An endothelin receptor antagonist, SB-217242, inhibits airway hyperresponsiveness in allergic mice

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There is a growing body of evidence from human and animal studies supporting a mediator role for endothelin-1 (ET-1) in several characteristic features of the allergic airway response, including airway inflammation and variable airflow obstruction. In allergic asthmatic patients, increased ET-1 immunoreactivity has been demonstrated in the airway epithelium of bronchial biopsies and elevated levels of ET-1 have been detected in bronchoalveolar lavage fluid. Consistent with these findings, several recent studies in rats have demonstrated that allergen induced ET-1 immunoreactivity in bronchial epithelium, and elevated ET-1 peptide levels in lung tissue and bronchoalveolar lavage fluid.

Animal studies using ET-1 antibodies and endothelin receptor antagonists point to endogenous ET-1 contributing to several processes involved in the allergic inflammatory response. For example, the endothelin receptor antagonist BQ-123 reduced the late, but not the early, antigen-induced reduction in airflow response in allergic sheep (26) and guinea pigs (38). The involvement of ET-1 in the late-phase allergic response, which is associated with the migration of inflammatory cells into the airways, is in accord with the recent findings from several independent groups that endothelin receptor antagonists inhibit the antigen-induced influx of eosinophils into the airways and bronchoalveolar lavage fluid (12, 15, 31).

In addition to its effects on eosinophil recruitment into the airways, ET-1 has many other purported actions within the airways that may promote inflammation and airflow obstruction. For example, ET-1 stimulates the release of a range of proinflammatory cytokines [such as interleukin (IL)-6, IL-1β, and tumor necrosis factor-α] and promotes microvascular leakage, mucous secretion, and collagen deposition within the airways (16). Furthermore, ET-1 is a potent and powerful spasmogen of airway smooth muscle in most animal species studied, including humans. The bronchoconstrictor actions of ET-1 are probably due to a combination of its direct actions on airway smooth muscle and the augmented release of the spasmodenic neurotransmitter acetylcholine from postganglionic parasympathetic nerves (10, 18, 20). In addition to its spasmodenic action on airway smooth muscle, ET-1 has also been demonstrated to contribute to the synthesis of airway smooth muscle DNA after repeated antigen exposure in sensitized rats (31). These findings are consistent with the previously identified comitogenic influence of ET-1 in airway smooth muscle cell cultures (28) and suggest that ET-1 may contribute to the airway wall remodeling that is associated with allergic inflammatory processes.

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Although this profile of effects is consistent with a substance that would be expected to promote the development of airway hyperresponsiveness, which is a characteristic feature of allergic inflammatory airway diseases such as asthma, there is no strong evidence supporting such a role for ET-1 in allergen-induced airway hyperresponsiveness. To further evaluate a possible link between ET-1 and airway hyperresponsiveness, we have investigated the effects of an endothelin receptor antagonist, SB-217242 (27, 39), on development of airway hyperresponsiveness and eosinophil recruitment into the airways and bronchoalveolar lavage fluid in mice sensitized and challenged with the house dust mite (*Dermatophagoides pteronyssinus*) allergen Der P1.

**METHODS**

**Preparation of Der P1.** Der P1 was extracted from *D. pteronyssinus* (Commonwealth Serum Laboratory, Parkville, Australia) and purified on affinity columns prepared by coupling monoclonal anti-Der P1 antibody to cyanogen bromide activated Sepharose 4B.

**Antigen exposure and drug treatment of mice.** Groups of 6-wk-old male CBA/CaH mice (Animal Resource Center, Murdoch, Australia) were sensitized with an intraperitoneal injection of 5 μg of Der P1 + 1 mg of alhydrogel or were sham sensitized with Der P1-free solution (Fig. 1). Airway hyperresponsiveness was assessed following three intranasal instillations, 25-μl aliquots containing 5 μg of Der P1 or sterile saline were applied to the noses of anesthetized mice (methoxyflurane). Alzet microosmotic pumps (Alza, Palo Alto, CA) containing CB-217242 (30 μg/kg·day−1) or vehicle (0.25 M NaHCO3) were implanted subcutaneously into anesthetized mice (100 mg/kg ketamine + 10 mg/kg xylazine ip). Each experiment, 24 mice were studied, 6 mice in each of 4 groups: sham/vehicle, sham/SB-217242, allergic/vehicle, and allergic/SB-217242. Of the six mice from each group, two were used for in vivo lung function measurements, two were used for bronchoalveolar lavage, and two were fixed in situ with paraformaldehyde for histological and immunohistochemical studies (see below). Experiments were performed on three separate occasions, and the data were combined.

**In vivo lung function measurements.** Mice were anesthetized intraperitoneally with ketamine (100 mg/kg) + xylazine (10 mg/kg), and cannulas were placed in the trachea (to measure airflow), jugular vein (for drug administration), and esophagus (for transpulmonary pressure measurement). Spontaneous breathing was stopped by pancuronium bromide (0.4 mg/kg iv), and thereafter mice were ventilated. Breath-to-breath measurement of airway resistance was recorded (model PR 800, Mumed, London, UK) according to the principles of Amdur and Mead (1). A bronchoconstrictor stimulus, methacholine (50, 100, 200, 400, and 800 μg/kg iv), was administered as a bolus dose at 5-min intervals.

**Bronchoalveolar lavage.** Murine lungs were lavaged with five 0.5-ml aliquots of PBS. Bronchoalveolar lavage fluid was centrifuged (1,500 rpm for 10 min), and total cell number and viability were calculated using a hemocytometer and trypan blue exclusion, respectively. Differential cell counts of macrophages/monocytes, lymphocytes, neutrophils, and eosinophils were performed on cytospin preparations stained with Diff-Quick.

**Eosinophil major basic protein immunohistochemistry.** Murine lungs were fixed in situ with picric acid-paraformaldehyde fixative. Tissue sections (5 μm thick) were sequentially incubated with 100% normal goat serum (overnight at 4°C), polyclonal rabbit anti-mouse major basic protein (1:1,000 for 1 h at 37°C), and goat anti-rabbit Alexa 488 cross-absorbed secondary antibody (1:500 for 1 h at 22°C). Confocal microscope (model 1024, Bio-Rad) images were taken of all 50- to 300-μm-diameter airways, and major basic protein-positive cells were counted using Image-Pro Plus software.

**Lung ET-1 content.** Lungs were homogenized in 1 M acetic acid solution containing 1 mg/ml pepstatin (10 ml/g wet tissue wt), incubated in a boiling water bath for 10 min, cooled to 4°C, and centrifuged at 100,000 g for 20 min. Supernatants were passed through Sep-Pak Plus C-18 columns (Waters), and eluted ET-1 was assayed using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI).

**Data and statistical analyses.** Values are means ± SE. Differences between treatment groups were determined using one-way ANOVA. Differences between pairs of treatment groups were then determined using the modified t-statistic (40). *P* < 0.05 was considered statistically significant.

**RESULTS**

**Cell numbers in bronchoalveolar lavage fluid.** Bronchoalveolar lavage fluid recovered from sham/vehicle mice contained, on average, 243 × 10^3 cells per mouse (*n* = 6; Table 1). As expected, macrophages were the predominant cell type (making up ~95% of total cells recovered), and no eosinophils were detected. Challenge with Der P1 in sensitized animals (allergic/vehicle) caused a 137% increase in total cells recovered. The majority of this increase in cell number was due to the presence of eosinophils (37.6% of total cells), although the numbers of macrophages and lymphocytes were also elevated. SB-217242 treatment of allergic mice caused a significant reduction in total cell number (19% lower than allergic/vehicle mice). This reduction could be entirely accounted for by a >50% reduction in the number of eosinophils. SB-217242 treatment had no effect on the number of macrophages, neutrophils, or lymphocytes in sham or allergic mice.

**Number of major basic protein-positive cells in lung sections.** Immunohistochemical studies revealed the presence of large numbers of major basic protein-positive cells (eosinophils) in peribronchial and perivascul ar regions of the allergic murine lung (Fig. 2B). However, the mean number of major basic protein-positive cells in allergic mice that received SB-217242 treatment (69 ± 12 cells/airway, *n* = 6 mice; Fig. 2C) was less than half of that observed in allergic mice that...
received vehicle (142 ± 28 cells/airway, n = 6 mice, P < 0.05; Fig. 2B). A total of 88 airways (43 airways from 6 allergic/vehicle mice and 45 airways from 6 allergic/SB-217242 mice) were analyzed. The mean diameter of airways used for determination of major basic protein-positive cell number was similar for the allergic/vehicle mice (138 ± 19 μm, n = 6 mice) and the allergic/SB-217242 mice (149 ± 12 μm, n = 6 mice). No major basic protein-positive cells were detected in lung sections from nonallergic mice that received SB-217242 or vehicle (Fig. 2A).

Airway responsiveness to methacholine. Intravenous administration of bolus doses of methacholine induced dose-dependent increases in airway resistance. In sham/vehicle mice, the threshold dose of methacholine for increasing airway resistance was 100–200 μg/kg (Fig. 3A), and the mean effective dose that produced a 300 cmH2O·L−1·s increase in airway resistance (ED300) was 390 μg/kg (95% confidence limit = 270–560, n = 6 mice; Fig. 3B). In allergic/vehicle mice, the threshold dose (50–100 μg/kg) and the ED300 (200 μg/kg, 95% confidence limit = 155–260, P < 0.05; Fig. 3) for methacholine were lower, indicative of the development of airway hyperresponsiveness in these mice. However, airway hyperresponsiveness to methacholine was not observed in allergic mice treated with SB-217242. In these allergic/SB-217242 mice, airway responsiveness to methacholine (threshold dose = 100–200 μg/kg, ED300 = 410 μg/kg, 95% confidence limit = 320–530, n = 6 mice) was not significantly different from that observed in sham/vehicle mice (Fig. 3). This effect of SB-217242 was not due to inhibition of methacholine-induced bronchoconstriction per se, since the responsiveness of sham/SB-217242 mice to methacholine (threshold dose = 100–200 μg/kg, ED300 = 470 μg/kg, 95% confidence limit = 380–570, n = 6 mice) was not significantly different from that observed in sham/vehicle mice (Fig. 3).

ET-1 levels in murine lungs. The ET-1 content in the lungs of allergic/vehicle mice (1,820 ± 120 pg/g, n = 6 mice) was 35% higher than that detected in sham/vehicle mice (1,350 ± 80 pg/g, n = 6 mice, P < 0.05).

DISCUSSION

In the present study, administration of a mixed ETα and ETβ receptor antagonist, SB-217242, to sensitized mice during challenge with the house dust mite allergen Der P1 significantly attenuated airway eosinophilia and airway hyperresponsiveness. These findings provide compelling evidence that endogenous generation of ET-1 contributes to the increased numbers of airway eosinophils and increased airway responsiveness to spasmogens, which are characteristic features of the allergic inflammatory response in the airways.

The relationship between ET-1 expression and airway eosinophilia has recently been examined in several animal models of allergic inflammation. In rats, increased ET-1 mRNA and/or peptide levels have been observed in the airway epithelium after antigen challenge (31, 32) and intratracheal instillation of Sephadex particles to which rats are allergic (12). These marked increases in ET-1 mRNA expression and tissue ET-1 peptide levels occurred very early after antigen challenge (15 min–2 h) and preceded the influx of eosinophils into the airways (11, 31, 32). The cause-and-effect relationship between ET-1 expression and airway eosinophilia in allergic animals has been more fully evaluated in studies using endothelin receptor antagonists and endothelin antibodies. In the present study, treatment of allergic mice with a nonpeptidic, mixed ETα and ETβ receptor antagonist, SB-217242, during antigen challenge caused a >50% reduction in the number of eosinophils recovered from bronchoalveolar lavage fluid and revealed in lung tissue using immunohistochemical techniques. These findings are in agreement with previously published data in mice (15) and rats (12, 31) and, together, provide strong evidence in favor of a prominent role for endogenous ET-1 in the airway eosinophilia associated with the allergic inflammatory response.

Despite the apparent strong association between ET-1 and airway eosinophilia, the underlying mechanisms have not been elucidated. The recruitment of eosinophils from the circulation to sites of inflammation is a complex process involving many steps, including the cell surface expression of complementary adhesion molecules that promote adhesive interactions between circulating eosinophils and vascular endothelial cells (4). The influence of ET-1 on adhesion molecule-dependent events such as eosinophil rolling on, and firm adhesion to, the vascular endothelium is not known. Nevertheless, ET-1 has been reported to enhance the expression of adhesion molecules, including vascular cell adhesion molecule-1 (19) and intercellular cell adhesion molecule-1 (17, 23, 42), which have been proposed to play a significant role in eosinophil migration into allergic airways (5, 25, 36). Furthermore, in vivo application of ET-1 has been shown to increase...
rolling and adhesion of leukocytes to rat mesenteric microvessels (3, 33), perhaps via ET\textsubscript{A} receptor-mediated expression of endothelial adhesion molecules such as P-selectin. These and other potential mechanisms, including the generation of secondary mediators such as cysteinyl leukotrienes, which are chemotactic for human eosinophils in vitro and in vivo, remain to be fully investigated.

Airway hyperresponsiveness to the bronchoconstrictor substance methacholine was a characteristic feature of the murine model of Der P1-induced allergic inflammation used in the present study. Moreover, airway hyperresponsiveness was significantly inhibited by pretreatment of allergic mice with an endothelin receptor antagonist, SB-217242. These findings provide direct evidence of a link between ET-1 and

Fig. 2. Immunohistochemical detection of eosinophils using an antibody to major basic protein. Lung sections from sham/vehicle (A), allergen/vehicle (B), and allergen/SB-217242 (C) mice are shown. a, Airway; v, blood vessel. Scale bar, 50 μm.

Fig. 3. In vivo airway responsiveness to methacholine. A: dose-response curves for methacholine-induced increases in airway resistance in sham/vehicle (○), sham/SB-217242 (●), allergic/vehicle (▲), and allergic/SB-217242 (●) mice. Values are means ± SE from 6 mice. B: doses of methacholine producing a 300 cmH\textsubscript{2}O/L·sec increase in airway resistance in sham and allergic mice treated with SB-217242 or vehicle. Values are means ± SE from 6 mice. *P < 0.05 vs. sham/vehicle.
increased airway responsiveness to bronchoconstrictor stimuli and are consistent with several previously published studies that provided indirect evidence of such a link. For example, inhalation of aerosolized ET-1 produced an ET\textsubscript{A} receptor-mediated increase in airway responsiveness to histamine in rabbits (7, 8) and to carbachol in sheep (26). Furthermore, studies in allergic guinea pigs and sheep indicate that endogenous ET-1 contributes to antigen-induced late responses (26, 38), which are thought to be closely associated with airway inflammation and airway hyperresponsiveness. However, contrary to our findings, a recent study has reported that airway hyperresponsiveness in allergen (ovalbumin)-exposed rats was not affected by pretreatment with a dual endothelin receptor antagonist (31). The precise reasons for the differences between our findings and those of Salmon and coworkers (31) is not known, but contributing factors may include species differences and the marked difference in antigen sensitization and challenge protocols (Salmon and coworkers used a model of repeated allergen exposure). Nevertheless, our findings in Der P1-sensitized and -challenged mice provide direct evidence in support of a mediator role for ET-1 in the development of airway hyperresponsiveness during allergic inflammation.

In the present study, administration of the endothelin receptor antagonist SB-217242 to allergic mice inhibited airway eosinophilia and airway hyperresponsiveness. Typically, but not universally (21), agents that promote eosinophil trafficking into the lung (e.g., IL-5) also promote airway hyperresponsiveness (22). Inhibition of eosinophil trafficking, as seen in IL-5 knockout mice, reduces airway hyperresponsiveness (13). It is proposed that eosinophil-associated hyperresponsiveness is mediated by eosinophil-derived proteins, such as major basic protein, which exert potent actions on airway epithelial cells and/or neural receptors (41). Thus it is tempting to speculate that the inhibition of eosinophilia produced by SB-217242 in the present study is causally linked to inhibition of airway hyperresponsiveness, although there is no direct evidence for such a link.

In the present study, SB-217242 administration to allergic mice reduced by 50% the number of eosinophils present within tissue or recovered from bronchoalveolar lavage but completely abrogated the development of airway hyperresponsiveness. One possible interpretation of these data is that airway eosinophilia is not causally related to airway hyperresponsiveness. However, in a recent review, which carefully and critically dissected a number of studies using murine models of allergic inflammation, Foster and colleagues (14) proposed that the degree of residual eosinophilia correlates positively with the induction of airway hyperresponsiveness. If this is the case, then the presence of residual eosinophils, but not airway hyperresponsiveness, in SB-217242-treated allergic mice might be explained by proposing that SB-217242 has two effects within allergic airways: 1) inhibition of the influx of eosinophils into the airways and 2) inhibition of eosinophil-induced airway hyperresponsiveness. Consistent with this secondary action, activated eosinophils have been shown to interact with airway epithelial cells to stimulate the release of ET-1 (9), and ET-1 exerts many actions within the airways that may contribute to the development of airway hyperresponsiveness (see below). An alternative explanation is that eosinophil-dependent and -independent factors contributed to airway hyperresponsiveness and that SB-217242 attenuated eosinophil migration into compartment(s) that promoted airway hyperresponsiveness and the remaining SB-217242-insensitive eosinophils migrated into a compartment where they did not contribute to airway hyperresponsiveness. Consistent with this is the concept that it is the location of the eosinophils, rather than their absolute number, that determines the extent of airway hyperresponsiveness (37). Finally, it is possible that eosinophilia and airway hyperresponsiveness are causally linked; however, a threshold level of eosinophils is required to develop airway hyperresponsiveness.

It is important to appreciate that ET-1 has been reported to exert many other actions within the airways, in addition to promoting eosinophil trafficking, that could conceivably contribute to the development of airway hyperresponsiveness. ET-1 is a potent, effective, and long-lasting spasmogen of airway smooth muscle and a bronchoconstrictor in patients with asthma (6), animal models of allergic inflammation (2), and mice (24). In addition, studies in animal models suggest that ET-1 increases mucous glycoprotein secretion (34) and reduces tracheal mucous velocity (30). Together, these effects are likely to promote airway mucous plugging and airway obstruction. ET-1 may also increase airway microvascular permeability, leading to airway wall edema (29). ET-1 may also promote airway remodeling, via its proliferative effects on airway smooth muscle and fibroblasts, although the significance of remodeling in a relatively acute model of allergic inflammation is likely to be minor. Nonetheless, the combined effects of ET-1-induced bronchoconstriction, bronchial obstruction, and airway wall edema are likely to contribute significantly to the development of airway hyperresponsiveness.

In the present study, SB-217242 administration was commenced before antigen challenge and maintained for the duration of the study protocol. The rationale for starting SB-217242 administration before antigen challenge is that previous studies (11) have shown that synthesis and release of ET-1 occur during the initial phase of airway inflammation, within 15–30 min of challenge. Although these studies have demonstrated that SB-217242 can inhibit the development of eosinophilia and airway hyperresponsiveness in allergic inflammation, the effectiveness of endothelin receptor antagonists in reversing established allergic inflammatory responses is not known.

Consistent with these findings, the lungs of Der P1-sensitized and -challenged mice contained elevated levels of ET-1, as has also been shown in allergic rats after antigen challenge (11, 31, 32). Although statistically significant, the magnitude of the increase in ET-1
content in lungs of allergic mice was relatively small (35% higher than in lungs from nonallergic mice). This was not unexpected, because time-course studies have demonstrated that lung ET-1 levels peak at ~3 h after antigen challenge (11), whereas the present study determined ET-1 levels 24 h after the final antigen challenge. Although not determined in the present study, immunohistochemical studies of allergic (31) and asthmatic (35) airways indicate that the principal cellular source of elevated ET-1 is the airway epithelium.

In summary, administration of an endothelin receptor antagonist, SB-217242, significantly reduced airway hyperresponsiveness and eosinophilia in a murine model of allergic inflammation induced by the house dust mite antigen Der P1. These findings are fully consistent with epithelium-derived ET-1 playing a significant mediator role in two characteristic features of allergic inflammation: airway eosinophilia and airway hyperresponsiveness.

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