Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 507 (2005) 261–271

www.elsevier.com/locate/ejphar

Effects of bemiparin on airway responses to antigen in sensitized Brown–Norway rats

Jana Suchankova[a], Manuel Mata[a], Julio Cortijo[a,b], Esteban J. Morcillo[a,*]

[a]*Department of Pharmacology, University of Valencia, Avenida Blasco Ibanez 15, E-46010 Valencia, Spain*
[b]*Research Foundation of University General Hospital, Faculty of Medicine, University of Valencia, Avenida Blasco Ibanez 15, E-46010 Valencia, Spain*

Received 4 November 2004; accepted 10 November 2004
Available online 23 December 2004

Abstract

Heparins have demonstrated activity in asthma. The effects of bemiparin, a low molecular weight heparin, were examined on antigen-induced responses in sensitized Brown–Norway rats. Inhaled bemiparin (1 mg/ml) reduced the acute bronchospasm produced by aerosol antigen, prevented airway hyperresponsiveness to 5-hydroxytryptamine postantigen exposure, and reduced the eosinophil count (from 0.205 ± 0.062 to $0.054\pm0.016\times10^6$ cells/ml in antigen and antigen+bemiparin groups, respectively; $P<0.05$), eosinophil peroxidase activity, and proteins in the bronchoalveolar lavage fluid (BALF), as well as the transiently augmented mucin Muc5ac expression. Hyperresponsiveness to adenosine was not affected by bemiparin. In similar experiments, inhaled fondaparinux (1 mg/ml) did not affect the antigen-induced responses, while a low-anticoagulant low molecular weight heparin was effective. In conclusion, bemiparin showed beneficial effects in experimental asthma, probably unrelated to its anticoagulant activity, which extends the previous positive findings obtained with other heparins.

Keywords: Experimental asthma; Brown–Norway (rat); Bemiparin; Fondaparinux

1. Introduction

Asthma is a chronic inflammatory disease characterized by reversible bronchial obstruction and airways hyperresponsiveness, eosinophil accumulation, and plasma exudation. The mast cell is an abundant inflammatory cell present in the airways in asthma, which is activated by cross-linking of immunoglobulin E, to cause degranulation, with the subsequent release of inflammatory mediators, such as histamine, prostaglandin D_2, leukotrienes, cytokines, and platelet-activating factor (Page and Minshall, 1993). Human lung mast cells also release heparin following allergen exposure (Green et al., 1993). The simultaneous release of heparin and inflammatory mediators from the airway mast cell suggests that heparin, further to its anticoagulant properties, may act as a regulatory mechanism since it inhibits mast cell degranulation and has a wide spectrum of antiinflammatory properties (Lever and Page, 2001). It is plausible, therefore, that the administration of heparin prior to allergen challenge may prevent the degranulation of the mast cells and thus inhibit the release of mediators, which may be responsible, at least in part, for the development of airway bronchoconstriction, hyperresponsiveness, and inflammation. There is clinical and experimental evidence that unfractioned and low molecular weight heparins may be beneficial in asthma (Diamant and Page, 2000).

Although unfractionated heparin and various low molecular weight heparins have been examined in experimental models of allergic asthma (Ahmed et al., 1992, 1997, 2000; Howell and Woeppel, 1993; Seeds et al., 1993, 1995; Martinez-Salas et al., 1998; Campo et al., 1999; Preuss and Page, 2000; Wang et al., 2000; Seeds

* Corresponding author. Tel.: +34 96 3864623; fax: +34 96 3864622.
E-mail address: Esteban.Morcillo@uv.es (E.J. Morcillo).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.11.014

and Page, 2001; Yahata et al., 2002), bemiparin, a new second-generation, low molecular weight heparin in clinical use for venous thromboembolism (Chapman and Goa, 2003; Planes 2003), has not been studied in asthma models. The average molecular weight of bemiparin, 3.6 kDa, is lower than the mean molecular weights of other low molecular weight heparins (Chapman and Goa, 2003; Planes, 2003). Since it has been reported that the lower the molecular weight of heparins, the greater their effect on immediate and late antigen responses in experimental asthma (Martinez-Salas et al., 1998; Molinari et al., 1998; Campo et al., 1999; Ahmed et al., 2000), we were interested in extending the available information on the antiasthma effects of low molecular weight heparins by examining also the effects of bemiparin. In addition, to avoid repetition of experimental animal models in which low molecular weight heparins had been already tested (sheep, rabbit, and guinea pig), we selected the actively sensitized Brown–Norway rat, a widely used model in which the effects of heparins have not been yet examined, to our knowledge.

The antiinflammatory and antiallergic effects of heparins appear elicited by mechanisms unrelated to their anticoagulant activity. This has been previously ascertained by the use of heparin derivatives lacking anticoagulant activity (Seeds and Page, 2001). Therefore, a low-anticoagulant fraction of heparin (compound 1FMF-52/3) with a molecular weight close to that of bemiparin was included for comparison in this study. In addition, experimental evidence in vitro suggests that a minimum change length exists for heparin to be able to exert antiinflammatory effects (Tones et al., 1989; Lever et al., 2003). Fondaparinux is a synthetic pentasaccharide that replicates the sequence of the site of heparin that binds to antithrombin III, thus producing a specific antifactor Xa activity clinically useful for the prevention of venous thromboembolism (Bauer et al., 2002; Samama and Gerotziafas, 2003). Therefrom, we studied fondaparinux to test whether heparin-like molecules of as small as five saccharides in length possess antiinflammatory effects in the sensitized rat model of asthma.

Therefore, the objective of the present work was to compare the effects of inhaled bemiparin and fondaparinux on the airway constriction and microvascular leakage immediately after antigen challenge, as well as on the airways hyperresponsiveness to direct (5-hydroxytryptamine; 5-HT) and indirect (adenosine) bronchoconstrictors (Hannon et al., 2001) and on the eosinophilia and protein extravasation observed after antigen provocation in actively sensitized Brown–Norway rats. The effect of compound 1FMF-52/3 on antigen-induced hyperresponsiveness and airway inflammation was also studied. In addition, the effect of bemiparin and fondaparinux on the antigen-induced expression of mucin Muc5ac was also examined in this experimental model of allergic asthma.

2. Methods

2.1. Animals

Male Brown–Norway rats (Harlan Interfauna Iberica SL, Barcelona, Spain) weighing 200–300 g were used for this study. The rats were housed four to a cage, in standard cages, and were fed on standard lab chow (Panlab, Barcelona, Spain) with drinking water freely available. They were kept in a room maintained at 22±2 °C, 55±5% humidity, and 12-h light–dark cycle in the Central Research Unit of the Faculty of Medicine. The protocol and experimental design of this study were approved by the local Ethics Committee and comply with the Regulations on Animal Care established by the European Community and by the Spanish and Regional Governments.

2.2. Drugs

The following drugs were used: Bemiparin (sodium salt from porcine intestinal mucosa, average mol. wt. of 3.6 kDa; anti-Xa activity 95 IU/mg) and compound 1FMF-52/3 (heparin fraction of average mol. wt. of 3.5 kDa; anti-Xa activity 3 IU/mg) were from Rovi Laboratory (Madrid, Spain); fondaparinux sodium (mol. wt. of 1.73 kDa; anti-Xa activity 639 IU/mg) was from Sanofi-Synthelabo (Toulouse, France); adenosine hemisulphate was from Sigma–Aldrich Química (Madrid, Spain). Other drugs were from sources previously stated (Cortijo et al., 2001). Water purified on a Milli-Q (Millipore Iberica, Madrid, Spain) system was used throughout. Bemiparin and fondaparinux were dissolved in saline immediately before use.

2.3. Sensitization procedure

Animals were actively sensitized by an intraperitoneal injection of 1 ml of a suspension of 1 mg ovalbumin and 100 mg of aluminium hydroxide [$Al(OH)_3$] in 0.9% (wt./vol.) saline for three consecutive days. The sensitized animals were used for the experiments 21 days after the initial intraperitoneal injection. This procedure has previously been shown to result in the development of immunoglobulin E-type antibody (Elwood et al., 1992).

2.4. Experimental groups

Animals were randomly distributed into the experimental groups indicated in Table 1. Treatments (vehicle or drugs) were administered for 15 min and given 1 h before the antigen challenge. These experimental groups were studied for airway responses at 3 (hyperresponsiveness to adenosine) and at 24 h (hyperresponsiveness to 5-HT). Aerosols were generated by a DeVilbiss ultrasonic nebulizer. Inhalation route was selected as a usual way of drug administration in the clinical setting. The dose level and timing of the administration were based on previous studies with other

Table 1
Summary of the different experimental groups in this study

Experimental groups		Pretreatment	Aerosol exposure
Untreated controls	Negative control	Drug vehicle	Saline
	Positive control	Drug vehicle	Antigen
Drug treated	Bemiparin	Bemiparin (1 mg ml^{-1})	Saline
		Bemiparin (1 mg ml^{-1})	Antigen
		Bemiparin (5 mg ml^{-1})	Saline
		Bemiparin (5 mg ml^{-1})	Antigen
	Fondaparinux	Fondaparinux (1 mg ml^{-1})	Saline
		Fondaparinux (1 mg ml^{-1})	Antigen
	Compound 1FMF-52/3	Compound 1FMF-52/3 (1 mg ml^{-1})	Saline
		Compound 1FMF-52/3 (1 mg ml^{-1})	Antigen

low molecular weight heparins (Seeds and Page, 2001; Yahata et al., 2002).

2.5. Animal instrumentation

The animals were anæsthetised and instrumented as previously outlined (Advenier et al., 1979), with modifications (Cortijo et al., 2001). In brief, the trachea was cannulated for mechanical ventilation with room air by means of an Ugo Basile ventilator at a rate of 90 breaths min^{-1}, with a stroke volume of 1 ml 100 g^{-1} body weight. A heated (37 °C) pneumotachograph (Fleish 000) was positioned in the ventilator circuit to measure the inspiratory and expiratory flow rates. A side arm from the tracheal cannula was attached to the positive port of a differential pressure transducer (Celesco model LCVR) and used to measure pulmonary inflation pressure, while the negative port of the transducer was attached to a cannula inserted into the intrapleural space, to measure intrathoracic pressure. The difference between these two pressures is the transpulmonary pressure. Arterial blood pressure was measured by a transducer (Spectramed Statham P23XL) connected to a saline-filled cannula inserted into the carotid artery. Body temperature was maintained at 37±0.5 °C by a heated blanket. Signals for airflow, transpulmonary pressure, and arterial blood pressure were amplified (PMS 800, Mumed, London, U.K.) and fed via an analogue to digital converter to a personal computer. Lung resistance (R_L; cm H_2O ml^{-1} s^{-1}) and dynamic compliance (C_{dyn}; ml cm H_2O^{-1}) were calculated by an online respiratory analyser (PMS software version 5.2; Mumed). Changes in airway responsiveness following drug administration or antigen challenge are expressed as the maximum percentage change in R_L and C_{dyn} from baseline.

2.6. Assessment of the immediate airway constriction following antigen challenge

After 10 min stabilisation, anaesthetized and instrumented animals were challenged with inhaled antigen (100 mg ml^{-1}, 5 min) delivered to the animal via the tracheal cannula, and changes in lung resistance were monitored for 15 min. The dose of antigen was selected from Olivenstein et al. (1997). Animals pretreated with aerosol drug vehicles and then receiving antigen or its vehicle were used as controls.

2.7. Assessment of airway hyperresponsiveness and of cell counts, eosinophil peroxidase activity, and proteins in bronchoalveolar lavage

Sensitized conscious rats were exposed to antigen aerosol in a clear plastic chamber (approximate volume=4 l), which was connected to the output of an ultrasonic nebuliser (Ultra-Neb 99; DeVilbiss Health Care, Heston, Middlesex, U.K.). The nebuliser chamber was filled with an ovalbumin (1% in saline) or saline solution. Nebuliser output was 10 ml h^{-1}. The duration of the antigen challenge was 15 min. The time course of airway hyperresponsiveness in antigen-exposed Brown–Norway rats has been previously examined (Elwood et al., 1992; Hannon et al., 2001), and the response at 3 h for adenosine, and at 24 h for 5-HT, was selected on this basis. Thus, 24 h after exposure to the aerosol, airway reactivity was determined from the dose–response curves to 5-HT, administered intravenously (5–50 μg kg^{-1}) to animals anaesthetized and instrumented as indicated above. 5-HT has been used to assess airway hyperresponsiveness in other studies in rats since it produces a direct and reproducible bronchoconstrictor response (Pauwels et al., 1990; Carvalho et al., 1999). In additional experiments, the airway hyperresponsiveness to adenosine (1 mg kg^{-1}, intravenous) and the airway responses to 5-HT (5–50 μg kg^{-1}, intravenous) were measured at 3 h post antigen exposure, as previously outlined (Hannon et al., 2001).

After the measurement of airway responsiveness, animals were sacrificed with an overdose of thiopental sodium. Bronchoalveolar cells were collected in two successive lavages using 6-ml aliquots of sterile saline, at room temperature, injected and recovered through a polyethylene tracheal cannula. Cell suspensions were concentrated by low-speed centrifugation, and the cell pellet was resuspended. Total cell counts were made in a haemocytometer. Differential cell counts were determined from cytospin preparations by counting 300 cells stained with May–Grünwald–Giemsa. Because the yield of the injected fluid was equivalent in all experimental groups (≥85%), the results are expressed as absolute cell counts per milliliter of lavage fluid. The cell-free supernatant was used for biochemical determinations. The total protein concentration was measured by a standard technique (BCA protein assay reagent kit; Pierce, Rockford, USA). The levels of free eosinophil peroxidase in the supernatant from the bronchoalveolar lavage fluid (BALF) was determined as a marker of eosinophil activation, according to the method of Strath et al. (1985), with modifications (Pons et al., 2000), and the results are expressed as the optical density

Table 2
Primers and probes for real-time quantitative RT–PCR

Gene	Primers and probes	Sequence	Product size (bp)	Genbank
MUC5AC	Forward	5′-CTGCCACATGTTGGACTTGG-3′	102	U83139
	Reverse	5′-TTGGTATGGCTTCTCGAGGG-3′		
	TaqMan probe	5′-CATCACTATGTGCAGCCCAAGGCG-3′		
GAPDH	Forward	5′-CCTGGAGAAACCTGCCAAGTATG-3′	103	NM017008
	Reverse	5′-ACAACCTGGTCCTCAGTGTAGCC-3′		
	TaqMan probe	5′-CAAGAAGGTGGTGAAGCAGGCGGC-3′		

bp: Base pairs.
For Muc5ac, a reverse transcription of RNA to generate cDNA was done using Taqman RT reagents (N808-0234, PE Biosystems). The specificity of the PCR primers was tested under normal PCR conditions, and the products of the reaction were electrophoresed into a 2.5% Nusieve GTG agarose (BMA, Rockland, ME) gel. One single band with the expected molecular size was observed for Muc5ac and GAPDH. For the validation of the $\Delta\Delta C_t$ method, the C_t values for target and reference genes were measured at different input amounts of total RNA (25–3000 ng); then, ΔC_t values (target vs. reference) were plotted against log total RNA, and the absolute value of the slope was found to be less than 0.1 (not shown), thus indicating a similar efficiency of the two systems.

measured at 490 nm in a Microplate Autoreader (EL309, Bio-Tek Instruments).

2.8. Determination of mucin Muc5ac expression

The mucin Muc5ac mRNA transcript was measured using a real-time quantitative RT–PCR as previously described (Blesa et al., 2003; Mata et al., 2003). The method used for obtaining the quantitative data of relative gene expression is the comparative C_t method ($\Delta\Delta C_t$ method), as described by the manufacturer (PE-ABI PRISM 7700 Sequence Detection System). Glyceraldehide 3-phosphate dehydrogenase (GAPDH) was chosen as the endogenous control gene. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The PCR primers for rat MUC5AC and rat GAPDH were designed using the Primer Express (PE Biosystems, Morrisville, NC) according to the published rat MUC5AC and GAPDH cDNA sequences (Table 2).

2.9. Statistical analysis

Data are presented as mean±S.E.M. Statistical analyses of the results were carried out by analysis of variance (ANOVA), followed by appropriate post hoc tests, including Bonferroni correction and Student's *t*-test (GraphPad Software, San Diego, CA, USA). Significance was accepted when $P<0.05$.

3. Results

3.1. Effects of bemiparin and fondaparinux on the immediate airway constriction in response to antigen exposure

No statistically significant difference was found for the baseline values of pulmonary resistance and dynamic compliance between the experimental groups (not shown). Saline challenge in the negative control group did not significantly alter the pulmonary resistance, but the challenge of sensitized untreated animals with aerosol antigen (positive control) provoked an acute rise in lung resistance, with a peak in about 2–3 min (153±13%; n=5). Inhaled bemiparin (1 mg ml^{-1}) inhibited the antigen-induced bronchoconstriction (the increase from baseline amounted 114±8%, n=5; $P<0.05$ from positive control). Inhaled fondaparinux (1 mg ml^{-1}) did not alter the antigen-induced augmentation of pulmonary resistance (152±10%, n=5; $P>0.05$ from positive control). The decreases in dynamic compliance after antigen challenge (49±5% in the positive control) were attenuated in bemiparin-treated animals (34±5%; $P<0.05$ from positive control) but remained unaffected by fondaparinux (50±4%). Neither bemiparin nor fondaparinux altered the pulmonary resistance and dynamic compliance values in saline-challenged rats (not shown). No significant changes in arterial blood pressure were observed in bemiparin- and fondaparinux-treated animals compared with the controls (data not shown).

3.2. Effects of bemiparin, fondaparinux, and compound 1FMF-52/3 on airway responses at 24-h postantigen challenge

The intravenous administration of 5-HT (5–50 μg kg^{-1}) 24 h after saline (negative control) or antigen (positive control) exposure resulted in a dose-dependent increase of pulmonary resistance and decrease in dynamic compliance in sensitized, untreated rats. Statistical difference between groups, i.e., 'hyperresponsiveness', was reached from 10 to 50 μg kg^{-1} (Fig. 1). When the dose–response curve to 5-HT was reproduced in animals challenged with antigen but pretreated with inhaled bemiparin (1 or 5 mg ml^{-1}), a decreased responsiveness (i.e., an anti-hyperresponsiveness effect) was apparent. A similar decreased responsiveness was found for compound 1FMF-52/3 (1 mg ml^{-1}; Fig. 1). Inhaled fondaparinux (1 mg ml^{-1}) did not alter the 5-HT airway responses in antigen-challenged animals (Fig. 1). Treatment with bemiparin (1 or 5 mg ml^{-1}), compound 1FMF-52/3 (1 mg ml^{-1}), or fondaparinux (1 mg ml^{-1}) did not modify the 5-HT dose–response curve obtained in sensitized rats challenged with saline (not shown). No significant changes in arterial blood pressure were observed

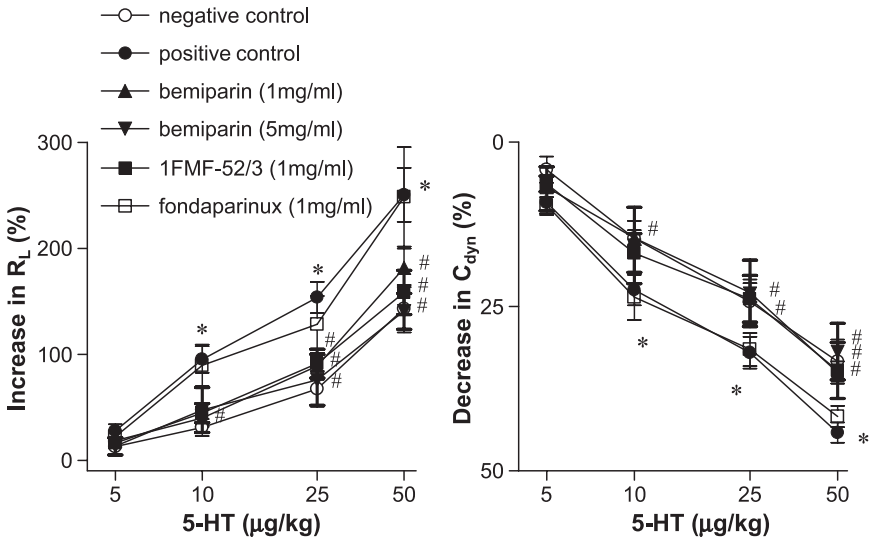

Fig. 1. Dose–response curves for intravenous 5-hydroxytryptamine (5-HT) were obtained in Brown–Norway rats previously (24 h before) exposed to aerosol saline (negative control) or antigen (1%, 15 min). The antigen-challenged animals received, by inhalation, drug vehicle (positive control), bemiparin (1 or 5 mg ml^{-1} for 15 min, 1 h prechallenge), compound 1FMF-52/3 (1 mg ml^{-1} for 15 min, 1 h prechallenge), or fondaparinux (1 mg ml^{-1} for 15 min, 1 h prechallenge) as indicated. The increase in pulmonary resistance (R_L; left panel) and decrease in dynamic compliance (C_{dyn}; right panel) are presented as percent change from baseline values. The airway hyperresponsiveness to 5-HT observed in antigen-challenged sensitized rats was prevented by bemiparin, but not after fondaparinux. Points are mean±S.E.M. of 8–10 animals for each group; *P<0.05 from the negative control; $^{\#}P$<0.05 from the positive control.

in bemiparin-, compound 1FMF-52/3-, and fondaparinux-treated animals compared with the controls (not shown).

Bronchoalveolar lavage was carried out 24 h after saline or antigen exposure of sensitized rats. In rats exposed to antigen, there was a marked increase in the total number of cells in BALF. This increase in total cell number was not significantly modified in treated animals (Fig. 2). The differential cell count showed an increase of each of the different cell types present in the BALF of the antigen-challenged rats compared with that of the saline-exposed animals. The increase in the eosinophil number was reduced by bemiparin and compound 1FMF-52/3, while no significant inhibitory effect was produced on the rest of cell types. Fondaparinux did not alter cell numbers in the BALF (Fig. 2).

There was also a significant increase in eosinophil peroxidase in BALF supernatant recovered from immunized rats challenged with antigen compared with saline-chal-

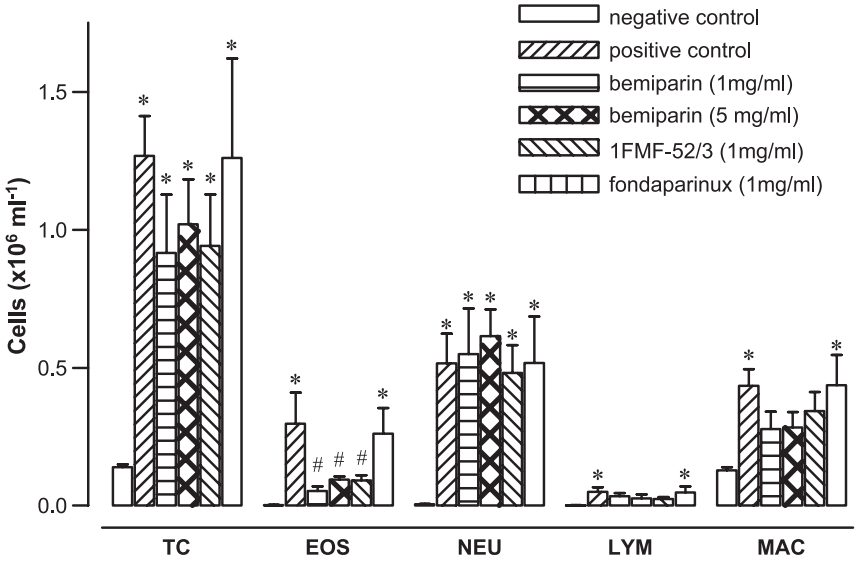

Fig. 2. The total cell (TC), eosinophil (EOS), neutrophil (NEU), lymphocyte (LYM), and macrophage (MAC) numbers in bronchoalveolar lavage fluid are shown for sensitized Brown–Norway rats previously (24 h before) exposed to aerosol saline (negative control) or antigen (1%, 15 min). The antigen-challenged animals received, by inhalation, drug vehicle (positive control), bemiparin (1 or 5 mg ml^{-1} for 15 min, 1 h prechallenge), compound 1FMF-52/3 (1 mg ml^{-1} for 15 min, 1 h prechallenge), or fondaparinux (1 mg ml^{-1} for 15 min, 1 h prechallenge), as indicated. Inhaled bemiparin, but not fondaparinux, inhibited the augmented eosinophils in the bronchoalveolar lavage fluid of antigen-challenged sensitized Brown–Norway rats. Columns are mean±S.E.M. of 8–10 animals for each group; *P<0.05 from the negative control; $^{\#}P$<0.05 from the positive control.

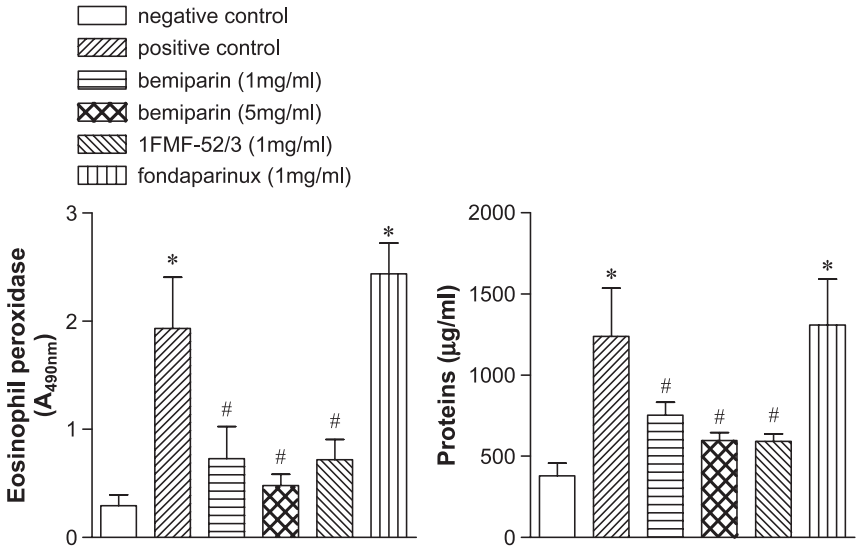

Fig. 3. Eosinophil peroxidase activity and proteins were determined in supernatants of bronchoalveolar lavage fluid of sensitized Brown–Norway rats previously (24 h before) exposed to aerosol saline (negative control) or antigen (1%, 15 min). The antigen-challenged animals received, by inhalation, drug vehicle (positive control), bemiparin (1 or 5 mg ml^{-1} for 15 min, 1 h prechallenge), compound 1FMF-52/3 (1 mg ml^{-1} for 15 min, 1 h prechallenge), or fondaparinux (1 mg ml^{-1} for 15 min, 1 h prechallenge) as indicated. Eosinophil peroxidase activity is shown as absorbance values at 490 nm (A_{490nm}), and proteins, as µg ml^{-1}. Inhaled bemiparin, but not fondaparinux, inhibited the augmented eosinophil peroxidase activity (left panel) and protein (right panel) in antigen-challenged sensitized Brown–Norway rats. Columns are mean±S.E.M. of 8–10 animals for each group; *P<0.05 from the negative control; $^{\#}P$<0.05 from the positive control.

lenged rats (Fig. 3). In sensitized animals challenged with antigen but pretreated with bemiparin (1 or 5 mg ml^{-1}) or compound 1FMF-52/3 (1 mg ml^{-1}), the eosinophil peroxidase values were significantly diminished, while no decrease was observed in fondaparinux-treated (1 mg ml^{-1}) rats (Fig. 3).

Proteins in the BALF were also augmented in antigen-exposed rats compared with rats challenged with saline (Fig. 3), thus indicating airway microvascular leakage. Inhaled bemiparin (1 or 5 mg ml^{-1}) or compound 1FMF-52/3 (1 mg ml^{-1}) attenuated the antigen-induced increase of BALF proteins, and fondaparinux (1 mg ml^{-1}) was not effective (Fig. 3).

3.3. Effects of bemiparin and fondaparinux on airway responses at 3 h postantigen challenge

The dose–response curves to 5-HT (5–50 µg kg^{-1}) constructed at 3 h postchallenge did not significantly differ between saline- and antigen-exposed rats, and pretreatment

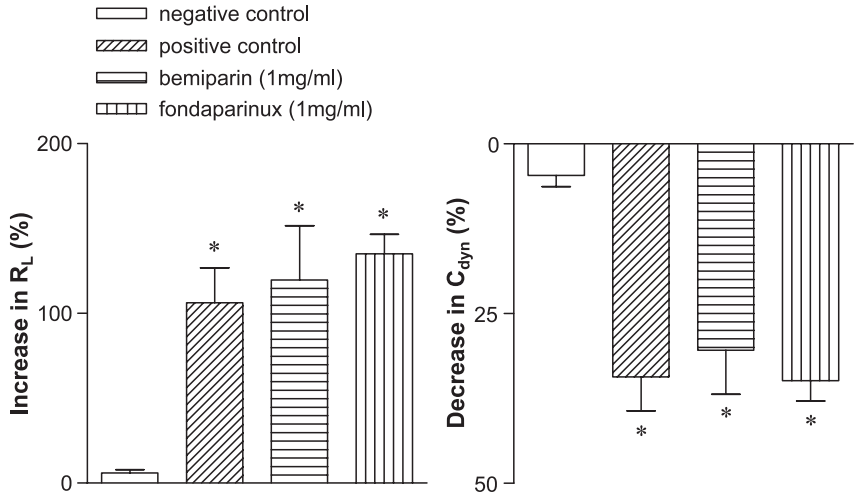

Fig. 4. Adenosine (1 mg kg^{-1}) was given intravenously to sensitized Brown–Norway rats previously (3 h before) exposed to aerosol saline (negative control) or antigen (1%, 15 min). The antigen-challenged animals received, by inhalation, drug vehicle (positive control), bemiparin, or fondaparinux (each at 1 mg ml^{-1} for 15 min, 1 h prechallenge) as indicated. The increase in pulmonary resistance (R_L; left panel) and decrease in dynamic compliance (C_{dyn}; right panel) are presented as percent change from baseline values. Neither bemiparin nor fondaparinux altered the airway hyperresponsiveness to adenosine in rats. Points are mean±S.E.M. of five animals for each group; *P<0.05 from the negative control.

with inhaled bemiparin or fondaparinux did not significantly alter 5-HT airway responses (not shown). By contrast, at this time point, a significant augmentation of the airway response to adenosine (1 mg kg^{-1}) was noted (Fig. 4). This hyperresponsiveness to adenosine was not decreased in animals pretreated with inhaled bemiparin or fondaparinux (Fig. 4).

No significant changes in total and differential cell counts in BALF were observed at 3 h postantigen in either untreated rats or in rats treated with bemiparin or fondaparinux (Fig. 5). Eosinophil peroxidase activity was not significantly augmented at this time point, but proteins in BALF were increased; this change was not modified by bemiparin or fondaparinux (Fig. 5).

3.4. Effects of bemiparin and fondaparinux on mucin Muc5ac expression

A significant, near twofold, increase in Muc5ac expression in the antigen-exposed group of untreated animals was observed at 3 h postchallenge. This enhancement was transient since, at 24 h, the expression had decreased to values below control levels. The antigen-induced augmentation of Muc5ac expression was attenuated in the bemiparin-treated group but not in rats receiving fondaparinux (Fig. 6).

4. Discussion

Heparin and heparin-derived oligosaccharides have been reported to reduce antigen-induced airway responses in experimental models of allergic asthma in sheep (Ahmed et al., 1992, 1997, 2000; Martinez-Salas et al., 1998; Molinari et al., 1998; Campo et al., 1999), rabbit (Preuss and Page, 2000), and guinea pig (Howell and Woeppel, 1993; Seeds et al., 1993, 1995; Wang et al., 2000; Seeds and Page, 2001; Yahata et al., 2002). In this study, we have extended these observations to show that bemiparin, a fractionated heparin with a molecular weight lower than that of the other clinically used low molecular weight heparins, is also endowed with these inhibitory effects on antigen-induced bronchospasm, as well as on postantigen airway hyperresponsiveness and inflammatory cell augmentation in the

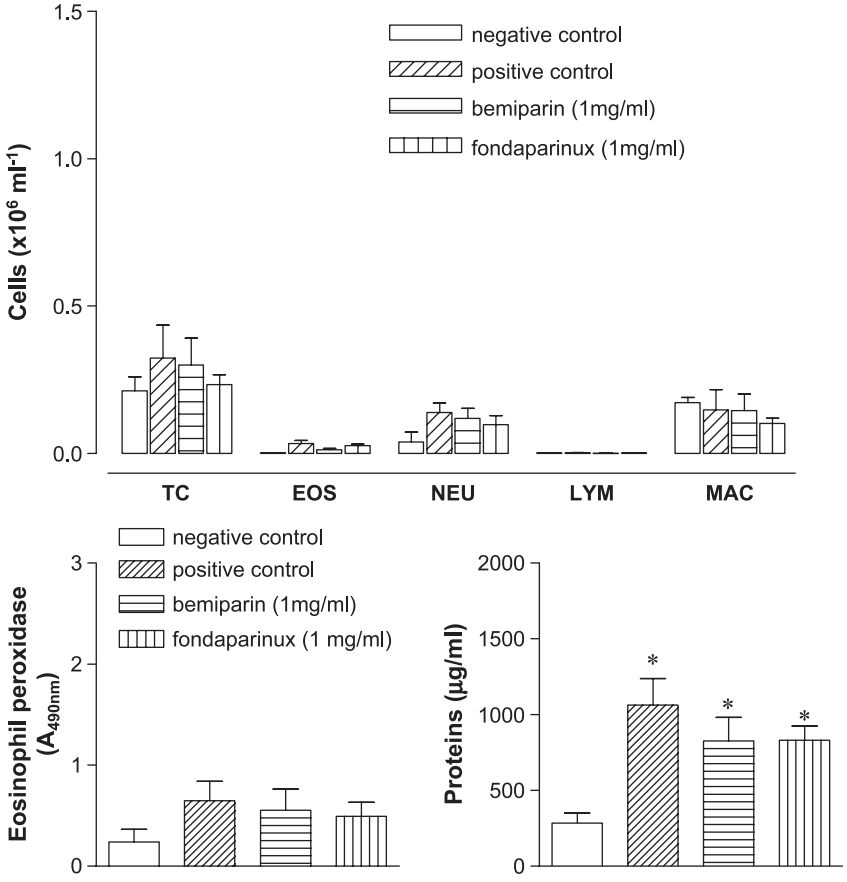

Fig. 5. Upper panel: total cell (TC), eosinophil (EOS), neutrophil (NEU), lymphocyte (LYM), and macrophage (MAC) numbers in bronchoalveolar lavage fluid of sensitized Brown–Norway rats at 3 h postchallenge. Lower panels: Eosinophil peroxidase activity (left) and proteins (right) in the bronchoalveolar lavage fluid of sensitized rats at 3 h postchallenge. Rats were exposed to aerosol saline (negative control) or antigen (1%, 15 min). The antigen-challenged animals received, by inhalation, drug vehicle (positive control), bemiparin, or fondaparinux (each at 1 mg ml^{-1} for 15 min, 1 h prechallenge) as indicated. Scales of the *y*-axis are as in Fig. 2 to ease the comparison between 24- and 3-h values. No effect of bemiparin or fondaparinux was detected. Columns are mean±S.E.M. of five animals for each group; *P<0.05 from the negative control.

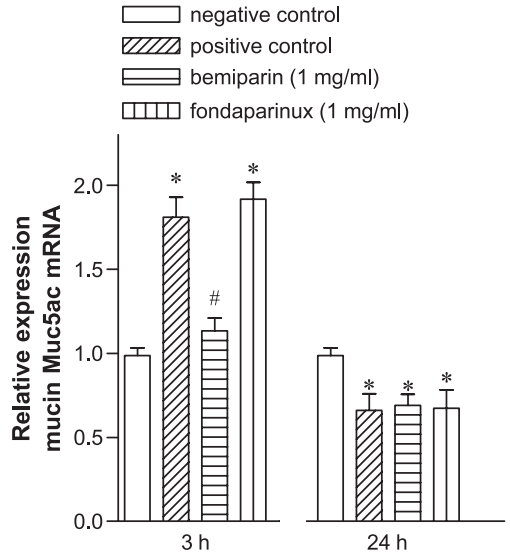

Fig. 6. Relative quantification of mucin Muc5ac and glyceraldehide 3-phosphate dehydrogenase (GAPDH) mRNA in rat bronchial tissue, 3 and 24 h after antigen aerosol challenge, by real-time quantitative RT–PCR using the comparative threshold cycle (C_t) method ($\Delta\Delta C_t$ method). Rats were previously exposed to aerosol saline (negative control) or antigen (1%, 15 min). The antigen-challenged animals received, by inhalation, drug vehicle (positive control), bemiparin, or fondaparinux (each at 1 mg ml^{-1} for 15 min, 1 h prechallenge) as indicated. The C_t values for GAPDH were similar in the different samples, confirming the value of this housekeeping gene as endogenous control. Data are presented as mean±S.E.M. of $2^{-\Delta\Delta Ct}$ relative to GAPDH (n=3 for each group). *P<0.05 from the negative control; $^{\#}P$<0.05 from the positive control.

actively sensitized Brown–Norway rats. Since the pulmonary antiallergic effects of heparins may be species dependent (Howell and Woeppel, 1993), it is interesting to confirm their activity in the sensitized rat, an established experimental model of asthma in which these effects of heparins had not been previously accounted.

The immediate bronchoconstrictor response to antigen in sensitized animals is thought to involve inflammatory mediators released from mast cells (Advenier et al., 1979). In the Brown–Norway rats, the mediators involved in the antigen-induced bronchospasm are predominantly 5-HT and leukotriene D_4 (Nagase et al., 1996; Hele et al., 2001). The acute increase in pulmonary resistance and decrease in dynamic compliance that follow the antigen challenge in sensitized rats were reduced by bemiparin, thus confirming that low molecular weight heparins are effective to inhibit mediator release from mast cells. This finding is consistent with the inhibition of the antigen-induced bronchoconstriction described with other low molecular weight heparins and ultralow molecular weight heparin in sensitized guinea pig (Yahata et al., 2002) and sheep (Ahmed et al., 1997, 2000), yet acute bronchoconstriction was unaltered by low molecular weight heparins in neonatally immunized rabbit (Preuss and Page, 2000). This discrepancy may be due to the differences in the dose of the low molecular weight heparin or in the experimental model and animal species studied, as previously suggested (Howell and Woeppel, 1993; Yahata et al., 2002). The ability of heparin to inhibit mast cell degranulation induced by antiimmunoglobulin E has also been demonstrated in vitro for rat peritoneal mast cell degranulation (Ahmed et al., 1997) and human uterine mast cells (Lucio et al., 1992) and is attributed to the capacity of heparins with chains above eight monosaccharide units to block inositol 1,4,5-triphosphate receptors in mast cells (Ghosh et al., 1988; Tones et al., 1989).

The presence of airway hyperresponsiveness at 24 h after the antigen challenge of sensitized animals is well established in the literature (Elwood et al., 1992; Carvalho et al., 1999). This hyperresponsiveness was accompanied by increased total and differential cell numbers in the airway lumen (Elwood et al., 1992). Bemiparin was effective at reducing the airway hyperresponsiveness to 5-HT, as well as the eosinophil counts and eosinophil peroxidase activity in bronchoalveolar lavage fluid. This finding confirms and extends previous reports showing that aerosolized heparin and low molecular weight heparins inhibit the airway hyperresponsiveness to cholinomimetics in sensitized guinea pigs (Yahata et al., 2002) and allergic sheep (Ahmed et al., 1997) and to histamine in immunized rabbits (Preuss and Page, 2000). Heparin and low molecular weight heparins inhibit airway the eosinophil infiltration induced by allergen in the guinea pig (Seeds et al., 1993, 1995; Wang et al., 2000; Yahata et al., 2002), as well as the eosinophil peroxidase activity and eosinophil cationic protein in bronchoalveolar lavage (Wang et al., 2000). In our experiments, we tested two dose levels of bemiparin, 1 and 5 mg ml^{-1}, and found similar inhibitory effects on airway hyperresponsiveness and eosinophil counts. This lack of a clear dose dependency has been attributed to the complex and heterogenous nature of heparin preparations and is typical of their biological actions (Seeds and Page, 2001).

The mechanisms whereby heparin and related molecules inhibit airway hyperresponsiveness and cell infiltration are not yet elucidated. In fact, both phenomena are not necessarily interrelated (Preuss and Page, 2000; Cortijo et al., 2001). A direct effect of heparin on airway smooth muscle is unlikely to contribute to its anti-hyperresponsiveness effect (Ahmed et al., 1992). The neutralization of chemical mediators and eosinophil cationic granuloproteins, such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase, by interaction with heparins as anionic polyelectrolyte substances has been pointed out (Lever and Page, 2001). Furthermore, it is now well recognised that heparin binds several classes of adhesion molecules on both leukocytes and endothelial cells, thus inhibiting the adhesion of leukocytes to vascular endothelial cells, a critical step necessary prior to the recruitment of infiltrating cells into tissues (Diamond et al., 1995; Lever and Page, 2001). In addition, heparin inhibits heparanase activity, and this action may contribute to the inhibitory effect on cell trafficking, as described for lymphocyte migration (Lider et al., 1990). Other potent antiinflamma-

tory activities have been also described for heparins, including the binding of various cytokines, the modulation of T lymphocytes, and the inhibition of neutrophil chemotaxis and free radical generation (Lever and Page, 2001).

The bronchoconstrictor response to adenosine is prominent in asthmatics and generally not present in control subjects (Cushley et al., 1983). The mechanism of adenosine-induced bronchoconstriction in asthmatics appears primarily related to mast cell activation (Meade et al., 2001). Hannon et al. (2001) described a marked augmentation of the bronchoconstrictor response to adenosine following allergen challenge in actively sensitized Brown–Norway rats, and therefore, it bears similarity to the bronchoconstrictor response to inhaled adenosine observed in asthmatics. This augmented bronchoconstrictor response to adenosine in Brown–Norway rats results from the activation of a population of lung mast cells containing and releasing mainly 5-HT and sensitive to compound 48/80 (Hannon et al., 2001). By contrast with the inhibitory effect on airway hyperresponsiveness to 5-HT, we found no effect of bemiparin on the augmented bronchoconstriction to adenosine in sensitized rats. This was an unexpected finding, considering the established inhibitory effect of heparin on mast cell degranulation. A possible explanation is the known mast cell heterogeneity in the airways (Damazo et al., 2001), with mucosal mast cells relatively insensitive to compound 48/80 and functionally more important for the production of the rapid inflammatory response produced by antigen challenge in airway tissue and connective mast cells sensitive to compound 48/80 and therefore potentially related with adenosine bronchoconstriction. This result would be consistent with the clinical finding that inhaled heparin failed to alter the reactivity to adenosine in mild asthma (Ceyhan and Celikel, 1997).

Plasma extravasation elicited by antigen challenge in rats is also an established feature in experimental models of asthma (Olivenstein et al., 1997), and we found that bemiparin was able to reduce the airway microvascular leakage that follows antigen provocation. Yet, the antiexudative effect of heparins has been scarcely studied in experimental asthma; this result would be in keeping with reports showing that heparin inhibits vascular leakage induced by various inflammatory mediators (Carr, 1979).

Muc5ac is an important mucin in the airway epithelium (Jackson, 2001), and its expression is augmented in asthmatics (Fahy, 2002), where it is considered part of the airway remodelling response (Becket and Howarth, 2003). The early increase in Muc5ac mRNA expression, preceding inflammatory cell infiltration, has been demonstrated in the guinea pig and rat models of allergic asthma (Li et al., 2001; Blesa et al., 2003), and it is of transient nature for single antigen provocation since the expression levels went below control baseline at few hours post-challenge (Li et al., 2001; this study). Bemiparin abolished this transient increase in mucin Muc5ac gene expression. To our knowledge, this is the first description of an inhibitory effect of a low molecular weight heparin on mucin gene expression. The enhanced Muc5ac gene expression in experimental asthma has been related to the activation of the epidermal growth factor receptor cascade (Takeyama et al., 1999). Heparins, in addition to their known ability to bind a variety of growth factors, may inhibit several kinases of the epidermal growth factor receptor cascade and suppress gene activation by inhibiting the formation of active transcription factors (Weigert et al., 2001; Newman et al., 2004). However, the precise mechanism(s) underlying the inhibitory effect of heparin on Muc5ac gene expression merits further research.

The antiinflammatory effects of heparins appear unrelated to their anticoagulant property, as previously reported for various heparin fractions devoid of anticoagulant activity (Campo et al., 1999; Seeds and Page, 2001). In this study, we further confirmed this notion by showing that compound 1FMF-52/3, a heparin fraction with molecular weight similar to that of bemiparin but with low anticoagulant activity, elicited anti-hyperresponsiveness and antiinflammatory activity similar to those found for bemiparin.

Fondaparinux is the active pentasaccharide endowed with the specific antifactor Xa activity clinically useful as an anticoagulant (Bauer et al., 2002; Samama and Gerotziafas, 2003). The recent availability of this synthetic compound offers the opportunity to study whether this short-length compound is endowed with antiinflammatory activity, since in vitro studies suggest that a minimum change length is required (Tones et al., 1989; Lever et al., 2003). In this study, we have demonstrated that fondaparinux was effectively absent of inhibitory effects on antigen-induced acute bronchoconstriction, as well as it was lacking any anti-hyperresponsiveness and antiinflammatory activities. It was also without effect to suppress the transient Muc5ac expression elicited by antigen exposure. These findings obtained with this new anticoagulant drug, fondaparinux, add further support to the notion that a minimum change length is required for in vivo antiallergic activities, while the anticoagulant property of this compound appears unrelated to the described antiinflammatory effects of heparins.

In summary, the results from this study demonstrate that the inhalation of a low molecular weight heparin, bemiparin, is effective to reduce a number of airway responses to antigen exposure in an animal model of allergic asthma, whereas the active pentasaccharide, fondaparinux, does share these antiinflammatory effects. Although animal models of asthma are not fully equivalent to the human disease (Coleman, 1999), these findings add experimental support to the few clinical studies carried out with low molecular weight heparins in clinical asthma (Ahmed et al., 1999; Ceyhan and Celikel, 2000) and further confirm the relevance of the non-anticoagulant component of the heparin molecule for these antiallergic effects.

Acknowledgements

This work was supported by grants SAF2002-04667 and SAF2003-07206-C02-01 from CICYT (Ministry of Science and Technology, Spain) and research funds from the local government ('Research Groups-03/166' of Generalitat Valenciana). Dr. Suchankova was supported by the Research Grant CEZ 13/98:11600002 (Charles University, Prague). This research was not funded by any drug company. We thank the gift of bemiparin and compound IFMF-52/3 by Rovi and of fondaparinux by Sanofi-Synthelabo. The technical assistance of Pedro Santamaria is also gratefully acknowledged.

References

Advenier, C., Mallard, B., Santais, M.C., Ruff, F., 1979. The effects of metiamide and H_1 receptor blocking agents on anaphylactic response in guinea-pigs. Agents Actions 9, 467–473.

Ahmed, T., Abraham, W.M., D'Brot, J., 1992. Effects of inhaled heparin on immunologic and nonimmunologic bronchoconstrictor responses in sheep. Am. Rev. Respir. Dis. 145, 566–570.

Ahmed, T., Campo, C., Abraham, M.K., Molinari, J.F., Abraham, W.M., Ashkin, D., Syriste, T., Andersson, L.O., Svahn, C.M., 1997. Inhibition of antigen-induced acute bronchoconstriction, airway hyperresponsiveness, and mast cell degranulation by a nonanticoagulant heparin: comparison with a low molecular weight heparin. Am. J. Respir. Crit. Care Med. 155, 1848–1855.

Ahmed, T., Gonzalez, B.J., Fanta, I., 1999. Prevention of exercise-induced bronchoconstriction by inhaled low-molecular-weight heparin. Am. J. Respir. Crit. Care Med. 160, 576–581.

Ahmed, T., Ungo, J., Zhou, M., Campo, C., 2000. Inhibition of allergic late airway responses by inhaled heparin-derived oligosaccharides. J. Appl. Physiol. 88, 1721–1729.

Bauer, K.A., Hawkins, D.W., Peters, P.C., Petitou, M., Herbert, J.M., van Boeckel, C.A., Meuleman, D.G., 2002. Fondaparinux, a synthetic pentasaccharide: the first in a new class of antithrombotic agents—the selective factor Xa inhibitors. Cardiovasc. Drug Rev. 20, 37–52.

Becket, P.A., Howarth, P.H., 2003. Pharmacotherapy and airway remodelling in asthma? Thorax 58, 163–174.

Blesa, S., Cortijo, J., Mata, M., Serrano, A., Closa, D., Santangelo, F., Estrela, J.M., Suchankova, J., Morcillo, E.J., 2003. Oral *N*-acetylcysteine attenuates the rat pulmonary inflammatory response to antigen. Eur. Respir. J. 21, 394–400.

Campo, C., Molinari, J.F., Ungo, J., Ahmed, T., 1999. Molecular-weight-dependent effects of nonanticoagulant heparins on allergic airway responses. J. Appl. Physiol. 86, 549–557.

Carr, J., 1979. The anti-inflammatory action of heparin: heparin as an antagonist to histamine, bradykinin and prostaglandin E_1. Thromb. Res. 16, 507–516.

Carvalho, C., Jancar, S., Mariano, M., Sirois, P., 1999. A rat model presenting eosinophilia in the airways, lung eosinophil activation, and pulmonary hyperreactivity. Exp. Lung Res. 25, 303–316.

Ceyhan, B.B., Celikel, T., 1997. Effect of inhaled heparin on adenosine-induced bronchial hyperreactivity. Int. J. Clin. Pharmacol. Ther. 35, 208–213.

Ceyhan, B.B., Celikel, T., 2000. Effect of inhaled low molecular weight heparin on methacholine-induced bronchoconstriction. Int. J. Clin. Pharmacol. Ther. 38, 446–451.

Chapman, T.M., Goa, K.L., 2003. Bemiparin. A review of its use in the prevention of venous thromboembolism and treatment of deep vein thrombosis. Drugs 63, 2357–2377.

Coleman, R.A., 1999. Current animal models are not predictive for clinical asthma. Pulm. Pharmacol. Ther. 12, 87–89.

Cortijo, J., Blesa, S., Martinez-Losa, M., Mata, M., Seda, E., Santangelo, F., Morcillo, E.J., 2001. Effects of taurine on pulmonary responses to antigen in sensitized Brown–Norway rats. Eur. J. Pharmacol. 431, 111–117.

Cushley, M.J., Tattersfield, A.E., Holgate, S.T., 1983. Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. Br. J. Clin. Pharmacol. 15, 161–165.

Damazo, A.S., Tavares de Lima, W., Perretti, M., Oliani, S.M., 2001. Pharmacological modulation of allergic inflammation in the rat airways and association with mast cell heterogeneity. Eur. J. Pharmacol. 426, 123–130.

Diamant, Z., Page, C.P., 2000. Heparin and related molecules as a new treatment for asthma. Pulm. Pharmacol. Ther. 13, 1–4.

Diamond, M.S., Alon, R., Parkos, C.A., Quinn, M.T., Springer, T.A., 1995. Heparin is an adhesive ligand for the leukocyte integrin Mac-1 (CD11b/CD1). J. Cell Biol. 130, 1473–1482.

Elwood, W., Barnes, P.J., Chung, K.F., 1992. Airway hyperresponsiveness is associated with inflammatory cell infiltration in allergic Brown–Norway rats. Int. Arch. Allergy Immunol. 99, 91–97.

Fahy, J.V., 2002. Goblet cell and mucin gene abnormalities in asthma. Chest 122, 320S–326S.

Ghosh, T.K., Eis, P.S., Mullaney, J.M., Ebert, C.L., Gill, D.L., 1988. Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. J. Biol. Chem. 263, 11075–11079.

Green, W.F., Konnaris, K., Woolcock, A.J., 1993. Effect of salbutamol, fenoterol and sodium cromoglycate on the release of heparin from sensitized human lung fragments challenged with *Dermatophagoides pteronyssinus* antigen. Am. J. Respir. Cell Mol. Biol. 8, 518–521.

Hannon, J.P., Tigani, B., Williams, I., Mazzoni, L., Fozard, J.R., 2001. Mechanism of airway hyperresponsiveness to adenosine induced by allergen challenge in actively sensitized Brown Norway rats. Br. J. Pharmacol. 132, 1509–1523.

Hele, D.J., Birrell, M.A., Webber, S.E., Foster, M.L., Belvisi, M.G., 2001. Mediator involvement in antigen-induced bronchospasm and microvascular leakage in the airways of ovalbumin sensitized Brown Norway rats. Br. J. Pharmacol. 132, 481–488.

Howell, R.E., Woeppel, S.L., 1993. Potential species dependency for the pulmonary antiallergic effects of aerosolized heparin. Pulm. Pharmacol. 6, 237–239.

Jackson, A.D., 2001. Airway goblet-cell mucus secretion. Trends Pharmacol. Sci. 22, 39–45.

Lever, R., Page, C., 2001. Glycosaminoglycans, airways inflammation and bronchial hyperresponsiveness. Pulm. Pharmacol. Ther. 14, 249–254.

Lever, R., Lo, W.T., Brown, R.A., Gallaher, J., Page, C.P., 2003. The effects of heparin fractions of defined chain length on human neutrophil functions in vitro. Br. J. Pharmacol. 140 (3P).

Li, Y., Martin, L.D., Minnicozzi, M., Greenfeder, S., Fine, J., Petterse, C.A., Chorley, B., Adler, K.B., 2001. Enhanced expression of mucin genes in a guinea pig model of allergic asthma. Am. J. Respir. Cell Mol. Biol. 25, 644–651.

Lider, O., Mekori, Y.A., Miller, T., Bar-Tana, R., Vlodavsky, I., Baharav, E., Cohen, I.R., Naparstek, Y., 1990. Inhibition of T lymphocyte heparanase by heparin prevents T cell migration and T cell-mediated immunity. Eur. J. Immunol. 20, 493–499.

Lucio, J., D'Brot, J., Guo, C.B., Abraham, W.M., Lichtenstein, L.M., Kagey-Sobotka, A., Ahmed, T., 1992. Immunologic mast cell-mediated responses and histamine release are attenuated by heparin. J. Appl. Physiol. 73, 1093–1101.

Martinez-Salas, J., Mendelssohn, R., Abraham, W.M., Hsiao, B., Ahmed, T., 1998. Inhibition of allergic airway responses by inhaled low-molecular-weight heparins: molecular-weight dependence. J. Appl. Physiol. 84, 222–228.

Mata, M., Ruiz, A., Cerda, M., Martinez-Losa, M., Cortijo, J., Santangelo, F., Serrano-Mollar, A., Llombart-Bosch, A., Morcillo, E.J., 2003. Oral

N-acetylcysteine reduces bleomycin-induced lung damage and mucin Muc5ac expression in rats. Eur. Respir. J. 22, 900–905.

Meade, C.J., Dumont, I., Worrall, L., 2001. Why do asthmatic subjects respond so strongly to inhaled adenosine? Life Sci. 69, 1225–1240.

Molinari, J.F., Campo, C., Shakir, S., Ahmed, T., 1998. Inhibition of antigen-induced airway hyperresponsiveness by ultralow molecular-weight heparin. Am. J. Respir. Crit. Care Med. 157, 887–893.

Nagase, T., Dallaire, M.J., Ludwig, M.S., 1996. Airway and tissue behavior during early response in sensitized rats: role of 5-HT and LTD4. J. Appl. Physiol. 80, 583–590.

Newman, D.R., Li, C.-M., Simmons, R., Khosla, J., Sannes, P.L., 2004. Heparin affects signaling pathways stimulated by fibroblast growth factor-1 and -2 in type II cells. Am. J. Physiol. 287, L191–L200.

Olivenstein, R., Du, T., Xu, L.J., Martin, J.G., 1997. Microvascular leakage in the airway wall and lumen during allergen induced early and late responses in rats. Pulm. Pharmacol. Ther. 10, 223–230.

Page, C.P., Minshall, E., 1993. Mast cells and the lung. In: Foreman, J.C. (Ed.), The Handbook of Immunopharmacology: Immunopharmacology of Mast Cells and Basophils. Academic Press, London, pp. 181–195.

Pauwels, R.A., Kips, J.C., Peleman, R.A., Van der Straeten, M.E., 1990. The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats. Am. Rev. Respir. Dis. 141, 540–545.

Planes, A., 2003. Review of bemiparin sodium—a new second-generation low molecular weight heparin and its applications in venous thromboembolism. Expert Opin. Pharmacother. 4, 1551–1561.

Pons, R., Santamaria, P., Suchankova, J., Cortijo, J., Morcillo, E.J., 2000. Effects of inhaled glaucine on pulmonary responses to antigen in sensitized guinea pigs. Eur. J. Pharmacol. 397, 187–195.

Preuss, J.M., Page, C.P., 2000. Effect of heparin on antigen-induced airway responses and pulmonary leukocyte accumulation in neonatally immunized rabbits. Br. J. Pharmacol. 129, 1585–1596.

Samama, M.M., Gerotziafas, G.T., 2003. Evaluation of the pharmacological properties and clinical results of the synthetic pentasaccharide (fondaparinux). Thromb. Res. 109, 1–11.

Seeds, E.A., Page, C.P., 2001. Heparin inhibits allergen-induced eosinophil infiltration into guinea-pig lung via a mechanism unrelated to its anticoagulant activity. Pulm. Pharmacol. Ther. 14, 111–119.

Seeds, E.A., Hanss, J., Page, C.P., 1993. The effect of heparin and related proteoglycans on allergen and PAF-induced eosinophil infiltration. J. Lipid Mediat. 7, 269–278.

Seeds, E.A., Horne, A.P., Tyrrell, D.J., Page, C.P., 1995. The effect of inhaled heparin and related glycosaminoglycans on allergen-induced eosinophil infiltration in guinea-pigs. Pulm. Pharmacol. 8, 97–105.

Strath, M., Warren, D.J., Sanderson, C.J., 1985. Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. J. Immunol. Methods 83, 209–215.

Takeyama, K., Dabbagh, K., Lee, H.M., Agusti, C., Lausier, J.A., Ueki, I.F., Grattan, K.M., Nadel, J.A., 1999. Epidermal growth factor system regulates mucin production in airways. Proc. Natl. Acad. Sci. U. S. A. 96, 3081–3086.

Tones, M.A., Bootman, M.D., Higgins, B.F., Lane, D.A., Pay, G.F., Lindahl, U., 1989. The effect of heparin on the inositol 1,4,5-trisphosphate receptor in rat liver microsomes. Dependence on sulphate content and chain length. FEBS Lett. 252, 105–108.

Wang, Q.L., Shang, X.Y., Zhang, S.L., Ji, J.B., Cheng, Y.N., Meng, Y.J., Zhu, Y.J., 2000. Effects of inhaled low molecular weight heparin on airway allergic inflammation in aerosol–ovalbumin-sensitized guinea pigs. Jpn. J. Pharmacol. 82, 326–330.

Weigert, C., Brodbeck, K., Häring, H.U., Gambado, G., Schleicher, E.D., 2001. Low-molecular-weight heparin prevents high glucose- and phorbol ester-induced TGF-β1 gene activation. Kidney Int. 60, 935–943.

Yahata, T., Nishimura, Y., Maeda, H., Yokoyama, M., 2002. Modulation of airway responsiveness by anionic and cationic polyelectrolyte substances. Eur. J. Pharmacol. 434, 71–79.