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EICOSANOID RELEASE AND MEPYRAMINE, LTC_4 AND LTD_4 BINDING IN PASSIVELY SENSITIZED HUMAN LUNG PARENCHYMA *IN VITRO*

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Abstract—*In vitro* passive sensitization of human lung parenchyma with hyper-immune serum did not affect the release of prostaglandin D_2 (PGD_2) or leukotriene (LT)-like activity upon challenge with anti-IgE antibody with respect to control lung, despite a marked difference in IgE levels between control (C) and sensitized (S) tissue. Binding studies with [^{3}H]LTC_4, [^{3}H]LTD_4 and [^{3}H]mepyramine (a histamine H_1 antagonist) showed a statistically significant increase in the amount bound in sensitized vs control lung for [^{3}H]mepyramine only. Contractile response to 5×10^{-5} M histamine (H) in C and S lung parenchymal strips did not correlate with binding data. It is concluded that *in vitro* elevated IgE levels do not affect the interaction of sulfidopeptide leukotrienes with their putative receptors. As for the observed increase in [^{3}H]mepyramine binding, this might not represent a true increase in histamine receptors on lung smooth muscle cells.

Non-specific bronchial hyper-responsiveness is a common characteristic of asthma and can be defined as an increased contractile response of the airways to several stimuli [1]. Symptomatic asthma appears to be the result of the release of several mediators leading to airway smooth muscle contraction, mucosal edema and airway narrowing [2].

The sulfidopeptide leukotrienes, formerly known as slow reacting substance of anaphylaxis (SRS-A¶), have a variety of biological actions that mimic those found during clinical asthma including bronchoconstriction and increased vascular permeability [3]. These powerful autacoids together with PGD_2 are released from a number of cells, including mast cells, present in the airways that participate in the airway responses to inhaled allergens [4, 5]. In addition, the existence of separate receptors for leukotriene C_4 and D_4 in lung parenchyma has been demonstrated previously by physiological and radioligand binding studies and LTE_4 has been found to cause airway hyper-responsiveness to histamine in guinea pig tracheal smooth muscle [6–8].

Exposure to allergens can increase airway responsiveness which is associated with increased symptoms of asthma. Atopic subjects are characterized by high circulating levels of IgE and show a marked tendency to develop allergic diseases such as asthma, rhinitis, urticaria etc. [9]. Although atopy should not be regarded as a synonym of allergic disease, the atopic subject is genetically susceptible to this type of disease and shows enhanced bronchial reactivity towards specific allergens (specific bronchial hyper-reactivity) as well as to non-allergenic substances such as histamine (H), methacholine, mist of H_2O etc. (aspecific bronchial hyper-reactivity) [10].

The purpose of the present work was to evaluate whether the condition of IgE hypersensitization *in vitro* was associated with changes in the release of eicosanoids and in binding to sulfidopeptide leukotriene and H receptors.

MATERIALS AND METHODS

Passive sensitization of human lung. Macroscopically normal human lung parenchyma from patients undergoing surgery for bronchial carcinoma was minced into fragments of approximately 100 mg each. The tissue was washed extensively with Tyrode's buffer of the following composition in g/L: NaCl 8.0, KCl 0.25, $NaHPO_4 \cdot H_2O$ 0.066, glucose 1.0, $NaHCO_3$ 1.0, $CaCl_2$ 0.15, $MgCl_2 \cdot 6\ H_2O$ 0.01, pH 7.4, divided into two aliquots and incubated (1g/5 mL) for 3 hr at 37° in buffer without (control, C) or with (passively sensitized, S) hyper-immune serum (kindly supplied by Dr S. Ahlstedt, Pharmacia AB, Uppsala, Sweden; final IgE titer 5 μg/mL). At the end of the incubation period both C and S lung fragments were washed again and aliquots (500 mg) frozen for binding and IgE assay. The remaining C and S parenchyma was preincubated for 10 min at 37° in Tyrode's (1 g tissue/10 mL) and subsequently challenged with an anti-human IgE antibody (0.11 mg protein/mL) kindly provided by Dr S. Ahlstedt. The reaction was stopped 15 min later and aliquots of

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¶ Abbreviations: SRS-A, slow reacting substance of anaphylaxis; H, histamine; EIA, enzyme immunoassay; BSA, bovine serum albumin; LT, leukotriene; PGD_2, prostaglandin D_2.

the supernatant frozen for enzyme immunoassay of PGD_2 or immediately used for bioassay of SRS-A (see below).

Bioassay of slow reacting substance of anaphylaxis (SRS-A) activity. The SRS-A activity in lung incubations was assessed on guinea-pig ileum with a bioassay system as described by Ferreira and Costa [11]. The assay was made specific by pretreating guinea-pig ileum strips with a mixture of receptor antagonists (0.5 μM scopolamine, 3 μM propranolol, 0.5 μM mepyramine, 0.5 μM methysergide and 0.5 μM phenoxybenzamine) and 10 μM indomethacin. The smooth muscle contraction was standardized with different doses of LTC_4 (ranging from 0.25 to 2 ng), and test solutions were given as boluses in volumes not exceeding 0.2 mL. The pattern of responses obtained with test solutions and LTC_4 standards developed over a period of approximately 1 min and lasted for about 5 min before a complete return to baseline tone. In order to verify the specificity of the assay, the same LTC_4 standards as well as the test solutions were assayed in the presence of 10 μM FPL 55712 which fully antagonized the response (data not shown).

EIA for PGD_2. PGD_2 was measured by enzyme immunoassay (EIA) [12]. This method has been developed using the stabilized 11-methoxime derivate (PGD_2-MO) obtained by treating PGD_2 with methoxamine–HCl [13]. Initially, aliquots (200 μM) of samples, evaporated to dryness, were treated with 100 μL of pyridine saturated with methoxamine–HCl. After 1 hr at 35°, the pyridine was taken to dryness and the samples diluted in EIA incubation buffer (phosphate buffer 0.1 M, pH 7.4, containing NaCl 0.4 M, EDTA 1 mM, BSA 0.1% and sodium azide 0.01%).

Solid-phase EIA was performed with specialized Titertek microtitration equipment including an automatic washer (Microplate Washer 120), an automatic dispenser (Autodrop) and a spectrophotometer (Multiskan MC) from Flow Laboratories (Helsinki, Finland). Microtitre plates (96F immunoplate I with certificate) were from Nunc (Denmark). Monoclonal anti-rabbit IgG resuspended in phosphate buffer 5×10^{-2} M, pH 7.4, was immobilized on 96-well microtitre plates with the Autodrop apparatus. After overnight incubation at room temperature, 100 μL of EIA incubation buffer were added to each well and the plates stored at 4° for 24 hr prior to use.

The plates were washed with 10^{-2} M phosphate buffer, pH 7.4, containing 0.05% Tween 20 in the Multiwash apparatus before use. The assay was performed in a total volume of 150 μL with each of the following components being added in a 50 μL volume: standard PGD_2-MO or biological samples, enzymatic tracer (PGD_2-MO-AchEsterase) and an appropriate dilution (1:60,000) of specific antiserum. The PGD_2 antibody was a generous gift from Dr J. Maclouf (Unitè 150, INSERM, Hospital Laribosière, Paris) and Dr Philippe Pradelles (Laboratories Analytical Immunology, CEA, Saclay, France). After overnight incubation at room temperature, the plates were washed and 200 μL of Ellman's reagent was automatically dispensed into each well with the Autodrop apparatus. After 1–2 hr, the absorbance at 414 nm of each well was measured with a Multiskan MC spectrophotometer. In order to evaluate the concentration of PGD_2-MO, a standard curve from 15 pg/mL to 1 ng/mL was used.

Results are calculated in terms of per cent of B/Bo where B and Bo represent the absorbance measured for the bound fractions in the presence and absence of PGD_2-MO competitor, respectively, and are expressed in nmol/g tissue. The standard curve and quantitative determination of PGD_2-MO in biological samples were analysed with an APPLE IIC computer using a linear log–logit transformation.

Assay of tissue-bound IgE. The tissue was homogenized in Tyrode's buffer (1 g/10 mL, w/v) and an aliquot (0.35 g) centrifuged for 10 min at 10,000 *g* at 4°; pellets and supernatants were separated and processed independently.

Three 100 μL aliquots of each supernatant were incubated for 3 hr at room temperature with paper discs covalently saturated with sheep anti-human IgE (Pharmacia IgE PRIST paper discs). After the incubation, each sample was washed three times, with an interval of 10 min between each wash, with saline solution (2.5 mL) containing freshly prepared 2% Tween 20. Rabbit anti-human ^{125}I-IgE (100 μL, approx. 0.05 μCi) was then added to each tube and the tubes were incubated for 12 hr at room temperature. Each sample was then washed again as described and the radioactivity was counted for 2 min using a Packard γ Counter. Results are expressed in I.U. (International Units, where 1 I.U. is approximately equivalent to 1.8–2.4 μg/mL) compared with a commercially available standard curve (Pharmacia–Phadebas IgE PRIST).

Each pellet was resuspended using 300 μL of the rabbit anti-human ^{125}I-IgE solution, divided into three aliquots and incubated for 3 hr at room temperature with vigorous shaking (1200 rpm). Each tube was then washed three times with 2.5 mL of the washing buffer mentioned above and centrifuged at 10,000 *g* for 10 min at 4° between each wash. Finally each sample was counted as previously described for the supernatant. Measuring radioactivity associated with the pellet is just an internal control and the values are seldom detectable.

Binding assays. These were performed on membranes obtained from both C and S parenchymal fragments before immunological challenge. The membranes were prepared as described previously [14]. Briefly, C and S frozen lung fragments were thawed, finely minced, homogenized at 4° in 50 mM Tris–HCl (pH 7.4, 1:24 w:v) with a Polytron homogenizer and centrifuged at 770 *g* for 10 min. The supernatant was centrifuged at 27,000 *g* for 20 min and the resulting pellet was resuspended in the same buffer and recentrifuged under the same conditions. The final pellet was resuspended in 50 mM Tris–HCl containing 50 mM serine-borate.

The incubations were performed at 4° for 20 min for [^{3}H]LTC_4 and at 25° for 40 min for [^{3}H]LTD and [^{3}H]mepyramine. The assay mixture in 50 mM Tris–HCl (pH 7.4) and in a total volume of 0.25 mL, contained the following: for LTC_4 [14], 50 nM [^{3}H]LTC_4 (sp. act. 1.5–2 Ci/nmol), 20 mM serine-borate, 10 mM $CaCl_2$ and 20 μM LTC_4 to measure non-specific binding; for LTD_4 [15], 2 nM [^{3}H]LTD_4 (sp.

Table 1. Release of PGD_2 and of LTC_4-like compounds from control (C) and passively sensitized (S) human lung parenchyma compared to IgE levels

	PGD_2 (nmol/g tissue)	LTC_4-like compounds (nmol/g tissue)	IgE (I.U./g tissue)
	Net increase over basal		
Control lung (C)	1.23 ± 0.19	0.84 ± 0.15	386 ± 134
Sensitized lung (S)	1.03 ± 0.16	0.84 ± 0.17	3524 ± 609

Values represent means ± SE of 23 experiments.

Eicosanoid release and IgE levels in the supernatants were measured following anti-IgE challenge, as described under Materials and Methods.

Basal PGD_2 levels ranged from 0.1 to 0.3 nmol/g tissue. LTC_4-like activity was invariably undetectable in unstimulated samples.

act. 20–60 Ci/nmol), 20 mM serine-borate, 10 mM cysteine, 10 mM glycine, 5 mM $MgCl_2$ and 1 mM LY 171883 to measure non-specific binding; and for mepyramine [16], 5 μM [^{3}H]mepyramine (sp. act. 0.4–0.6 Ci/nmol), 20 mM serine-borate, 25 mM $MgCl_2$ and 10 mM mepyramine to measure non-specific binding. The incubation was started by addition of the membrane preparation (0.08–0.15 mg protein/sample). Separation of bound from free ligand was achieved by vacuum filtration through Whatman GF/C glass fiber filters and rapid washing with two 4 mL aliquots of ice-cold 50 mM Tris–HCl, pH 7.4. The radioactivity was extracted from filters with 10 mL Filtercount (Packard, Groningen, Netherlands) and then counted in the same scintillation fluid.

Contractile activity of lung parenchymal strips. Strips of human lung parenchyma approximately 4 cm long and 0.5 cm wide (total tissue weight approximately 500 mg) were set up for isotonic recording in a 20 mL organ bath using Tyrode's buffer with a counterweight of 1 g (corresponding to an effective tension of 0.5 g) and gassed using 95% O_2/5% CO_2. After 1 hr equilibration at 37°, the strips were challenged using cumulative concentrations of histamine (50 nM–50 μM). This concentration range allowed invariably attainment of maximal contractile response. After extensive washing and return to basal tone, the strips were stimulated further with 10^{-2} M $BaCl_2$ and the contractile response to histamine expressed as the per cent of maximal contraction elicited by $BaCl_2$.

Materials. [14, 15-^{3}H]LTC_4 (20–60 Ci/mmol) and [14,15-^{3}H]LTD_4 (20–60 Ci/mmol) were from New England Nuclear Du Pont (Dreieich, F.R.G.); [pyridinyl-5-^{3}H]mepyramine (20–30 Ci/mmol) was from Amersham International (Amersham, U.K.). LTC_4, LTD_4 and FPL 55712 were kind gifts of Dr A. F. Welton, Hofmann-La Roche (Nutley, NJ, U.S.A.). Mepyramine, serine, boric acid, cysteine, glycine, histamine, PGD_2, methoxamine–HCl, scopolamine, propranolol, methysergide, phenoxybenzamine, and indomethacin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Serine-borate was the equimolar solution of serine and boric acid in 50 mM Tris–HCl buffer, pH 7.4. LY 171883 was a kind gift of Dr J. H. Fleisch, Lilly Research Laboratories (Indianapolis, IN, U.S.A.). PGD_2-AchE, monoclonal anti-rabbit IgG and Ellman's reagent were from the Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Anti-human ^{125}I-IgE was from Pharmacia Diagnostic AB (Phadebas RAST), Uppsala, Sweden.

RESULTS

The formation of PGD_2 and LTC_4-like compounds from endogenous arachidonic acid has been studied in control (C) and passively sensitized (S) human lung parenchymal fragments from 23 individuals following an *in vitro* immunological challenge using an anti-human IgE antiserum. Passive sensitization increased markedly the level of tissue-bound IgE by approximately 10-fold. However, the increase in levels of both eicosanoids released due to anti-IgE challenge was not statistically different between C and S fragments (Table 1).

Because there was a marked difference in lung IgE levels between C and S fragments, indicating that sensitization had taken place, it was of interest to investigate whether modifications in the binding to sulfidopeptide leukotriene (LTC_4, LTD_4) and histamine sites might have occurred. The results shown in Fig. 1 indicate that the amount of LTC_4 or LTD_4 bound/mg protein is similar in C and S parenchymal membranes. On the contrary, a small but statistically significant increase ($P < 0.02$, paired Student's t-test) in [^{3}H]mepyramine (H_1 antagonist) binding could be observed (mean binding: 64.45 ± 43.14 and 74.67 ± 46.04 pmol/mg protein in C and S, respectively). The increase occurred in 18 out of 23 specimens following passive sensitization (Fig. 1).

In order to establish whether the enrichment of tissue-bound IgE induced by passive sensitization and the concomitant changes in binding parameters observed for the H_1-histamine receptor could represent an *in vitro* model of airway hyperresponsiveness, we have studied whether the binding data were matched by an increased contractile response to histamine in sensitized human lung.

Two strips from each of five lung specimens were excised; one was kept as a control (C) while the other was passively sensitized (S). They were set up for isotonic recording of histamine-induced contractions as described in Materials and Methods. Fragments from both C and S strips from the same

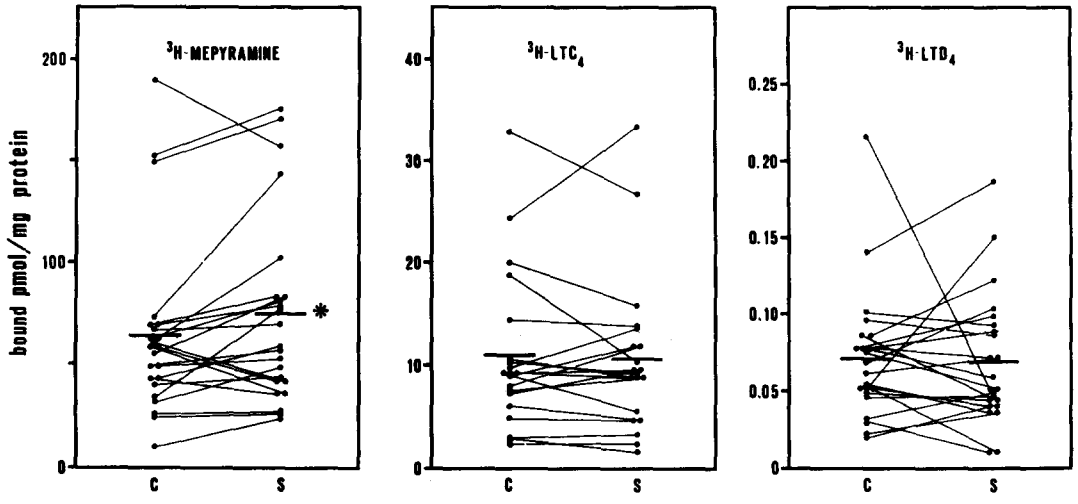

Fig. 1. Binding of [^{3}H]mepyramine, [^{3}H]LTC$_4$ and [^{3}H]LTD$_4$ to membranes from control (C) and passively sensitized (S) human lung parenchyma. Values are expressed as pmol bound/mg protein. [^{3}H]Mepyramine, [^{3}H]LTC$_4$ and [^{3}H]LTD$_4$ were 5 μM, 50 and 2 nM, respectively, Horizontal bars represent the means. The increase in [^{3}H]mepyramine binding in S vs C was statistically significant (* $P < 0.02$, paired Student's t-test).

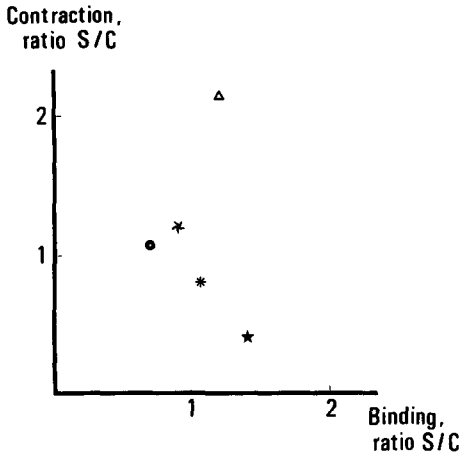

Fig. 2. Lack of correlation between the increase, induced by sensitization, in contractile response to 50 μM histamine (y-axis) and in binding of 5 μM [^{3}H]mepyramine (x-axis). S = sensitized, C = control. A ratio S/C > 1 indicates a greater contractile response or a higher amount bound in sensitized vs normal lung.

lung specimens were excised and used for binding experiments.

The data reported in Fig. 2 demonstrate that the contractile response to 5×10^{-5} M histamine in S strips varied between 40 and 220% of the response obtained in C strips. A ratio S/C higher than 1 indicates a greater contractile response to H or a higher amount of [^{3}H]mepyramine bound in sensitized than in control lung strips. The parallel binding studies show that the [^{3}H]mepyramine binding in S and C fragments does not correlate with the contractile response. Although the number of cases is limited by the reduced supply of human tissue, it is evident that when an increase in [^{3}H]mepyramine binding in S compared with C fragments is observed (i.e. S/C ratio higher than 1 for binding), it is not necessarily accompanied by a higher contractile response to H of S compared with C fragments (Fig. 2).

DISCUSSION

The cause of airway hyper-responsiveness in asthmatic patients is unknown but there is convincing evidence to incriminate airway inflammation [2]. Two major groups of arachidonic acid derived lipid mediators, namely prostaglandins and leukotrienes, are produced during an inflammatory response providing a mechanism through which inflammation can alter airway responsiveness [17, 18].

LTE_4 has been shown to increase the responsiveness of guinea-pig tracheal strips to histamine but these findings were not confirmed by other investigators [8, 19]. In addition, Kern *et al.* [20] found that in normal subjects precontraction of the airways with LTD_4 is accompanied by an increased responsiveness to inhaled methacholine.

A number of other clinical studies have also addressed the problem of bronchial hyper-reactivity to leukotrienes and have obtained conflicting results [21]. Smith *et al.* [22] reported that asthmatic subjects were more sensitive to LTD_4 than normal subjects, a conclusion shared by Bisgaard *et al.* [23]. On the other hand, Griffin *et al.* [24] and Weiss *et al.* [25] demonstrated that a group of asthmatic subjects were only 3.3-fold more sensitive to inhaled LTD_4 than normal subjects and suggested that patients did not have the degree of expected hyper-responsiveness to LTD_4.

Recently, the airways of asthmatic subjects have

been shown to develop a selective and marked hyper-responsiveness to LTE_4 possibly reflecting the existence of LT receptor subtype heterogeneity within the human airway [26]. However, to date, neither binding activity nor functional "receptors" for LTE_4 in the human airway have been described. In contrast, a large number of studies have been performed using human lung membranes and specific sites have been identified for LTC_4 and LTD_4 [14, 15, 27]. The results obtained with selective antagonists indicate that true receptors, probably different for LTC_4 and LTD_4, exist [17, 27].

Our results show that even in the presence of a marked enrichment of tissue IgEs there was no difference in $[^3H]LTC_4$ or $[^3H]LTD_4$ binding parameters. Although binding studies were performed at a fixed ligand concentration, such a "single point" binding analysis would have revealed changes in affinity and/or number of binding sites.

None of the human lung specimens used in our study came from asthmatic patients but it is possible that they all had a sufficient basal tissue IgE titer to guarantee maximal eicosanoid releasability. This might be the reason why we do not observe any increase in eicosanoid formation upon passive sensitization. This process carried out for 3 hr at 37° caused a 10-fold increase in membrane-bound IgE providing the lung fragments with an immunoglobulin titer that is quite high and within the range attainable by atopic subjects [28]. Another explanation for the absence of any effect of lung tissue fragment incubation with IgE on anti-IgE-induced PGD_2 and LT release is that, under the conditions used, IgE may not have gained access to the receptors on mast cells. This is, however, extremely unlikely due to the very high receptor density on this cell type (10^6/cell, as compared to 4×10^4/cell on basophils) [29]. In addition, the conditions used for sensitization were mild enough to prevent IgE degradation. These immunoglobulins are fairly stable; they are inactivated by heating at 56° for 2–4 hr [30]. The length of incubation and the temperature at which it was carried out might have led to the *de novo* synthesis of new receptor protein and would have eventually altered the binding parameters.

It should be noted, however, that LTC_4 binding sites might have limited biological relevance since studies carried out using cytosolic fractions from the rat liver have shown that the "sites" represent binding of LTC_4 to the enzyme glutathione *S*-transferase [31]. Tissue as well as species differences seem to play an important role; in the longitudinal smooth muscle of the guinea-pig LTC_4 binds to sites that are unlikely to be glutathione *S*-transferase [32]. However, the evidence that these structures are functional receptors is not compelling and they may represent carriers or uptake sites [33]. The biological significance of LTC_4 binding sites in human lung parenchyma still remains to be elucidated.

The binding sites identified for LTD_4 are generally more accepted as being true receptors. This was clearly the case for guinea-pig lung where radioligand competition activity correlated well with agonist and antagonist smooth muscle contractile activity [34].

Our present results, therefore, indicate that *in vitro* elevated IgE levels in the range characteristic of asthma or atopy do not affect the interaction of certain sulfidopeptide LTs with their putative "receptors", and tend to rule out the possibility that the clinical observation of bronchial hyper-reactivity to LTs (at least LTC_4 and LTD_4) is mediated by changes in the stimulus–response coupling occurring at receptor level.

The process of passive sensitization did cause a small but significant increase in $[^3H]$mepyramine binding. Hyper-reactivity to histamine has been shown in patients with exogenous asthma [35], an observation which may find its rationale in our results. However, the attempt to correlate the radioligand competition activity observed for the H_1 receptor with parallel changes in smooth muscle contractile activity failed. The reasons for this apparent discrepancy are purely a matter of speculation. The binding sites identified for $[^3H]$mepyramine might not represent true functional receptors or, as is more likely, the complex cellular heterogeneity of human lung parenchyma indicates that increased $[^3H]$mepyramine binding might not necessarily be taking place at smooth muscle level.

Inflammation and inflammatory mediators appear to play a critical role in the pathogenesis of airway hyper-responsiveness but a lot more basic research is certainly needed to clarify the still elusive relationship between the immunological history of the asthmatic patient and the mechanisms of stimulus translation into pathological responses.

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