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ABSTRACT

Asthma is an allergic lung disease can be modulated by drugs that modify the activity of central nervous system (CNS) such as amphetamine (AMPH). AMPH is a highly abused drug that exerts potent effects on behavior and immunity. In this study we investigated the mechanism involved in the effects of long-term AMPH treatment on the increased magnitude of allergic lung response. We evaluated mast cells degranulation, cytokines release, airways responsiveness and, expression of adhesion molecules. Male Wistar rats were treated with AMPH or vehicle (PBS) for 21 days and sensitized with ovalbumin (OVA) one week after the first injection of vehicle or AMPH. Fourteen days after the sensitization, the rats were challenged with an OVA aerosol, and 24 h later their parameters were analyzed. In allergic rats, the treatment with AMPH exacerbated the lung cell recruitment due increased expression of ICAM-1, PECAM-1 and Mac-1 in granulocytes and macrophages recovered from bronchoalveolar lavage. Elevated levels of IL-4, but decreased levels of IL-10 were also found in samples of lung explants after AMPH treatment. Conversely, the ex-vivo tracheal hyper-responsiveness to methacholine (MCh) was reduced by AMPH treatment, whereas the force contraction of tracheal segments due to in vitro antigen challenge remained unaltered.

Our findings suggest that lung inflammation and airway hyper-responsiveness due to OVA challenge are under the distinct control of AMPH during long-term treatment. Our data strongly indicate that AMPH positively modulates allergic lung inflammation via the increase of ICAM-1, PECAM-1, Mac-1 and IL-4. AMPH also abrogates the release of the anti-inflammatory cytokine IL-10.

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1. Introduction

Several environmental chemicals and drugs that act on the CNS such as amphetamine (AMPH) are recognized as potential immunotoxicants. The clinical use of AMPH is presently restricted to the treatment of particular disorders such as attention deficit disorder, narcolepsy and obesity [1]. However, AMPH is a widely abused recreational drug [2,3] and, induces chemical dependence [4]. AMPH has potent effects on the CNS and can also induce behavioral and immune alterations [5]. Studies of our group and others have revealed that AMPH interferes with the hypothalamo-pituitary axis (HPA) by modulating the secretion of corticosterone, one of the mechanisms by which AMPH regulates allergic lung inflammation, including asthma [6–8].

Asthma is a chronic inflammatory disorder, characterized by, among other characteristics, pulmonary cellular infiltration and airway hyper-responsiveness. The incidence of asthma has increased greatly in the last few decades [9]. Varying degrees of mononuclear cell and eosinophil infiltration, mucus hypersecretion, epithelium desquamation, smooth muscle hyperplasia and airway remodeling have been reported in asthma patients [10].

In previous studies, we showed that the development of allergic lung inflammation can be suppressed by a single treatment with AMPH before OVA challenge. This effect is mediated by increased corticosterone production, which subsequently reduces cytokine release and the expression of adhesion molecules on the lung tissue [6,11].

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On the other hand, utilizing a different protocol, we found that repeated (21 days) AMPH treatment exacerbated the lung inflammatory response of allergic rats, an effect not detected 72 and 120 h after abrupt withdrawal [6].

Because AMPH is an abused drug that induces chemical dependence and in previous studies we found that repeated AMPH treatment exacerbates the allergic lung inflammation, in the present work we investigated the mechanism involved in the effects of long-term AMPH treatment on the increased magnitude of allergic lung response. Accordingly, we examined the modulatory role of AMPH on important parameters that regulate the allergic response, including mast cells degranulation, cytokines release, airways responsiveness and, expression of adhesion molecules. The data obtained reinforce the relevance of studies that associate abused drugs and airway diseases as a public health problem.

2. Materials and methods

2.1. Animals

Male Wistar rats (180–200 g) from our departmental facilities were used. The animals were maintained under controlled temperature (21–23 °C) and 12 h light/12 h dark cycle conditions with free access to food and water. They were housed in plastic cages and handled in accordance with the guidelines of the Bioethical Committee of Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo.

2.2. Amphetamine treatment

Amphetamine sulfate (Sigma Chemical Company®, USA) was administered long-term intraperitoneally (i.p.) at a dose of 1.0 mg/kg once a day for 21 consecutive days. The control groups were treated with PBS (AMPH vehicle). This dose of AMPH was chosen because it has been shown to induce anxiety-like symptoms [12]. In addition, the dosage used has been shown to decrease hamster resistance to *Mycobacterium bovis* infection [13] and the number of lung inflammatory cells and blood leukocytes in OVA-sensitized and challenged rats (OVA grade II, Sigma Company®, USA) [7].

2.3. Sensitization procedures

Rats were sensitized by subcutaneous injection of a suspension of 10 μg of ovalbumin (OVA) with 10 mg of aluminum hydroxide (day 0). One week later (day 7), the rats received 10 μg of OVA dissolved in saline by subcutaneous injection (booster). At day 14, the animals were subjected to a single 15-min exposure of aerosolized OVA (1% in phosphate-buffered saline, PBS) using an ultrasonic nebulizer device (Icel®, SP, Brazil) coupled to a plastic inhalation chamber (18.5 cm× 18.5 cm×13.5 cm).

2.4. Experimental design

The experiments were performed in accordance with good laboratory practice and quality assurance methods.

Adult (60 days old) male rats were randomly assigned to 3 groups. The rats of the first group (allergic) were treated with the AMPH vehicle (PBS) for 21 consecutive days. OVA sensitization was performed one week after the first injection of vehicle. Fourteen days after the sensitization, the rats were challenged with OVA by aerosol (see protocol above). The second group (allergic + AMPH) rats were treated with amphetamine for 21 consecutive days and sensitized with ovalbumin (OVA) one week after the first injection of AMPH. One week after the first injection, the rats were subjected to an OVA aerosol challenge. The third group rats (naïve, non-manipulated rats) were used to investigate basal parameters. These rats were euthanized by sectioning the
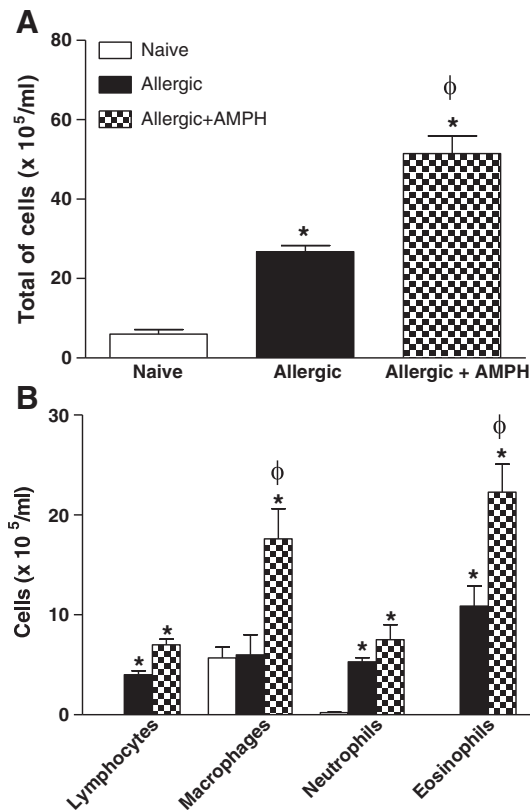
Fig. 1. Effects of long-term amphetamine treatment on allergic lung inflammation. Group of rats were treated with amphetamine (AMPH, 1 mg/kg, ip) or vehicle (PBS, ip), once a day, for 21 consecutive days, and sensitized with ovalbumin (OVA) one week after the first injection of vehicle or AMPH. Seven days after the sensitization, the rats were challenged with OVA by aerosol. Lung inflammation was assessed by the quantification of recruited cells present in the bronchoalveolar lavage (BAL) 24 hours after the OVA aerosol challenge. The control group consisted of non-manipulated rats (*naive group*). Panel A represents the number of total cells, whereas panel B represents the number of differential cells. Data are the mean± SEM from 5 animals. *$p<0.05$ relative to *naive* group and $^\phi p<0.05$ relative to allergic group (ANOVA followed by Student–Newman–Keuls test).

abdominal aorta under deep chloral hydrate anesthesia (>400 mg/kg, i.p.) 24 h after the OVA challenge.

2.5. Bronchoalveolar lavage (BAL)

BAL fluid was taken from sacrificed animals according to the procedure described by De Lima et al. [14]. The trachea was cannulated with a polyethylene tube (1-mm inner diameter) and the lungs were washed by flushing with PBS (20 ml). The recovered BAL fluid was centrifuged (170 ×g for 10 min at 20 °C), and the resulting cell pellet was then re-suspended in PBS (1 ml). Cell suspensions (90 μl) were stained with 10 μl of 0.2% crystal violet, and the total cell number was determined in Neubauer chambers. The differential cell counts were carried out with cytocentrifuge preparations (Cytospin®, Fanem, Brazil) stained with May–Grünwald–Giemsa solution.

2.6. Determination of cytokines in lung explants

Interleukin-10, 4 and tumor necrosis factor alpha (TNF-**α**) levels were determined for samples of supernatants of lung explants maintained in culture [15]. Briefly, the lungs of the studied groups of rats were flushed through the right heart with 5 ml of PBS to remove the intravascular blood. The remaining parenchyma was
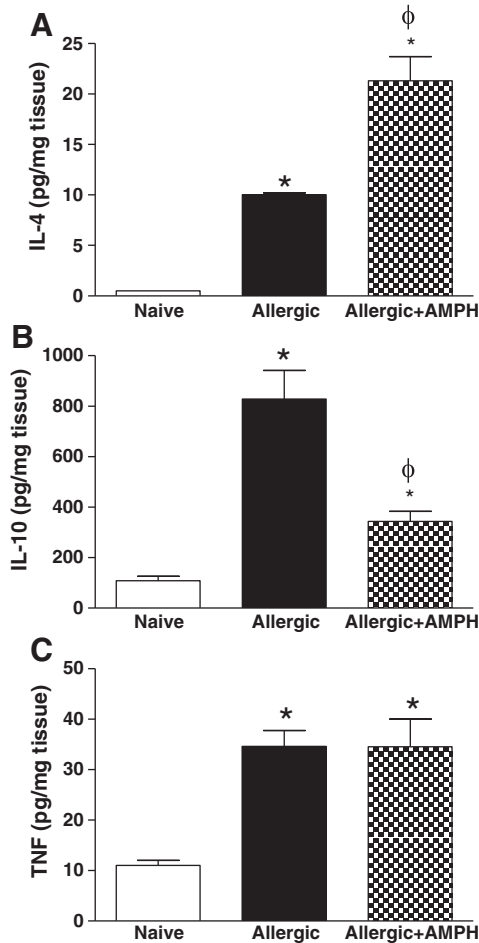
Fig. 2. Cytokines IL-4 (A), IL-10 (B) and TNF-α (C) levels released by lungs in explant culture after long-term AMPH treatment. One group of rats were OVA-sensitized and then OVA-challenged 14 days after the start of the experiment (Allergic group). In a parallel set of experiments, a group of rats were long-term treated with AMPH (1 mg/kg) one week before OVA-sensitization, once a day, for 21 days (Allergic + AMPH group). The control group consisted of non-manipulated rats (*Naïve*). Twenty-four hours after OVA-challenge, the cytokines were quantified by ELISA. Data are the mean ± SEM from 5 animals. *$p < 0.05$ relative to *naive* group and $^\phi p < 0.05$ relative to allergic group (ANOVA followed by Student–Newman–Keuls test).

chopped randomly into four small pieces per well and distributed into 24-well plates filled with 1 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.5% penicillin-streptomycin (10.000 UI − 10 mg/ml) and incubated for 24 h at 37 °C with 5% CO_2. The results were expressed as pg of cytokine produced per mg of dry-weight lung tissue. All determinations were made using standard curves according to the manufacturer's specifications.

2.7. Flow cytometry

BAL cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 2% FCS. After BAL fluid centrifugation, the cells were recovered after washing with Hank's balanced salt solution (HBSS). To quantify the expression of adhesion molecules, leukocytes (1×10^5) were incubated for 20 min in the dark at 4 °C with 100 μl of 1:100 monoclonal antibody dilution (Ly6G or F4/80 conjugated with FITC and Mac-1, ICAM-1 or PECAM-1 conjugated with PE). After that, the cells were analyzed in a FACS Calibur flow cytometer (Becton & Dickinson, San Jose, CA, USA). Data from 10,000 events were obtained and only the morphologically viable leukocytes were considered for analysis. The results are presented as arbitrary units of fluorescence and marked cells percentage.

2.8. Tracheal responsiveness to methacholine (MCh) and anaphylaxis in vitro

Tracheal rings were mounted for the measurement of isometric force quantification by means of two steel hooks in a 30-ml organ bath [16]. Force contraction was registered using a force displacement transducer and a chart recorder (Powerlab®, Labchart, AD Instruments). Briefly, the tracheal rings were suspended in an organ bath filled with KH at 37 °C. The tissues were continuously aerated (95% O_2 and 5% CO_2) and after the equilibrium period (40 min), the tracheal tension was adjusted to 1 g. Tissue viabilities were assessed by replacing KH solution with KCl buffer (60 mM) in the bath and comparing the contraction force generated with those obtained previously. Next, cumulative dose–response curves to methacholine (MCh) were constructed [17]. In a parallel set of experiments, we evaluated the effects of AMPH on mast cell degranulation. For this purpose, the Shultz–Dale reaction was performed. After the equilibrium period (40 min), an ovalbumin solution (4 mg/ml) was added to a 30-ml organ bath and the contraction force was measured.

2.9. Histological analysis

Rat lungs were excised before being fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 4 °C. Next, they were fragmented, washed, dehydrated in ethanol, clarified in xylene and embedded in Histosec™ (MERK, USA). Sections were cut (3 μm; HYRAX M60, Zeiss, GR), mounted on slides, and stained with 0.25% toluidine blue and 0.25% borate sodium solution. The quantification of mast cell number and activation in tissue samples was performed with a high-power objective (×40) by measuring the area of analysis with the *Axiovision* software (Zeiss, GR). Data were reported as cells/mm² (analyzing at least 10 distinct sections per rat).

2.10. Statistical analysis

Data are expressed as means ± SEM, and comparisons among the experimental groups were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons using GraphPad software V.2.01, GraphPad Instat™ (1990–1993). *p*-Values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of long-term treatment with amphetamine on allergic lung inflammation induced by OVA challenge

A significant increase in the number of leukocytes was observed in the BAL fluid of the allergic group when compared to the naïve group. However, the treatment with AMPH caused a significant increase in the number of leukocytes recovered in BAL compared to the allergic and naïve groups (Fig. 1, panel A). Differential cell analyses indicated that the OVA challenge increased the number of recruited lymphocytes, neutrophils and eosinophils in BAL fluid compared to the naïve group. The AMPH treatment in allergic rats (Allergic + AMPH group) increased the number of macrophages and eosinophils compared to the rats of the allergic group. Moreover, rats of the allergic + AMPH group presented an increase in the number of lymphocytes, macrophages, neutrophils and eosinophils when compared to the naive group (Panel B).

3.2. Quantification of IL-4, IL-10 and TNF-α in the lung tissue of allergic rats after chronic treatment with amphetamine

As seen in Fig. 2 (panel A), there were increased levels of IL-4 in the allergic and allergic + AMPH groups compared to the naive group. Moreover, the treatment with AMPH caused an additional increase in IL-4 levels in relation to untreated rats (allergic group). On the other hand, we observed, as shown in panel B, that treatment with AMPH in
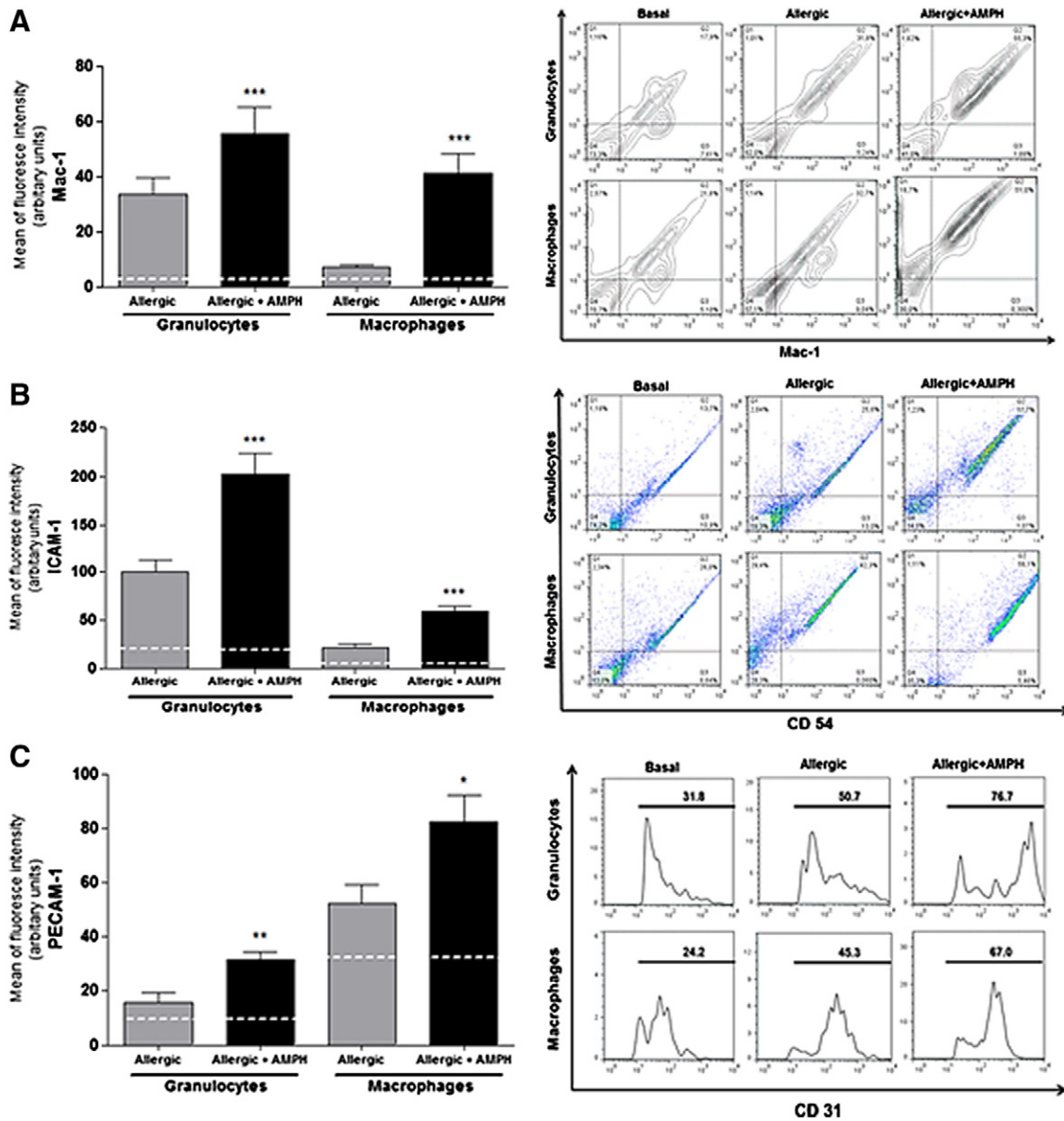
Fig. 3. Expression of MAC-1 (A), ICAM-1 (B) and PCAM-1 (C) in the leukocytes present in the BAL of allergic rats treated or not treated with amphetamine. The expression of adhesion molecules was determined by flow cytometry 24 hours after OVA challenge. Data are the mean ± SEM from 5 animals. ***$p < 0.001$; **$p < 0.01$ and *$p < 0.05$ relative to allergic group (ANOVA followed by Student–Newman–Keuls test).

allergic rats caused a decreased in the levels of IL-10 compared to allergic group. As seen in panel C, TNF-α concentrations measured in the supernatants of 24-h cultured lung tissue from the allergic group did not differ from those observed in the allergic + AMPH group.

3.3. Evaluation of Mac-1, ICAM-1 and PECAM-1 expression after chronic amphetamine treatment in allergic rats

As shown in Fig. 3 (panels A, B and C), AMPH treatment caused an increase in Mac-1 expression (CD11b/CD18), ICAM-1 and PECAM-1 in both cellular types (macrophages and granulocytes) compared to rats not treated with AMPH (Allergic group).

3.4. Effects of long-term treatment with amphetamine on tracheal reactivity to methacholine and anaphylaxis in vitro in allergic rats

As shown in Fig. 4 (panel A), the tracheal hyper-responsiveness to MCh observed in the allergic group was reduced by treatment with AMPH. Moreover, AMPH caused a reduction in tracheal reactivity to

MCh relative to the naive group. In panel B, we can observe that treatment with AMPH did not alter the tracheal reactivity induced by OVA challenge *in vitro*.

3.5. Effects of long-term amphetamine treatment on mast cells degranulation after OVA challenge

As shown in Fig. 5 (panels A, B and C), long-term AMPH treatment did not modify mast cells degranulation in allergic rats, as the number (Panel B) and the percentage of mast cells degranulated (Panel C) was similar between the allergic and allergic + AMPH groups. Moreover, we observed that allergic rats treated or not treated with AMPH presented an increase number in mast cells degranulation (Panel B) as well as of the percentage of degranulation (Panel C) when compared to the naive group.

4. Discussion

AMPH is an abused drug that exerts effects on the immunological systems [18,5] and, modulates the course of allergic lung response
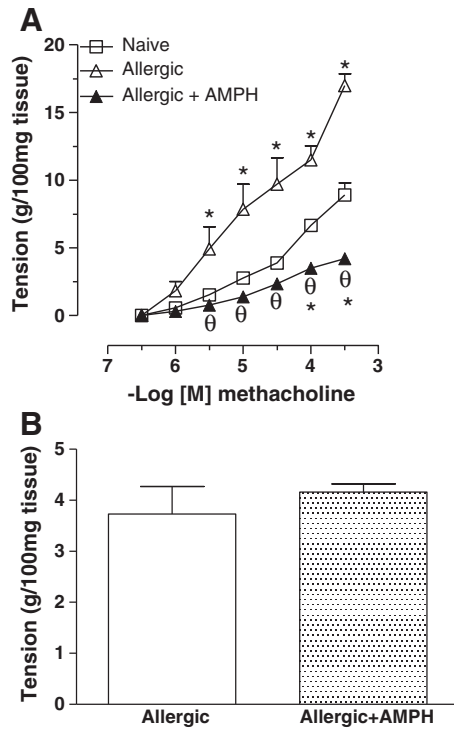
Fig. 4. Tracheal responsiveness to methacholine (MCh) (A) and antigen specific response (B) in allergic rats treated by long-term with amphetamine. Groups of rats were treated or not treated with AMPH during 21 days and sensitized with OVA one week after the first treatments (AMPH or vehicle). Seven days after, the rats were challenged with OVA by aerosol. The non-manipulated rats were used as controls (*naive group*). Tracheal rings were maintained in the organ bath system and the contractile responses to MCh were recorded. Anaphylactic reactions were determined by addition of OVA (4 mg/ml) in the organ bath system. Data are the mean ± SEM from 5 animals. *$p<0.05$ relative to *naïve* group and $p<0.05$ relative to allergic group (ANOVA followed by Student–Newman–Keuls test).

[11,6]. We demonstrated herein that long-term AMPH treatment potentiates the allergic lung inflammation by adhesion molecules expression and cytokines release mediated-mechanism. In parallel, reduces the tracheal hyper-responsiveness to cholinergic stimulus (methacholine).

We confirmed in the present study that treatment with AMPH for 21 days increased leukocyte infiltration into the lungs, as demonstrated by an elevated number of macrophages and eosinophils. In contrast to what we observed concerning the increase in allergic lung inflammation after long-term AMPH treatment, we have reported in other studies, using an experimental asthma model, that single AMPH treatment from 12 h before OVA challenge down-regulates several parameters of allergic lung inflammation, including cellular migration, vascular permeability and tracheal hyper-responsiveness to methacholine (MCh). Moreover, we also demonstrated that the reduced lung inflammation induced by acute AMPH treatment (before OVA challenge) might not be attributable to a direct effect of this drug, but due to a corticosterone action on bone marrow activity [7]. Interestingly, corticosterone serum levels measured in these AMPH repeated treated rats were statistically similar to those of their respective controls [6]. Thus, we can infer that after repeated AMPH treatment, the corticosterone levels return to basal levels suggesting an adaptive mechanism that can contribute to the development of the allergic lung response.

Cytokines, including IL-4 and IL-10 are critical in inflammatory disease, contributing to the recruitment and activation of inflammatory leukocytes. These cytokines are produced in abundance by activated CD4þ Th2 cells, mast cells, eosinophils and, basophils. Alternatively, activated alveolar macrophages also release substantial amounts of IL-4 [19,20]. IL-4 induces production of IgE antibodies and increases bronchial responsiveness, while IL-10 causes suppression of mast cell and eosinophil activity [21,22].

In the present study, we observed elevated levels of IL-4 and reduced levels of IL-10 after treatment with the AMPH, a phenomenon that coexisted with increased cells influx into the lung. On the other hand, TNF levels did not modify by AMPH treatment. Thus, we suggest that a high level of IL-4 associated with a low level of IL-10 might have contributed to cell mobilization into the lung in allergic rats after AMPH treatment. Overall, these data might indicate that AMPH alters the endogenous control of inflammatory response.

Leukocyte migration into inflamed tissues involves complex interactions of leukocytes with the endothelium through regulated expression of surface adhesion molecules. ICAM-1 and PECAM-1 are well established as molecules involved in neutrophil, macrophage and lymphocyte vascular transmigration [23,24]. The integrin αMβ2 (Mac-1) is expressed on the surface of many leukocytes involved in the innate immune system, including monocytes, granulocytes, macrophages, and natural killer cells [25]. We demonstrated that long-term AMPH treatment increases cellular expression of Mac-1, ICAM-1 and PECAM-1, a fact that could justify the augmented allergic inflammatory response after OVA challenge.

Cholinergic bronchial hyper-responsiveness can be observed in eosinophil-mediated lung inflammation models [26,27]. In this context, it was interesting to find that a significant lung inflammation elicited by long-term AMPH treatment coexisted with a reduction in tracheal hyper-responsiveness. In fact, other studies demonstrated that inflammatory events (pulmonary cell influx) can be dissociated from those governing the bronchial smooth muscle tonus (hypo-responsiveness) [28]. Moreover, similar to the result of a recent study of our group, we demonstrated that single AMPH treatment 12 h before an OVA challenge also reduced tracheal hyper-responsiveness to MCh [11]. AMPH exerts its effects on peripheral and central catecholamine neurotransmitter systems, which play a key role in the high abuse potential of this drug. The molecular products of nervous and immune system cells provide a means of communication between these two systems and constitute what is currently being investigated in the neuroimmunomodulation field [29,30]. Within this context, AMPH has been shown to modulate asthma through neuronal (adrenergic) and endocrine (corticosterone) mediation [6,7]. It is reasonable to admit that the tracheal hypo-responsiveness observed in our studies could be related to the release of catecholamines, which are known to exert relaxant effects on the airways smooth muscle by acting on the beta 2 adrenergic receptors [31].

As the long-term AMPH treatment began before the OVA sensitization, we investigated whether the effect induced by AMPH on lung inflammation could correlate to changes in the synthesis of anaphylactic immunoglobulin. Our results indicated that AMPH treatment did not alter tracheal responsiveness to the OVA challenge *in vitro*, indicating that AMPH did not interfere with antibody generation.

Mast cells exert a pivotal role on airway responsiveness caused by allergic disorders [32]. Moreover, recent studies of our group found that OVA-induced tracheal hypo-responsiveness might explain the regulation of AMPH on mast cell degranulation without modifications to anti-OVA IgE synthesis [11]. Our previous data suggest that the treatment with AMPH decreased the percentage of mast cells that presented degranulation, as indicated by lower activation and release of the cytoplasmic granules [11]. In contrast, in the current work, we demonstrated that allergic rats treated long-term or not with AMPH presented a similar percentage of mast cells degranulation. Thus, we can infer that the increase in lung inflammation and the decrease in tracheal reactivity observed after long-term AMPH treatment might not be attributed to changes in activation of mast cells. Indeed, the percentage of mast cells degranulated in both groups (allergic and allergic + AMPH) was similar.

In conclusion, our findings suggest that lung inflammation and upper airway reactivity due to OVA-challenge are under the distinct control of this long-term AMPH treatment. Thus, AMPH treatment increases allergic lung inflammation and reduces the tracheal responsiveness. Our
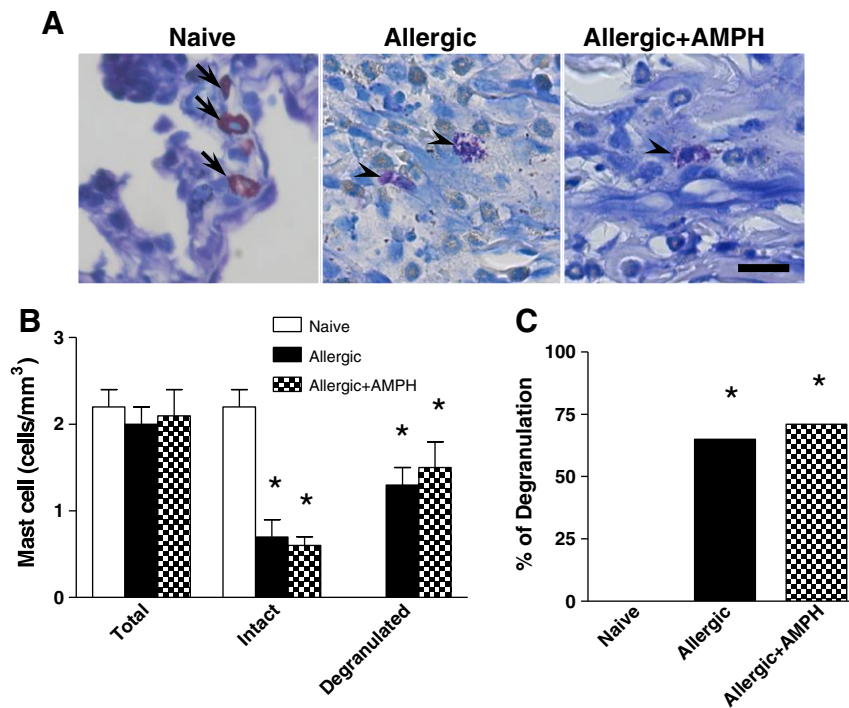
Fig. 5. Mast cell degranulation in lung tissue after long-term amphetamine treatment. Quantitative analysis of histological lung sections presenting intact (arrows) and degranulated mast cells (arrowheads). The *naïve* group presenting intact mast cells, while the allergic and allergic + AMPH groups present degranulated mast cells in the lung parenchyma as evidenced by metachromatic granules in the extracellular matrix (Panel A). In panel B, the number of mast cells and in panel C the percentage of degranulation are presented. Toluidine blue stain. Bar: 5 μm. Data are the mean ± SEM from 5 animals. $*p < 0.05$ relative to *naïve* group (ANOVA followed by Student–Newman–Keuls test).

data strongly indicate that the effect of AMPH on the lung inflammatory response involves an increase in ICAM-1, PECAM-1, Mac-1 and IL-4 and a decrease of IL-10. Therefore, individuals that abuse drugs, such as amphetamines, are more sensitive to the development of an allergic lung response, presenting a serious health public problem.

Acknowledgments

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