ELSEVIER

European Journal of Pharmacology 434 (2002) 71–79

ejp

www.elsevier.com/locate/ejphar

Modulation of airway responsiveness by anionic and cationic polyelectrolyte substances

Tomoyuki Yahata, Yoshihiro Nishimura*, Hitoshi Maeda, Mitsuhiro Yokoyama

Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Received 9 October 2001; received in revised form 8 November 2001; accepted 13 November 2001

Abstract

To elucidate the effects of anionic and cationic polyelectrolyte substance on bronchoconstriction, we examined the serial changes in respiratory resistance (Rrs) in ovalbumin-sensitized guinea pigs after antigen exposure with or without preinhalation of low-molecular-weight heparin, poly-L-glutamic acid, poly-L-lysine and dextran, and with or without oral intake of dalteparin. Both immediate and late responses after antigen exposure were significantly decreased after pretreatment with inhaled low-molecular-weight heparin and poly-L-glutamic acid compared with saline alone. The late response was significantly decreased after pretreatment with oral dalteparin. Both low-molecular-weight heparin and poly-L-glutamic acid significantly decreased the airway response to methacholine in sensitized guinea pigs. In sensitized guinea pigs, the airway response to methacholine was significantly increased after pretreatment with inhaled poly-L-lysine. Pretreatment with inhaled low-molecular-weight heparin before poly-L-lysine exposure significantly suppressed the airway hyperresponsiveness after inhaled poly-L-lysine. These findings indicated that the "cationic–anionic interaction" plays an important role in airway responsiveness.

Keywords: Bronchial asthma; Low-molecular-weight heparin; Poly-L-glutamic acid; Poly-L-lysine; Cationic–anionic interaction

1. Introduction

In bronchial asthma, after the immediate asthmatic response activated mast cells release inflammatory cell migrating factors, which include platelet-activating factor (PAF), leukotriene B_4, neutrophil chemotactic factor of anaphylaxis (NCF-A), eosinophil chemotactic factor of anaphylaxis (ECF-A) and so on (Salvi et al., 2001). Then, monocytes, neutrophils, eosinophils, and basophils migrate to the airway. Major basic protein (MBP) and eosinophil cationic protein, which are eosinophil-derived cytotoxic cationic granuloproteins, damage the bronchial epithelium and cause airway inflammation (Salvi et al., 2001). It has been demonstrated that highly negative-charged proteoglycans such as heparin and chondroitin sulfate are contained in secretary granules of mast cells and may regulate the stability and activity of many chemical mediators (Schwartz and Huff, 1991). Barker et al. (1988) demonstrated by analysis of cDNA that MBP was translated as a slightly acidic preproprotein (proMBP) with an acidic propart and that MBP was a 13.8-kDa single polypeptide rich in arginine with a calculated isoelectric point (pI) of 10.9, while proMBP itself had a calculated pI of 6.2 because the 9.9-kDa proportion of proMBP was rich in glutamic and aspartic acids and had a calculated pI of 3.9. Furthermore, they (Barker et al., 1991) demonstrated that acidic polyamino acids inhibited MBP toxicity in a charge-dependent manner in vitro, and suggested that the acidic propart of proMBP functioned to mask mature MBP toxicity. Similarly, Motojima and Makino (1994) demonstrated that the damaging effect to the guinea pig tracheal epithelium caused by eosinophil peroxidase was prevented when eosinophil peroxidase was mixed with anionic polyelectrolyte substances, such as heparin, chondoroitin sulfate, dextran sulfate, and mucin. However, it has been demonstrated that synthetic cationic proteins such as poly-L-lysine and poly-L-arginine induce airway hyperresponsiveness when applied intratracheally or by inhalation in guinea pigs (Coyle et al., 1993a; Shirotani et al., 1999), rats (Uchida et al., 1993), and sheep (Ahmed et al., 1992). Intratracheal injection of poly-L-arginine produces an important increase in eosinophil and neutrophil numbers, and in β-glucuronidase, histamine, eosinophil peroxidase and albumin levels in bronchoalveolar lavage fluid (Arseneault et al., 1999). Moreover, cationic

* Corresponding author. Tel.: +81-78-382-5846; fax: +81-78-382-5859.
E-mail address: nishiy@med.kobe-u.ac.ip (Y. Nishimura).

0014-2999/02/$ - see front matter
PII: S0014-2999(01)01528-X

proteins increase the permeability of cultured rabbit tracheal epithelial cells (Uchida et al., 1996). Furthermore, it has been demonstrated that inhaled or intratracheal pretreatment with negatively charged heparin inhibits the increased epithelial cell permeability induced by cationic (Coyle et al., 1993a; Shirotani et al., 1999; Ahmed et al., 1992; Arseneault et al., 1999; Uchida et al., 1996). Coyle et al. (1993b) demonstrated that two cationic proteins, platelet factor 4 and cathepsin G, increased airway responsiveness, on effect that was inhibited by low-molecular-weight heparin. Sasaki and Shioya (1995) showed that the airway hyperresponsiveness to histamine and inflammatory cell migration caused by platelet PAF inhalation was suppressed by low-molecular-weight heparin inhalation. Martinez-Salas et al. (1998) suggested that fractionated low-molecular-weight heparins attenuate antigen-induced acute bronchoconstrictor responses and airway hyperresponsiveness, and that the lower the molecular weight of heparin the greater the effect. Recently, Ahmed et al. (2000) demonstrated that only ultralow-molecular-weight heparins caused a dose-dependent inhibition of the antigen-induced immediate asthmatic response and late asthmatic response and postantigen airway hyperresponsiveness, whereas low- and medium-molecular-weight heparins were ineffective.

These findings suggest that charge interaction is one of the important factors not only in the immediate asthmatic response, but also in the late asthmatic response. However, the precise effects of anionic and cationic polyelectrolyte substances on bronchoconstriction, especially in the late asthmatic response, after antigen exposure remain to be determined. Therefore, we investigated the effects of low-molecular-weight heparin, dalteparin, and poly-L-glutamic acid, which are anionic polyelectrolyte substances, and poly-L-lysine, a cationic polyelectrolyte substance, on the bronchoconstriction and the non-specific bronchoconstriction in ovalbumin-sensitized guinea pigs after antigen exposure.

2. Materials and methods

2.1. Animals

Male and female specific pathogen-free Hartley guinea pigs (Japan SLC, Hamamatsu, Japan) weighing 250–350 g were used for this study. The guinea pigs were housed four to a cage in standard cages and were fed on standard lab chow (CG-7, Japan Kurea, Osaka, Japan). They were kept in a room maintained at 23 ± 2 °C and at 55 ± 5% humidity. All animal experiments were conducted according to the Guidelines for Animal Experimentation of the Kobe University School of Medicine.

2.2. Drugs

Low-molecular-weight heparin (sodium salt from porcine intestinal mucosa, mol. wt. 4000–6000), poly-L-glutamic acid (sodium salt, mol. wt. 14300), poly-L-lysine (hydrobromide, mol. wt. 9800), dextran (produced by Leuconostoc mesenteroides, mol. wt. 9300), ovalbumin (Grade III) and pyrilamine maleate were obtained from Sigma (St. Louis, MO, USA). Dalteparin was donated by Kissei Pharmaceutical (Tokyo, Japan). Methacholine was obtained from Wako (Osaka, Japan) and saline was obtained from Otsuka Chemical (Tokyo, Japan).

2.3. Measurement of respiratory resistance (Rrs)

Respiratory resistance was measured by a modification of Mead's forced oscillation method (Mead, 1960). Briefly, to observe serial changes in respiratory resistance, the guinea pigs were restrained in an enclosed animal chamber, with the head and neck protruding. The neck was fixed in this chamber using a rubber ring like a doughnut. A 30-Hz sine wave oscillation was administered from the posterior part of the chamber via a loudspeaker through an amplifier. A conical plastic face mask with an opening in the top was fixed to the head of the guinea pig, and the expiratory flow rate was measured via this mask using a respiratory flow control box (RY 111S, Nihon Koden, Japan) and a differential transducer (TR-602T, Nihon Koden). The expiratory flow rate was measured by removing only the amplitude of vibration with the same 30 Hz components as generative pressure waves through the amplifier (RMP-6008, Nihon Koden). The respiratory resistance (cm H_2O/l/s) was, therefore, calculated from the pressure in the chamber and the expiratory flow.

2.4. Sensitization

Guinea pigs were sensitized by Terashi's method (Terashi et al., 1988). Briefly, we made on acrylic antigen-inhalation system, in which four guinea pigs could be exposed to antigen equally. Using this system, guinea pigs inhaled nebulized 1% ovalbumin produced by an ultrasonic nebulizer once a day for 10 min, and for 10 consecutive days. In addition, they inhaled 1% ovalbumin for 5 min twice every seventh day after sensitization. All guinea pigs showed immediate allergic airway responses and their respiratory resistance reached more than twice the baseline value on the 9th or 10th day after ovalbumin inhalation. On the basis of

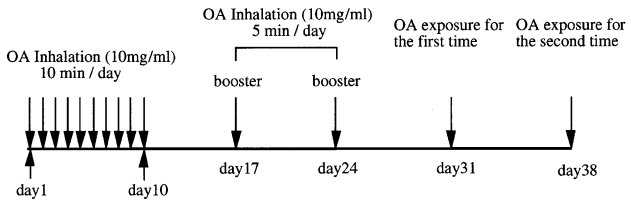

Fig. 1. Experimental protocol for sensitization to ovalbumin (OA) of guinea pigs. LMWH: Low-molecular-weight heparin; P-L-G: Poly-L-glutamic acid; P-L-L: Poly-L-lysine.

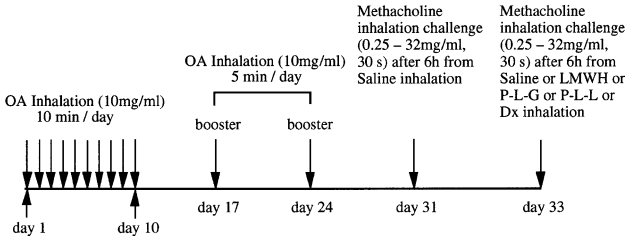

Fig. 2. Experimental protocol for the effects of charge interaction on airway responsiveness after methacholine inhalation by ovalbumin (OA)—sensitized guinea pigs. LMWH: Low-molecular-weight heparin; P-L-G: Poly-L-glutamic acid; P-L-L: Poly-L-lysine; Dx: Dextran.

these findings, they were judged to be sensitized. Non-sensitized guinea pigs inhaled saline instead of 1% ovalbumin.

2.5. Experimental protocol in the study of the effects of polyelectrolyte substance on bronchoconstriction induced by antigen exposure

Guinea pigs received the first antigen exposure, 2% ovalbumin inhalation for 10 min, on the 31st day, which was 1 week after the booster, and received the second antigen exposure on the 38th day.

To elucidate the effects of inhalation of polyelectrolyte substances, guinea pigs inhaled saline for 6 min 1 h before antigen exposure on the 31st day, and inhaled saline ($n=14$), low-molecular-weight heparin ($n=15$), poly-L-glutamic acid ($n=11$), poly-L-lysine ($n=7$) or dextran ($n=6$) 1 h before antigen exposure on the 38th day (Fig. 1). Low-molecular-weight heparin, poly-L-glutamic acid, poly-L-lysine and dextran were dissolved in saline and prepared at the final concentrations of 10, 5, 5 and 10 mg/ml, respectively.

In experiments with oral dalteparin, we divided the guinea pigs into two groups at random. Guinea pigs in group A ($n=5$) received tap water from the 25th day to the 31st day and water containing dalteparin from the 32nd day to the 38th day. The guinea pigs in group B ($n=6$) initially received dalteparin water and then tap water in reverse order to group A. Dalteparin was dissolved in tap water and prepared at a final concentration of 25 U/ml. We performed ovalbumin provocation on the 31st day and the 38th day in both groups.

To prevent fatal bronchoconstriction, we injected 10 mg/kg of pyrilamine maleate, a histamine H_1 receptor antagonist, intraperitoneally at 30 min before antigen exposure.

Guinea pigs inhaled 2% ovalbumin for 10 min after measurement of baseline respiratory resistance. We measured respiratory resistance at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 30 min and at 1, 2, 3, 4, 6, 7, 8, 10, 11, 12 and 24 h after antigen exposure. When the respiratory resistance showed an increase greater than 100% of the baseline within 1 h after ovalbumin exposure, the increase was judged to reflect the immediate response. Since non-sensitized guinea pigs did not show an increase greater than 30% of the baseline respiratory resistance from 4 to 24 h after antigen exposure, increases greater than 30% of the baseline respiratory resistance during this period were judged to be the late response.

2.6. Experimental protocol in the study of the effects of polyelectrolyte substance on the non-specific bronchoconstriction induced by methacholine inhalation

Six hours before the methacholine inhalation challenge test, ovalbumin-sensitized guinea pigs inhaled saline for 6 min on the 31st day, and inhaled saline, low-molecular-weight heparin ($n=5$), poly-L-glutamic acid ($n=5$), poly-L-lysine ($n=9$) or dextran ($n=6$) similarly on the 33rd day. Low-molecular-weight heparin, poly-L-glutamic acid, poly-L-lysine and dextran were dissolved in saline and prepared at final concentrations of 5, 2.5, 2.5 and 2.5 mg/ml, respectively.

To clarify whether the pretreatment with inhaled low-molecular-weight heparin could inhibit airway hyperresponsiveness induced by poly-L-lysine inhalation ($n=5$), ovalbumin-sensitized guinea pigs inhaled saline for 6 min twice 6 and 7 h before methacholine challenge on the 31st day. They inhaled low-molecular-weight heparin for 6 min at 7 h before and poly-L-lysine for 6 min at 6 h before methacholine challenge on the 33rd day.

The baseline respiratory resistance was measured after saline inhalation for 2 min, and doubled concentrations of methacholine (0.25–32 mg/ml) were nebulized and inhaled by the guinea pigs. Each dose was inhaled for 30 s and peak respiratory resistance was determined during the following 2 min. Increased concentrations of methacholine were delivered until the respiratory resistance reached more than twice the baseline value (Fig. 2). The dose of methacholine re-

Table 1
Serial changes in Rrs after antigen exposure of sensitized guinea pigs treated with inhaled saline, P-L-L and dextran are expressed in percent of change in Rrs

		1 min	2 min	5 min	10 min	2 h	4 h	8 h	12 h	24 h	Peak IR	Peak LR
Saline at the 31st day	$n=14$	383 ± 208	178 ± 40	163 ± 23	230 ± 41	61 ± 17	94 ± 29	54 ± 19	64 ± 20	37 ± 19	538 ± 198	120 ± 30
Saline at the 38th day		320 ± 74	178 ± 54	174 ± 34	250 ± 46	66 ± 17	66 ± 17	46 ± 35	65 ± 36	38 ± 15	496 ± 79	120 ± 44
Saline	$n=7$	200 ± 41	168 ± 46	162 ± 59	206 ± 93	70 ± 26	52 ± 23	57 ± 17	59 ± 25	34 ± 14	305 ± 78	92 ± 23
P-L-L		676 ± 188[a]	290 ± 56	320 ± 77	212 ± 56	77 ± 19	13 ± 6	23 ± 19	22 ± 19	20 ± 19	786 ± 150[a]	45 ± 15
Saline	$n=6$	478 ± 161	293 ± 62	296 ± 61	173 ± 42	65 ± 18	13 ± 6	39 ± 18	45 ± 8	66 ± 15	715 ± 72	77 ± 24
Dextran		357 ± 94	256 ± 45	300 ± 68	186 ± 31	40 ± 15	11 ± 9	38 ± 20	51 ± 7	62 ± 19	652 ± 48	77 ± 28

The results are means ± S.E.M. P-L-L: Poly-L-lysine. IR: Immediate response. LR: Late response. Rrs: Respiratory resistance.
[a] $P<0.05$ compared with control (saline).

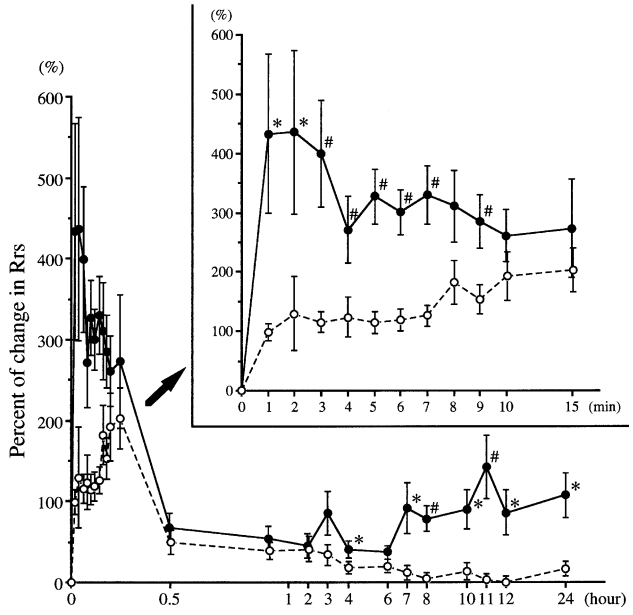

Fig. 3. Serial change in Rrs after antigen exposure of sensitized guinea pigs treated with inhaled LMWH (○) and with inhaled saline (●). Inserted figure shows percentage changes in Rrs up to 15 min after antigen exposure. #$P<0.01$ compared with LMWH. *$P<0.05$ compared with LMWH. LMWH: Low-molecular-weight heparin; Rrs: Respiratory resistance.

quired to increase respiratory resistance to twice its baseline value was used to determine the methacholine concentration provoking a 100% increase in respiratory resistance from baseline (PC100).

In non-sensitized guinea pigs, methacholine challenge tests were carried out twice at 48-h intervals (1st day and 3rd day). The pretreatment with saline or polyelectrolyte substances (saline: $n=4$, low-molecular-weight heparin: $n=7$, poly-L-glutamic acid: $n=6$, poly-L-lysine: $n=5$, dextran: $n=5$) was administered on either day at random.

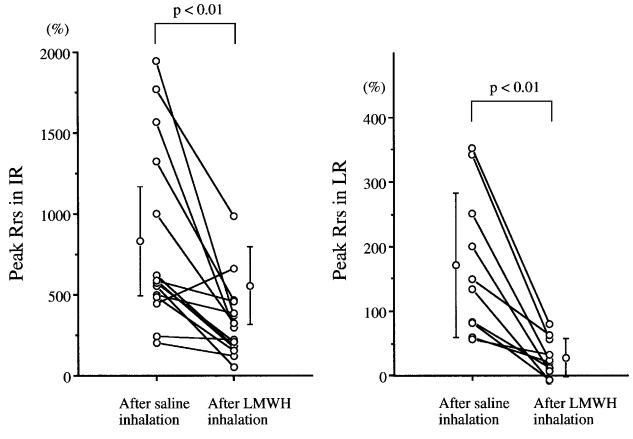

Fig. 4. Effects of inhaled LMWH on peak Rrs in IR and LR after antigen exposure in sensitized guinea pigs. LMWH: Low-molecular-weight heparin; Rrs: respiratory resistance; IR: Immediate as response; LR: Late response.

2.7. Statistics

Values concerning the serial changes in respiratory resistance in ovalbumin-sensitized guinea pigs after antigen exposure are expressed as the geometric means ± geometric standard error of the means (S.E.M.). The other values are expressed as the geometric means ± geometric standard deviations (S.D.). Comparisons between two groups were performed using a two-way analysis of variance (ANOVA) with repeated measures or Student's paired *t*-tests. Changes in respiratory resistance during allergen challenge were analyzed by a two-way ANOVA with repeated measure. Peak immediate response, peak late response and PC100 values were compared using Student's paired *t*-tests. A *P*-value <0.05 was considered to be significant.

3. Results

3.1. The effects of polyelectrolyte substances on the bronchoconstriction induced by antigen exposure

There were no significant differences in respiratory resistance changes between the first antigen exposure (31st day) and the second exposure (38th day) (Table 1). Furthermore, there were no significant differences in the peak respiratory resistance in the immediate response (peak immediate response) and the peak respiratory resistance in the late response (peak late response) between the first and the second antigen exposure. Fig. 3 shows the effects of low-molecular-

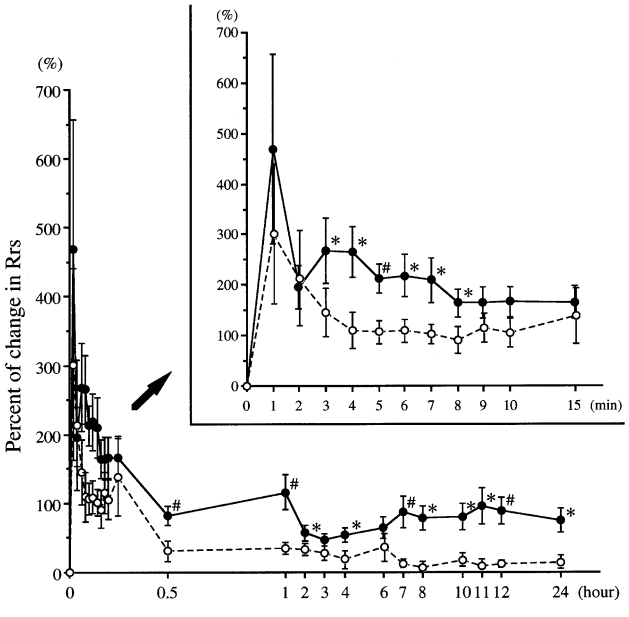

Fig. 5. Serial changes in Rrs after antigen exposure of sensitized guinea pigs treated with inhaled P-L-G (○) and with inhaled saline (●). Insert shows percentage changes in Rrs up to 15 min after antigen exposure. #$P<0.01$ compared with P-L-G. *$P<0.05$ compared with P-L-G. P-L-G: Poly-L-glutamic acid; Rrs: Respiratory resistance.

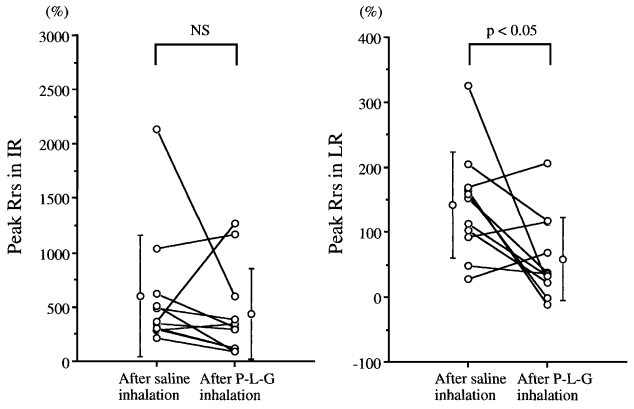

Fig. 6. Effects of inhaled P-L-G on peak Rrs in IR and LR after antigen exposure in sensitized guinea pigs. P-L-G: Poly-L-glutamic acid; Rrs: Respiratory resistance; IR: Immediate response; LR: Late response.

weight heparin inhalation on the bronchoconstriction induced by antigen exposure. Pretreatment with low-molecular-weight heparin significantly inhibited the increase in respiratory resistance induced by antigen exposure compared with the effect of pretreatment with saline. Significant inhibitory effects on bronchoconstriction were seen at 1, 2, 3, 4, 5, 6, 7 and 9 min (immediate response), and 4, 7, 8, 10, 11, 12 and 24 h (late response). Furthermore, pretreatment with low-molecular-weight heparin significantly decreased both the peak immediate response and the peak late response after antigen exposure compared with the effect of pretreatment with saline (Fig. 4). Fig. 5 shows the effects of poly-L-glutamic acid inhalation on the bronchoconstriction induced by antigen exposure. Pretreatment with poly-L-glutamic acid also significantly inhibited the increase in respiratory resistance induced by antigen exposure compared with the effect of pretreatment with saline. Significant inhibitory effects on bronchoconstriction were seen during both the immediate response and late response phases. Pretreatment with poly-L-glutamic acid had a significant inhibitory effect on the peak late response, but not on the peak immediate response (Fig.

Table 2
Log PC100 in sensitized guinea pigs and control guinea pigs treated with saline, with P-L-G, with P-L-L and with dextran, and that in sensitized guinea pigs treated with saline twice and with P-L-L after LMWH inhalation

	Sensitized guinea pigs		Control guinea pigs	
Saline at the 31st day	NS (n=8)	0.29±0.50	NS (n=4)	0.15±0.35
Saline at the 33rd day		0.42±0.33		0.12±0.30
Saline	P<0.05 (n=8)	0.43±0.37	NS (n=6)	0.13±0.49
P-L-G		0.58±0.36		0.29±0.38
Saline	P<0.05 (n=9)	0.43±0.36	P<0.01 (n=5)	0.49±0.29
P-L-L		0.20±0.25		0.28±0.37
Saline–Saline	NS (n=5)	0.25±0.36		
LMWH–P-L-L		0.28 ±0.50		
Saline	NS (n=6)	0.48 ±0.70	NS (n=5)	0.28± 0.40
Dextran		0.44 ±0.36		0.35± 0.54

The data are expressed as means ± S.D. PC100: The provoking methacholine concentration causing a 100% increase from baseline in Rrs. P-L-G: Poly-L-glutamic acid. P-L-L: Poly-L-lysine. LMWH: Low-molecular-weight heparin. Rrs: Respiratory resistance.

6). Pretreatment with inhaled poly-L-lysine significantly increased respiratory resistance only at 1 min, whereas it significantly decreased respiratory resistance only at 12 h after antigen exposure compared with the effect of pretreatment with saline. Pretreatment with poly-L-lysine significantly enhanced the peak immediate response, but not the peak late response (Table 1). There were no significant dif-

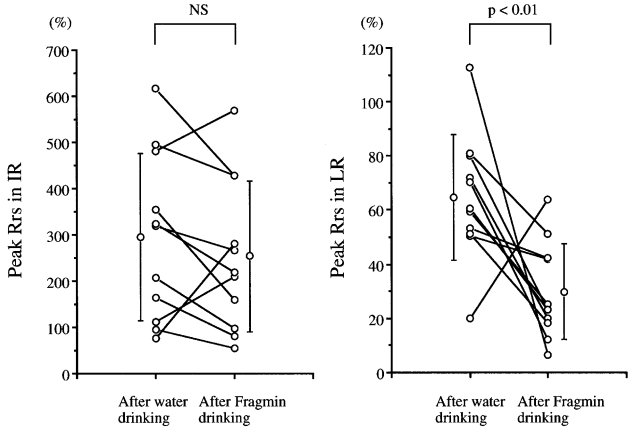

Fig. 7. Effects of oral dalteparin on peak Rrs in IR and LR after antigen exposure in sensitized guinea pigs. Rrs: Respiratory resistance; IR: Immediate response: LR: Late response.

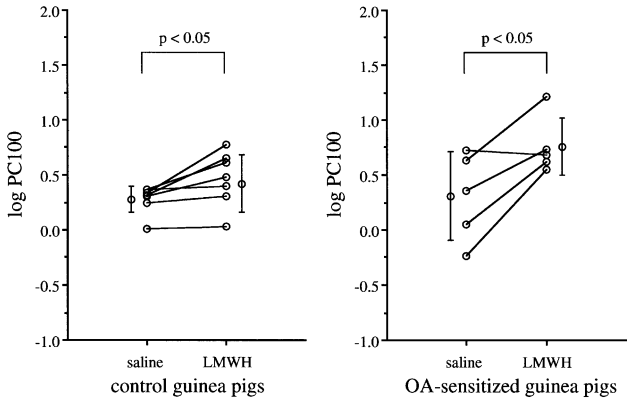

Fig. 8. Log PC100 in control guinea pigs and sensitized guinea pigs treated with saline and with LMWH. PC100: The provoking methacholine concentration causing a 100% increase from baseline in Rrs; LMWH: Low-molecular-weight heparin; Rrs: Respiratory resistance.

ferences in the serial changes in respiratory resistance after antigen exposure between pretreatment with inhaled dextran or saline. Dextran did not have a significant effect on either the peak immediate response or the peak late response (Table 1).

3.2. The effects of oral dalteparin on the bronchoconstriction induced by antigen exposure

Pretreatment with oral dalteparin significantly inhibited the increase in respiratory resistance induced by antigen exposure at 8 and 10 min and 6, 7, 8, 10, 11, 12 and 24 h compared with the effect of pretreatment with tap water alone. Oral dalteparin completely suppressed the late response, but only partially suppressed the immediate response. Fig. 7 shows the effects of oral dalteparin on the peak immediate response and the peak late response.

3.3. The effects of polyelectrolyte substances on the non-specific bronchoconstriction induced by methacholine inhalation

Since there were no significant differences in the airway responsiveness to methacholine between the 31st and the 33rd day (Table 2), we confirmed the reproducibility of the methacholine challenge test.

Fig. 8 shows the effects of low-molecular-weight heparin inhalation on the airway responsiveness to methacholine. Low-molecular-weight heparin significantly inhibited the airway responsiveness to methacholine in both sensitized guinea pigs and non-sensitized guinea pigs.

The effects of inhalation of poly-L-glutamic acid, poly-L-lysine alone, poly-L-lysine after low-molecular-weight heparin and dextran on the airway responsiveness to methacholine are summarized in Table 2. Poly-L-glutamic acid significantly inhibited the airway responsiveness to methacholine in sensitized guinea pigs, but had no significant effects in non-sensitized guinea pigs. Although poly-L-lysine significantly enhanced the airway responsiveness to methacholine in both sensitized and non-sensitized guinea pigs, the airway hyperresponsiveness induced by poly-L-lysine was significantly suppressed after pretreatment with inhaled low-molecular-weight heparin 1 h before poly-L-lysine inhalation in sensitized guinea pigs. Dextran had no significant effects on the airway responsiveness to methacholine in either sensitized or non-sensitized guinea pigs (Fig. 9).

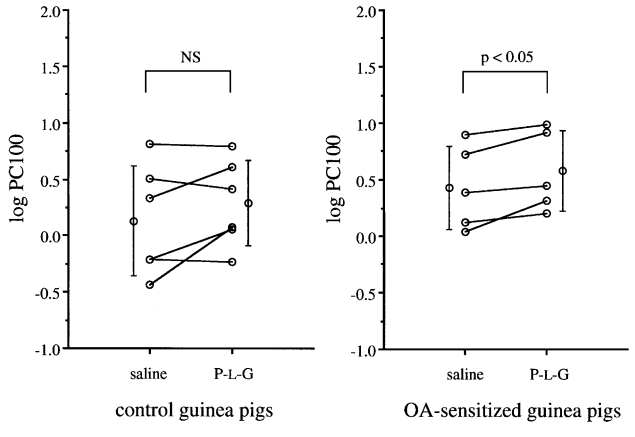

Fig. 9. Log PC100 in control guinea pigs and sensitized guinea pigs treated with saline and with P-L-G. PC100: The provoking methacholine concentration causing a 100% increase from baseline in Rrs; P-L-G: Poly-L-glutamic acid; Rrs: Respiratory resistance.

4. Discussion

The present findings showed that low-molecular-weight heparin and poly-L-glutamic acid, which are anionic polyelectrolyte substances, inhibited the bronchoconstriction induced by antigen exposure and the airway responsiveness to methacholine, while poly-L-lysine, a cationic polyelectrolyte substance, induced airway hyperresponsiveness to methacholine, an effect which was suppressed by pretreatment with inhaled low-molecular-weight heparin. We have shown previously that pretreatment with inhaled low-molecular-weight heparin inhibits both the immediate response and the late response induced by antigen exposure and decreases the percentage of eosinophils in bronchoalveolar lavage fluid and the number of infiltrated eosinophils in the airway wall (Maeda et al., 1995). Although there have been several studies of the anti-inflammatory and anti-allergic properties of heparin, and of the inhibitory effects of heparin on the immediate bronchoconstriction elicited by antigen exposure and airway hyperresponsiveness, we believe this is the first study showing that low-molecular-weight heparin inhibits the late response after antigen exposure.

In preliminary experiments, most of the sensitized guinea pigs died of asphyxiation within 10 min after antigen exposure if a histamine antagonist was not injected before antigen exposure. Therefore, we performed preliminary experiments in which pyrilamine maleate was injected at concentrations of 1, 2, 5, 10, 20 or 50 mg/kg before antigen exposure, and used 10 mg/kg for the present study, as this was the minimal concentration providing a survival rate greater than 80%. Therefore, this concentration was considered insufficient to completely antagonize the effects of released histamine. Thus, the immediate response in our animal model was considered to be due to histamine, leukotrienes and prostaglandins.

As indicated above, because most of the sensitized guinea pigs died of asphyxiation within 10 min after antigen exposure if pyrilamine maleate was not injected before antigen exposure, histamine may play an important role in the immediate response in our animal model. Moreover, in our animal model there is an increase in the percentage of eosinophils in bronchoalveolar lavage fluid and an increase in the number of infiltrated eosinophils in the airway wall (Maeda et al., 1995). In light of these experimental results,

allergic airway inflammation in our animal model may be similar to that in human asthma.

In the present study, both inhaled low-molecular-weight heparin and inhaled poly-L-glutamic acid significantly inhibited the immediate response and the late response, and weakened wheezes in sensitized-guinea pigs after antigen exposure. The effects of these anionic polyelectrolyte substances may be due to neutralization of chemical mediators and the eosinophil cationic granuloprotein by these anionic substances. In addition, low-molecular-weight heparin inhibited the immediate response more strongly than poly-L-glutamic acid did. Previous studies indicated that low-molecular-weight heparin acts as a competitive inhibitor of inositol 1,4,5-triphosphate (IP_3) receptors in mast cells (Ghosh et al., 1988; Lucio et al., 1992) and has an inhibitory effect on histamine release preventing mast cell degranulation (Ahmed et al., 1997). IP_3 is one of the second messengers involved in stimulus-secretion coupling in mast cells, and it has been demonstrated that heparin inhibits mast cell-mediated reactions (Lucio et al., 1992). Ahmed et al. (1994) suggested that heparin prevents antigen-induced bronchoconstriction and airway hyperresponsiveness by inhibiting IP_3-dependent mast cell mediator release. The inhibitory effects of poly-L-glutamic acid on the late response were equivalent to those of low-molecular-weight heparin. It was suggested that anionic polyelectrolyte substances neutralized chemical mediators and eosinophil cationic granuloproteins such as MBP, eosinophil cationic protein and eosinophil peroxidase and diminished epithelial edema and dysfunction, and that consequently the late asthmatic response was inhibited. Recently, Ahmed et al. (2000) demonstrated that only ultralow-molecular-weight heparins caused a dose-dependent inhibition of the antigen-induced immediate and late asthmatic response and postantigen airway hyperresponsiveness, whereas low-molecular-weight heparins were ineffective. The discrepancies between their and our results may be caused by the difference in the dose of low-molecular-weight heparin administered. In their experiments, low-molecular-weight heparins (1.25 mg/kg) were dissolved in 3 ml of bacteriostatic injection water and administered into the trachea as an aerosol over 15–20 min. The guinea pigs inhaled low-molecular-weight heparin (about 240 mg/kg) by mean of an ultrasonic nebulizer in our study. We administered higher doses of low-molecular-weight heparin than Ahmed et al. The inhibition of IP_3 binding to its receptors by heparin is molecular weight-dependent, and the inhibitory activity decreases as the size of the heparin chain is reduced below 18 monosaccharide units (Tones et al., 1989). Whereas 10- to 14-monosaccharide fractions had substantially lower activity, the 8-monosaccharide fractions (mol. wt. <2500) had none. Ahmed et al. (2000) focused on the antiallergic and anti-inflammatory activities of heparin-derived oligosaccharides and speculated that these activities might be inversely related to the chain length (mol. wt. <2500), as was found to be the case in the inhibition of elastase activity (Redini et al., 1988). In our study, however, low-molecular-weight heparin significantly inhibited the antigen-induced immediate response, the late response and airway hyperresponsiveness to methacholine. Therefore, it is assumed that the "cationic–anionic interaction" is important, as are heparin-derived oligosaccharides, to the mechanism of antiallergic activity of heparin.

In the present study, both low-molecular-weight heparin and poly-L-glutamic acid suppressed airway responsiveness to methacholine. These protective effects may be due to the ability of both compounds to reverse antigen-induced dysfunction of muscarinic M_2 receptors in guinea pigs (Fryer and Jacoby, 1992, 1993).

Barker et al. (1991) showed that inhaled polyglutamic acid inhibited bronchoconstriction and airway hyperresponsiveness to methacholine after intratracheal instillation of MBP, whereas Sasaki et al. (1993) reported that the intravenous administration of polyglutamic acid did not significantly affect PAF-induced airway hyperresponsiveness to histamine. It was suggested inhalational administration had a more potent inhibitory effect on bronchoconstriction and airway hyperresponsiveness after antigen exposure than intravenous administration. Preuss and Page (2000) reported that inhaled polyglutamic acid failed to inhibit antigen-induced bronchoconstriction and airway hyperresponsiveness to histamine. The discrepancies between their and our results may be caused by the difference in molecular-weight of polyglutamic acid. The molecular-weight of polyglutamic acid used in their study was 50 000–100 000, whereas ours was 14 300. Barker et al. (1991) demonstrated that the negative charge of poly-L-glutamic acid was stronger as the molecular-weight increased, and that a high-molecular-weight poly-L-glutamic acid had a more potent ability to inhibit MBP, eosinophil cationic protein and poly-L-arginine toxicity to K562 cells in vitro. The possibility remains that there is a molecular-weight appropriate for inhibitory effects of poly-L-glutamic acid following inhalation, and that this may be related to not only the strength of the negative charge, but also to the diffusion of aerosolized poly-L-glutamic acid in the airway. It was shown in the present study that poly-L-glutamic acid did not suppress airway responsiveness to methacholine in non-sensitized guinea pigs, while low-molecular-weight heparin suppressed airway responsiveness to methacholine in non-sensitized guinea pigs. The former finding is compatible with a previous study (Coyle et al., 1993a), whereas the latter is contradictory to the findings of previous studies. Although the mechanism of the latter finding remains unexplained, the possibility remains that low-molecular-weight heparin can directly affect airway smooth muscle (Johnson et al., 1995; Ceyhan and Celikel., 1995).

Our aim was to study the inhibitory effects of oral low-molecular-weight heparin because low-molecular-weight heparin showed inhibitory effects superior to those of poly-L-glutamic acid in the present study, and we had not been aware of reports of the effects of oral low-molecular-weight

heparin. Therefore, we performed experiments with dalteparin, which is a commercially available low-molecular-weight heparin. It was shown in the present study that oral dalteparin significantly inhibited the late response, but not the immediate response after antigen exposure. Although it has been demonstrated that inhaled dalteparin inhibits the immediate asthmatic response and airway hyperresponsiveness induced by antigen exposure (Ahmed et al., 1997), it is not yet known whether oral dalteparin affects the immediate and late asthmatic responses after antigen exposure. Sue et al. (1976) demonstrated that heparin was absorbed from the gastrointestinal tract, and that the absorption was poor when heparin was ionized. This poor absorption may be one of reasons for the reduced effect of oral dalteparin compared with inhaled dalteparin. New agents to aid the absorption of heparin in the gastrointestinal tract and to prevent its migration into the airways may hold the key to the possible future therapeutic use of oral heparin.

The present findings showed that inhaled poly-L-lysine significantly affected neither the immediate nor the late response after antigen exposure, while inhaled poly-L-lysine elicited a significant increase in airway responsiveness to methacholine in both sensitized and non-sensitized guinea pigs. Pretreatment with inhaled low-molecular-weight heparin before poly-L-lysine exposure suppressed the airway hyperresponsiveness induced by poly-L-lysine in sensitized guinea pigs. These findings were compatible with those of previous studies (Shirotani et al., 1999; Uchida et al., 1996) and may be attributed to the charge interaction (Gleich et al., 1974). We speculate that the major mechanisms responsible for the increased response to methacholine following pretreatment with poly-L-lysine may be airway epithelial dysfunction and damage induced by cationic proteins. Epithelial denudation and infiltration of inflammatory cells were seen when we dissected the lungs of non-sensitized guinea pigs 12 h after inhalation of poly-L-lysine or poly-L-arginine, which is also a cationic protein. Pretreatment with cationic protein may neutralize and remove negative charges on the airway epithelial cell surface. Chang and Voelkel (1989) reported that neutralization and/or removal of surface negative charges would promote the adhesion of inflammatory cells to the lung microvascular endothelial surface, allowing an increased area of direct contact between other injurious species and endothelial cells, and that cationic compounds might further stimulate the release of proteolytic enzymes and oxidants from neutrophils. The same would apply to epithelial cells. In an intact guinea pig tracheal tube preparation in vitro, perfusion of the luminal surface with polycations increased responsiveness to intraluminally, but not to extraluminally, applied methacholine, directly demonstrating that cationic peptides may indeed induce airway hyperreactivity by changing the epithelial layer (Coyle et al., 1993b). Moreover, there is increasing evidence that cationic proteins can exert a cytotoxic effect on airway epithelial cells and also alter airway functions through "non-cytotoxic" mechanisms. In addition, cationic proteins appear to activate sensory nerves (Coyle et al., 1994), to liberate bradykinin (Shirotani et al., 1999; Coyle et al., 1995), and to enhance parasympathetic neurotransmission (Costello et al., 1997). Recently, Meurs et al. (1999) suggested that the enhanced sensitivity to intraluminal methacholine in poly-L-arginine-treated airways may reflect an enhanced epithelial permeability for the agonist due to disruption of the diffusion barrier, and that a deficiency of nitric oxide, which is one of the epithelium-derived relaxing factors, was involved in polycation-induced airway hyperreactivity. One possible mechanism of polycation-induced nitric oxide deficiency could be polycation-induced inhibition of the cellular uptake of L-arginine by cationic amino acid transporters, as indicated in rat and guinea pig alveolar macrophages and tracheal epithelial cells (Hammermann et al., 1999). Moreover, it has been shown that the presence of heparin together with cationic proteins blocked the inhibitory effect of cationic proteins on L-arginine uptake (Hammermann et al., 1999).

We showed that inhaled dextran did not significantly affect either bronchoconstriction after antigen exposure or airway responsiveness to methacholine in both sensitized and non-sensitized guinea pigs. Coyle et al. (1993a) reported that instillation of dextran sulfate increases airway responsiveness, and that this may be related to the well-documented ability of dextran to elicit mast cell degranulation (Ankier et al., 1968). However, our neutral dextran showed no significant effects. It is suggested that this ability of dextran to elicit mast cell degranulation is less important than the charge interaction in the present experiments.

We believe this is the first study to investigate the effects of anionic polyelectrolyte substances, except heparin and cationic polyelectrolyte substances, on serial bronchoconstriction for as long as 24 h after airway challenge with antigen. The findings of the present investigation showed that the "cationic–anionic interaction" may play an important role in the immediate and late asthmatic responses and airway hyperresponsiveness in bronchial asthma. How charge interaction contributes to the pathophysiology of bronchoconstriction in bronchial asthma needs further investigation.

Acknowledgements

We would like to thank the Laboratory of Kissei Pharmaceutical for analyzing dalteparin.

References

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

Ahmed, T., Syriste, T., Mendelssohn, R., Sorace, D., Mansour, E., Lansing, M., Abraham, W.M., Robinson, M.J., 1994. Heparin prevents antigen-induced airway hyperresponsiveness: interference with IP_3-mediated mast cell degranulation? J. Appl. Physiol. 76, 893–901.

Ahmed, T., Campo, C., Abraham, M.K., Morinari, 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. Am. J. Respir. Crit. Care Med. 155, 1848–1855.

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.

Ankier, S.I., West, G.B., Harris, J.M., Luscombe, D.K., 1968. Reactivity of Wistar rats to dextran. J. Pharm. Pharmacol. 17, 118–119.

Arseneault, D., Maghni, K., Sirois, P., 1999. Selective inflammatory response induced by intratracheal and intravenous administration of poly-L-arginine in guinea pig lungs. Inflammation 23, 287–304.

Barker, R.L., Gleich, G.L., Pease, L.R., 1988. Acidic precursor revealed in human eosinophil granule major basic protein cDNA. J. Exp. Med. 168, 1493–1498.

Barker, R.L., Gundel, R.H., Gleich, G.J., Checkel, J.L., Loegering, D.A., Pease, L.R., Hamann, K.J., 1991. Acidic polyamino acids inhibit human eosinophil granule major basic protein toxicity; evidence of a functional role for proMBP. J. Clin. Invest. 88, 793–805.

Ceyhan, B., Celikel, T., 1995. Effect of inhaled heparin on methacholine-induced bronchial hyperreactivity. Chest 107, 1009–1012.

Chang, S.W., Voelkel, N.F., 1989. Charge-related lung microvascular injury. Am. Rev. Respir. Dis. 139, 534–545.

Costello, R.W., Schofield, B.H., Kephart, G.M., Gleich, G.J., Jacoby, D.B., Fryer, A.D., 1997. Localization of eosinophils to airway nerves and effect on neuronal M-2 muscarinic receptor function. Am. J. Physiol. 17, L93–L103.

Coyle, A.J., Ackerman, S.J., Irvin, C.G., 1993a. Cationic proteins induce airway hyperresponsiveness dependent on charge interactions. Am. Rev. Respir. Dis. 147, 896–900.

Coyle, A.J., Mitzner, W., Irvin, C.G., 1993b. Cationic proteins alter smooth muscle function by an epithelium-dependent mechanism. J. Appl. Physiol. 74, 1761–1768.

Coyle, A.J., Perretti, F., Manzini, S., Irvin, C.G., 1994. Cationic protein-induced sensory nerve action. J. Clin. Invest. 94, 2301–2306.

Coyle, A.J., Ackerman, S.J., Burch, R., Proud, D., Irvin, C.G., 1995. Human eosinophil-granule major basic protein and synthetic polycations induce airway hyperresponsiveness in vivo dependent on bradykinin generation. J. Clin. Invest. 95, 1735–1740.

Fryer, A.D., Jacoby, D.B., 1992. Function of pulmonary M_2-muscarinic receptors in antigen-challenged guinea pigs is restored by heparin and poly-L-glutamate. J. Clin. Invest. 90, 2292–2298.

Fryer, A.D., Jacoby, D.B., 1993. Effect of inflammatory cell mediators on M_2 muscarinic receptors in the lung. Life Sci. 52, 529–536.

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

Gleich, G., Loegering, D.A., Kuppers, F., Bajaj, S.P., Mann, K.G., 1974. Physiochemical and biological properties of the major basic protein from guinea pig eosinophil granules. J. Exp. Med. 140, 313–331.

Hammermann, R., Hirschmann, J., Hey, C., Mossner, J., Folkerts, G., Nijkamp, F.P., Wessler, I., Racke, K., 1999. Cationic proteins inhibit L-arginine uptake in rat alveolar macrophages and tracheal epithelial cells. Am. J. Respir. Cell Mol. Biol. 21, 155–162.

Johnson, P.R., Armour, C.L., Carey, D., Black, J.L., 1995. Heparin and PGE2 inhibit DNA synthesis in human airway smooth muscle cells in culture. Am. J. Physiol. 269, L514–L519.

Lucio, J., D'Brot, J., Guo, C., 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.

Maeda, H., Yahata, T., Satouchi, M., Takenaka, K., Yokoyama, M., 1995. Inhibitory effect of low molecular weight heparin on the bronchoconstriction in ovalbumin-sensitized guinea pigs after antigen exposure. Arerugi 44, 1282–1289.

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.

Mead, J., 1960. Control of respiratory frequency. J. Appl. Physiol. 15, 325–336.

Meurs, H., Schuurman, F.E., Duyvendak, M., Zaagsma, J., 1999. Deficiency of nitric oxide in polycation-induced airway hyperreactivity. Br. J. Pharmacol. 126, 559–562.

Motojima, S., Makino, S., 1994. Effect of anionic polyelectrolyte substance on damages to respiratory epithelium induced by eosinophil peroxidase. Dokkyo J. Med. Sci. 21, 123–134.

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.

Redini, F., Tixier, J., Petitou, M., Choay, J., Robert, L., Hornebeck, W., 1988. Inhibition of leukocyte elastase by heparin and its derivates. Biochem. J. 252, 515–519.

Salvi, S.S., Krishna, M.T., Sampson, A.P., Holgate, S.T., 2001. The anti-inflammatory effects of leukotriene-modifying drugs and their use in asthma. Chest 119, 1533–1546.

Sasaki, M., Shioya, T., 1995. Asthma and heparin therapy. Respir. Res. 14, 509–516.

Sasaki, M., Herd, C.M., Page, C.P., 1993. Effect of heparin and low-molecular weight heparinoid on PAF-induced airway responses in neonatally immunized rabbits. Br. J. Pharmacol. 110, 107–112.

Schwartz, L.B., Huff, T.F., 1991. Mast cells. In: Crystal, R.G., West, J.B., et al. (Eds.), The Lung: Scientific Foundations. Raven Press, New York, pp. 601–616.

Shirotani, T., Maeda, H., Nishimura, Y., Yokoyama, M., 1999. Thromboxane A2 mediates cation-induced airway hyperresponsiveness through the bradykinin B2 receptor in guinea pigs. Allergol. Int. 48, 61–68.

Sue, T.K., Jaques, L.B., Yuen, E., 1976. Effects of acidity, cations and alcoholic fractionation on absorption of heparin from gastrointestinal tract. Can. J. Physiol. Pharmacol. 54, 613–617.

Terashi, Y., Yukawa, T., Fukuda, T., Makino, S., 1988. Late phase response in the guinea pig airway caliber following inhaled antigen exposure. Arerugi 37, 980–991.

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-triphosphate receptor in rat liver microsomes. Dependence on sulphate content and chain length. FFBS Lett. 252, 105–108.

Uchida, D.A., Ackrmann, S.J., Coyle, A.J., Laesen, G.L., Weller, P.F., Freed, J., Irvin, C.G., 1993. The effect of human eosinophil granule major basic protein of airway responsiveness in the rat in vivo. Am. Rev. Respir. Dis. 147, 982–988.

Uchida, D.A., Irvin, C.G., Ballowe, C., Larsen, G., Cott, G.R., 1996. Cationic proteins increase the permeability of cultured rabbit tracheal epithelial cells; modification by heparin and extracellular calcium. Exp. Lung Res. 22, 85–99.