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Inflammation Research

Modulation of allergic and immune complex-induced lung inflammation by bradykinin receptor antagonists

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Abstract. *Objective*: The effect of bradykinin (B_1 or B_2) receptor antagonists was studied in allergic and immune-complex-induced lung inflammation.
Methods: Lungs of BALB/c mice were examined 24 h after induction of lung inflammation, either allergic (ovalbumin-sensitized submitted to two aerosol of antigen, one week apart) or immune-complex induced (intratracheal instillation of IgG antibodies followed by intravenous antigen). The bradykinin B_2 receptor antagonist, HOE-140 or bradykinin B_1 receptor antagonist, R-954 were given intraperitoneally (100 μg/kg), 30 min before induction.
Results: In allergic inflammation, pre-treatment with R-954 reduced eosinophil infiltration into the lungs, mucus secretion and the airway hyperreactivity to methacholine. Pre-treatment with HOE-140 increased eosinophil infiltration but did not affect the other parameters. In immune-complex inflammation, HOE-140 increased neutrophil infiltration but not their activation nor the hemorrhagic lesions. R-594 pre-treatment did not change the parameters examined.
Conclusion: These results show important modulatory effects of bradykinin B_1 and B_2 receptor antagonists in both models of lung inflammation.

Key words: Bradykinin – bradykinin receptor antagonist – lung inflammation – asthma – immune complexes.

Introduction

Bradykinin, a potent proinflammatory nonapeptide synthesized *de novo* at the sites of tissue damage, reproduces many of the characteristic features of inflammation, such as plasma extravasation, vasodilation and inflammatory cell infiltration [1–3]. Bradykinin effects can be mediated directly by its action on bradykinin receptors located on target tissues or indirectly by the release of nitric oxide, neuropeptides or prostaglandins [4, 5]. Two subtypes of bradykinin receptors have been identified, B_1 and B_2: The B_2 receptor is expressed constitutively in several tissues and mediates most of bradykinin effects; the B_1 receptor is generally absent in normal tissues but is expressed in some inflammatory conditions [6, 7].

Experimental evidence suggests that bradykinin and its biologically active metabolite des-Arg^9-bradykinin play a role in asthma. Elevated levels of bradykinin were found in bronchoalveolar lavage fluid from asthmatic patients [8]. Inhalation of bradykinin caused strong bronchoconstriction but only in asthmatic patients. However the inhalation of bradykinin B_1 receptor agonist des-Arg^9-bradykinin, did not cause bronchoconstriction in asthmatic patients [9, 10]. Bradykinin B_2 receptors mediate the bronchoconstriction induced by bradykinin in humans [9, 11, 12]. Injection of bradykinin induces airway eosinophilia in several animal species. In guinea pig, allergen-induced eosinophilia seems to be mediated by both bradykinin receptors [13]. We have recently reported that the allergen-induced eosinophil and lymphocyte lung infiltration and mucus secretion in C57Bl/6 mice are enhanced by treatment with bradykinin B_2 receptor antagonist [14].

A number of inflammatory diseases are triggered by immune complexes. Inhalation of antigen by previously sensitized individuals can lead to deposition of immune complexes at the level of alveoli, causing local inflammation. This is characterized by dry caught, fever and respiratory distress that appear few hours after exposure to the antigen. The incidence of immune complex-induced pneumonitis is particularly high in individuals exposed to organic dusts or to thermophilic fungi present in moldy materials or in air conditioning systems [15]. However, the role of bradykinin receptors in this type of lung inflammation was never investigated. In immune complex-induced inflammation in the peritoneal cavity, bradykinin has an important role in the mechanism of plasma extravasation and this action seems to be mediated directly by bradykinin B_2 receptors [16].

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Clearly, more studies are needed to elucidate the role of bradykinin and its receptor subtypes in lung inflammation. In the present study we compared the effect of bradykinin receptor antagonists R-954 and HOE-140, respectively B_1 and B_2 receptor antagonists, in allergic and immune complex-induced lung inflammation in BALB/c mice. In the allergic model we focused on eosinophil infiltration, airway reactivity to methacholine and mucus production. In the immune complex-induced model, neutrophil infiltration, myeloperoxidase activity and lung hemorrhagic lesions were studied.

Materials and methods

Animals

Male BALB/c mice weighing 20–25 g, 6–8 weeks old, from our own animal facilities were housed in a room with 12 h light-dark cycle with water and food *ad libitum.* 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 Biomedical Sciences Institute/USP – Ethical Committee for Animal Research (CEEA).

Induction of the allergic inflammation

Mice were sensitized on days 0 and 7 by intraperitoneal injection of a mixture containing 50 µg of ovalbumin and 1 mg of Al_2O_3 in saline (a total volume of 0.2 ml). At 14 and 21 days after first immunization the animal 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% wt/vol.

The control group consisted of animals immunized as described and challenged with saline solution, or non-immunized animals challenged with ovalbumin aerosol as above.

Induction of immune complex-induced inflammation – passive reverse Arthus reaction.

Mice were anesthetized with an intraperitoneal injection of chlorhydrate of 2-(2,6-xilidine)-5,6-dihydro-4-h-1,3-tiasine (40 mg/kg) and chloral hydrate (250 mg/kg). A volume of 30 µl of rabbit immunoglobulin G antibodies to bovine serum albumin containing 15 mg/ml of specific antibody protein was instilled intratracheally followed by an intravenous injection of 2 mg of bovine serum albumin in a volume of 100 µl. As control, groups of mice received the antibody intratracheally as described above and saline intravenously. A group of naive animals was also included.

Treatments

Groups of mice received an intraperitoneal injection of 100 µg/kg of the bradykinin B_1 receptor antagonist (R-954) or bradykinin B_2 receptor antagonist (HOE-140), 30 min before each aerosol of ovalbumin in the allergic model or 30 min before triggering of the immune complex inflammation.

Bronchoalveolar lavage

The animals were killed by an intraperitoneal injection of ketamine/xylasine (50 µl of a 100 mg/ml solution) 24 h after exposure to the second aerosol challenge. A tracheal cannula was inserted via a mid-cervical incision and the airways were lavaged three times with 1 ml of phosphate buffered saline (PBS, pH 7.4 at 4 °C).

Total and differential cell counts

The bronchoalveolar lavage fluid was centrifuged at 170 g for 10 min at 4 °C, the supernatant was removed, and the cell pellet was resuspended 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 hematocytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin-eosin (Hema 3).

Evaluation of hemorrhagic lesions

The concentration of hemoglobin in the bronchoalveolar lavage fluid was determined by incubating the bronchoalveolar lavage fluid with Drabkin's solution volume/volume for 1 h at room temperature. The concentration of hemoglobin was determined colourimetrically at 550 nm wavelength and the results are expressed as µg of hemoglobin per milliliter of bronchoalveolar lavage fluid.

Assay of myeloperoxidase activity

The myeloperoxidase activity was assayed in the first milliliter of bronchoalveolar lavage fluid after centrifugation (300 g) to remove cells. The assay was adapted from Henson [17]. Briefly, to 175 µl of cell-free bronchoalveolar lavage fluid were added 125 µl of phosphate buffer (0.1 M, pH = 6.2 containing 0.35% of bovine serum albumin), 25µl of θ-dianisidine (4 mM) and 25 µl of H_2O_2 (0.05%). After 15 min the reaction was stopped with 25 µl of sodium azide (1%) and the absorbance of the samples determined at 490 nm.

Evaluation of airway reactivity

Mice were immunized and submitted to two antigen aerosol exposures as described above. Twenty-four hours after the second antigen challenge, mice were given an injection of ketamine/xylasine (50 µl of a 100 mg/ml solution, i.p.), the peritoneal cavity was cut open and animals were exsanguinated by section of the abdominal aorta. The thoracic cavity was then opened; the pulmonary artery was cannulated and perfused with 10 ml Kreb's 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 Kreb's (37ºC, 95% O_2 and 5% CO_2) solution. A small incision was made in the lower end of each lobe to permit the outflow of the solution. 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 (cmH_2O) as a function of the dose of methacholine (µg) was measured for the entire recording period. Areas under the curve were calculated and results were expressed as mean area under the curve (mm^2).

Evaluation of mucus

Lungs were removed after bronchoalveolar lavage collection, perfused via the right ventricle with 10 ml PBS to remove residual blood, immersed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced in 5 micron sections and stained 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 4 were attributed: 0 when none of the bronchi show any sign of mucus and 1, 2 and 3 when 25%, 50% and more than 50% of the bronchi epithelium was covered by mucus and 4 when the lumen was obliterated by a mucus plug. Values represent the sum of 10 bronchi scored randomly at × 250 magnification.

Drugs and reagents

The antagonists of bradykinin receptors, R-954 (Ac-Orn [Oic^2, αMePhe^5, βD-Nal^{7}Ile^8] des-Arg^9-bradykinin) and HOE-140 (D-Arg [Hyp^3, Thi^5, Dtic^7-Oic^8]- bradykinin) were synthesized in the Department of Pharmacology, Medical School, University of Sherbrooke, Quebec, Canada. Ovalbumin grade III, methacholine, bovine serum albumin, rabbit immunoglobulin G antibody to bovine serum albumin, hexadecyl trimethyl ammonium bromide, EDTA, and θ-dianisidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Hema 3 from Biochemical Sciences Inc. (Swedesboro, NJ) and aluminum hydroxide gel (Rehydragel) from Reheis Inc. (Berkley Heights, NJ, USA).

Statistical analysis

Data are expressed as the means ± S.E.M. Statistical evaluation 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. A P value lower than 0.05 was considered to be significant.

Results

Effect of bradykinin receptor antagonists on allergic lung inflammation

Cell infiltration

Mice (BALB/c) immunized with ovalbumin were submitted to two ovalbumin aerosol challenges (experimental group) or saline aerosol (control group). A bronchoalveolar lavage was performed 24 h after the second aerosol challenge. A significant increase in eosinophil number in bronchoalveolar lavage fluid (BAL) was observed in the experimental group compared to the control group (Fig. 1A). Eosinophils constituted around 40% of bronchoalveolar cells at this time period.

Groups of immunized mice were treated intraperitoneally with the bradykinin antagonists of B_1 or B_2 receptors, R-954 or HOE-140 respectively, 30 min before each of the aerosol antigen challenges. Figure 1A shows that treatment with HOE-140 significantly increased the number of eosinophils (from 28.2 to 86.7 × 10^4 cells) in the bronchoalveolar lavage whereas treatment with R-954 markedly reduced the numbers of eosinophils (from 28.2 to 2.0 × 10^4 cells).

Lung reactivity to methacholine

Lungs from immunized and antigen challenged mice (experimental group) or immunized and saline challenged mice (control group) were removed and perfused through the trachea as described in Materials and Methods. The bronchoconstriction induced by bolus injection of increasing doses of methacholine (0.1 to 100 µg) was measured as increase in perfusion pressure. In the experimental group the reactivity of the airways to methacholine was significantly higher

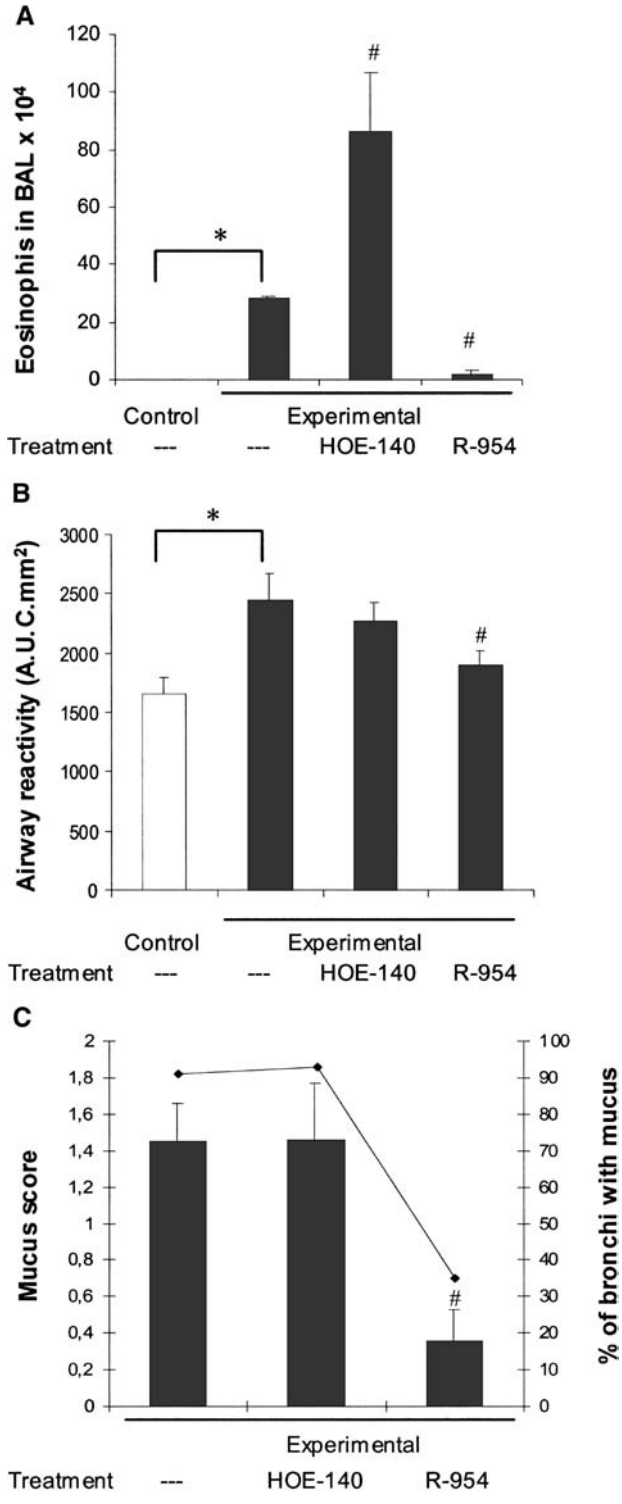

Fig. 1. Effect of Bradykinin B_1 and B_2 receptor antagonists on allergic lung inflammation. BALB/c mice were immunized with an intraperitoneal injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Bradykinin receptor antagonists were given intraperitoneally (100 µg/kg), 30 min before each aerosol challenge. Experiments were performed 24 h after the second challenge. (**A**) eosinophil numbers in bronchoalveolar lavage fluid; (**B**) airway reactivity to methacholine; (C) mucus score. Results are the mean ± S.E.M of 6–8 animals group. *P < 0.01 comparing experimental with control groups; # P < 0.01 comparing experimental non-treated with treated groups.

than that of the control group (around 47% higher comparing areas under the curves). Airway hyperreactivity was significantly reduced by pre-treatment of the mice with compound R-954 and unaffected by treatment with HOE-140 (Fig. 1B).

Mucus secretion

The bronchi were examined for mucus and scored from 0 to 4 as described in Materials and Methods. In the control group none of the bronchi contained measurable mucus (score 0) in contrast to the experimental group where the mean of ten bronchi scores was 1.45. Pre-treatment with R-954 significantly inhibited mucus production whereas treatment with HOE-140 had no effect (Fig. 1C).

When analyzing the percentage of bronchi which contained mucus, it was found that 91% were positive in the experimental group, 93% were positive in HOE-140 treated group and only 35% of the bronchi were positive for mucus in the R-954 treated group.

Effect of bradykinin receptor antagonists on immune-complex – induced lung inflammation

Cell infiltration

Mice were given an intratracheal instillation of IgG antibodies to bovine serum albumin followed by intravenous injection of bovine serum albumin (experimental group). The control group was given the intratracheal instillation of antibodies followed by intravenous injection of saline. After 24 h, there was a significant increase in total cell number in bronchoalveolar lavage fluid in the experimental group compared to the level in the control group. This increase was mainly due to infiltration of neutrophils, which averaged 74% of bronchoalveolar lavage fluid cells at this time period (Fig. 2A).

Treatment of mice with the bradykinin B_2 receptor antagonist HOE 140, 30 min before induction of the immune complex-reaction, increased significantly (58%) the neutrophil infiltration whereas treatment with the bradykinin B_1 receptor antagonist R-954 had no effect.

Myeloperoxidase activity (MPO)

The myeloperoxidase activity was measured in the cell free bronchoalveolar lavage fluid, 24 h after the induction of the immune-complex reaction. As showed in the figure 2B, myeloperoxidase activity was significantly higher in the experimental group (from 0.0658 to 0.814 optical density) than in the control group. The level of myeloperoxidase activity in the bronchoalveolar lavage fluid in mice pre-treated with the compounds HOE-140 or R-954 was similar to the non-treated animals.

Hemorrhagic lesions

The intensity of the hemorrhagic lesions was evaluated by measuring the amount of hemoglobin present in the bron-

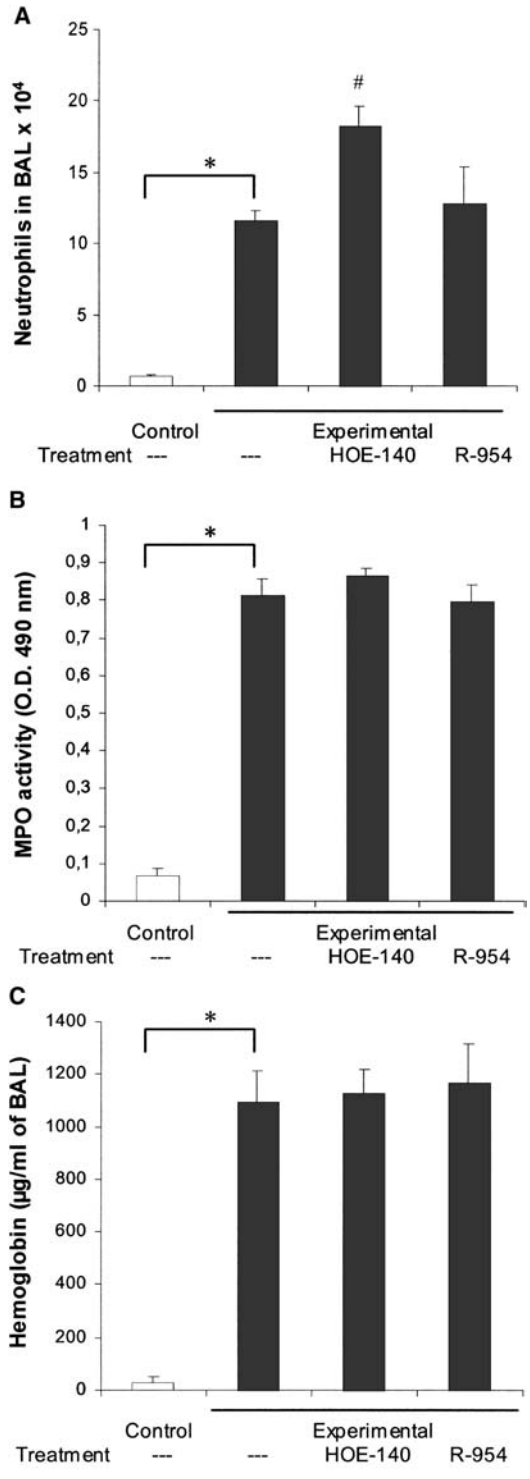

Fig. 2. Effect of Bradykinin B_1 and B_2 receptor antagonists on immune complex-induced lung inflammation. Balb/c mice received an instillation of rabbit immunoglobulin G antibodies to bovine serum albumin into the airways followed by intravenous injection of bovine serum albumin. Groups of mice were treated with bradykinin receptor antagonists (100 µg/kg) intraperitoneally, 30 min before induction of the reaction. Bronchoalveolar lavage (BAL) was performed 24 h after induction of the reaction. (**A**) number of neutrophils; (**B**) myeloperoxidase activity in cell free BAL; (**C**) hemoglobin content. Results are the mean ± S.E.M of 6–8 animals group. *$P < 0.001$ comparing experimental with control groups; #$P < 0.05$, comparing experimental non-treated with the treated groups.

choalveolar lavage fluid. Figure 2C shows that, 24 h after induction of immune-complex reaction in the lung, a significant increase (from 30 to 1094 µg/ml of BAL) in the concentration of hemoglobin was detected. Treatment of the mice with the bradykinin antagonists of B_1 or B_2 receptors, R-954 or HOE-140 respectively, did not change the hemoglobin content present in the bronchoalveolar lavage fluid (Fig. 2C).

Discussion

The results of the present study showed that in the murine (BALB/c) model of allergic lung inflammation employed, which presents the characteristic features of asthma, the bradykinin B_1 receptor antagonist (R-954), administered before each antigen challenge, reduced the number of eosinophils in the bronchoalveolar lavage fluid. The decrease of the eosinophil counts was associated with a decrease in lung hyperreactivity to methacholine, as well as a significant reduction in the amount of mucus in the bronchi. On the other hand, the treatment of the BALB/c mice with the bradykinin B_2 receptor antagonist (HOE-140) increased eosinophil numbers in the bronchoalveolar lavage fluid but these changes were not associated with increased hyperreactivity to methacholine or with increased mucus in the bronchi.

The results presented here are similar to those found recently in C57Bl/6 mice using the same model of allergic inflammation [14]. The C57Bl/6 and Balb/c mice have been extensively used in allergic lung inflammation studies and differences were observed regarding the intensity of pulmonary inflammatory response and the pattern of cytokines produced. In some studies, BALB/c mice developed a more intense airway hyperreactivity and lymphocyte/eosinophil influx into the lungs than C57Bl/6 mice [18, 19]. In our model of allergic lung inflammation we found that the intensity of the peribronchovascular cell infiltrate was similar in BALB/c and C57Bl/6 but the Balb/c presented 3 times less cells in the bronchoalveolar lavage fluid than C57Bl/6 (80×10^4 versus 242×10^4 respectively). This was due to a lower influx of eosinophils and neutrophils in BALB/c; the most striking difference being in eosinophil numbers, 28×10^4 in Balb/c and 129×10^4 in C57Bl/6 (35% and 54% of BAL cells in BALB/c and C57Bl/6, respectively). Regarding mucus formation, there was no difference in the mucus score among the strains, however, mucus plugs were frequently seen in BALB/c and never in C57Bl/6. The intensity of the hyperreactivity to methacholine was similar in both strains. However, despite these differences the contribution of the bradykinin receptors to the pathological events seems to be similar. Treatment of C57Bl/6 mice with HOE-140 increased eosinophil infiltration, but did not affect mucus production and airway reactivity to methacholine as observed in Balb/c mice. The other antagonist, R-954, decreased eosinophil infiltration, airway reactivity and mucus production in BALB/c mice as observed previously in C57Bl/6 mice [14].

In guinea pigs the bradykinin B_1 receptor antagonist [Leu^8] des-Arg^9-bradykinin also inhibited the leukocyte migration in airway inflammation induced by antigen [20]. Similar results were described by Perron [13] using another bradykinin B_1 receptor antagonist the LysLys [Hyp^3, Igl^5, Digl7, Oic^8] des-Arg^9-bradykinin (code name B9858).

Our results obtained with the bradykinin B_2 receptor antagonist (HOE-140) are in agreement with those of Woisin [21]. Using a rabbit model of allergic lung inflammation these authors found that pre-treatment with a potent and selective bradykinin B_2 receptor antagonist (D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-Dtic-*N*Chg-Arg) (code name CP-0597), did not affect antigen-induced acute bronchoconstriction, airway hyperresponsiveness or pulmonary eosinophilia. On the other hand, our results differ from those obtained in guinea pig and rabbit models of allergic lung inflammation. Farmer [20], Mashito [22] and Perron [13], found that the bradykinin B_2 receptor antagonists D-Arg- [Hyp^3, Thi$^{5-8}$, D-Phe^7]-bradykinin (code name NPC-349) or the D-Arg [Hyp^3, Thi^5, D-tic^7, Oic^8]-bradykinin (code name HOE-140) inhibited antigen-induced eosinophilia and neutrophilia and decreased eosinophil peroxidase activity in guinea-pigs. Another bradykinin B_2 receptor antagonist D-Arg [Hyp^3, D-Phe^7] bradykinin (code name NPC 567) abolished the bronchial hyperresponsiveness and reduced the airway inflammation in sheep with natural hypersensitivity to *Ascaris suum* [23]. These discrepancies could be related to the use of different species. It is noteworthy that species differences were reported by Regoli [24], particularly between human, rabbit and mouse bradykinin B_1 receptors and between human, rabbit and guinea pig bradykinin B_2 receptors.

The role of bradykinin in lung inflammation induced by immune complexes, characterized by an early infiltration of neutrophils and vascular damage was also studied. Our results showed that pre-treatment of BALB/c mice with HOE-140, the bradykinin B_2 receptor antagonist increased the neutrophil infiltration in the lung. However, it did not affect the activation of these cells, as indicated by the level of myeloperoxidase activity in bronchoalveolar lavage fluid. This might explain why this treatment did not affect the intensity of hemorrhagic lesions despite the high levels of neutrophils recovered in the bronchoalveolar lavage fluid.

Previous results showed that bradykinin has direct effects on the recruitment or activation of inflammatory cells, although it may act indirectly through the release of mediators from structural cells. For example, bradykinin induced the release of neutrophil and monocyte chemotactic factors from airway epithelial cells [25]. Bradykinin was shown to stimulate the release of selected mediators including leukotriene B_4 and platelet-activating factor from alveolar macrophages of asthma patients [26]. Since our previous study showed that the lung hemorrhagic lesions are dependent on leukotriene B_4 [27] and since the treatment of mice with bradykinin receptor antagonists did not affect the lung lesions, it appears that the production of leukotriene B_4 is independent of bradykinin in this model.

In conclusion, our results showed clearly that the role played by bradykinin in lung inflammation is dependent on the type of immunological reaction. They suggest that bradykinin contributes only marginally to the lung inflammation induced by immune complexes whereas in allergic lung inflammation, activation of the constitutive bradykinin B_2 receptor seems to down-regulate the inflammation, and activation of the inducible bradykinin B_1 receptor causes pro-inflammatory effects.

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