligosaccharides based on their molecular size (34). In the first affinity fractionation experiment (Fig. 3*A*), the SLPI-Sepharose column was deliberately underloaded, resulting in the binding of the entire sample and its release with NaC1 (from 0.05 to 0.96 M, peak at 0.26 M). Fractions eluting in the early, middle, and late portions of the peak were analyzed by gradient PAGE (Fig. 3*C*, *lanes a–c*, respectively). Oligosaccharides ranging in size from disaccharide through octasaccharide bound to SLPI-Sepharose. The sample eluting at the highest salt concentration (Fig. 3*C, lane c*) was enriched in larger oligosaccharides and disenriched in disaccharide (degree of polymerization (dp) 2) and tetrasaccharide (dp 4). Of particular note is the enrichment of an undersulfated hexasaccharide in the higher affinity fractions (Fig. 3*C*, *lanes b* and *c*) migrating just below the fully saturated hexasaccharide standard. Next, the SLPI-Sepharose column was overloaded with the same oligosaccharide mixture (Fig. 3*B*). The affinity chromatogram showed two peaks, unbound oligosaccharides eluting in the column wash (1–11 ml) and SLPI-bound oligosaccharides eluting again at 0.26 M NaC1. Gradient PAGE analysis of the nonbinding oligosaccharides eluting in the wash (not shown) contained all of the oligosaccharides present in the original sample. Oligosaccharides eluting in the early middle and late portion of the peak were collected and analyzed (Fig. 3*D*, *lanes*

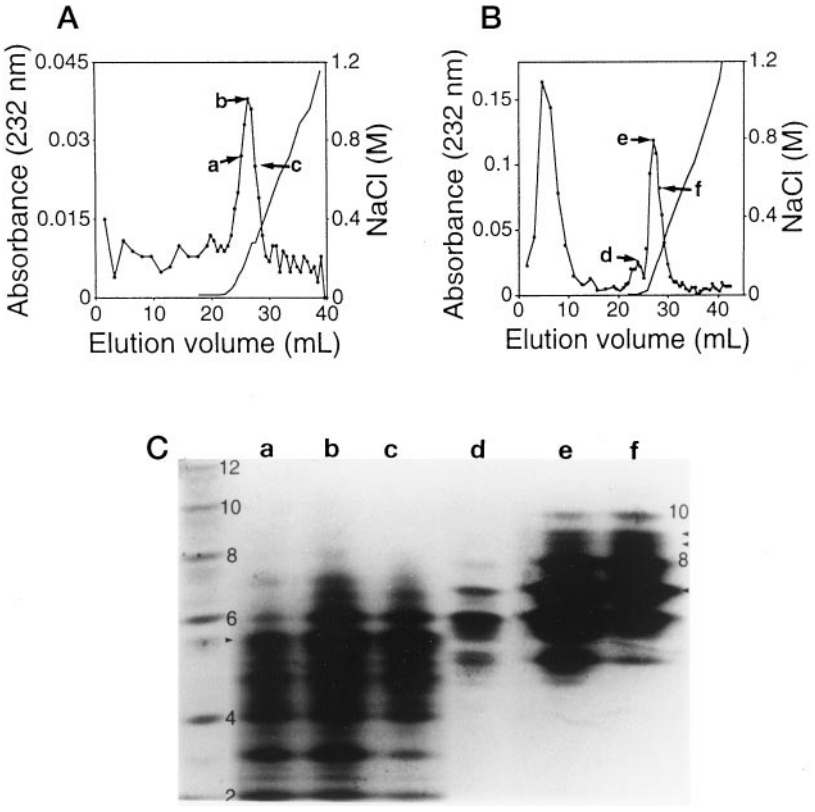

FIG. 3. **Gradient PAGE analysis of SLPI-Sepharose fractionated heparin-derived sized oligosaccharide mixture.** *A,* affinity chromatography of 50 μg oligosaccharide mixture (average dp 6) on SLPI-Sepharose. *B*, affinity chromatography of 500 μg of oligosaccharide mixture (average dp 6) on SLPI-Sepharose. *C*, gradient PAGE analysis of *fractions a–c* prepared in *A* and *fractions d–f* prepared in *B*. On the *far left* are fully sulfated oligosaccharide standards of the structure ΔUAp2S (1→[4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp2S(1→]$_n$4)-α-D-GlcNpS6S are shown: dp 2 (disaccharide, $n = 0$), dp 4 (tetrasaccharide, $n = 1$), dp 6 (hexasaccharide, $n = 2$), dp 8 (octasaccharide, $n = 3$), and dp 10 (decasaccharide, $n = 4$) (13).

d–f). The interacting fractions contained oligosaccharides of dp ≥ 6, with both di- and tetrasaccharides conspicuously absent in the PAGE analyses. The larger oligosaccharides (dp ≥ 6) apparently displace the weaker binding smaller oligosaccharides (dp 2–4) from the SLPI-Sepharose column. It was also clear that largest oligosaccharides in the mixture (dp 8 and 10) are enriched in the later fractions (Fig. 3*D, lanes e and f*). Finally, it is interesting that the octasaccharide and decasaccharide most enriched in the high affinity fractions (Fig. 3*D, lanes e* and *f*) migrate just below the fully sulfated octa- and decasaccharide standards, suggesting that they contain a slightly lower level of sulfation.

A higher molecular weight, heparin-derived oligosaccharide mixture (average dp 16, containing dp 12–26) was next loaded on the SLPI-Sepharose column. The affinity chromatogram again showed two peaks, one in the column wash (the result of overloading the column) and a second peak (from 0.05 to 1 M, peak at 0.32 M NaC1) of high affinity oligosaccharides (Fig. 4*A*). Gradient PAGE analysis again showed a trend with the larger oligosaccharides eluting in fractions collected at higher salt concentrations (Fig. 4*B, lanes a–c*). No interaction was observed below dp 14, suggesting that this represents the minimum sequence within heparin for optimal interaction with SLPI. Again, the most interesting observation is that heparin oligosaccharides binding the tightest to SLPI migrate just below the intense bands corresponding to the fully sulfated oligosaccharides in the standard heparin oligosaccharide mixture. The enriched oligosaccharides are again undersulfated, thus supporting the data obtained from the disaccharide analysis showing that the heparin fractions binding most tightly to SLPI are enriched in unsulfated iduronic and glucuronic acid residues (Fig. 2).

To further understand the structural requirements for oligosaccharide interaction with SLPI, selected members of a small collection of purified heparin oligosaccharides previously prepared in our laboratory were next examined. A fully sulfated tetrasaccharide ΔUAp2S(1→4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S (Fig. 1) was the smallest interacting oligosaccharide (eluting with 0.15 M NaC1). An undersulfated decasaccharide, having the structure ΔUAp2S(1→4)-α-D-Glc-

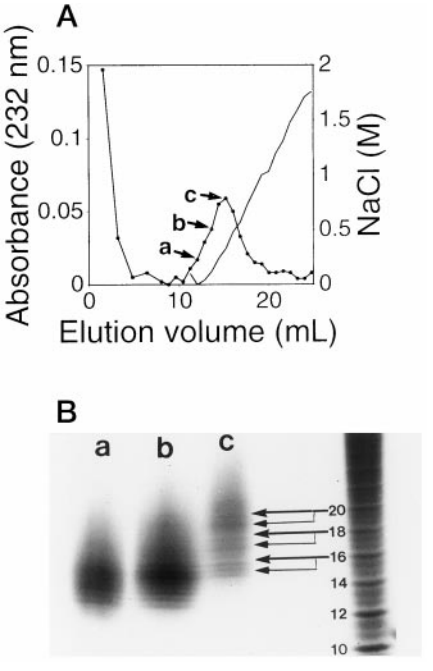

FIG. 4. **Gradient PAGE analysis of SLPI-Sepharose fractionated heparin-derived oligosaccharide mixture (average dp 16).** *A,* affinity chromatography of 1-mg-sized oligosaccharide mixture on SLPI-Sepharose. *B*, gradient PAGE analysis of *fractions a–c* prepared in *A*. Heparin partially (30%) depolymerized with heparin lyase I affords an oligosaccharide mixture that serves as standards (34) (*lane* on for *right*). The bands staining darkest in the standard ladder correspond to the fully sulfated heparin oligosaccharides (see Fig. 3 legend for structures, where $n = 1–18$, dp 4–20) based on co-migration studies using structurally defined oligosaccharide standards (34).

TABLE II
Effects of polysaccharides on kinetic constants for inhibition of proteases by SLPI

Protease	Polysaccharide	K_i	k_a	Ratio of k_a with and without polysaccharide
		nM	$M^{-1}\cdot s^{-1} \times 10^7$	
Human neutrophil elastase	Heparin	0.10 ± 0.05	2.7 ± 0.2	5
	Decasaccharide	0.14 ± 0.04	3.2 ± 1.0	5
	Tetrasaccharide	0.13 ± 0.05	0.4 ± 0.3	1
	None (control)	0.21 ± 0.08	0.6 ± 0.7	1
Human neutrophil cathepsin	Heparin	11 ± 0.9	0.42 ± 0.07	3
	Decasaccharide	9.0 ± 0.8	0.17 ± 0.08	1
	Tetrasaccharide	11 ± 0.6	0.21 ± 0.02	1
	None (control)	9.1 ± 0.5	0.15 ± 0.01	1
Bovine pancreatic chymoltrypsin	Heparin	0.17 ± 0.10	0.74 ± 0.05	5
	Decasaccharide	0.17 ± 0.10	0.79 ± 0.19	5
	Tetrasaccharide	0.25 ± 0.10	0.21 ± 0.02	1
	None (control)	0.18 ± 0.10	0.16 ± 0.01	1

NpS6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpS6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNpS6S (where GlcAp is glucopyranosyluronic acid) (Fig. 1), was identified that bound tightly (~0.3 M) to SLPI. The unsulfated glucuronic acid residue, found in this decasaccharide, is probably the same residue enriched in SLPI affinity fractionated heparin giving rise to the ΔUAp containing disaccharides (Fig. 2).

ITC affords useful data on the thermodynamics of binding including ΔH, K_a and n (the number of ligand interactions per mol of macromolecule) (17). The interaction of the fully sulfated, heparin-derived tetrasaccharide (Fig. 1) with SLPI was first examined by ITC. This tetrasaccharide bound weakly, affording a K_d of 197 μM, ΔH of −2.7 kcal/mole and an n of 3 (3 mol of tetrasaccharide/mol of SLPI). These data suggested that the heparin binding domain might be a dodeca- to tetradecasaccharide (dp 12–14) in size. SLPI interaction with LMW heparin (M_r(avg) 5,000, corresponding to dp ~15) had been previously examined using intrinsic fluorescence spectroscopy and a K_d of 0.5 μM in similar ionic strength buffer (50 mM Hepes, 150 mM NaC1, pH 7.4) (16), and a 1:1 binding stoichiometry was reported for this interaction (1). ITC was used to explore this interaction in more detail. Titrations of LMW heparin (M_r(avg) 4,800) into SLPI was not possible, due to precipitation of SLPI. In the reverse experiment, titrating SLPI into LMW heparin, no precipitation was observed. The fitted heats of interaction yielded a K_d of 2.4 nM, ΔH of -6.9 kcal/mol and an n of 1. The binding of the undersulfated decasaccharide (Fig. 1) to SLPI was next examined by ITC. This decasaccharide showed tight binding with a K_d of 13 nM and displayed an n of 1.4. The ITC data suggest that a tetradecasaccharide (dp 14) represents the minimum SLPI binding domain on heparin, consistent with the minimum high affinity oligosaccharide observed in the SLPI affinity chromatography experiment (Fig. 4*B*).

Record analysis was performed using the weakly binding tetrasaccharide, which was available in sufficient quantities to perform multiple titrations. The contribution of non-ionic interaction to binding was determined using NaC1 concentrations ranging from 10 to 150 mM in 50 mM sodium phosphate buffer, pH 7.4. These data show a 40% ionic component when comparing the extrapolated ΔG (K_d 573 μM) at the y intercept (1 M NaC1) in 50 mM sodium phosphate buffer, pH 7.4, with the ΔG (K_d 4.3 μM) under physiologic conditions (150 mM NaC1 in the same buffer). The remaining non-ionic component (~60%) of the interaction probably results from hydrogen bonding of the unsubstituted hydroxyl groups present in this tetrasaccharide (Fig. 1).

Heparin is found in the granules of mast cells present in substantial quantities in the human lung tissue (6–9) where

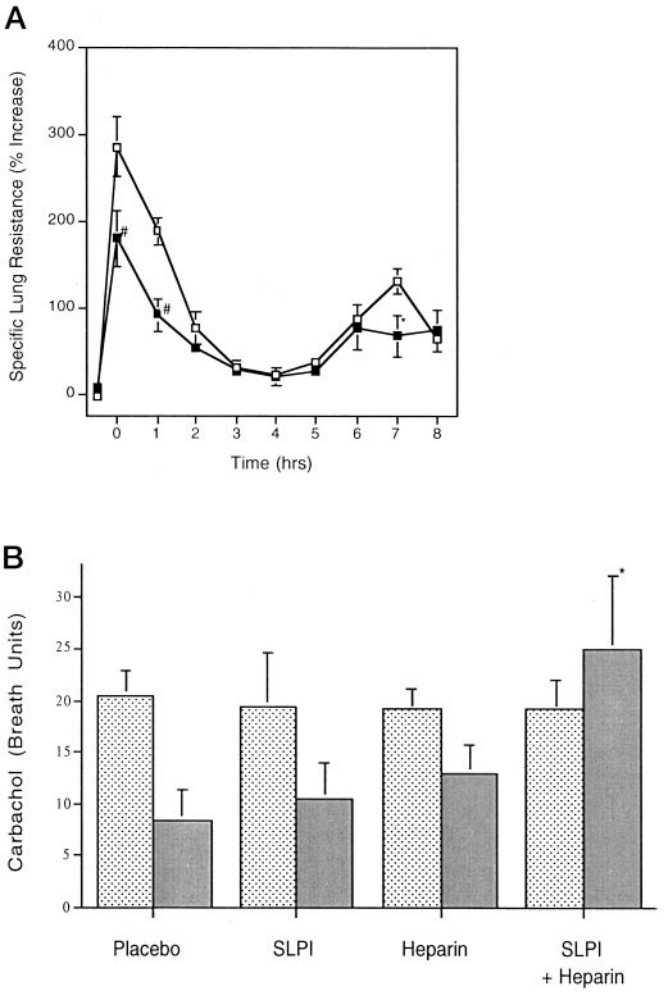

FIG. 5. **Effect of SLPI-heparin combination therapy on antigen-stimulated bronchial responses in sheep.** Animals were treated with placebo, SLPI (10 mg), heparin (16.7 mg, 2,500 IU in PBS), or SLPI (10 mg in PBS) + heparin (16.7 mg, 2,500 IU in PBS) by aerosol 30 min before antigen challenge. *A*, early and late phase bronchoconstriction are assessed as the percent increase of specific lung resistance over an 8-h period following antigen challenge (mean ± S.E., n = 3) (*, $p < 0.05$ effect of SLPI *versus* antigen-stimulated response; #, $p < 0.1$ effect of SLPI *versus* antigen-stimulated response). *Open squares* are placebo-treated with PBS and *filled squares* are treated with SLPI and heparin. *B*, airway hyperresponsiveness is assessed as the change in the carbochol dose required to induce a 400% change in airway resistance (PC_{400}) 24 h after antigen challenge (mean ± S.E., n = 3) (*, $p <$ 0.05 effect of SLPI *versus* antigen-stimulated response). Base line is shown as *dot-filled bars* and after antigen challenge as *solid bars*.

SLPI is found. Thus, the observed binding affinity between heparin and SLPI probably plays a biologically important role. Other negatively charged glycoconjugates, prevalent in the lung include heparan sulfate and mucin (35, 36). Both heparan sulfate (Table I) and mucin (35) bind to SLPI and, thus, might also be of physiologic importance. While porcine intestinal heparin binds with substantially higher affinity for SLPI than does porcine intestinal heparan sulfate (Table I), a highly sulfated heparan sulfate (10) might bind with comparable affinity.

Heparin and the heparin-derived tetrasaccharide and decasaccharide were examined for their effect on SLPI inhibition of human lung elastase as well as cathepsin and chymotrypsin (Table II). In the absence of heparin or heparin-derived oligosaccharide, SLPI is a potent (K_i ~200 pM) inhibitor of both human neutrophil elastase and bovine pancreatic chymotrypsin but considerable less inhibitory of neutrophil cathepsin G activity (K_i ~9 nM). The addition of heparin, decasaccharide or tetrasaccharide had no significant effect on the K_i of SLPI for any of these three proteases. Stopped-flow kinetics permits the measurement of the association rate (k_a) of SLPI and proteases (18). In the absence of heparin or heparin-derived oligosaccharide, the association rate of SLPI for human neutrophil elastase was five times faster than SLPI association with human neturophil cathepsin G and bovine pancreatic chymotrypsin. Taken together with the low K_i of SLPI for neutrophil elastase, this fast association rate suggests neutrophil elastase is an important protease target for this inhibitor. The k_a of SLPI for all three enzymes increased by 3–5-fold on the addition of heparin. The undersulfated decasaccharide also showed a 5-fold enhancement for the k_a of SLPI with both human neutrophil elastase and bovine pancreatic chymotrypsin but not for human neutrophil cathepsin G. The tetrasaccharide had no effect on the k_a of SLPI for any of the three proteases when compared with control. These data demonstrate that heparin, and to a lesser extent the undersulfated decasaccharide, enhance SLPI inhibition of protease by enhancing the k_a of SLPI with protease without effecting a change in the K_i. Furthermore, the effect of heparin on the k_a of the SLPI-protease interaction, particularly human lung elastase, suggests its therapeutic application to elastase based diseases of the lung such as asthma.

The effect of SLPI-heparin combination therapy against antigen-induced airway hyperresponsiveness was next evaluated in a sheep bronchoprovocation model. In saline-treated animals, antigen challenge resulted in the development of airway hyperresponsiveness, as demonstrated by the decreased amount of carbachol required to induce a 4-fold increase in specific lung resistance (Fig. 5). Aerosol treatment with SLPI (10 mg) or heparin (16.7 mg of corresponding to 2,500 units) alone had no effect on the development of hyperresponsiveness. In contrast, combination therapy with SLPI (10 mg) and heparin (16.7 mg of corresponding to 2,500 IU) provided protection against the development of hyperresponsiveness ($p < 0.05$ *versus* antigen-stimulated response). In comparison, the individual treatments had no inhibitory effect on peak early and late phase bronchoconstriction following antigen challenge, while the combination therapy inhibited the peak responses by 37 ($p < 0.1$) and 48% ($p < 0.05$), respectively. These results demonstrate that heparin increases the therapeutic activity of SLPI against antigen-induced airway hyperresponsiveness. Recombinant human-SLPI has been shown to inhibit ovine elastase (37). The identification of other ovine protease(s) inhibited by recombinant human-SLPI to promote this pharmacologic effect requires further investigation.

The sheep bronchoprovocation model has been used extensively to study the pathobiology of asthma (19). Epidemiological data suggests that patients who suffer from more severe asthma are the same ones that develop late antigen-induced response in the laboratory. Thus, the effect of SLPI-heparin combination therapy, which largely eliminates late phase bronchoconstriction in sheep, may represent a useful therapeutic approach to the treatment of severe asthma in humans.

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