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C-fibers, but not the transient potential receptor vanilloid 1 (TRPV1), play a role in experimental allergic airway inflammation

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ABSTRACT

The activation of C-fibers in the airways induces coughing, mucus production and bronchoconstriction, which are also symptoms of airway diseases. In this study, we evaluated the role of the C-fibers and the TRPV1 (transient receptor potential vanilloid 1) receptor in an experimental mouse model of allergic airway inflammation. To study the role of C-fibers, we either degenerated the C-fibers persistently (capsaicin administration in neonate mice) or transiently (capsaicin administration in adult mice). No alteration was observed in eosinophil recruitment to the bronchoalveolar lavage fluid in animals treated with capsaicin in the neonatal period. However, in adult animals, capsaicin treatment after the first ovalbumin challenge (in the establishment of the inflammatory process) decreased the eosinophil numbers. This effect was more pronounced in adult animals treated with capsaicin before beginning the ovalbumin immunization (in the development of the inflammatory process). In addition, interleukin (IL)-5 and chemokine ligand 11 (CCL11) levels in the bronchoalveolar lavage fluid, as well as P-selectin expression and p65 nuclear factor κB (NF-κB) activation in the lung were also decreased. No alterations were observed in the IL-10 and IL-13 levels. Next we determined the effect of TRPV1 receptor blockade on allergic airway inflammation. SB366791 administrated in mice by intraperitoneal (500 μg/kg) or intranasal (0.1, 1 or 10 nmol/site) route failed to decrease eosinophil recruitment to the bronchoalveolar lavage fluid or alter any other metrics cited above. Thus, the present results confirm and extend previous data supporting the involvement of C-fibers, but not the TRPV1 receptor, in allergic airway inflammation.

1. Introduction

Allergic asthma is a complex inflammatory disorder characterized by airway hyperresponsiveness, eosinophilic influx and the hypersecretion of mucus by goblet cells. This disease is frequently accompanied by high serum levels of immunoglobulin (Ig) E and by intrapulmonary production of certain interleukins such as interleukin (IL)-4, IL-5 and IL-13 by allergen-specific T-helper 2 (Th2) cells (Murphy and O'Byrne, 2010). Integrated signaling events between IL-4 and IL-13 are thought to regulate pulmonary eosinophilia by stimulating eosinophil-specific adhesion pathways and by modulating the local production of both IL-5 and CCL11 (eotaxin), which in turn selectively drive eosinophil recruitment. IL-5, in particular, plays a critical role in the regulation of bone marrow and blood eosinophilia (Foster et al., 2001).

The airways are innervated by branches of the trigeminal and vagal afferents nerves (nodose and jugular ganglia). The majority (75%) of afferent innervations of the respiratory tract are bronchopulmonary afferent C-fibers (Coleridge and Coleridge, 1984; Lee, 2006). In the airways, C-fibers are located in the mucosa, in the lung parenchyma and in the space between epithelial cells (Adriaensen et al., 1998; Watanabe et al., 2006). These fibers respond to thermal, chemical (such as capsaicin, the principal pungent ingredient of red pepper) and acidic stimuli. In addition, cigarette smoke, air pollutants and endogenous inflammatory mediators are also C-fiber stimuli (Ho et al., 2001). Capsaicin stimulates afferent C-fibers by activating the transient potential receptor vanilloid 1 (TRPV1). In addition to the expression of TRPV1 on sensory fibers, the TRPV1 receptor is also expressed in airway smooth muscle cells, epithelial cells, vascular endothelial cells and submucosal glands as well as in inflammatory cells (Agopyan et al., 2004; Jia and Lee, 2007; Ni et al., 2006; Reilly et al., 2003; Seki et al., 2006; Watanabe et al., 2005). TRPV1 receptor activation induces the release of neuropeptides such as neurokinins, calcitonin gene-related peptide (CGRP) and substance P. These neuropeptides are known to elicit neurogenic inflammation in the airways by inducing potent effects on cells of the respiratory tract (Barnes et al., 1991) and on inflammatory cells (Barnes, 1990).

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Our understanding of the function and localization of TRPV1 and other TRP members, such as TRPA1 (transient receptor potential ankyrin 1), in the airways and the relationship between these ion channels and airways diseases has dramatically increased in the last decade. Patients with chronic cough, for example, show an increased number of TRPV1 immunoreactive axons (Groneberg et al., 2004). It has also been reported that in asthmatic patients, treatment with a cyclooxygenase 2 (COX-2) inhibitor attenuated the cough reflex sensitivity in the airways to inhaled capsaicin (Ishiura et al., 2009).

Despite the growing number of studies, the involvement of C-fibers and TRPV1 in airway inflammation is not entirely clear. Therefore, the present study was carried out to evaluate the role of C-fibers and the TRPV1 receptor in a murine model of allergic airway inflammation induced by allergen (ovalbumin).

2. Materials and methods

2.1. Animals

Experiments were conducted using female BALB/c mice (8 weeks, weighing 20–25 g). The administration of capsaicin or vehicle in neonatal mice was performed on postnatal day 2, and the animals were used at adult ages. Mice were kept in a room with controlled temperature (22 ± 2 °C) and humidity (60–80%) under a 12:12 h light–dark cycle (lights on at 06:00 h). At appropriate time intervals, mice were killed by an isoflurane overdose. The animals were put in an acrylic chamber, and a controlled amount of the gas anesthetic was added to the oxygen (2% isoflurane in O_2) that was flowing through the machine. All of the procedures used in the present study complied with the guidelines on animal care of the UFSC Ethics Committee on the Use of Animals (protocol number PP00154), which follows the *Guide for the Care and Use of Laboratory Animals* (DHEW [DHHS] publication no. [NIH] 85–23, rev. 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205, U.S.A.).

2.2. Antigen immunization, booster and airway challenge

Mice (8 weeks old) were immunized on days 0 and 7 by subcutaneous injection of 4 μg ovalbumin plus 1.6 mg aluminum hydroxide in 0.4 mL saline. Next the animals were challenged twice by intranasal route (on post-immunization days 14 and 21) with 10 μg ovalbumin in 50 μL saline, which was delivered into the nostrils under gaseous anesthesia (2% isoflurane in O_2), with the aid of a micropipette. The control group consisted of non-immunized mice that received two intranasal instillations of ovalbumin (on days 14 and 21). Animals were killed 24 h after the second ovalbumin challenge (on post-immunization day 22), and the bronchoalveolar lavage fluids or lungs were collected (Rogerio et al., 2009).

2.3. Neonatal capsaicin treatment

Mice were given capsaicin (50 mg/kg, s.c.) or vehicle alone (1:1:8 vol/vol/vol ethanol-Tween 80-saline) at postnatal day 2, as previously described (Jancso et al., 1977). Next, adult animals (8 weeks after capsaicin pretreatment) were ovalbumin-immunized and -challenged as described above. To assess whether a complete degeneration of the C-fibers had occurred after neonatal treatment with capsaicin, the animals were first submitted to an eye-wiping test (Ikeda et al., 2001) 1 day before the second ovalbumin challenge (on post-immunization day 20, ~11 weeks after the capsaicin administration). For this test, 20 μl of a 0.01% capsaicin solution was administered to the eye, and the number of wiping movements that occurred for 1 min was counted. The maximal final concentration of ethanol in this solution was 0.001%, and it had no effect per se. The adult animals that wiped their eyes no more than five times were considered to have been desensitized by the neonatal capsaicin treatment, and the others were not used in further experiments.

2.4. Capsaicin treatment in adult animals

Mice (8 weeks old) received 25 mg/kg capsaicin subcutaneously on day 1 and 75 mg/kg on day 2 (both diluted in the proportion 1:1:8 vol/vol/vol ethanol-Tween 80-saline). For each day of treatment with capsaicin, the animals were pretreated with theophylline (100 mg/kg, s.c.) and terbutaline (1 mg/kg, i.p.) diluted in saline. After 30 min, the animals were anesthetized with isoflurane and then received capsaicin or vehicle (1:1:8 vol/vol/vol ethanol-Tween 80-saline) (Morris et al., 2003; Symanowicz et al., 2004). The capsaicin treatment in adult animals was done in two different periods. Animals allocated to the first period were injected with capsaicin on day 14 (30 min after the first ovalbumin challenge) and on day 15 after the first immunization. Animals allocated to the second period were injected with capsaicin during the 2 days before initiating the first ovalbumin immunization. To assess whether desensitization of the C-fibers had occurred, the animals were first submitted to an eye-wiping test as described above 1 day before the second ovalbumin-challenge (on post-immunization day 20).

2.5. Treatment with TRPV1 antagonist SB 366791

Two protocols were carried out to evaluate the participation of the TRPV1 receptor in the allergic airway inflammatory model. For these, SB366791 (a TRPV1 selective antagonist) was dissolved in dimethyl sulfoxide (DMSO). In the first protocol, animals were treated by intraperitoneal route with SB366791 (500 μg/kg) or its vehicle (DMSO 0.2%) (Varga et al., 2005) 1 h before the second ovalbumin challenge (day 21 after the first ovalbumin immunization). In the second protocol, SB366791 was administered directly into the lungs of the mice by the intranasal route (0.1, 1 or 10 nmol/site, 50 μL, 1 h prior and 5, 11, 17, and 23 h after exposure to the second ovalbumin challenge) (Kassuya et al., 2008). An ovalbumin-immunized and -challenged mice group was treated with vehicle (DMSO 0.2%) by the same route and timing as described above.

2.6. Evaluation of leukocyte influx into the bronchoalveolar space

Mice were killed by isoflurane overdose. A polyethylene cannula was subsequently introduced into the trachea, and phosphate-buffered saline (PBS) containing heparin (10 UI/ml) was instilled in three aliquots (0.3, 0.3 and 0.4 mL) for a total of 1 mL. The bronchoalveolar lavage fluid was recovered and placed on ice. Total cell and differential leukocyte counts were made according a previous study (Rogerio et al., 2009). Following centrifugation (400 × *g*, 5 min, 4 °C), supernatants of the bronchoalveolar lavage fluids were collected and stored at −70 °C for subsequent cytokine and chemokine determinations.

2.7. Measurement of IL-5, IL-10, IL-13 and CCL11

The IL-5, IL-10, IL-13 and CCL11 levels were assayed according to the manufacturer's instructions (R&D Systems: Minneapolis, USA) by specific ELISA (RayBiotech: Georgia, USA). The sensitivities were >10 pg/mL.

2.8. Immunohistochemical studies

The animals were intracardially perfused with 4% of the fixative solution formaldehyde in 0.1 M phosphate buffer (pH 7.4). Lungs were removed, post-fixed for 24 h in the same solution, placed in ethanol (70% v/v) and then submerged in paraffin. Tissues embedded in paraffin were cut into thick sections (5-μm). Slides were deparaffinized through a series of xylene baths and rehydrated through graded alcohol

solutions. High-temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98 °C in 10 mM trisodium citrate buffer, pH 6.0, for 45 min. After overnight incubation at 4 °C with primary antibody [polyclonal rabbit anti-phospho-p65 NF-κB (1:100, Cell Signaling Technology, Massachusetts, USA) or polyclonal goat anti-P-selectin (1:2000, Santa Cruz Biotechnology, California, USA)], the slides were washed with PBS and incubated with the secondary antibody Envision plus (DakoCytomation, California, USA), for 1 h at room temperature. The sections were washed in PBS, and the visualization was completed by use of 3,3′-diaminobenzidine (DAB) (DakoCytomation) in chromogen solution and light counterstaining with Harris's hematoxylin solution (Merck, Darmstadt, Germany). Images were obtained with a microscope (Nikon Eclipse 50i) and Digital Sight Camera (DS-5M-L1, Nikon, New York, USA). Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental tissues. For each mouse lung, three images were obtained. The images were transferred to a computer, and the average pixel color intensity of phospho-p65 NF-κB or P-selectin staining was calculated as described in a previous study (Rogerio et al., 2009).

2.9. Statistical analysis

The data are reported as the mean ± S.E.M. The means from different treatments in each individual experiment were compared by ANOVA. When significant differences were identified, individual comparisons were subsequently made with Tukey's test. Values of $P<0.05$ were considered to be statistically significant.

2.10. Drugs and reagents

The following drugs were used: capsaicin, aluminum hydroxide, ovalbumin, theophylline and terbutaline (Sigma Chemical: Missouri, USA), SB366791 (Tocris Cookson Inc., Mississippi, USA), isoflurane (anaesthesic, Forane®, Abbott: Abbott Park, USA), and heparin (anticoagulant, Liquemine®, Roche: Basel, Switzerland). The reagents Tween-80 (Merck: Darmstadt, Germany), DMSO and ethanol were purchased from Vetec Química Fina (Rio de Janeiro, Brazil).

3. Results

3.1. Effect of neonatal capsaicin treatment on leukocyte numbers in the bronchoalveolar lavage fluid

Because no difference in neutrophil and mononuclear cell influx after afferent denervation was observed in either neonatal or adult animals (data not shown), we will focus on eosinophil numbers. The control mice (non-immunized) that were treated in the neonatal period with capsaicin or with vehicle did not present eosinophils in the bronchoalveolar lavage fluid (Fig. 1A). However, mice that were treated during the neonatal period with capsaicin or its vehicle and were ovalbumin-immunized and then ovalbumin-challenged showed an increase of eosinophil numbers in the bronchoalveolar lavage fluid when compared with the respective control group (Fig. 1A). No significant difference was observed between mice treated with vehicle or capsaicin and ovalbumin-immunized and -challenged.

3.2. Effect of capsaicin treatment in adult animals in the allergic airway inflammatory model

To investigate the effect of the depletion of neuropeptide stores induced by capsaicin injection in adult mice, we used two different protocols: mice allocated to the first protocol started the capsaicin treatment 30 min after the first ovalbumin challenge (see Methods). No eosinophils were found in the bronchoalveolar lavage fluid of the control adult mice (non-immunized) treated with either vehicle or capsaicin (Fig. 1B). Ovalbumin-immunized and -challenged mice treated with vehicle showed an increase in the eosinophil recruitment to the bronchoalveolar lavage fluid compared to the non-immunized vehicle-treated group. Capsaicin-treated and ovalbumin-immunized and -challenged mice exhibited a significant decrease in eosinophil recruitment to the bronchoalveolar lavage fluid when compared with the vehicle-treated and ovalbumin-immunized and -challenged mice. Eosinophil numbers decreased by approximately 59% from $1.47 \pm 0.27 \times 10^6$ cells/mL (vehicle) to $0.47 \pm 0.23 \times 10^6$ cells/mL (capsaicin) (mean ± S.E.M., $p<0.05$) (Fig. 1B).

Animals allocated to the second protocol received capsaicin for 2 days before the first ovalbumin-immunization. Vehicle-treated and ovalbumin-immunized and -challenged mice exhibited a significant increase in eosinophil recruitment to the bronchoalveolar lavage fluid when compared with the vehicle-treated and non-immunized group

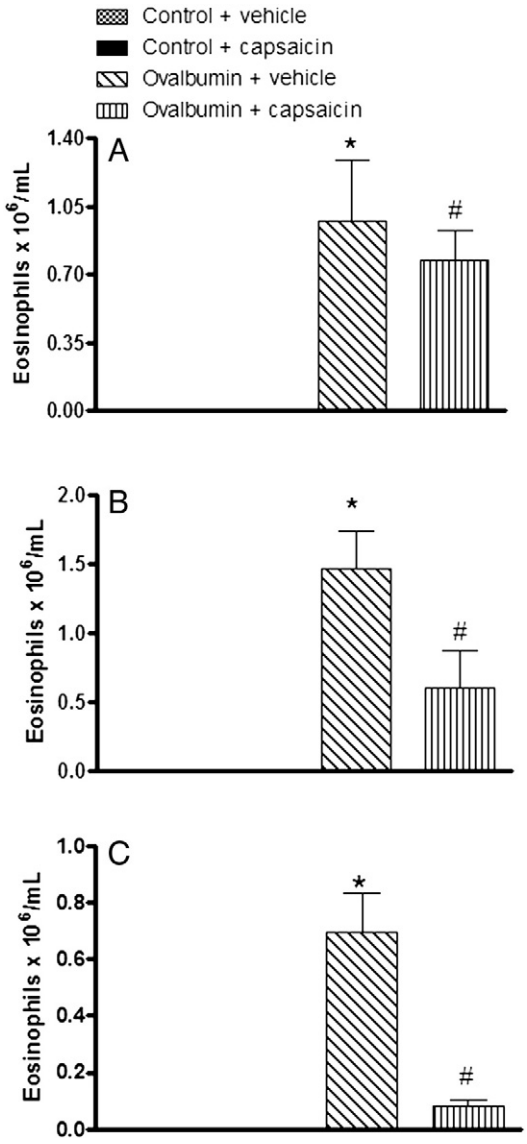

Fig. 1. Effect of capsaicin treatment on eosinophil recruitment to the bronchoalveolar lavage fluid in neonatal mice (A), adult mice after the first ovalbumin challenge (B) and adult mice before the first immunization (C) in an experimental allergic airway inflammation model. Control groups were treated with vehicle or capsaicin but were not ovalbumin-immunized. Samples were collected 24 h after the second ovalbumin challenge. Values are presented as the mean ± S.E.M. ($n=6$ per treatment). $^{*}P<0.05$ compared with control + vehicle group versus ovalbumin + vehicle group; $^{\#}P<0.05$ compared ovalbumin + vehicle group versus ovalbumin + capsaicin group.

(Fig. 1C). Similar to what was found for the first protocol, capsaicin-treated and ovalbumin-immunized and -challenged mice exhibited a significant decrease in eosinophil recruitment to the bronchoalveolar lavage fluid when compared with the vehicle-treated and ovalbumin-immunized and -challenged mice. Eosinophil numbers were decreased by approximately 88% from $0.7 \pm 0.14 \times 10^6$ cells/mL (vehicle) to $0.08 \pm 0.02 \times 10^6$ cells/mL (capsaicin) (mean ± S.E.M., $p<0.05$) (Fig. 1C).

3.3. IL-5, CCL11, IL-10 and IL-13 levels in the bronchoalveolar lavage fluid of adult animals treated with capsaicin before beginning the immunization process with ovalbumin

Next, we assessed the IL-5, CCL11, IL-10 and IL-13 levels in the bronchoalveolar lavage fluid of the adult animals allocated to the second treatment protocol with capsaicin because this protocol was more efficient in decreasing the eosinophil recruitment to the bronchoalveolar lavage fluid. No significant alteration was observed in IL-5, CCL11, IL-10 and IL-13 levels in the bronchoalveolar lavage fluid of control (non-immunized) animals treated with capsaicin compared to animals treated with vehicle (non-immunized) (Fig. 2). In agreement with the increase of eosinophil recruitment found in the bronchoalveolar lavage fluid, animals that were vehicle-treated and ovalbumin-immunized and -challenged showed a significant increase in the levels of IL-5, CCL11, IL-10 and IL-13 in the bronchoalveolar lavage fluid, when assessed 24 h after the second ovalbumin-challenge compared to vehicle-treated and non-immunized animals (Fig. 2A–D). Capsaicin-treated and ovalbumin-immunized and -challenged mice showed significantly decreased IL-5 and CCL11 levels when compared with the vehicle-treated and ovalbumin-immunized and -challenged mice. IL-5 levels were decreased by approximately 91% from 296.16 ± 47.52 pg/mL (vehicle) to 25.93 ± 12.02 pg/mL (capsaicin) (mean ± S.E.M., $p<0.05$) (Fig. 2A). CCL11 levels were decreased by approximately 52% from 20.29 ± 3.68 pg/mL (vehicle) to 8.02 ± 0.94 pg/mL (capsaicin) (mean ± S.E.M., $p<0.05$) (Fig. 2B). However, no alteration was observed in the IL-10 and IL-13 levels between these groups (Fig. 2C and D).

3.4. Effect of the capsaicin treatment in adult animals on NF-κB activation and P-selectin expression in the lung

We then assessed the effect of the depletion of neuropeptides in adult animals submitted to the second treatment protocol with capsaicin (before beginning the immunization process with ovalbumin) on the expression of P-selectin and the phosphorylation state of the p65 NF-κB subunit by means of an immunohistochemistry technique.

Constitutive P-selectin expression was observed in vehicle-treated mice or in capsaicin-treated and non-immunized mice (Fig. 3). In the vehicle-treated and ovalbumin-immunized and -challenged group, we observed an upregulation of P-selectin expression along the bronchial epithelium. Capsaicin-treated and ovalbumin-immunized and -challenged mice exhibited a decrease in P-selectin expression when compared with the vehicle-treated and ovalbumin-immunized and -challenged mice. P-selectin expression was decreased by approximately 37% from 53.20 ± 5.89 arbitrary units (vehicle) to 33.54 ± 2.56 arbitrary units (capsaicin) (mean ± S.E.M., $p<0.05$) (Fig. 3).

No alteration in the phosphorylation state of the p65 NF-κB subunit was found in the nucleus of the lung cells of vehicle-treated and non-immunized animals. A discrete staining was observed in capsaicin-treated and non-immunized animals. In the bronchial epithelium of vehicle-treated and ovalbumin-immunized and -challenged mice, we observed the activation of the p65 NF-κB subunit when compared to vehicle-treated and non-immunized animals. Capsaicin-treated and ovalbumin-immunized and -challenged mice exhibited a decrease in

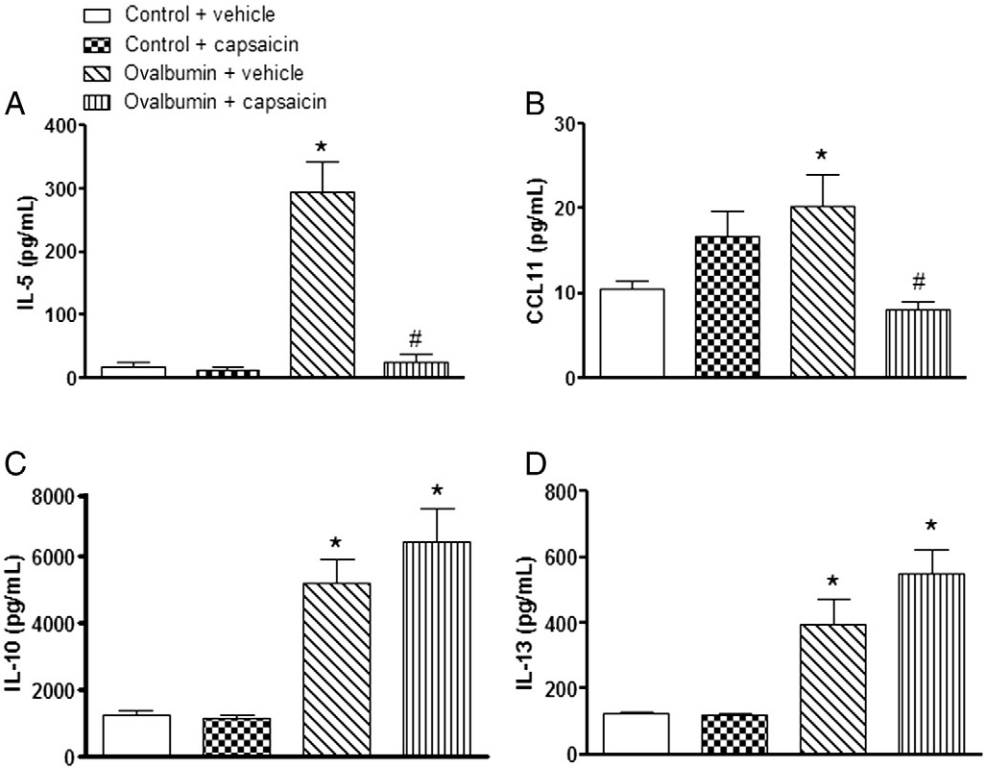

Fig. 2. Effect of the depletion of neuropeptides by capsaicin treatment on IL-5 (A), CCL11 (B), IL-10 (C) and IL-13 (D) levels in the bronchoalveolar lavage fluid of adult mice before the first immunization with ovalbumin. Control groups were treated with vehicle or capsaicin but were not ovalbumin-immunized. Samples were collected 24 h after the second ovalbumin challenge. Values are presented as the mean ± S.E.M. ($n = 6$ per treatment). $^*P<0.05$ compared with control + vehicle group versus ovalbumin + vehicle group; $^\#P<0.05$ compared ovalbumin + vehicle group versus ovalbumin + capsaicin group.

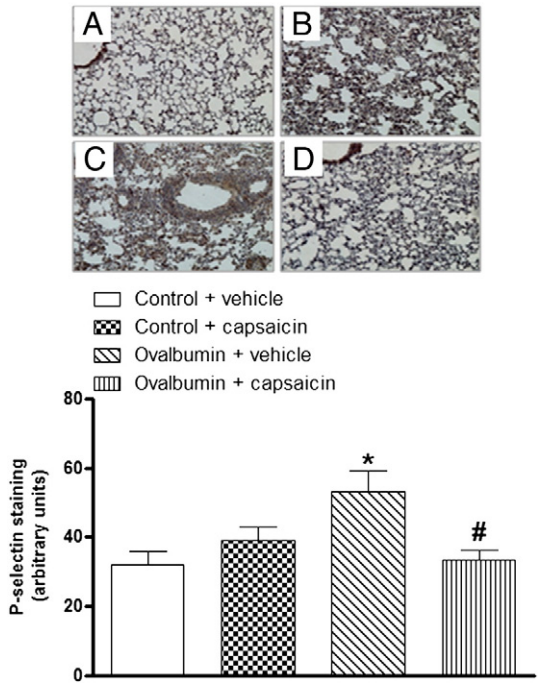

Fig. 3. Effect of the depletion of neuropeptides by capsaicin treatment in adult mice on P-selectin expression before the first immunization with ovalbumin. Representative images of P-selectin immunohistochemistry staining of the control + vehicle (A), control + capsaicin (B), ovalbumin + vehicle (C) and ovalbumin + capsaicin (D) groups (magnification: ×200). The mean intensity of P-selectin staining was determined from image analysis and is represented as arbitrary units (E). Control groups were treated with vehicle or capsaicin but were not ovalbumin-immunized. Values are the mean ± S.E.M. ($n = 3$ per group) for immunohistochemistry analysis. $^{*}P<0.05$ compared with control + vehicle group versus ovalbumin + vehicle group; $^{\#}P<0.05$ compared ovalbumin + vehicle group versus ovalbumin + capsaicin group.

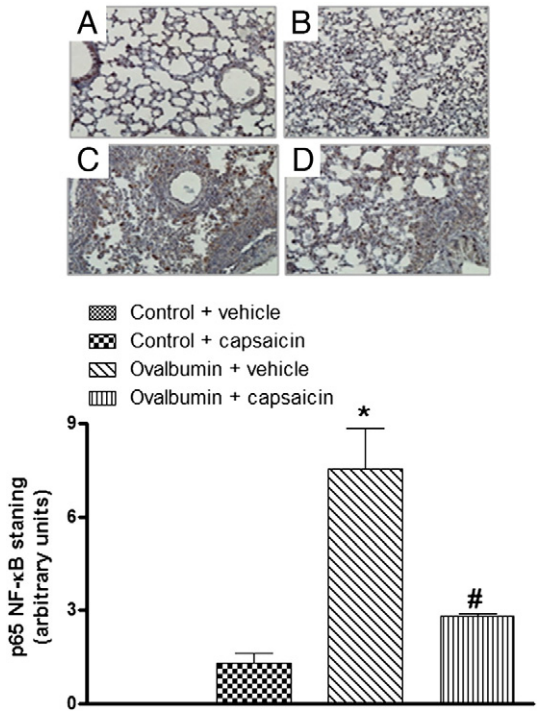

Fig. 4. Effect of the depletion of neuropeptides by capsaicin treatment in adult mice on the activation of p65 NF-κB before the first immunization. Representative images of phospho-p65 NF-κB immunohistochemistry staining of the control + vehicle (A), control + capsaicin (B), ovalbumin + vehicle (C) and ovalbumin + capsaicin (D) groups (magnification: x 200). The mean intensity of p65 NF-κB was determined from image analysis and is represented as arbitrary units (E). Control groups were treated with vehicle or capsaicin but were not ovalbumin-immunized. Values are the mean ± S.E.M. ($n = 3$ per group) for immunohistochemistry analysis. $^{*}P<0.05$ compared with control + vehicle group versus ovalbumin + vehicle group; $^{\#}P<0.05$ compared ovalbumin + vehicle group versus ovalbumin + capsaicin group.

p65 NF-κB expression when compared with the vehicle-treated and ovalbumin-immunized and -challenged mice. The p65 NF-κB expression was decreased by approximately 62% from 7.53 ± 1.30 arbitrary units (vehicle) to 2.81 ± 0.05 arbitrary units (capsaicin) (mean ± S.E.M., $p<0.05$) (Fig. 4).

3.5. Effect of the TRPV1 receptor antagonist SB366791 on the allergic airways inflammatory model

In view of the protective actions of depletion of neuropeptides by capsaicin in C-fibers in the airways, we next determined the effect of TRPV1 receptor (capsaicin receptor) blockade on allergic airway inflammation. Ovalbumin-immunized and -challenged mice that were treated with vehicle or untreated showed increased eosinophil numbers in the bronchoalveolar lavage fluid when compared with the control group. No significant alteration in eosinophil recruitment to bronchoalveolar lavage fluid was observed in the ovalbumin-immunized and -challenged mice that were treated with SB366791 by intraperitoneal (500 μg/kg; data not shown) or intranasal (0.1, 1 or 10 nmol/site) route when compared to ovalbumin-immunized and -challenged mice treated with vehicle (Fig. 5). In addition, the treatment with SB 366791 (10 nmol/site, intranasal route) did not reduce IL-5 and CCL11 levels in the bronchoalveolar lavage fluid and did not alter the P-selectin expression and the p65 NF-κB activation in the lung (data not shown).

4. Discussion

Here, we have provided evidence of the anti-inflammatory actions induced by the depletion of neuropeptides in adult animals, but not the degeneration of C-fibers, in neonate mice in allergic airways responses. When given before ovalbumin-immunization and -challenge, capsaicin blocked the development of allergic airway responses. The responsiveness to capsaicin in desensitized adult

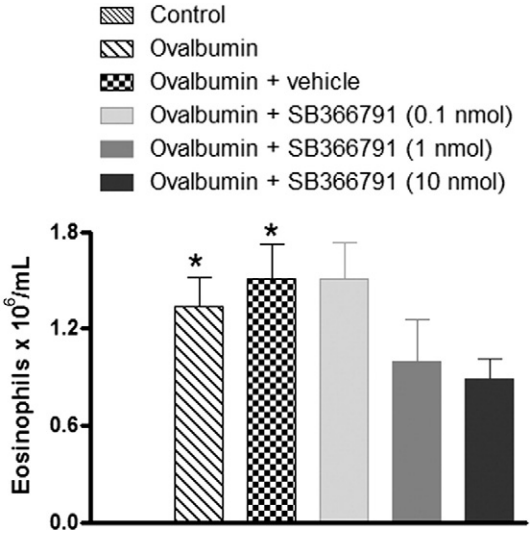

Fig. 5. Effect of intranasal treatment with SB366791 (0.1, 1 or 10 nmol/site) on the recruitment of eosinophils to the bronchoalveolar lavage fluid of ovalbumin-immunized and -challenged mice. An ovalbumin-immunized and -challenged mice group was treated with vehicle (DMSO 0.2%). Control groups were treated with vehicle but were not ovalbumin-immunized. Samples were collected 24 h after the second ovalbumin-challenge. Values are presented as the mean ± S.E.M. ($n = 12$ per treatment). $^{*}P<0.05$ compared with control group.

animals regulated the eosinophilia with marked decreases in the levels of IL-5 (an important Th2 cytokine that serves as a chemoattractant and activator of eosinophils) and CCL11 (a selective chemokine of eosinophils). In addition, P-selectin (an adhesion molecules involved in leukocyte control including eosinophils) expression in the lung was also decreased; these changes were associated with the inhibition of NF-κB pathways. TRPV1 appears not to be required for allergic airway inflammation, suggesting that other receptors expressed in C-fibers, such as the TRPA1, might be involved in asthma development.

Research regarding the role of C-fibers and the TRPV1 receptor in pathological conditions of the airways has had recent developments (Caceres et al., 2009; Jia and Lee, 2007; Watanabe et al., 2008). When airway C-fibers are activated by several stimuli (e.g., capsaicin, bradykinin, tobacco smoke, allergens, ozone and inflammatory mediators), these fibers release numerous mediators, including neuropeptides such as substance P, neurokinin A and CGRP (Barnes, 2001), which in turn cause inflammatory effects in the airways. Thus, these neuropeptides play an important role in human respiratory diseases, such as asthma, because they exert critical effects on airway smooth muscle, secretory glands and the airway epithelium. Moreover, they also act on inflammatory and immune cells by inducing the release of several lipids (e.g., prostaglandins) in addition to the pro-inflammatory cytokines (Rogers, 1995; Reynolds et al., 1997) and by inducing chemotaxis (Dunzendorfer and Wiedermann, 2000).

Eosinophilia is a hallmark of parasitic and allergic diseases. Eosinophils are important sources of cytokines, chemokines, lipid mediators (Rothenberg and Hogan, 2006) and cationic proteins, which are involved in the pathophysiology of asthma (Kubo et al., 1999). In addition, eosinophils release nerve growth factor (NGF) and neuropeptides such as substance P (Garland et al., 1997) that are known to activate and sensitize the C-fibers. Therefore, there is a putative positive feedback loop between eosinophils and C-fibers in the development and maintenance of the pathophysiology of asthma. The present results show that the degeneration of primary afferent C-fibers by capsaicin treatment in neonate animals submitted to allergic airway inflammation did not cause a significant alteration to eosinophil recruitment to the bronchoalveolar lavage fluid compared to vehicle-treated mice submitted to allergic airway inflammation. Nonetheless, the temporary depletion of sensory neuropeptide stores caused by capsaicin administration in adult animals, either before the ovalbumin immunization or during the ovalbumin challenge phase, resulted in a marked decrease in eosinophil recruitment to the bronchoalveolar lavage fluid, which confirmed and extended a previous study (Alessandri et al., 2003). These results demonstrate the inflammatory role of C-fibers in primary systemic sensitization as well as in pulmonary antigenic challenge. The differences in the effect in the neonatal (degeneration of C-fibers) and adult mice (temporary depletion of C-fibers) might be related to the fact that in neonatal animals, a compensatory mechanism may induce a phenotypic alteration in sensory fibers specifically neuropeptides-free fibers, as primary afferent neurons of nodose ganglion, which might contribute to neuropeptides production (Fischer et al., 1996). In contrast, the responsiveness to capsaicin in desensitized adult animals (in our protocol) is markedly reduced for at least 5 weeks (Morris et al., 2003; Symanowicz et al., 2004), and the short time between the temporary depletion, the inflammation and the end of experiment (3 weeks) is not sufficient to permit the C-fibers to have normal neuropeptide stores.

Next, we showed, for the first time, that the depletion of neuropeptide stores by capsaicin in adult mice reduced the concentration of IL-5 and CCL11 in the bronchoalveolar lavage fluid. IL-5 is essential for eosinophil migration from the bone marrow to the blood (Faccioli et al., 1996; Rogerio et al., 2003) and specifically supports terminal differentiation and proliferation of eosinophil precursors as well as activating mature eosinophils (Clutterbuck and Sanderson, 1988; Coeffier et al., 1991; Sanderson et al., 1985; Yamaguchi et al., 1988). Early studies have shown the important role played by CCL11 in eosinophil recruitment to tissues during certain physiological and inflammatory conditions (Shinkai et al., 1999; Zimmermann et al., 2000). In fact, CCL11 cooperates with IL-5 in the induction of tissue eosinophilia (Zimmermann et al., 2003). These mediators have a central role in the eosinophil biology of allergic diseases, and their consequent reduction is associated with the control of allergic inflammation, as was observed in the adult animals that were treated with capsaicin and ovalbumin-immunized and -challenged.

Previous studies demonstrated that IL-10 exhibits key regulatory effects on immune activation, including Th2 cell, mast cell, and eosinophil activation. IL-10 also serves as a key effector of regulatory T cell activity (Lloyd and Hawrylowicz, 2009; Royer et al., 2001). IL-13 is also involved in the pathophysiology of asthma: regulation of IgE synthesis, mucus hypersecretion, subepithelial fibrosis and eosinophil infiltration. However, in our results, no differences were observed concerning the concentrations of IL-10 and IL-13 in the capsaicin-treated adult animals and ovalbumin-immunized and -challenged compared to the vehicle-treated animals and ovalbumin-immunized and -challenged, which suggests that the anti-eosinophilic effect of the depletion of neuropeptide stores by capsaicin in adult mice is IL-10 and IL-13 independent.

As there is a great amount of evidence indicating that adhesion molecules are critically involved in leukocyte control, we next evaluated the expression of P-selectin in the lungs, which is also considered to be an important target for modulating eosinophilic influx to the inflammatory tissue (Wardlaw, 2001). Our findings revealed that the depletion of neuropeptides by capsaicin in adult animals consistently decreased the expression of P-selectin in the bronchial epithelium of ovalbumin-immunized and -challenged mice. Therefore, the reported inhibition of P-selectin expression is expected to contribute to anti-inflammatory actions.

Evidence suggests that in certain allergic diseases, the expression of some relevant genes encoding chemokines (e.g., CCL11), cytokines (e.g., IL-5) and adhesion molecules (e.g., P-selectin) is critically regulated by NF-κB (Anrather et al., 1997; Rothenberg et al., 1995; Yang et al., 1998). Therefore, the inhibition of NF-κB activation in the lungs observed following neuropeptide store depletion in capsaicin-treated adult animals in this study might be directly related to the reduction of CCL11 and IL-5 levels in the bronchoalveolar lavage fluid and P-selectin expression in the lungs as well as the consequent reduction in eosinophil recruitment to the bronchoalveolar lavage fluid.

Accumulated evidence shows that in the airways, the TRPV1 receptor (which is activated by capsaicin) is present in sensory nerves, smooth muscle cells, epithelial cells, vascular endothelial cells, submucosal glands and in inflammatory cells (Agopyan et al., 2004; Jia and Lee, 2007; Ni et al., 2006; Reilly et al., 2003; Seki et al., 2006; Watanabe et al., 2005). Animals treated intratracheally with capsaicin increased the eosinophil, mainly neutrophil and mononuclear cell influx to the bronchoalveolar lavage fluid. In addition, the recruitment of these cells was prevented by pre-treatment with capsazepine, a TRPV1 receptor antagonist (Karmouty-Quintana et al., 2007). Concerning the sensorial innervations of the airways, van den Worm et al. (van den Worm et al., 2005) reported a decrease in the tracheal rings contraction isolated from ovalbumin-immunized guinea pigs with capsazepine. Moreover, capsazepine was able to prevent the activation of NF-κB in lipopolysaccharide (LPS)-stimulated macrophages (RAW 267.4) (Oh et al., 2001).

To evaluate the role of the TRPV1 receptor in eosinophilic inflammation, we administrated SB366791, a TRPV1 selective antagonist, by intraperitoneal and intranasal routes. Both routes of treatment with SB366791 did not significantly decrease the eosinophil recruitment in any other metric (IL-5 and CCL11 levels as well as P-selectin and NF-κB expression) (data not shown). This result might be explained by robust the Ca^{2+} influxes after nervous depolarization, triggering

neuropeptide release through other ionic channels present in the C-fibers innervating the airways. Further reinforcing this idea, a recent study showed that vagal sensory neurons innervating the mouse lungs express, in addition to TRPV1, the TRPA1 channels (Nassenstein et al., 2008). Moreover, Caceres et al. (2009) have shown that the TRPA1 receptor blockade improves the phenotypes of allergic airway inflammation induced by ovalbumin, whereas TRPV1 was not required.

In conclusion, the results presented here reveal that the depletion of neuropeptides by capsaicin in adult animals, but not the degeneration of C-fibers in neonate mice, reduced some of the most important phenotypes of allergic airway inflammation, namely eosinophil recruitment, IL-5 and CCL11 levels in the bronchoalveolar lavage fluid and P-selectin expression in the lung, most probably through the modulation of transcription factor NF-κB. Together, such results largely extend previous findings and confirm the anti-inflammatory activity of the depletion of neuropeptides by capsaicin in C-fibers, and they suggest that TRPV1 might not contribute to the eosinophilic inflammatory process in the allergic airway inflammation.

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