



Endothelin A receptor antagonist modulates lymphocyte and eosinophil infiltration, hyperreactivity and mucus in murine asthma

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ABSTRACT

Levels of endothelins are particularly high in the lung, and there is evidence that these peptides are involved in asthma. Asthma is a chronic inflammatory disease associated with lymphocyte infiltration. In the present study, we used a murine model of asthma to investigate the role of endothelins in lymphocyte and eosinophil infiltration into the airway hyperreactivity and mucus secretion. Sensitized C57Bl/6 mice were treated with endothelin ET_A receptor antagonist (BQ123) or endothelin ET_B receptor antagonist (BQ788) 30 min before an antigen aerosol challenge. After 24 h, dose response curves to methacholine were performed in isolated lungs, FACS analysis of lymphocytes and eosinophil counts were performed in bronchoalveolar lavage fluid and mucus index was determined by histopathology. In sensitized and antigen-challenged mice there is a marked increase in the T CD₄⁺, T CD₈⁺, B220⁺, Tγδ⁺ and NK1.1⁺ lymphocyte subsets. Treatment with BQ123 further increased these cell populations. The number of eosinophils, airway hyperreactivity and mucus were all reduced by BQ123 treatment. The BQ 788 had no significant effect on the parameters analyzed. Treatment with BQ123 reduced the endothelin concentration in lung homogenates, suggesting that endothelins exert a positive feedback on their synthesis. We show here that in murine asthma the ET_A receptor antagonist up-regulates lymphocyte infiltration and reduces eosinophils, hyperreactivity and mucus.

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1. Introduction

Endothelins are a family of peptides (i.e., ET-1, ET-2, and ET-3) with a variety of biological activities in both vascular and non-vascular tissues, including hemodynamic, cardiac, renal and pulmonary effects [1]. In mammals, endothelins act by binding to two receptor subtypes: the ET_A and ET_B receptors [2]. The ET_A receptor has a higher affinity for ET-1 and ET-2 than for ET-3, whereas the ET_B receptor has similar affinity for the three endothelin ligands [3]. Endothelin receptors are widespread throughout the respiratory tract in animals and humans. Goldie et al. found that ET_A receptors preferentially localize in bronchial and pulmonary vasculature whereas the ET_B receptor is abundant in parenchyma [4]. However, the localization of the receptor subtypes may vary depending on the animal species. Levels of endothelins are particularly high in the lungs. Cellular origin of endothelins in the lungs includes airway epithelial cells and some inflammatory cells such as mast cells and macrophages, in addition to vascular endothelial cells [reviewed in 5].

Allergic asthma is a chronic inflammatory disease associated with the migration of T CD₄⁺ lymphocytes to the lungs, particularly those of the Th2 profile. Allergen-specific T cells are recruited into the airway by

mediators produced during the allergic inflammation and are responsible for the main features of asthma: airway eosinophilia, bronchial hyperreactivity and mucus hypersecretion. The lymphocyte infiltration in asthma consistently occurs in epithelial sites of the respiratory mucosa where the Tγδ and NKT cells are present in small numbers but exert important immunoregulatory functions. The Tγδ cells appear to play a major role in the protection of respiratory mucosa – they eliminate damaged epithelial cells and contribute to the resolution of lung inflammation by restoring the number of lung macrophages [6]. In experimental asthma the number of pulmonary Tγδ cells increase after prolonged airway stimulation with antigen [7]. In asthmatic patients the number of Tγδ lymphocytes also significantly increases following *in vivo* allergen provocation [8]. Studies using mice deficient in Tγδ cells suggest that these cells down-regulate lung allergic inflammation [9,10]. The NKT are cells of the innate immunity that play an important role in the stimulation of adaptive immunity. Upon activation they produce Th2 cytokines within minutes of stimulation. An increase in number of these cells was reported to occur in asthmatic patients, and there is evidence that they are involved in the development of airway hyperresponsiveness, either directly or by increasing the Th2 response to allergens [11]. Although substantial progress has been made in the elucidation of the role of the NKT and Tγδ lymphocytes in immunological homeostasis in the lung, understanding of their role in allergic asthma is still fragmentary [12].

We have previously shown in a murine model of lung inflammation a clear increase in the number of Tγδ⁺ and NK1.1⁺ (the mouse

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equivalent of NKT cells) as well as T CD₄⁺, T CD₈⁺, and B220⁺, in the bronchoalveolar lavage fluid of immunized mice (C57Bl/c and BALB/c) following antigen challenge [13]. In the present study, using the same model we investigated the effect of receptor antagonists of the endothelins on the number of these lymphocyte subsets in the bronchoalveolar lavage fluid. The effect of endothelin antagonists on airway eosinophilia, hyperreactivity and mucus secretion was also investigated.

2. Materials and methods

2.1. Animals

Male C57Bl/6 mice weighing 20–25 g, 6–8 weeks old, from our own animal facilities were housed in a room with a 12 h light–dark cycle with water and food *ad libido*. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences of the University of São Paulo. The animals were sacrificed by injection of ketamine/xylazine (50 µl of a 100 mg/ml solution, i.p.).

2.2. Immunization protocol

Mice were sensitized on days 0 and 7 by an intraperitoneal injection of a mixture containing 50 µg of ovalbumin and 1 mg of Al(OH)₃ in saline (a total volume of 0.2 ml). At 14 and 21 days after the first immunization the animals were challenged by exposure to an aerosol of ovalbumin (grade III, Sigma) generated by an ultrasonic nebulizer (ICEL US-800, SP, Brazil) delivering particles of 0.5–10 µm diameter at approximately 0.75 cc/min for 20 min. The concentration of ovalbumin in the nebulizer was 2.5% w/v. The control group consisted of animals immunized as described and challenged with saline solution.

Groups of animals were treated 30 min before each challenge with intraperitoneal injection of effective doses (1 mg/kg) of the ET_A receptor antagonist (BQ 123) or with the ET_B receptor antagonist (BQ 788). The control group consisted of animals treated with the drug vehicle (0.5% DMSO in saline).

2.3. Bronchoalveolar lavage

The animals were killed by injection of ketamine/xylazine (50 µl of a 100 mg/ml solution, i.p.) 24 h after exposure to the second aerosol challenge. A tracheal cannula was inserted via a midcervical incision, and the airways were lavaged twice with 1 ml of phosphate buffered saline (PBS, pH 7.4 at 4 °C).

2.4. Determination of total cells, eosinophils and lymphocytes in the bronchoalveolar lavage fluid

The bronchoalveolar lavage fluid was centrifuged at 170 ×g for 10 min at 4 °C, the supernatant was removed, and the cell pellet was re-suspended in 0.5 ml of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hemacytometer. Following cytocentrifugation of the bronchoalveolar lavage fluid, cells were stained with hematoxylin-eosin (Hema 3) for determination of eosinophils number. The total number of lymphocytes was determined by flow cytometry analysis, in which lymphocytes were defined on the basis of size and granulation.

2.5. Flow cytometric analyses of lymphocytes

Phenotypic analysis of lymphocyte subpopulations was assessed by three-color FACS, using a Facscalibur Cytometer equipped with

Cell Quest software (Becton & Dickinson, San Jose, CA, USA), using gates defined by forward and side light scatter properties. Bronchoalveolar lavage cells were incubated with Fluorescein Isothiocyanate (FITC), R-Phycoerythrin (PE) or Cy-chrome-labeled monoclonal antibodies anti-CD₄ (clone H129.19), anti-CD₈ (clone 53–6.7), anti-CD45R/B220 (clone RA3-6B2), anti-Tγδ (clone GL3) or anti-NK-1.1 (clone PK136) and adjusted to 1 × 10⁵ cells/ml in PBS supplemented with 5% fetal bovine serum and sodium azide (0.1%). Flow cytometry data are expressed as percentages of lymphocytes bearing specific markers, having gated on the general lymphocyte population using forward and right angle scatter.

2.6. Evaluation of airway reactivity

We used a method previously described [14] that measures whole airway responsiveness to agonists. Mice were immunized and submitted to two antigen aerosol exposures as described above. Twenty-four hours after the second antigen challenge, mice received an injection of ketamine/xylazine (50 µl of a 100 mg/ml solution, i.p.), the peritoneal cavity was cut open and animals were exsanguinated by sectioning of the abdominal aorta. The thoracic cavity was then opened; the pulmonary artery was cannulated and perfused with 10 ml of Krebs solution at 10 ml/min. A cannula was then inserted in the trachea; the lungs were removed carefully and perfused (5 ml/min) through the trachea with Krebs (37 °C, 95% O₂ and 5% CO₂) solution. A small incision was made in the lower end of each lobe to permit the outflow of the perfusate. The perfusion pressure was recorded in a Beckman R511A using Gould P23DB pressure transducers. Increases over basal levels of perfusion pressure following bolus injection of methacholine were taken as a measure of constriction of the airways. Increase in perfusion pressure (cmH₂O) as a function of the dose (µg) of methacholine was determined for the entire recording period. The areas under the curve were calculated, and the results were expressed as mean area under the curve (mm²).

2.7. Histological analysis of mucus

Lungs were dissected after the recovery of bronchoalveolar lavage, perfused via the right ventricle with 10 ml PBS to remove residual blood, immersed in 10% phosphate buffered formalin for 24 h and then kept in 70% ethanol until embedding in paraffin. Tissues were sliced (5 µm sections) and stained with hematoxylin/eosin for light microscopy examination or with periodic acid-Schiff (PAS)/hematoxylin for evaluation of mucus producing cells. The intensity of mucus production was evaluated in each preparation and scores from 0 to 3 were attributed: 0 when none of the bronchi showed any sign of mucus; 1, 2 or 3 when 25%, 50% or more than 50% of the bronchi epithelium was covered by mucus, respectively. Values shown for each lung represent the sum of 10 bronchi scored randomly at ×250 magnification.

2.8. Determination of immunoreactive endothelin

Lungs were homogenized with a Polytron homogenizer (20 s) in 2 ml of phosphate buffer pH 7.2, and the samples were centrifuged (3000 ×g, 15 min). The concentration of endothelin in lung tissue was measured by specific ELISA — (Endothelin-1 EIA kit, Cayman Chem., USA) after extraction. The antiserum had 100% cross-reactivity with endothelin-1, endothelin-2 and endothelin-3, and 0.1% cross-reactivity with human big-endothelin-1. The extraction of endothelins was performed using SepPak C18 cartridges as follows: The SepPak columns were equilibrated with 1 ml methanol and 1 ml water. To each ml of the sample 0.25 ml HCl (2 M) was added and the acidified samples were then applied to the cartridges. After washing with 10 ml aqueous trifluoroacetic acid (TFA; 0.1%, v/v), the endothelin was eluted with 3 ml methanol:water:TFA (90:10:0.1). After that the samples were evaporated under nitrogen and re-suspended in EIA buffer.

2.9. Drug treatments

The antagonists used in this study were injected intraperitoneally 30 min before the rats were killed and lungs removed. BQ 123 (ET_A receptor antagonist) was dissolved in PBS with 20% DMSO to obtain a 10 mg/ml stock solution and used at 1 mg/kg. BQ 788 (ET_B receptor antagonist) was dissolved in PBS with 5% DMSO to obtain a solution of 1 mg/ml and used at 1 mg/kg. DMSO has to be used because these antagonists are insoluble in aqueous solution at this concentration according to Brochu et al. [15]. The concentration of the antagonists was chosen from previous studies [16–19].

2.10. Drugs and reagents

Physiological salt solutions were prepared with salts of analytical grade in double-distilled water. BQ 788 (*N*-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-1-methoxycarbonyl-Trp-D-Nle) a selective ET_B receptor antagonist and BQ 123 (Cyclo(D-Asp-Pro-D-Val-Leu-D-Trp, Na) a selective ET_A receptor antagonist, were purchased from American Peptide (Sunnyvale, CA, USA); Endothelin-1 EIA kit from Cayman Chemical (Ann Arbor, MI, USA); Trifluoroacetic acid from Burdick & Jackson (Muskegon, MI, USA); Sep-Pak Cartridges from Waters Corporation (Milford, MA, USA), ovalbumin grade III, methacholine and fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA); monoclonal antibodies to lymphocyte markers (BD Pharmingen, San Diego, CA, USA); Hema 3 (Biochemical Sciences Inc., Swedesboro, NJ, USA) and aluminum hydroxide gel “Rehydregel” (Reheis Inc., Berkley Heights, NJ, USA).

2.11. Statistical analysis

Data are expressed as the mean±S.E.M. Statistical analysis of the data was carried out by analysis of variance (ANOVA), and sequential analysis of differences among means was done by Tukey's contrast analysis. Statistics for the mucus scores were performed by non-parametrical ANOVA with Kruskal Wallis post test. A *p*-value lower than 0.05 was considered significant.

3. Results

3.1. Endothelin A receptor antagonist increases lymphocytes and reduces eosinophils infiltration into airways

Mice immunized with ovalbumin were submitted to two ovalbumin aerosol challenges and bronchoalveolar lavage was performed 24 h after the second aerosol challenge. Immunized and saline challenged mice were used as control. Groups of immunized mice received intraperitoneal injections of selected effective doses of endothelin receptor antagonist BQ 123 (ET_A receptor antagonist) or BQ 788 (ET_B receptor antagonist) 1 mg/kg, 30 min before each challenge.

To evaluate the lymphocyte subpopulations, the cells from bronchoalveolar lavage were incubated with monoclonal antibodies to the cell markers NK 1.1⁺, Tγδ⁺, CD₄⁺, CD₈⁺, B220⁺ as described in Materials and methods and submitted to flow cytometry analysis. Fig. 1 shows that whereas these cell populations are either undetectable or found in very low numbers in the control group (immunized animals given saline aerosol), significant increases in all cell populations were observed in the immunized and challenged mice. Treatment with ET_A receptor antagonist further increased the number of CD₄⁺, CD₈⁺, B220⁺, Tγδ⁺ and NK1.1⁺ lymphocytes. Treatment with the ET_B receptor antagonist had no effect on lymphocytes infiltration.

When we analyzed the number of lymphocytes gated on the general lymphocyte population we found that they significantly increased in sensitized and challenged lungs (23.6±1.9 versus 0.4±0.2×10⁴ cells/ml in the control group). Treatment with the ET_A receptor antagonist did not significantly affect the number of

lymphocytes (30.0±2.1×10⁴ cells/ml); however, it markedly increased the relative number of lymphocytes, which accounted for 41.3% of total cells in the bronchoalveolar lavage fluid, when compared to the non-treated group, where lymphocytes accounted for 11.3% of the total cells. The ET_B receptor antagonist did not significantly affect the number nor the percentage of lymphocytes (Table 1). Eosinophil cell counts were performed in preparations stained with hematoxylin/eosin. The number of these cells in the bronchoalveolar lavage fluid significantly increased in the immunized and antigen-challenged group. Treatment with the ET_A receptor antagonist significantly reduced (65% inhibition) the number of eosinophils in the bronchoalveolar lavage fluid whereas treatment with the ET_B receptor antagonist had no significant effect (Table 1).

3.2. Endothelin A receptor antagonist reduces airway hyperreactivity and mucus secretion

Groups of immunized mice were submitted to two aerosol ovalbumin challenges, and 24 h later the lungs were removed and the airway perfusion pressure in response to bolus injections of increasing doses of methacholine (0.1 to 100 μg) was measured as described in Materials and methods. Fig. 2 shows that the reactivity of the airways to methacholine was significantly higher in the antigen-challenged group compared to the control group (around 30% higher comparing areas under the curves). This airway hyperreactivity was significantly reduced by treatment of the mice with the ET_A receptor antagonist (79%) whereas treatment with the ET_B receptor antagonist had no effect on the airway hyperreactivity.

The intensity of mucus production was evaluated in the bronchi (*n*=10) and scored from 0 to 3 as described in Materials and methods. In the control group none of the bronchi contained mucus (score 0), in contrast to the immunized and antigen-challenged group, where the scores averaged 2.5. Mucus plugs were not observed in any group. Pre-treatment with the ET_A receptor antagonist significantly inhibited mucus production. The ET_B receptor antagonist had no significant effect, although it showed a tendency to reduce mucus secretion (Fig. 3, panel A). This is illustrated in panel B, which shows photomicrographs of lung tissue preparations stained with PAS; (B1) shows bronchus from mice of the experimental group showing that most of the bronchus epithelium is covered by mucus; (B2) shows bronchus from mice of the experimental group pre-treated with the endothelin A receptor antagonist showing a marked reduction in the mucus-covered area; (B3) shows bronchus from mice of the experimental group pre-treated with the ET_B receptor antagonist where the mucus area is similar to the non-treated group.

3.3. Endothelin A receptor antagonist down regulates endothelin synthesis

The concentration of endothelins was measured in whole lung homogenates from sensitized mice 24 h after antigen challenge and was not significantly different from the concentration found in the control group (sensitized which received saline aerosol). These values were not significantly different from the basal levels of endothelins measured in non-manipulated mice (42.9±4.7 pg endothelin/g tissue). Treatment with the ET_A receptor antagonist significantly reduced endothelin levels (52%). Treatment with the ET_B receptor antagonist had no effect (Fig. 4).

4. Discussion

Our results show a clear increase in the number of CD₄⁺, CD₈⁺, B220⁺, Tγδ⁺ and NK1.1⁺ cells in the bronchoalveolar lavage fluid of immunized C57Bl/6 mice following antigen challenge, confirming a previous report in this mouse strain [13]. Treatment of the sensitized mice with the ET_A receptor antagonist, before the antigen challenge, significantly

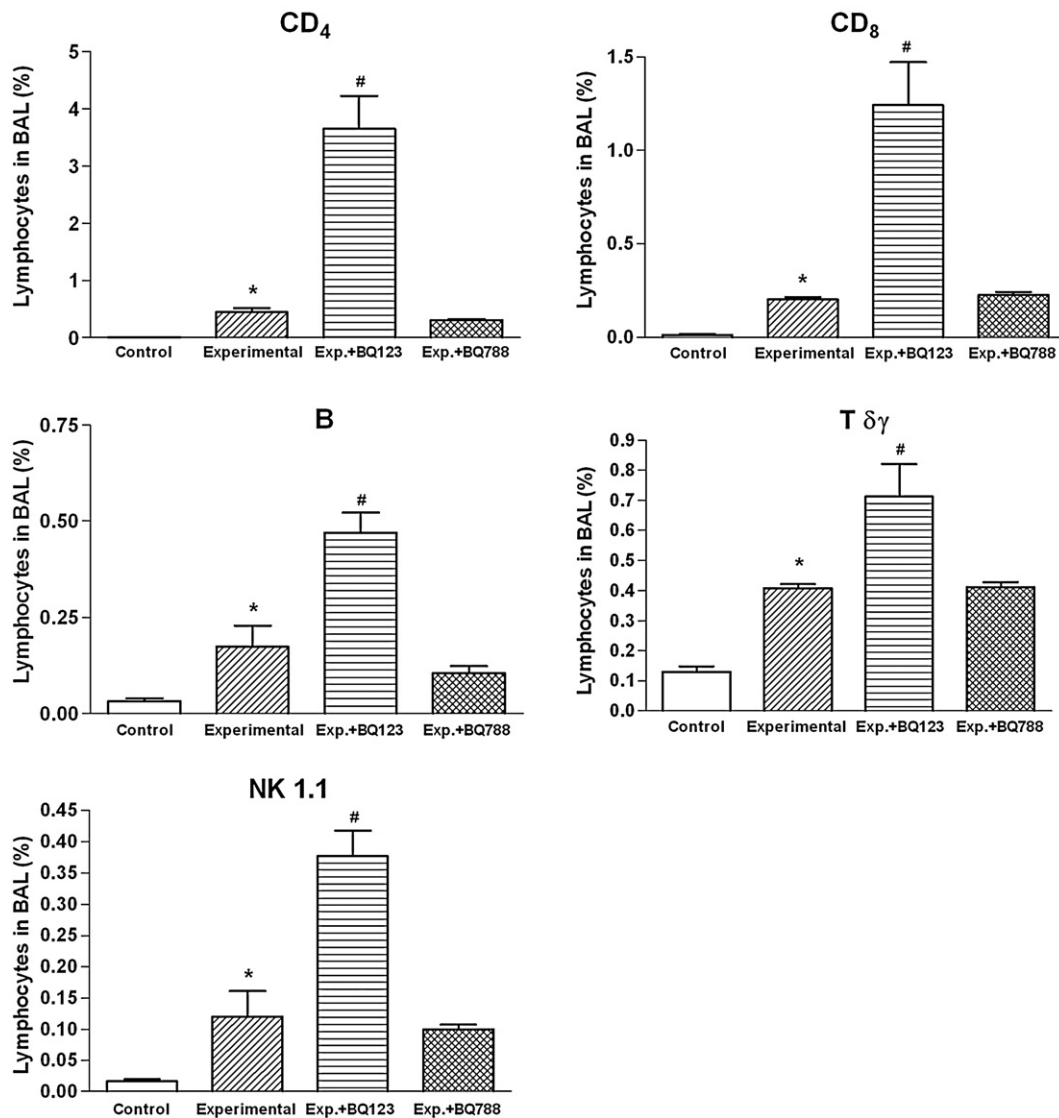


Fig. 1. Effect of endothelin receptor antagonists on the bronchoalveolar lavage lymphocyte subpopulations. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Selective endothelin A (BQ 123) or B (BQ 788) receptor antagonists (1 mg/kg, i.p.), were given 30 min before each aerosol challenge. Bronchoalveolar lavage was performed 24 h after the second challenge. The cells were incubated with fluorochrome-labeled monoclonal antibodies to CD₄, CD₈, B220, T $\gamma\delta$, NK1.1 and submitted to FACS analysis. Results are the mean $\pm$ S.E.M. of groups of 5–7 animals. * P < 0.05 in comparison with control group and # P < 0.05 in comparison with experimental group.

increased the percentage of CD₄⁺, CD₈⁺, B220⁺, T $\gamma\delta$ ⁺ and NK1.1⁺ cells in the bronchoalveolar lavage fluid, suggesting that endogenous endothelins down-regulate lymphocyte migration into the lung following allergic stimulation. This is the first demonstration that endothelin antagonist modifies lymphocyte infiltration in experimental asthma. In allergic pleurisy, which is a different situation, treatment with BQ123 reduced CD₄⁺ and CD₈⁺ cell numbers [20].

The antagonist of ET_A receptor markedly reduced the lung eosinophilia, corroborating previous reports [21–23].

Airway hyperreactivity is a characteristic feature of asthma. Enhanced response of bronchi to methacholine is found in several asthma models. In our model, we also found increased airway reactivity to methacholine after OVA challenge of OVA-sensitized mice. The hyperreactivity was abolished in mice treated with the ET_A

Table 1

Effect of endothelin receptor antagonists on the bronchoalveolar lavage cell infiltration

Cell $\times 10^4$ /ml	Control	Experimental	Exp.+BQ 123	Exp.+BQ 788
Total cells	32 $\pm$ 3.1	208.5 $\pm$ 12.5*	72.7 $\pm$ 5.2#	202.6 $\pm$ 19.6*
Eosinophils	0.9 $\pm$ 0.8 (2.8%)	124.4 $\pm$ 12.6* (59.7%)	7.1 $\pm$ 2.1# (9.8%)	99.6 $\pm$ 7* (49.2%)
Lymphocytes	0.4 $\pm$ 0.2 (1.2%)	23.6 $\pm$ 1.9* (11.3%)	30 $\pm$ 2.1* (41.3%)	25.1 $\pm$ 2.7* (12.4%)

Results represent the mean $\pm$ S.E.M. of groups of 6–8 animals. * P < 0.001 in comparison with the control group; # P < 0.001 comparing treated with non-treated groups. The total number of cells was determined by counting in a hemacytometer. Eosinophils cell counts were performed after cytocentrifugation and staining with hematoxylin-eosin. The number of lymphocytes was determined by flow cytometry analysis, lymphocytes being defined on the basis of size and granulation.

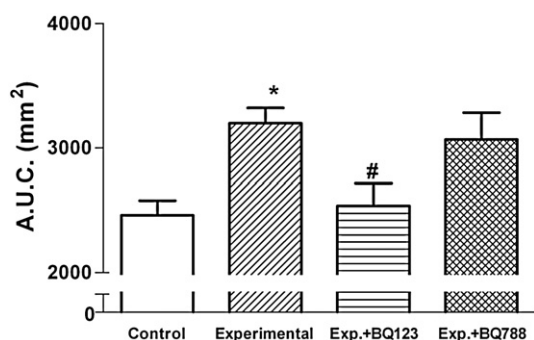


Fig. 2. Effect of endothelin receptor antagonists on airway reactivity to methacholine. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Selective endothelin A (BQ 123) or B (BQ 788) receptor antagonists (1 mg/kg, i.p.), were given 30 min before each aerosol challenge. Twenty-four hours after the second challenge, the lungs were removed and perfused via the trachea, and increases in perfusion pressure during bolus injection of methacholine were recorded (2A). Results are expressed as mean area under the curve $\pm$ S.E.M of groups of 6 animals. * $P < 0.05$ in comparison with the control group and # $P < 0.05$ in comparison with the experimental group.

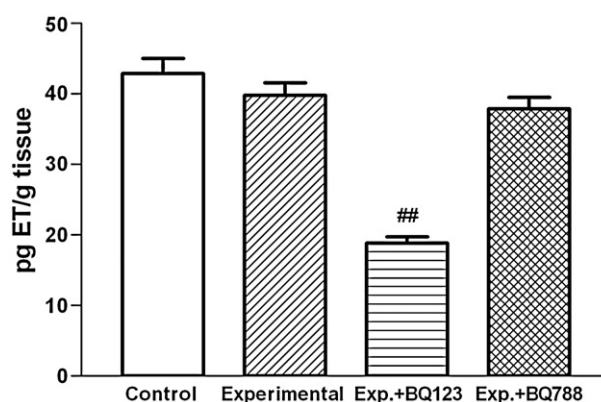


Fig. 4. Concentration of endothelin in lung homogenates. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Selective endothelin A (BQ 123) or B (BQ 788) receptor antagonists (1 mg/kg, i.p.), were given 30 min before each aerosol challenge. Lungs were removed 24 h after the second antigen challenge and the concentration of endothelins in lung homogenates was measured by ELISA. Results are expressed as pg endothelin/g lung $\pm$ S.E.M of groups of 5–8 animals. ## $P < 0.01$ in comparison with the experimental group.

receptor antagonist, BQ123. This is in agreement with Henry et al. [21] who showed that another ET_A receptor antagonist, SB217242, inhibited the development of hyperreactivity in mice sensitized to house dust mite allergen and challenged with the allergen.

We showed here that the ET_A receptor antagonist reduced the airway hyperreactivity while it increased the T $\gamma\delta$ ⁺ cell infiltration. This is consistent with previous findings from studies using T $\gamma\delta$ -deficient mice that suggested that the bronchial hyperreactivity is down-regulated by the T $\gamma\delta$ ⁺ cells [9,10]. The obstruction of airway caused by mucus hypersecretion is another important feature of asthma. In our study the ET_A receptor antagonist significantly reduced the mucus

index. Involvement of endothelins in mucus hypersecretion was shown for the first time in this study.

An interesting finding of this study was that the ET_A receptor antagonist reduced the lung levels of endothelins in immunized and antigen-challenged mice. This is in accordance with a previous publication [16] in which we found that the non-selective antagonist of endothelin receptors (Bosentan), significantly reduced (56%) the lung concentration of endothelins. In both studies ETs were measured in homogenates of lung tissue of C57Bl/6. This indicates that there is a basal production of endothelins in the lung and that there is a turnover

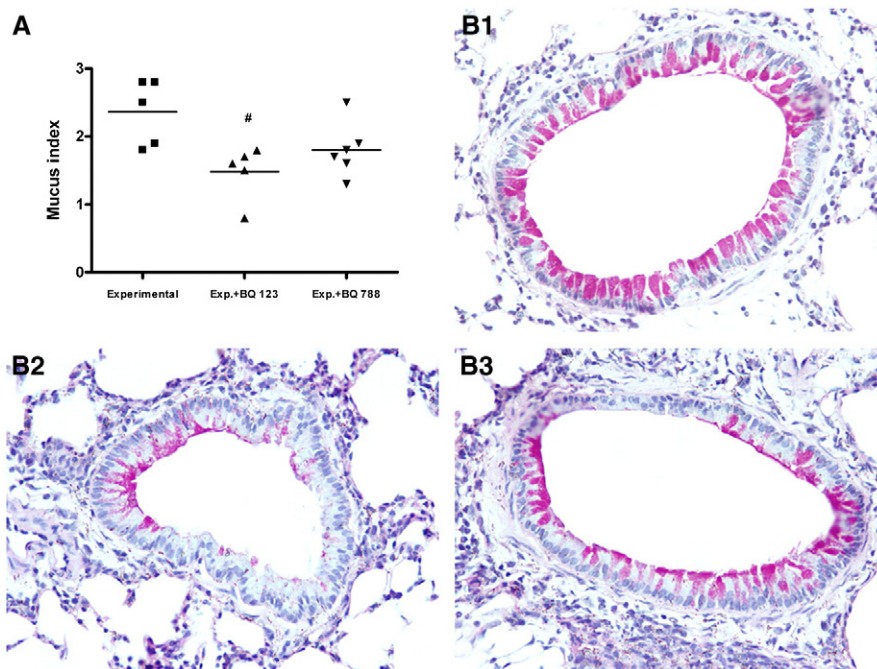


Fig. 3. Effect of endothelin receptor antagonists on mucus production. Panel A: C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization (experimental group). Selective endothelin A (BQ 123) or B (BQ 788) receptor antagonists (1 mg/kg, i.p.), were given 30 min before each aerosol challenge. Twenty-four hours after the second challenge, the lungs were removed, embedded in paraffin, sliced and stained with PAS/hematoxylin for evaluation of mucus as described in Materials and methods. The results are expressed as median of the scores. # $P < 0.05$ in comparison with the experimental group. Panel B: Photomicrographs of lungs stained with PAS/hematoxylin; (B1) bronchus from mice of the experimental group showing that most of the bronchus epithelium is covered by mucus; (B2) bronchus from the mice of the experimental group pre-treated with the endothelin A receptor antagonist showing that the area covered by mucus is reduced; (B3) bronchus from mice of the experimental group pre-treated with the endothelin B receptor antagonist with mucus-covered area similar to the non-treated group.

of endothelin synthesis and degradation, where endothelins exert a positive feedback on their synthesis. However, this effect seems to be strain-dependent since in the previous study this feedback was not observed in BALB/c mice [16]. Surprisingly, the concentration of endothelins in whole lung homogenates did not increase after antigen challenge of sensitized mice, compared to control mice (sensitized and not challenged) or to naive mice. The levels of endothelins in the lung tissue are particularly high compared to other tissues [5]. It is possible that the amount released by antigen challenge is too small to be detected. Also, endothelins were measured in whole lung homogenates, and release of endothelins seems to be restricted to bronchial epithelium and macrophages, at least in the Sephadex model of lung eosinophilic inflammation [24]. Another possibility is that the production would occur at earlier time points. Finsnes et al. [25] found in the Sephadex model of lung eosinophilic inflammation that the peak levels of ET1 mRNA expression were 30 min after provocation.

In a previous study we observed that the percentage of the CD4⁺, B220⁺ and $\gamma\delta$ ⁺ cells in sensitized and challenged mice was much higher in BALB/c than in C57Bl/6 [14]. Interestingly, we have recently shown that the BALB/c lungs present much lower levels of endothelin than C57Bl/6 under physiological conditions [15]. Thus, a low level of endothelins in BALB/c seems to be associated with high infiltration of lymphocytes. Moreover, blocking the ET_A receptor in C57Bl/6 mouse, increased lymphocytes infiltration into the airway. Taken together these results suggest that endogenous endothelin in the lung down regulates lymphocyte infiltration in experimental asthma.

In summary, the ET_A receptor antagonist was effective to reduce eosinophil infiltration, airway hyperreactivity and mucus hypersecretion in experimental asthma but it increased lymphocyte infiltration into the airway. It is important to further study the effect of ET_A treatment on later stages of lung allergic inflammation. We can only speculate that the increased infiltration of the $\gamma\delta$ ⁺ and NK1.1 cells promoted by the ET_A receptor antagonist would protect the respiratory mucosa and favor the resolution of lung inflammation.

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References

- [1] Rubanyi GM, Pokoloff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* 1994;46:325–415.
- [2] Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptors. *Trends Pharmacol Sci* 1992;13:103–8.

- [3] Masaki T, Yanagisawa M, Goto K. Physiology and pharmacology of endothelins. *Med Res Rev* 1992;12:391–421.
- [4] Goldie RG, Henry PJ, Knott GJ, Self MA, Luttmann MA, Hay DWP. Endothelin-1 receptor density, distribution, and function in human isolated asthmatic airways. *Am J Respir Crit Care Med* 1995;152:1653–8.
- [5] Goldie RG, Henry PJ. Endothelins and asthma. *Life Sci* 1999;65:1–15.
- [6] Kirby A, Newton D, Carding S, Kaye P. Pulmonary dendritic cells and alveolar macrophages are regulated by gammadelta T cells during the resolution of *S. pneumoniae*-induced inflammation. *J Pathol* 2007;212:29–37.
- [7] Cui X, Zeni F, Vodovitz Y, Correa-de-Araujo R, Quezada M, Roberts A, et al. TGF-beta1 increases microbial clearance but worsens lung injury during *Escherichia coli* pneumonia in rats. *Cytokine* 2003;24:115–27.
- [8] Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* 1995;373:255–7.
- [9] Zuany-Amorim C, Ruffie C, Haile S, Vargaftig BB, Pereira P, Pretolani M. Requirement for gamma delta T cells in allergic airway inflammation. *Science* 1988;240:1265–7.
- [10] Lahn M, Kanehiro A, Takeda K, Joetham A, Schwarze J, Kohler G, et al. Negative regulation of airway responsiveness that is dependent on gammadelta T cells and independent of alphabeta T cells. *Nat Med* 1999;5:1150–6.
- [11] Meyer EH, DeKruyff RH, Umetsu DT. T cells and NKT cells in the pathogenesis of asthma. *Annu Rev Med* 2007;59:281–92.
- [12] Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1T cells: development, specificity, and function. *Annu Rev Immunol* 1997;15:535–62.
- [13] Landgraf RG, Nossi DF, Sirois P, Jancar S. Prostaglandins, leukotrienes and PAF selectively modulate lymphocyte subset and eosinophil infiltration into the airways in a murine model of asthma. *Prostaglandins Leukot Essent Fatty Acids* 2007;77:163–72.
- [14] Landgraf RG, Sirois P, Jancar S. Differential modulation of murine lung inflammation by bradykinin B1 and B2 selective receptor antagonists. *Eur J Pharmacol* 2003;460:75–83.
- [15] Brochu I, Labonté J, Bkaily G, D'Orléans-Juste P. Role of endothelin receptors in the hypertensive state of kinin B₂ knockout mice subjected to a high-salt diet. *Clin Sci* 2002;103:380–4.
- [16] Landgraf RG, Jancar S. The role of endothelin pathway in modulation of airway reactivity to methacholine in C57Bl/6 and BALB/c mice. *Eur J Pharmacol* 2008;590:396–9.
- [17] Ihara M, Noguchi K, Saeki T, Fukuroda T, Tsuchida S, Kimura S, et al. Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci* 1992;50:247–55.
- [18] Bazil MK, Lappe RW, Webb RL. Pharmacological characterization of an endothelin A (ETA) receptor antagonist in conscious rats. *J Cardiovasc Pharmacol* 1992;20:940–8.
- [19] Ishikawa K, Ihara M, Noguchi K, Mase T, Mino S, Saeki T, et al. Biochemical and pharmacological profile of a potent and selective endothelin B receptor antagonist, BQ-788. *Proc Natl Acad Sci U S A* 1994;91:4892–6.
- [20] Sampaio AL, Rae GA, Henriques MM. Role of endothelins on lymphocyte accumulation in allergic pleurisy. *J Leukoc Biol* 2000;67:189–95.
- [21] Henry PJ, Mann TS, D'Aprile AC, Self GJ, Goldie RG. An endothelin receptor antagonist, SB-217242, inhibits airway hyperresponsiveness in allergic mice. *Am J Physiol Lung Cell Mol Physiol* 2002;283:1072–8.
- [22] Fujitani Y, Trifilieff A, Tsuyuki S, Coyle AJ, Bertrand C. Endothelin receptor antagonists inhibit antigen-induced lung inflammation in mice. *Am J Respir Crit Care Med* 1997;155:1890–4.
- [23] Gosselin M, Goulet S, Wu-Wong JR, Wessale JL, Opgenorth TJ, Boulet LP, et al. Effects of a selective ET(A)-receptor antagonist, atrasentan (ABT-627), in murine models of allergic asthma: demonstration of mouse strain specificity. *Clin Sci* 2002;103:367–70.
- [24] Finsnes F, Skjonsberg OH, Tonnessen T, Naess O, Lyberg T, Christensen G. Endothelin production and effects of endothelin antagonists during experimental airway inflammation. *Am J Respir Crit Care Med* 1997;155:1404–12.
- [25] Finsnes F, Christensen G, Lyberg T, Sejertstad OM, Skjonsberg OH. Increased synthesis and release of endothelin-1 during initial phase of airway inflammation. *Am J Respir Crit Care Med* 1998;158:1600–6.