

Time course of changes in adenosine 5'-monophosphate airway responsiveness with inhaled heparin in allergic asthma

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Background: Recent studies have shown that inhaled heparin exerts a protective effect against various bronchoconstrictor stimuli in asthma, possible through an inhibition of mast cell activation.

Objective: Because adenosine 5'-monophosphate (AMP) elicits bronchoconstriction by augmenting mast cell mediator release, we have investigated the effect of inhaled heparin (15,000 units USP/ml, 4 ml) on the bronchoconstrictor response to this agonist and to methacholine in a randomized, double-blind, placebo-controlled study of 10 subjects with asthma. We also carried out a separate randomized, double-blind study in seven additional volunteers with asthma to examine in more detail the time-course of change in bronchial reactivity to inhaled AMP after treatment with nebulized heparin.

Results: Inhaled heparin significantly increased the provocative concentration of AMP causing a 20% decrease in forced expiratory volume in 1 second ($PC_{20}FEV_1$ -AMP) from the postplacebo treatment value of 22.3 mg/ml (range, 5.7 to 68.9 mg/ml) to 48.1 mg/ml (range, 5.1 to 196.8 mg/ml) ($p < 0.01$). When compared with placebo, inhaled heparin failed to alter the airway responsiveness to methacholine; the mean (range) PC_{20} methacholine values were 1.00 mg/ml (0.44 to 4.76 mg/ml) and 1.08 mg/ml (0.46 to 5.08 mg/ml), respectively. After placebo administration, the PC_{20} AMP values at 15, 60, and 180 minutes did not differ significantly from each other; their geometric mean (range) values were 26.1 mg/ml (5.9 to 85.8 mg/ml), 26.6 mg/ml (6.3 to 87.8 mg/ml), and 24.9 mg/ml (5.2 to 80.2 mg/ml), respectively. When compared with placebo, the PC_{20} values for AMP after administration of inhaled heparin were significantly increased up to 57.3 mg/ml (14.7 to 176.0 mg/ml) and to 52.7 mg/ml (13.9 to 90.8 mg/ml) at 15 minutes and 60 minutes, respectively. At 180 minutes, inhaled heparin failed to affect AMP airway responsiveness; the PC_{20} AMP was not significantly different from that of placebo, with a value of 30.6 mg/ml (4.8 to 93.3 mg/ml).

Conclusion: Heparin administered by inhalation is effective in attenuating the airway response to AMP but not to methacholine. The time course of change in bronchial reactivity to AMP has a peak effect at 15 minutes and lasts up to 60 minutes. It is possible that the mechanism(s) underlying the protective effects of inhaled heparin in asthma may be related to

an inhibitory modulation of mast cell activation. (*J Allergy Clin Immunol* 1997;99:338-44.)

Key words: Asthma, bronchoconstriction, adenosine, heparin

Heparin, a highly sulfated unbranched glycosaminoglycan, possesses multiple nonanticoagulant properties, including modulation of various proteases,^{1,2} inhibition of cell growth,^{3,4} and antiinflammatory actions.^{5,6} Moreover, Page⁷ has suggested that heparin may play a role in limiting the allergic inflammatory events associated with asthma. Recently, heparin administered by inhalation has been shown to protect the asthmatic airways against various bronchoconstrictor stimuli, including allergen⁸ and exercise.⁹ Thus although heparin's anticoagulant properties dominate its use in medicine, heparin may also play a role in limiting the allergic inflammatory events associated with asthma.

Although the mechanisms underlying the protective effects of this drug in experimental models of clinical asthma are not yet understood, the inhibitory effects of inhaled heparin in the airways in the absence of bronchodilatation may be related to a suppressive action on mast cell degranulation.^{10,11} Thus because a component of the bronchoconstriction produced by both exercise and allergen is dependent on mediator release from airway mast cells,^{12,13} it is possible that heparin exerts its inhibitory effect on these cells. To test this hypothesis we have investigated the effect of inhaled heparin on the bronchoconstrictor response of subjects with asthma to adenosine 5'-monophosphate (AMP), a purine nucleotide, which is believed to act largely through augmenting mast cell degranulation within the tracheobronchial tree,¹⁴ and to methacholine, which provokes bronchoconstriction directly by stimulating specific muscarinic receptors on airway smooth muscle.

The study took the form of a randomized, double-blind, placebo-controlled study of 10 subjects with asthma, with heparin being administered as an aerosol. Most studies have measured changes in airway response after inhalation of nebulized heparin only at a single time point, and little is known of the change in bronchial reactivity with time. Therefore we undertook a separate randomized, double-blind trial in seven additional subjects with asthma to examine in more detail the time course of change in bronchial reactivity to inhaled AMP after treatment with inhaled heparin.

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Abbreviations used

AMP:	Adenosine 5'-monophosphate
PC ₂₀ FEV ₁ :	Provocative concentration causing a 20% decrease in forced expiratory volume in 1 second
IP ₃ :	1,4,5-Inositol triphosphate

METHODS

Subjects

A total of 17 subjects with asthma (4 men) with a mean ($\pm$ SEM) age of 27.2 ($\pm$ 1.7) years, participated in the study (Table I). All patients were nonsmokers, and all were atopic as defined by a positive skin prick test reaction (>2 mm wheal response)¹⁵ and class III or IV positive RAST responses to one or more common aeroallergens (*Dermatophagoides pteronyssinus*, *D. farinae*, *Parietaria* pollen—one of the most common inhalant allergens in Sicily,¹⁶ mixed grass pollen, mixed tree pollen, cat fur, and dog hair). All patients had baseline FEV₁ responses greater than 75% of their predicted values or greater than 2.01. No patient received oral corticosteroids, theophylline, or sodium cromoglycate in the preceding 8 weeks. Bronchodilators were withheld for 8 hours before each visit to the laboratory, although subjects were allowed to use inhaled corticosteroids as usual. Subjects were not studied within 4 weeks of an upper respiratory tract infection or of exacerbation of their asthma, and all visits to the laboratory took place outside the pollen season. The study was approved by the Ethical Sub-committee of the Department of Respiratory Diseases (University of Catania), and all subjects gave their informed consent.

Bronchial provocation

Pulmonary function was measured before and during the provocation as the FEV₁ by using a dry wedge spirometer (Vitalograph, Buckinghamshire, U.K.); the first of two consecutive measurements was used for analysis. Methacholine and AMP (both from Sigma Chemical Co., St. Louis, Mo.) were made up in 0.9% sodium chloride to produce a range of increasing doubling concentrations from 0.03 to 64.00 mg/ml (0.2 to 327 mmol/L) and 0.78 to 800 mg/ml (8.96 to 1151.4 mmol/L), respectively. The solutions were administered as aerosols generated from a starting volume of 3 ml in a disposable Inspiron mininebulizer (C.R. Bard International, Sunderland, U.K.) driven by compressed air at 8 L/minute. Under these conditions, the nebulizer has an output of 0.48 ml/minute and generates an aerosol with a mass median particle diameter of 4.7 μ m.¹⁷ While wearing a nose clip, each subject inhaled the aerosolized solutions through a mouthpiece as five breaths from end-tidal volume to full inspiratory capacity.¹⁸⁻²⁰

Study design

The study consisted of two distinct phases.

Phase 1. Ten patients with asthma attended the laboratory on six separate visits, at least 72 hours apart. On the first two visits, subjects attended the laboratory to undertake concentration-response studies with inhaled methacholine and AMP in the absence of any drug treatment. On the first visit, after a 15-minute rest, three baseline measurements of FEV₁ were made at intervals of 3 minutes, followed by inhalation of 0.9% sodium chloride and repeat FEV₁ measurements at 1 and 3

minutes, the higher value was recorded. Provided that FEV₁ did not fall by more than 10% of the baseline value, a methacholine concentration-response study was carried out. After administration of each methacholine concentration-response study was carried out. After administration of each methacholine concentration, FEV₁ was measured at 1 and 3 minutes. Increasing doubling concentrations of methacholine were inhaled at 5-minute intervals until the FEV₁ had fallen by more than 20% of the postsaline inhalation value. The fall in FEV₁ after each concentration of agonist was expressed as a percentage of the higher of the two postsaline inhalation baseline FEV₁ recordings. The percentage fall in FEV₁ was plotted against the cumulative concentration of agonist on a logarithmic scale, and the provocation concentration required to produce a 20% decrease in FEV₁ from the postsaline treatment baseline value (PC₂₀) was determined by linear interpolation. On the second occasion, a bronchial provocation test with inhaled AMP was undertaken in a manner similar to that described for methacholine. FEV₁ measurements were recorded 1 and 3 minutes after inhalation of each concentration of AMP, and the corresponding PC₂₀ FEV₁ values were derived.

On the following four visits, subjects attended the laboratory to undertake concentration-response studies with inhaled AMP and methacholine after nebulized heparin or matched placebo was administered in a double-blind manner in random order 15 minutes before challenge. Both the heparin and the placebo solutions were freshly prepared by an independent investigator on the basis of a randomized code and were then returned to the conducting physician to administer to the attending subject. On each occasion, after a 15-minute rest, three baseline measurements of FEV₁ were recorded at intervals of 3 minutes. This was followed by inhalation of nebulized heparin (Sigma Chemical Co.) at a concentration of 15,000 units USP/ml or placebo, consisting of 0.9% sodium chloride adjusted to a pH and tonicity similar to the drug solution used. The aerosol solutions were generated from a starting volume of 4.0 ml in an Inspiron mininebulizer driven by compressed air at 8 L/minute, and inhaled to dryness by deep tidal breathing over a 7- to 9-minute period. The same nebulizer was used for all studies in all subjects. The amount of heparin delivered to the mouth was calculated by differential weighing (taking into account the heparin exhausted to the ambient air during patient expiration), and on five occasions amounted to 40,000 $\pm$ 4.20 units USP (mean $\pm$ SEM). Further FEV₁ measurements were repeated at 2, 5, 10, and 15 minutes after drug or placebo inhalation, and dose-response studies with increasing concentrations of AMP or methacholine were carried out in a manner similar to that described in the initial two visits.

Phase 2. Seven subjects (nos. 11 to 17) attended the laboratory on six separate occasions 3 to 5 days apart in order to investigate the time course of changes in AMP bronchial reactivity with inhaled heparin (15,000 units USP/ml). On each occasion, the subject received nebulized heparin or matched placebo 15, 60, and 180 minutes before a bronchial provocation test with AMP in a randomized, double-blind fashion. Thus on the six visits required to complete this phase of the study, each subject received: (1) placebo at 15, 60, and 180 minutes and (2) heparin at 15, 60, and 180 minutes. The administration of the aerosol solutions and the challenge procedure used were identical to those described in phase 1 of the study.

Data analysis

Results are expressed as mean $\pm$ SEM values unless otherwise stated, and a *p* value less than 0.05 was accepted as

TABLE I. Demographic details of subjects studied

Subject No.	Sex	Age (yr)	Baseline FEV ₁ (% predicted)	Atopy*	PC ₂₀ methacholine (mg/ml)	Regular medication
1	F	20	96	D	0.90	BDP (1000 µg)
2	F	33	99	W	4.58	
3	F	22	84	W-D	0.88	
4	F	26	126	D	0.75	S, BDP (1000 µg)
5	M	23	91	D	0.51	
6	F	23	80	W-D	2.65	
7	M	19	99	W	0.45	BDP (1000 µg)
8	M	38	90	D	0.72	
9	F	25	87	W-D	0.45	S, BDP (2000 µg)
10	F	36	107	W-G	0.65	
11	F	37	100	W	1.21	
12	M	22	83	W	0.65	BDP (1000 µg)
13	F	18	78	W-D	0.31	S, BDP (1000 µg)
14	F	23	97	W	1.78	S, SCG
15	F	27	94	D-G	1.56	
16	F	40	90	W	1.60	S
17	F	31	95	D	0.25	
		27.2	93.9		0.87‡	
		± 1.7†	± 2.8†		(0.25-4.58)	

D, *Dermatophagoides*; W, *Parietaria* spp.; G, grass; BDP, beclomethasone dipropionate. S, salbutamol as needed; SCG, sodium cromoglycate.

*Atopic; positive immediate skin test response to one or more allergens.

†Mean ± SEM.

‡Geometric mean (range).

the minimum level of statistical significance. Pretreatment and posttreatment baseline values of FEV₁ before bronchial challenges were compared within each study day by using Student's *t* test for paired data and between study days by two-way analysis of variance. The repeatability of the AMP challenge procedure was determined according to the method described by Altman and Bland²¹ of plotting the difference against the mean of the logarithmically transformed PC₂₀ values obtained on the placebo and open-study days. The mean and standard deviation of the difference between these values were then derived and used to calculate the coefficient of repeatability between the results of the 2 study days.

The slopes of the AMP and the methacholine concentration-response curves were determined by least-squares linear regression analysis and compared between postplacebo and postheparin study days by using Student's *t* test for paired data. AMP and methacholine PC₂₀ values were logarithmically transformed to normalize their distribution and compared by means of two-factor analysis of variance followed by Neuman Keuls analysis when appropriate.

For each subject, concentration ratios for the protective effect of heparin against bronchoprovocation with each agonist were calculated individually by dividing the PC₂₀ value obtained after administration of active drug by that obtained after administration of the placebo. The relationship between PC₂₀ AMP and PC₂₀ methacholine values and their respective concentration ratios after heparin administration were investigated by least squares linear regression analysis.

Finally, analysis of the concentration ratio for AMP obtained from the six patients taking inhaled steroids (subjects 1, 4, 7, 9, 12, and 13), compared with that obtained from the remaining patients without inhaled steroids, was done with the Mann-Whitney U test.

RESULTS

Phase 1 studies

There were no significant differences in mean baseline values of FEV₁ between any of the study days. Inhaled heparin was well tolerated and had no effect on baseline FEV₁ values. After treatment with placebo and heparin, both AMP and methacholine provoked concentration-dependent falls in FEV₁, with slopes that did not depart significantly from parallel. For the 10 subjects studied, the geometric mean (range) PC₂₀ values with AMP and methacholine after treatment with placebo were 22.3 mg/ml (5.7 to 68.9 mg/ml) and 1.00 mg/ml (0.44 to 4.76 mg/ml), respectively, which did not differ significantly from the value of 21.7 mg/ml (5.4 to 71.0 mg/ml) and 0.90 mg/ml (0.45 to 4.58 mg/ml) obtained on the open study day. The challenge procedure in this group of subjects was found to be repeatable for both AMP and methacholine, with a coefficient of repeatability of 0.81 doubling dilutions for AMP and 1.08 doubling dilutions for methacholine. The inhalation test was repeatable to within a single doubling dilution in all the subjects receiving AMP and in eight of the 10 subjects receiving methacholine. These findings were consistent with the repeatability data obtained in previous studies with the AMP challenge.²²⁻²⁴

When compared with placebo, heparin protected the airways against the bronchoconstrictor effects of inhaled AMP. Heparin produced a displacement of the AMP concentration-response curve to the right in eight of 10 subjects (Table II). Inhaled heparin had a significant protective effect against the fall in FEV₁ provoked by

TABLE II. Effects of pretreatment with inhaled heparin and placebo on airway AMP and methacholine responsiveness

Subject no.	PC ₂₀ AMP (mg/ml)			PC ₂₀ Methacholine (mg/ml)		
	Baseline	Placebo	Heparin	Baseline	Placebo	Heparin
1	26.0	25.4	81.1	0.90	0.55	0.74
2	29.9	29.2	64.4	4.58	4.76	5.08
3	26.6	25.3	36.7	0.88	1.69	1.31
4	32.0	30.4	125.0	0.75	1.58	1.76
5	17.2	27.0	68.0	0.51	0.54	0.58
6	71.0	68.9	196.8	2.65	1.99	1.92
7	36.9	33.0	63.0	0.45	0.48	0.46
8	18.9	15.6	15.6	0.72	0.91	0.94
9	7.6	5.7	5.1	0.45	0.44	0.59
10	5.4	9.8	41.4	0.65	0.71	0.84
Geometric mean	21.7	22.3	48.1	0.90	1.00	1.08
Range	(5.4-71.0)	(5.7-68.9)	(5.1-196.8)	(0.45-4.58)	(0.44-4.76)	(0.46-5.08)

AMP (Table II); the geometric mean (range) PC₂₀ value increased from 22.3 mg/ml after placebo to 48.1 mg/ml (5.1 to 196.8 mg/ml) after treatment with drug $p < 0.01$. Thus when expressed as a concentration ratio, heparin afforded a 2.4-fold (0.9- to 4.2-fold) protection of the airways against AMP. No significant relationship could be found between concentration ratio for AMP and the baseline levels of airway responsiveness measured both as PC₂₀ AMP and PC₂₀ methacholine. The same dose of inhaled heparin had no significant effect on the methacholine-provoked fall in FEV₁; the geometric mean (range) PC₂₀ was 1.00 mg/ml and 1.08 mg/ml (0.46 to 5.08 mg/ml) after placebo and heparin administration, respectively (Table II).

Phase 2 studies

There were no significant differences in mean baseline values of FEV₁ between any of the 6 study days. Inhaled heparin did not significantly alter baseline FEV₁ values at any of the time points studied. When compared with placebo, inhaled heparin reduced the airway responsiveness to AMP significantly at 15 and 60 minutes in all the subjects studied, with a peak effect at the 15-minute time point. After placebo administration, the PC₂₀ AMP at 15, 60, and 180 minutes did not differ significantly from each other; their geometric mean (range) values were 26.1 mg/ml (5.9 to 85.8 mg/ml), 26.6 mg/ml (6.3 to 87.8 mg/ml), and 24.9 mg/ml (5.2 to 80.2 mg/ml) respectively (Fig. 1). Pretreatment with inhaled heparin increased the PC₂₀ AMP values up to 57.3 mg/ml (14.7 to 176.0 mg/ml) and 52.7 mg/ml (13.9 to 90.8 mg/ml) ($p < 0.01$) at 15 and 60 minutes, respectively (Fig. 1). However, at 180 minutes, heparin failed to protect against AMP-induced bronchoconstriction; the PC₂₀ value for AMP was 30.6 mg/ml (4.8 to 93.3 mg/ml), which was not significantly different from the value of 24.9 mg/ml obtained with placebo at the same time point (Fig. 1).

Finally, a separate analysis shows that when the data obtained from the six patients taking inhaled steroids (patients 1, 4, 7, 9, 12, and 13) are pooled, a similar

degree of protection with heparin is obtained on airway AMP responsiveness compared with the group as a whole. Inhaled heparin increased the concentration ratio for AMP in both groups, affording protection of 2.2-fold and 2.4-fold in the groups with and without inhaled steroids, respectively ($p = \text{NS}$).

DISCUSSION

We have demonstrated that administration of heparin by inhalation protects the airways of subjects with asthma against bronchoconstriction provoked by inhaled AMP. The protection afforded by heparin occurred in eight of the 10 subjects studied in phase 1 and in all of the subjects studied in phase 2 and amounted to displacement of the concentration-response curves of approximately 2.5-fold to the right, which is similar to that reported previously for other bronchoprovocants.^{8,9} We also failed to show a significant change in airway responsiveness to methacholine after heparin exposure. In addition, we have shown that the time course of change in bronchial reactivity to AMP has a peak effect at 15 minutes for inhaled heparin and lasts up to 60 minutes.

The dosage of inhaled heparin used in this study and the timing of administration before bronchial challenge were chosen on the basis of previous studies that have been shown to effectively reduce the bronchospastic response to specific and nonspecific stimuli in subjects with asthma.^{8,9}

AMP causes dose-related bronchoconstriction when inhaled by atopic and nonatopic subjects with asthma.^{19,20} Although the mode of action of adenosine in causing bronchoconstriction in asthma is not known with certainty, activation of mast cells with degranulation within the tracheobronchial tree is likely.¹⁴ Indeed, adenosine augments mediator release from immunologically stimulated human lung mast cells.^{25,26} The enhancement of mediator release from activated mast cell activation by adenosine in vitro is compatible with our recent observation in vivo that airways of patients with asthma response to endobronchial instillation of AMP

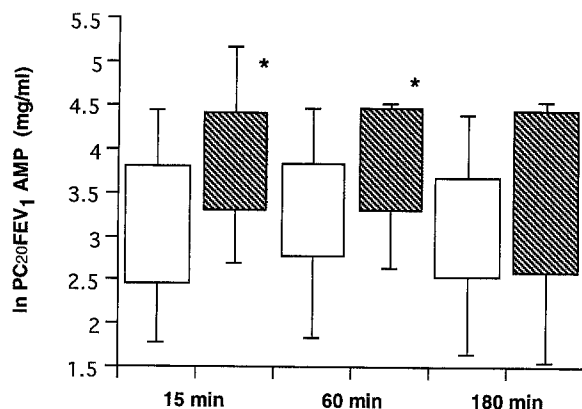


FIG. 1. Time-dependent inhibition of provocation concentrations of AMP required to provoke a 20% decrease in FEV₁ (PC₂₀FEV₁) after administration of inhaled heparin (hatched bars) and placebo (open bars) in seven subjects with asthma. Data are shown as box plots with mean $\pm$ SEM values. *Significant difference at $p < 0.01$ versus placebo.

with bronchial narrowing, and this is paralleled by a significant rise in bronchoalveolar lavage fluid levels of histamine and prostaglandin D₂.²⁷ Thus potentiation of mast cell mediator release may account for the bronchoconstriction provoked by inhaled AMP.

The mechanism by which heparin attenuates the bronchoconstrictor response to AMP is unclear and requires some investigation. We have shown no effect of inhaled heparin on baseline FEV₁ values, and this is in agreement with previous work with this drug in allergic sheep and in patients with asthma.^{8,9,11} In addition, a reduction in nonspecific airway responsiveness is unlikely because we have also shown that inhaled heparin had no effect on responsiveness to methacholine in the subjects studied. Similar lack of effect has been reported for the bronchoconstrictor response to both carbachol and histamine in allergic sheep¹¹ and for histamine-induced bronchoconstriction in subjects with asthma.⁹

Inhaled heparin seems to exert a preferential protective effect against stimuli such as allergen,⁸ exercise,⁹ and AMP (this study) that cause bronchoconstriction mainly by releasing spasmogenic mediators from airway mast cells. In support of this view, the antiallergic actions of heparin may be related to the inhibition of mediator release from mast cells.^{8,10,11} In addition, heparin has the ability to bind histamine,²⁸ and therefore it is plausible that heparin administered by inhalation may limit the biologic activity of endogenously released histamine.

To speculate further, the protective effect of inhaled heparin in adenosine-induced asthma may also be ascribed to blockade of intracellular mechanisms of mast cell degranulation. Lucio et al.¹⁰ have recently shown that heparin inhibits anti-IgE-provoked histamine release from human uterine mast cells in vitro without modifying the effect of the potent mast cell degranulator calcium ionophore A23187. Multiple signaling pathways control both immunologic and nonimmunologic stimu-

lus-secretion coupling in mast cells.²⁹ One of these pathways includes the breakdown of inositol phospholipids, which in turn leads to generation of 1,4,5-inositol triphosphate (IP₃), causing internal release of calcium in various cell types through specific binding to IP₃ receptors.^{30,31} It has also been demonstrated that IP₃ may degranulate mast cells and elicit histamine release.^{32,33} Heparin has been suggested to act as a specific blocker of IP₃ receptors and inhibits IP₃-induced release of calcium in various tissues including vascular and airway smooth muscle, the cerebellum, and the liver.³⁴⁻³⁷ More recently, light and electron microscopy findings showed that heparin prevented anti-IgE- and IP₃-mediated degranulation of purified rat peritoneal mast cells without attenuating the effects of the calcium ionophore A23187,³⁸ suggesting that the protective effect of heparin in asthma is likely to be ascribed to the inhibition of IP₃-dependent mast cell mediator release. It has been proposed that stimulation of cell-surface adenosine receptors may generate IP₃ either directly or indirectly through coupling to G proteins.^{39,40} Thus although further investigations on highly purified human lung mast cells are necessary before definitive conclusions can be drawn, the protective effect of inhaled heparin in adenosine-induced asthma may also be ascribed to blockage of IP₃ receptors in airway mast cells.

The contribution of alternative mechanisms (other than mast cell mediator release) in AMP-induced bronchoconstriction must be considered when analyzing heparin's mode of action. Adenosine has been reported to enhance the contractile response to transmural nerve stimulation in isolated rabbit bronchial smooth muscle.⁴¹ In subjects with asthma, both cholinergic and peptidergic neural pathways may contribute to the airway effects of adenosine.^{42,43} There is now some evidence that heparin may modulate neural activity in the airways. In anesthetized guinea pigs, antigen-induced dysfunction of inhibitory M2 muscarinic autoreceptors on parasympathetic nerves in the lungs can be reversed by heparin.⁴⁴ Thus in this animal model heparin vagally attenuates induced bronchoconstriction because acetylcholine from the vagus nerves is now able to stimulate M2 receptors to reduce further release of acetylcholine. When these findings are considered, the effect of heparin on AMP-induced bronchoconstriction can also be viewed as a modulating effect on presynaptic neural mechanisms. However, the lack of protective effect of inhaled heparin against sodium metabisulfite-induced,⁴⁵ and methacholine-induced (this study) bronchoconstriction argues against an inhibitory effect on neural pathways or airway smooth muscle and suggests that inhibition of airway mast cells may be the likely mechanism of action of heparin in human asthma.

In this study we have also extended the findings of phase 1 of the study and investigated the pharmacodynamic characteristics of bronchoprotective properties of inhaled heparin by studying the duration of the change in AMP airway responsiveness with heparin in a separate group of asthmatic subjects. Again heparin elicited