



Effects of MK-801 and amphetamine treatments on allergic lung inflammatory response in mice

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ABSTRACT

Glutamate acts as a neurotransmitter within the Central Nervous System (CNS) and modifies immune cell activity. In lymphocytes, NMDA glutamate receptors regulate intracellular calcium, the production of reactive oxygen species and cytokine synthesis. MK-801, a NMDA receptor open-channel blocker, inhibits calcium entry into mast cells, thereby preventing mast cell degranulation. Several lines of evidence have shown the involvement of NMDA glutamate receptors in amphetamine (AMPH)-induced effects. AMPH treatment has been reported to modify allergic lung inflammation. This study evaluated the effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg), given alone or in combination, on allergic lung inflammation in mice and the possible involvement of NMDA receptors in this process. In OVA-sensitized and challenged mice, AMPH and MK-801 given alone decreased cellular migration into the lung, reduced IL-13 and IL-10 levels in BAL supernatant, reduced ICAM-1 and L-selectin expression in granulocytes in the BAL and decreased mast cell degranulation. AMPH treatment also decreased IL-5 levels. When both drugs were administered, treatment with MK-801 reversed the decrease in the number of eosinophils and neutrophils induced by AMPH in the BAL of OVA-sensitized and challenged mice as well as the effects on the expression of L-selectin and ICAM-1 in granulocytes, the IL-10, IL-5 and IL-13 levels in BAL supernatants and increased mast cell degranulation. At the same time, treatment with MK-801, AMPH or with MK-801 + AMPH increased corticosterone serum levels in allergic mice. These results are discussed in light of possible indirect effects of AMPH and MK-801 via endocrine outflow from the CNS (i.e., HPA-axis activity) to the periphery and/or as a consequence of the direct action of these drugs on immune cell activity, with emphasis given to mast cell participation in the allergic lung response of mice.

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1. Introduction

Asthma is a multifactorial disease characterized by pulmonary cellular infiltration, plasma exudation and airway hyperresponsiveness, the latter of which is globally related to the toxic effects of mediators released in the lungs by alveolar macrophages, neutrophils, eosinophils and mast cells [1]. We and others have previously shown that drugs that modify central nervous system (CNS) activity, such as amphetamines (AMPH), methamphetamines (ecstasy) and cocaine, can modulate the burden and course of several immune diseases, including allergic lung inflammation [2–4].

This is mainly due to the fact that immune cells may express several different neurotransmitter receptors. Just to mention a few examples, leukocyte may express β 2-adrenoceptors [5], α 7 nicotinic receptors [6] and both ionotropic and metabotropic glutamate receptors [7]. Naïve T cells express the ionotropic NMDA glutamate receptor. Moreover, glutamate transporters have also been found in mast cells and antigen presenting cells (APCs), including dendritic cells and macrophages [7]. Although widely found in the CNS as the most abundant and most important excitatory neurotransmitter, glutamate is also found in the periphery. It has been recently shown that splenic dendritic cells secrete high amounts of glutamate after proper stimuli [8]. More interesting, this release implicates in tuning adaptive immune response. Besides DCs, thymic epithelial cells also secrete glutamate, whose role is important in thymocyte selection [9]. “Neuroactive” non-IgE-dependent ligands, such as nerve growth factor and neuropeptides, trigger the release of potent vasoactive and inflammatory mediators from rat

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mast cells within the CNS and in the periphery [10]. In lymphocytes, NMDA receptors have been reported to be involved in the regulation of intracellular calcium levels [5], reactive oxygen species levels [11] and modulating cytokine synthesis [12]. These observations reveal the “cross-talk” between immune and neural cells in the periphery and in the CNS by common mediators and represent the hallmark of the neuroimmunomodulation field.

Our group showed that acute and chronic AMPH treatments have opposite effects on the outcome of allergic lung inflammation in rats. Acute AMPH treatment appears to act directly on the CNS, activating the Hypothalamus Pituitary Adrenal (HPA) axis and the Sympathetic Nervous System (SNS), thus inducing the secretion of glucocorticoids and catecholamines, respectively, which suppress allergic lung inflammation [13,14]. In contrast, chronic AMPH treatment resulted in exacerbated allergic lung inflammation, an effect not observed 72 and 120 h after abrupt withdrawal from similar repeated AMPH treatment [4,14].

Several lines of evidence indicate the involvement of the NMDA subtype of glutamate receptors on AMPH-induced effects. The administration of NMDA antagonists prevents a variety of responses induced by AMPH, such as behavioral sensitization, neuronal degeneration and gene expression [15]. Furthermore, it was shown that: 1) Excessive activation of NMDA receptors may provoke acute edematous lung injury as seen in the “adult respiratory distress syndrome” [16]; 2) endogenous glutamate mediates newborn rat lung damage induced by hypoxia through NMDA receptors [17]; 3) dextromethorphan, a noncompetitive NMDA receptors antagonist reduced acute lung injury including edema, neutrophils infiltration, and hemorrhage scores in rat [18] and 4) ovalbumin-induced airway hyperreactivity in guinea pigs is influenced by NMDA receptors agonist and antagonist [19].

These data support the idea that the effects of AMPH on allergic lung inflammation might be modified by NMDA receptor antagonists acting on neuronal circuits and/or directly on immune cells. For this purpose, we investigated the putative role of previous treatment with MK-801 on the effects of AMPH on allergic lung response.

2. Material and methods

2.1. Animals

Male Balb/c mice (25–35 g, 8 weeks) from our departmental facilities were used. The animals were maintained under a controlled temperature (22–24 °C) and a 12-h light/dark cycle with free access to food and water. The animals were housed in plastic cages and were handled and used in accordance with the guidelines of the Bioethical Committee for the Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, Brazil (protocol No.1633/2009); these guidelines are similar to those of the National Institute of Health (NIH), USA.

2.2. Pharmacological treatments

Amphetamine sulfate (Sigma Chemical Company®, USA) was administered intraperitoneally (i.p.) at a dose of 2.0 mg/kg. This AMPH dose was chosen because it has been shown to abrogate allergic lung inflammation symptoms [13,14]. MK-801 (Research Biochemical International RBI®, USA) was also administered i.p. at a dose of 0.25 mg/kg. This dose has been reported to block NMDA glutamatergic receptors [20].

2.3. Induction of allergy

On experimental day 1 (ED₁), mice were sensitized by i.p. administration of a suspension containing 10 µg OVA (Egg Albumin Grade V, Sigma Chemical Company®, USA) and 10 mg of aluminum hydroxide. On ED₇, the animals were treated i.p. with the same solution. One

week later, the mice were exposed to an OVA aerosol challenge (2.5%, 20 min, twice a day) for two consecutive days (ED₁₄ and ED₁₅), using an ultrasonic nebulizer device (Inalamax NS model S3®, Brazil) coupled to a plastic inhalation chamber (18.5 cm × 18.5 cm × 13.5 cm).

2.4. Group formation

Three identical replications were performed. In each replication, 30 Balb/C mice were equally and randomly assigned to 1 of the following 5 groups: 1) Naïve group: non-manipulated mice that were used to determine basal parameters; 2) Allergic group: OVA-sensitized mice that received saline i.p. (0.1 mL/10 g) 1 h before the 1st and 2nd OVA challenges; 3) Allergic + AMPH group: animals that were sensitized with OVA and subjected to treatment with AMPH (2.0 mg/kg) 1 h before the 1st and 2nd OVA challenges; 4) Allergic + MK-801 group: mice were sensitized with OVA and subjected to treatment with MK-801 (0.25 mg/kg) 1 h before the 1st and 2nd OVA challenges and 5) Allergic + MK-801 + AMPH group: mice that were sensitized with OVA and subjected to treatment with MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) 1.5 h and 1 h before the 1st and 2nd OVA challenges, respectively. All mice were euthanized with ketamine and xylazine (100 and 10 mg/kg, i.p., respectively) 12 h after the 2nd OVA challenge. Peripheral blood, bone marrow and bronchoalveolar lavage fluid (BAL) were examined after the 1st replication; corticosterone plasma levels, interleukin levels and adhesion molecules expression in BAL were evaluated after the 2nd replication, and lung mast cell and tracheal reactivity analyses were performed after the 3rd replication.

2.5. Leukocyte recruitment in bronchoalveolar lavage fluid (BAL)

The lungs were lavaged with 1.5 mL of phosphate-buffered saline (PBS) solution through a cannula inserted into the trachea. The recovered BAL (approximately 1 mL) was centrifuged (250 ×g for 5 min at 20 °C), and the resulting cell pellet was resuspended in 1 mL of PBS. Cell suspensions (90 µL) were stained with 10 µL of 0.2% crystal violet, and the total cell number was determined in Neubauer chambers using a light microscope. Differential cell counts were performed with cytocentrifuge preparations (Cytospin, Fanem, São Paulo, Brazil) that were stained with Rosenfeld's dye using standard morphological criteria.

2.6. Quantification of blood leukocytes and bone marrow cells

Blood aliquots taken from the abdominal aorta of the mice of all groups were diluted (1:20) in Turk's fluid (3% acetic acid) for total white blood cell counting with an automatic cell counter (ABC Vet®, São Paulo, Brazil). The total number of bone marrow cells taken from the mice was quantified from femoral marrow lavage fluid. The recovered bone marrow lavage (5 mL) was centrifuged (250 ×g for 5 min at 20 °C). The supernatant was discarded, and the pellet was resuspended in 1 mL PBS, stained with crystal violet (0.2%) and quantified by optical microscopy as described above.

2.7. Analysis of adhesion molecules in the BAL

BAL cells were resuspended in Dulbecco-modified Eagle's medium (DMEM) containing 2% FCS. The cells were blocked with anti-CD16/anti-CD32 at a 1:100 dilution at 4 °C for 20 min to prevent nonspecific binding via the Fc receptor. After Fc blocking, the cells were stained with FITC (VCAM-1) and PE (ICAM-1 and L-selectin) for 30 min at 4 °C according to the manufacturer's specifications. The cells were then washed twice with cold HBSS, resuspended in paraformaldehyde (1%) and analyzed using a FACalibur cytometer (BD Bioscience, San Diego, CA). Data were analyzed using FlowJo software.

2.8. Quantification of interleukins in BAL supernatant

IL-5, IL-10 and IL-13 levels were determined in samples of BAL supernatants. The results were expressed in pg of interleukin produced per mL. An ELISA kit (R&D Systems, Minneapolis, USA) was used to measure IL-5, IL-10 and IL-13 in the BAL supernatant, and the assays were performed according to the manufacturer's instructions. The detection limit for IL-5 and IL-10 was 15.6 pg/mL, and that for IL-13 was 7.8 pg/mL.

2.9. Determination of mast cell degranulation

Lungs were excised and fixed in 2% paraformaldehyde and 2% glutaraldehyde in PBS, pH 7.4, for 24 h at 4 °C. The lungs were then fragmented, washed, dehydrated in ethanol, clarified in xylene and embedded in Histosec (Merck, USA). Sections were cut (3 µm; HYRAX M60, Zeiss, GR), mounted on slides and stained with a 0.25% toluidine blue and 0.25% borate sodium solution. Quantification of mast cell number and degranulation in the lung tissue was performed using a high-power objective (×40) through the measurement of the area of analysis with Axiovision software (Carl Zeiss Canada Ltd., Toronto, Canada). Data were reported as cells/mm², and at least 10 distinct sections per mouse were analyzed.

2.10. Determination of corticosterone plasmatic levels

An ELISA kit (Life Science, Plymouth Meeting, PA, USA) was used to measure corticosterone in the plasma. The assays were performed according to the manufacturer's instructions. The detection limit for corticosterone was 27.0 pg/mL.

2.11. Statistical analysis

GraphPad Prism 5.0 (GraphPad Software) was used for statistical analysis. Because all data obtained were parametric, they were analyzed by a one-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons. In all experiments, $P \leq 0.05$ was considered significant. The data are presented as the mean $\pm$ SD.

3. Results

3.1. MK-801 and AMPH effects on allergic lung inflammation

As depicted in Fig. 1, differences in the total number of leukocytes in the BAL taken from the different groups of mice were observed ($F = 25.67$; $df = 4/25$; $P < 0.05$). An increase ($p < 0.05$) in the total number of leukocytes was observed in the BAL of allergic mice compared to that of the naïve animals. Treatments with AMPH (allergic + AMPH group) or with MK-801 (allergic + MK-801 group) before OVA challenge caused a significant decrease ($p < 0.05$) in the total number of leukocytes recovered in the BAL compared to the allergic group and did not differ from naïve group (Fig. 1, Panel A). Further analysis showed that the association between MK-801 and AMPH (allergic + MK-801 + AMPH group) increased the total number of leukocytes ($p < 0.05$) when compared to values obtained from allergic animals treated with each drug alone and did not differ from allergic group.

OVA-sensitized and challenge animals is clearly able to induce lung allergic response, which was evaluated by cytospin. Differential cell count demonstrates increased number of eosinophils ($F = 64.29$; $df = 4/25$; $p < 0.05$; Fig. 1, Panel B) and neutrophils ($F = 49.02$; $df = 4/25$; $p < 0.05$; Fig. 1, Panel C) in the BAL. Interestingly, our analysis also showed that treatment with AMPH or MK-801 alone in allergic mice decreased ($p < 0.05$) eosinophils and neutrophils infiltration to the lungs when compared to allergic group. Fig. 1 (Panels B and C) also shows that the number of eosinophils and neutrophils of allergic + AMPH + MK-801 group was higher ($p < 0.05$) than those of the allergic + AMPH or allergic + MK-801 groups and did not differ from allergic group. Finally, the number of macrophages in the BAL did not differ among the different groups studied ($F = 0.94$; $df = 4/25$; $p > 0.05$; Fig. 1, Panel D).

3.2. MK-801 and AMPH effects on peripheral blood leukocytes and bone marrow cells of allergic mice

As depicted in Fig. 2 (Panel A), the total number of leukocytes in the peripheral blood were decreased ($F = 6.64$; $df = 4/25$; $p < 0.05$) in allergic mice in relation to naïve animals. The previous treatment with

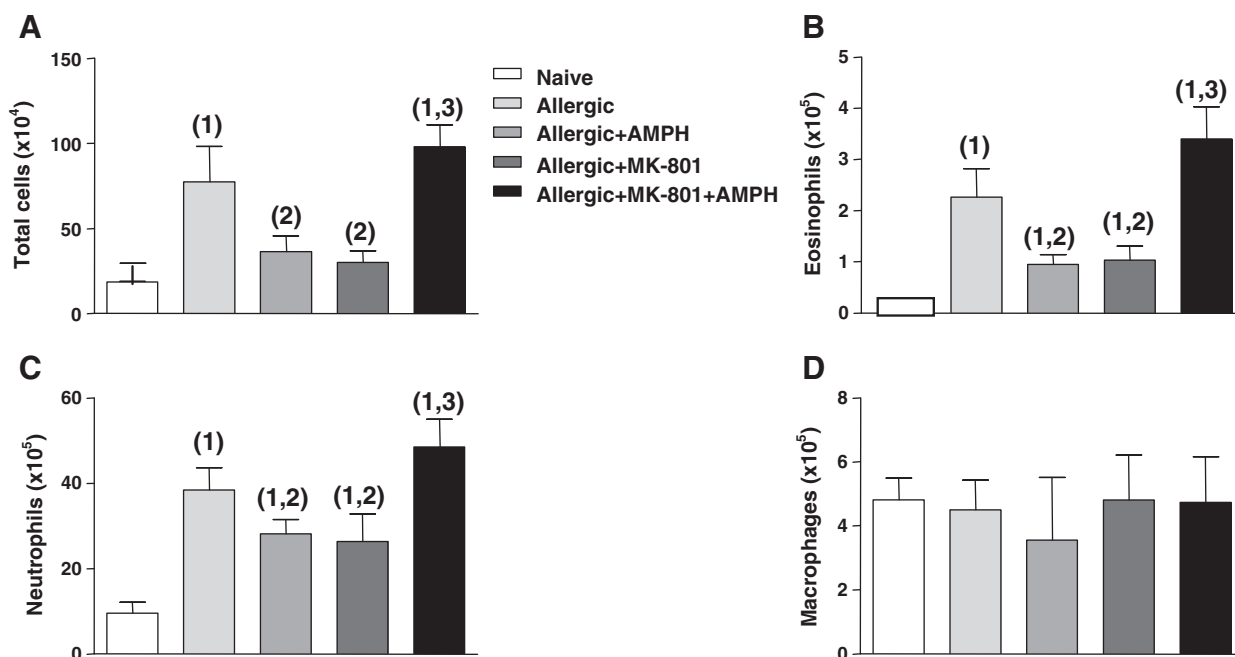


Fig. 1. Effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) given alone or in combination on the total BAL count (A) and the levels of eosinophils (B), neutrophils (C) and macrophages (D) in OVA-sensitized and challenged mice. ⁽¹⁾ $p < 0.05$ compared to the naïve group; ⁽²⁾ $p < 0.05$ compared to the allergic group; ⁽³⁾ $p < 0.05$ compared to the allergic + AMPH and allergic + MK-801 groups. Data are expressed as the mean $\pm$ SD ($n = 6$ mice/group). ANOVA followed by Student–Newman–Keuls test.

AMPH and MK-801 alone or in combination did not modify the reduced number of leukocytes present in the blood. However, as shown in Fig. 2 (Panel B), allergic lung inflammation mobilized leukocytes from the bone marrow. The total number of bone marrow cells measured in the allergic mice of all groups was smaller ($F = 14.27$; $df = 4/25$; $p < 0.05$) than that in the naïve animals. Moreover, the total number of bone marrow cells obtained from the allergic + AMPH group was significantly higher ($p < 0.05$) than those measured in the allergic + MK-801 + AMPH groups (Fig. 2, Panel B). MK-801 alone did not have any effects on bone marrow cells.

3.3. MK-801 and AMPH effects on IL-5, IL-13 and IL-10 levels in the BAL

As shown in Fig. 3, BAL obtained from all allergic groups show increased levels of IL-5 ($F = 27.62$; $df = 4/25$; $p < 0.05$; Panel A), IL-10 ($F = 4.23$; $df = 4/25$; $p < 0.05$; Panel B) and IL-13 ($F = 9.02$; $df = 4/25$; $p < 0.05$; Panel C) compared to naïve group. Fig. 3 also shows that AMPH treatment of allergic mice decreased ($p < 0.05$) IL-5, IL-13 and IL-10 levels. Furthermore, MK-801 itself had no effect on IL-5 and IL-10 levels but slightly decreased IL-13 levels in the BAL. Finally, the effects of AMPH on IL-13 and IL-10 levels were abrogated by prior MK-801 treatment.

3.4. MK-801 and AMPH effects on the expression of the adhesion molecules L-selectin, VCAM-1 and ICAM-1 in BAL granulocytes

As shown in Fig. 4, BAL granulocytes from allergic mice displayed increased expression of L-selectin ($F = 17.38$; $df = 4/25$; $p < 0.05$; Panels A and D), decreased expression of VCAM-1 ($F = 21.49$; $df = 4/25$; $p < 0.05$; Panels C and E) and no changes were found on ICAM-1 expression ($F = 15.63$; $df = 4/25$; $p > 0.05$; Panels B and E) in relation to the naïve group. AMPH and MK-801 treatments given alone reduced ($p < 0.05$) the L-selectin and ICAM-1 expression in relation to the allergic group. Furthermore, regarding the allergic animals, VCAM-1 expression was not changed by AMPH treatment, but it was increased ($p < 0.05$) by MK-801. It is noteworthy that allergic + MK-801 + AMPH group shows a reduced expression of L-selectin and ICAM-1 when compared to AMPH and MK-801 alone. The VCAM-1 expression was reduced ($p < 0.05$) in allergic + MK-801 + AMPH group when compared to the allergic + MK-801 group.

3.5. MK-801 and AMPH effects on mast cell degranulation

Fig. 5 depicts data regarding mast cell degranulation on lung tissue. Histological analysis of lung sections indicated that, compared to naïve animals (Panel A), the allergic mice presented a high percentage of mast cell degranulation (panel B), as depicted by the scattered metachromatic cytoplasmic granules in the lung parenchyma. In line with this finding, a small number of intact mast cells

were found in the allergic mice. The allergic mice also presented high leukocyte migration into the tissue (Fig. 5, Panel B).

As shown in Table 1 and Fig. 5, mice of the allergic + MK-801 and allergic + AMPH groups presented an increased number of intact mast cells ($F = 6.06$; $df = 4/25$; $p < 0.05$) and a decreased number of degranulated mast cells ($F = 11.23$; $df = 4/25$; $p < 0.05$) compared to the allergic group (Panels C and D). Table 1 also shows that the allergic + MK-801 + AMPH group presented an increased number ($p < 0.05$) of degranulated mast cells compared to the allergic + MK-801 group. In summary, AMPH treatment modifies the effects of MK-801 on mast cell degranulation (Fig. 5, Panel E).

3.6. MK-801 and AMPH effects on plasma corticosterone levels

Table 2 shows the corticosterone plasma levels taken from naïve and allergic mice treated with AMPH, MK-801 or MK-801 + AMPH. No differences were found in the corticosterone plasma levels between the naïve and allergic animals ($F = 22.50$; $df = 4/25$; $p > 0.05$). However, MK-801 and AMPH treatments induced an increase in the plasma levels of corticosterone in allergic mice ($p < 0.05$), and the corticosterone levels were higher ($p < 0.05$) in allergic + AMPH group than in allergic + MK-801 group. Additionally, the plasma corticosterone levels of the allergic + MK-801 + AMPH group were also higher ($p < 0.05$) in relation to the naïve and allergic groups.

4. Discussion

Recently, more and more data are being generated concerning the modulatory role of different neurotransmitters over the immune response. Besides, elegant experiments have shown that neurotransmitters may also be secreted by immune cells, such as T CD4 cells and T regulatory cells. Recent research has clearly demonstrated that glutamate may be produced by antigen presenting cells, such as splenic dendritic cells as well as thymic epithelial cells [8]. More important is the fact that in both cases, glutamate exerted important biological functions. In this context, our research aimed to study the role of the ionotropic NMDA glutamate receptor during the allergic lung inflammation in mice treated or not with amphetamine.

The present data obtained in mice are in agreement, extend and reinforce our previous results derived from rats [3,4,13,14] and other data from different laboratories [16,18]. They showed an increased migration of leukocytes to the lungs of allergic mice after an antigen challenge. Furthermore, we observed that, a single AMPH treatment performed before the antigen challenge in mice was also able to modulate cellular recruitment to the lungs. On the other hand, we also observed that previous treatment with MK-801 decreased cell recruitment into the lungs in a mouse model of allergic inflammation. Specifically, we now report that MK-801 treatment in OVA-sensitized and challenged mice decreased the total number of

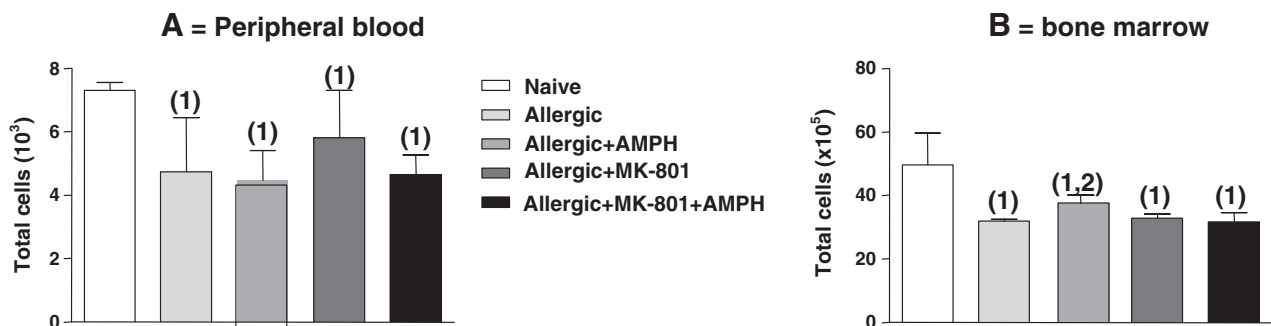


Fig. 2. Effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) given alone or in combination on the bone marrow (A) and white blood cell counts (WBC) (B) in OVA-sensitized mice. (1) $p < 0.05$ compared to the naïve group; (2) $p < 0.05$ compared to the allergic + MK-801 and allergic + MK-801 + AMPH groups. Data are expressed as the mean $\pm$ SD ($n = 6$ mice/group). ANOVA followed by Student–Newman–Keuls test.

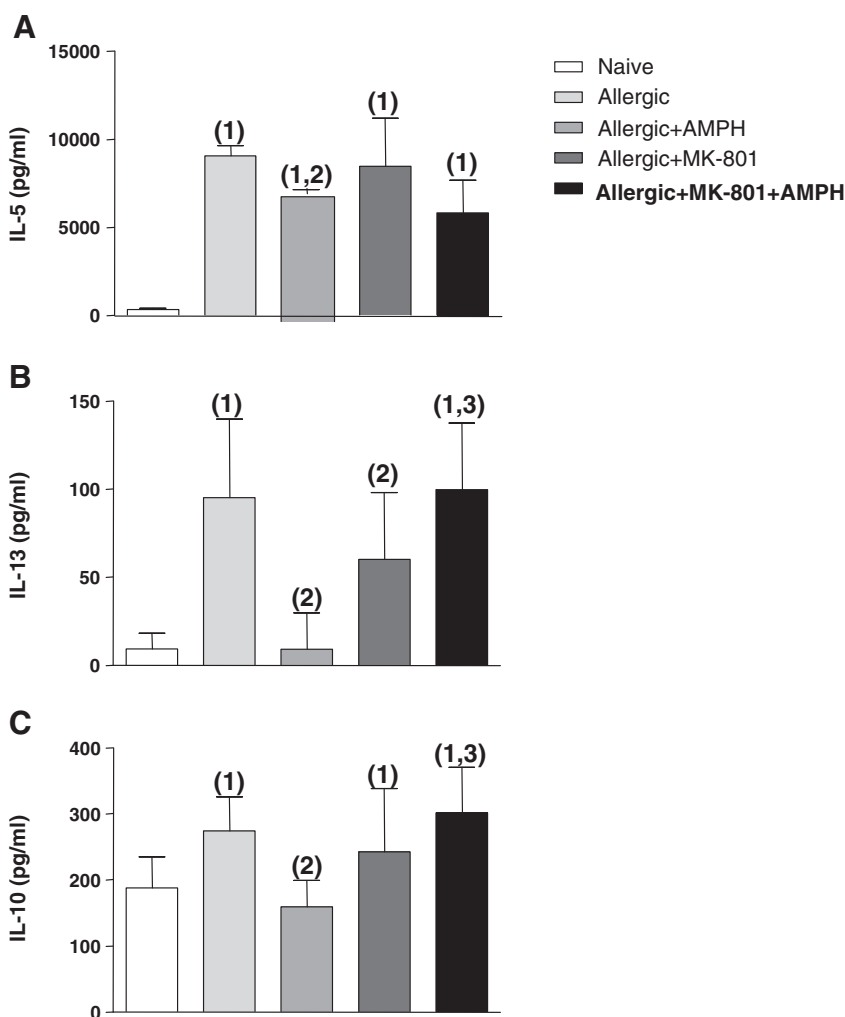


Fig. 3. Effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) given alone or in combination on IL-5 (A), IL-10 (B) and IL-13 (C) levels in the BAL supernatant. ⁽¹⁾ $p < 0.05$ compared to the naive group; ⁽²⁾ $p < 0.05$ compared to the allergic group ⁽³⁾ $p < 0.05$ compared to the allergic + MK-801 group. Data are expressed as the mean $\pm$ SD ($n = 6$ mice/group). ANOVA followed by Student–Newman–Keuls test.

eosinophils and neutrophils found in the BAL, increased the total number of bone marrow cells, increased plasma corticosterone levels, decreased IL-13 release from BAL supernatant, decreased L-selectin and ICAM-1 expression and decreased mast cell degranulation. Some of these MK-801 data obtained *in vivo* and in mice agree and reinforce others reported *in vitro* during lung excitotoxicity [16] and *in vivo* during a heat-induced acute lung inflammation [18] in rats. Furthermore, we observed that previous treatment with MK-801 abrogated the effects of AMPH on allergic lung inflammation.

AMPH and MK-801 given alone to allergic mice prior to the OVA challenge decreased immune cell migration into the lungs. These results clearly suggest an anti-inflammatory effect of both drugs. Indeed, one of the more important features of inflammation in the lung and other tissues is an increase in leukocyte migration. Additionally, leukocyte increase within a tissue, especially neutrophils, is commonly known to predict tissue damage. The following two lines of evidence might explain the observed reduction in leukocyte migration to the inflamed lungs after AMPH and MK-801 administration: indirect action via endocrine outflow from the CNS to the periphery, i.e. through hormones controlled by the hypothalamus or pituitary such as corticotrophin/glucocorticoids and sympathetic nervous system activation [21], and/or direct action on immune cells, particularly on mast cells. A relationship between the suppressive activity of AMPH and NMDA receptors both on central nervous system and on immune cells was already reported [22].

Using an experimental protocol similar to the one used in the current study, we have previously suggested that stress is a component of the lung anaphylactic response in rats sensitized to and challenged with OVA [23]. Thus, it seems reasonable to suggest that treatment with AMPH and MK-801 might also change HPA-axis responsiveness to the stress conditions imposed on mice by the OVA challenge. We found that both treatments increased corticosterone plasmatic levels in OVA-sensitized and challenged mice compared to allergic non-treated animals, a fact that, at least with regard to AMPH, agrees with our previous study and other studies [24]. There is also considerable evidence that glutamatergic systems are activated in response to stress stimuli [25], and intracerebroventricular injection of MK-801 has been shown to increase the plasma levels of adrenocorticotrophic hormone (ACTH) and corticosterone [26]. The observation that previous treatment with metyrapone, a drug that inhibits corticosterone synthesis abrogates AMPH-induced effects on cellular migration to the lung in allergic rats supports and strengthens our hypothesis. Corticosterone is known to exert potent anti-inflammatory and immunosuppressive effects [27], to modify bone marrow function [28], and to alter the cytokine profile and the expression of adhesion molecules [29]. The data now being reported on cytokine release, leukocyte migration and adhesion molecule expression after AMPH and MK-801 treatments seem to explain such an effect.

Cytokines and their activation of adhesion molecules contribute to cell migration and activation during allergic lung inflammation,

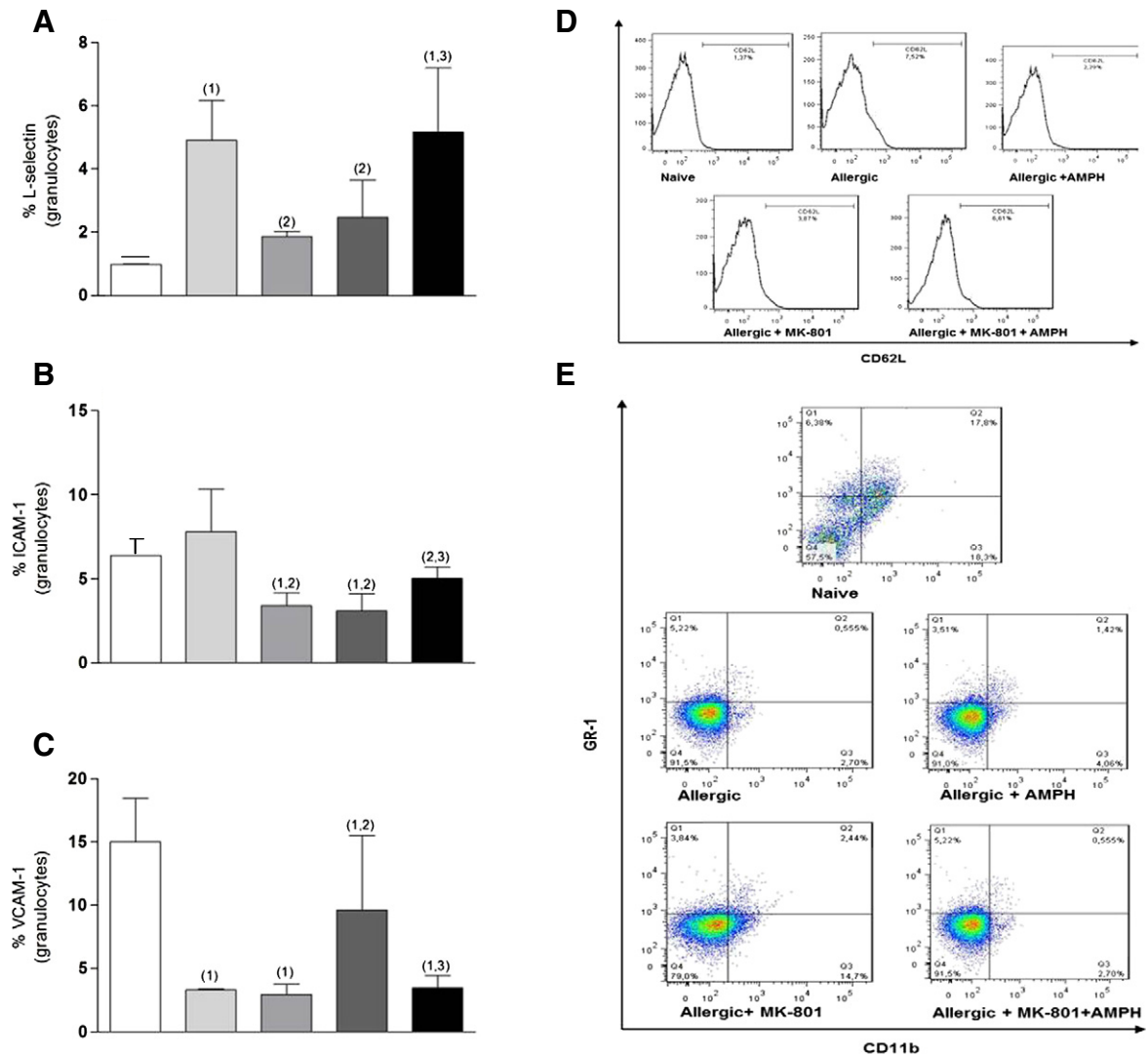


Fig. 4. Effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) given alone or in combination on the expression of the adhesion molecules L-selectin, ICAM-1 and VCAM-1 in granulocytes present in the BAL of OVA-sensitized and challenge mice. The expression of L-selectin (A), ICAM-1 (B) and VCAM-1 (C) was determined by flow cytometry in OVA-sensitized mice 12 h after an OVA challenge. Values are represented in histograms of L-selectin (D) and dot plots of ICAM-1 and VCAM-1 (E). ⁽¹⁾ $p < 0.05$ compared to the naïve group; ⁽²⁾ $p < 0.05$ compared to the allergic group ⁽³⁾ $p < 0.05$ compared to the allergic + MK-801 group. Data are expressed as the mean $\pm$ SD ($n = 6$ mice/group). ANOVA followed by Student–Newman–Keuls test.

which might explain the elevated levels of IL-5, IL-13 and IL-10 found in BAL of allergic mice. BAL supernatant of allergic mice treated with AMPH and MK-801 showed reduced levels of IL-13, a phenomenon that was concurrent with reduced pulmonary recruitment to the lung. Thus, it seems feasible to suggest that a low level of IL-13 after AMPH and MK-801 treatments is a result of reduced cell mobilization into the lungs after OVA challenge. Interestingly, and contrary to what was previously reported in rats [3], IL-10 levels in BAL remained unchanged in allergic mice after MK-801 treatment. IL-10, an anti-inflammatory cytokine, is suppressive to the immune system in allergic disease and suppresses mast cells and eosinophil activities [30]. IL-10 was also involved in the reduction of IgE synthesis [31], and the allergic responses performed herein depended on IgE and mast cell participation [32]. On the other hand, the levels of IL-5, a cytokine that favors eosinophil and mast cell recruitment to the lung [33], were significantly reduced only by AMPH treatment of allergic mice.

Leukocyte migration into inflamed tissues involves complex interactions between leukocytes and the endothelium through the regulated expression of surface adhesion molecules. The migration of mast cells is essential for their recruitment within target tissues, where they play an important role in immune responses. These processes rely on the

ability of mast cells to recognize appropriate chemotactic stimuli and react to them by producing chemoattractants, which results in the accumulation of hematopoietic cells at the sites of inflammation. In the present study, we showed that treatment with AMPH and MK-801 reduced the cellular expression of L-selectin and ICAM-1 in allergic mice, a fact that explains the reduced inflammatory response observed in allergic mice given each of these drugs alone.

ICAM-1 is well established as a molecule involved in neutrophil, macrophage and lymphocyte vascular transmigration [34]. Furthermore, ICAM-1 is a ligand for LFA-1 (integrin), a receptor found on leukocytes [35]. When activated, leukocytes bind to endothelial cells via ICAM-1/LFA-1 and then transmigrate into tissues [36]. In addition, L-selectin acts as a “homing receptor” for leukocytes to enter secondary lymphoid tissues via high endothelial venules [37]. With respect to allergic animals, the percentage of VCAM-1 expression by granulocytes remained unchanged after AMPH and AMPH + MK-801 treatments and was slightly but significantly increased by MK-801 treatment. VCAM-1 and eotaxin expression in the epithelia of the airways are induced by IL-1, favoring eosinophil and neutrophil recruitment to the lung [1].

However, our data showed that corticosterone serum levels were higher in allergic mice after MK-801 + AMPH treatments, and

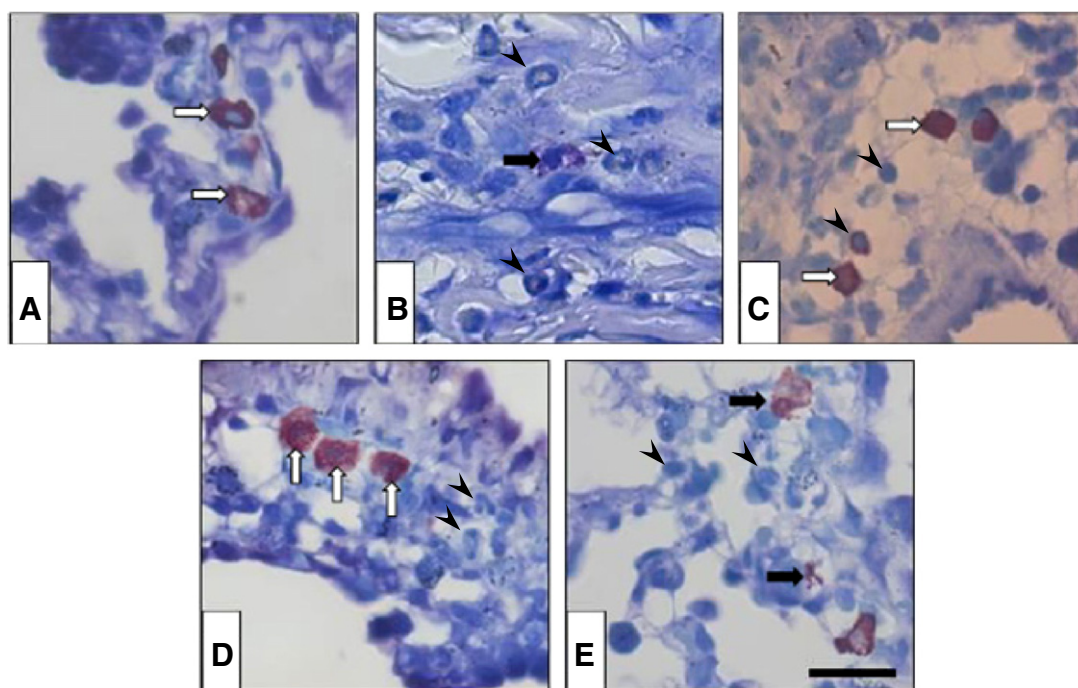


Fig. 5. Effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) given alone or in combination on intact (open arrow) and degranulated (closed arrow) mast cells. Arrowhead indicates infiltrated leucocytes. (A) Naïve group, (B) allergic group, (C) allergic + AMPH group, (D) allergic + MK-801 and (E) allergic + MK-801 + AMPH group. Toluidine blue stain. Bar: 10 μ m.

similarly treated mice showed no changes in cell migration to the lung, increased levels of IL-5, IL-13 and IL-10, no changes in L-selectin expression and reduced levels of ICAM-1 and VCAM-1 expression in comparison to allergic and non-treated animals. Thus, other mechanisms besides HPA axis activation and the subsequent increase in plasma corticosterone levels might also be activated as part of the AMPH and MK-801 effects on allergic lung inflammation.

Mast cells are pivotal to allergic responses. In the present set of experiments conducted in mice, mast cell degranulation was reduced by AMPH and MK-801 treatments but remained unchanged after the use of both drugs, i.e., one drug abrogated the effects of the other on mast cell degranulation. AMPH, through noradrenaline (NE) release, might reduce mast cell degranulation by acting on the β_2 receptor sites present on mast cell membranes, an effect reported both *in vivo* and *in vitro* studies for NE [38]. Furthermore, it was shown that β_2 receptor stimulation decreases cell migration to inflammatory sites [39], thus inducing anti-inflammatory effects. On the other hand, mast cells may be triggered to undergo exocytotic secretory processes by ligands other than IgE [33]. Neuroactive non-IgE-dependent ligands, including neuropeptides, trigger the release of potent vasoactive and inflammatory mediators from mast cells [10]. An influx of calcium accompanies NMDA receptor activation [7], and an increased intracellular calcium concentration initiates the exocytotic degranulation processes in mast cells.

MK-801 blocks calcium entry into the mast cells and thus inhibits mast cells degranulation [10], as observed in this experiment in allergic mice treated with MK-801. It seems feasible to suggest that mast cell degranulation is pivotal for MK-801 and AMPH effects as well as for the opposite effects they induced (MK-801 + AMPH group) on IL-13, L-selectin and ICAM-1 expression and cell migration into the lungs of allergic and challenged mice when administered together. According to Said et al., 1996, an over activation of NMDA glutamatergic receptor leads to influx of Ca^{2+} into the cells, which activates NOS in the presence of calmodulin. Excess of NO thus generated diffuses out of the cell, causing cytotoxicity. However, it is worth noting that NMDA receptors have been found on NE terminals [40] and that the activation of these receptors was found to modulate the release of NE *in vitro* [41]; and MK-801 was reported to be closely involved in the regulation of catecholaminergic functions [25]. The effects of MK-801 and AMPH on allergic lung allergic inflammation might also rely on NE release and effects. Reserpine treatment, a drug that inhibits vesicular amine uptake, antagonized the effects of AMPH on cell recruitment to the lung in OVA-sensitized and challenged rats [14]. Future experiments now being performed using pharmacological tools or RNA interference might confirm our suggestion.

In conclusion, our results point towards a “cross talk” between immune and neural systems and suggest the observed effects might

Table 1

Effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) given alone or in combination on mast cell morphology in the lungs of OVA-sensitized and challenged mice.

Groups	Mast cells/mm ²			
	Intact	Degranulated	% degranulation	Total
Naïve	2.2 $\pm$ 0.2	0.0 $\pm$ 0.0	0	2.2 $\pm$ 0.2
Allergic	0.7 $\pm$ 0.2 ⁽¹⁾	1.3 $\pm$ 0.2 ⁽¹⁾	65.0	2.0 $\pm$ 0.2
Allergic + AMPH	1.2 $\pm$ 0.2 ⁽¹⁾	0.8 $\pm$ 0.2 ^(1,2)	40.0	2.0 $\pm$ 0.2
Allergic + MK-801	1.5 $\pm$ 0.3 ⁽¹⁾	0.5 $\pm$ 0.1 ^(1,2)	25.0	2.0 $\pm$ 0.2
Allergic + MK801 + AMPH	1.2 $\pm$ 0.2 ⁽¹⁾	1.0 $\pm$ 0.1 ⁽¹⁾	45.5	2.2 $\pm$ 0.1

The presence of intact and degranulated mast cells, the relative percentage of degranulation and the total number of cells are shown. Data are expressed as the mean $\pm$ SD (n = 6 mice/group). ANOVA followed by Student–Newman–Keuls test.

⁽¹⁾ p < 0.05 in relation to the naïve group.

⁽²⁾ p < 0.05 in relation to the allergic group.

Table 2

Effects of MK-801 (0.25 mg/kg) and AMPH (2.0 mg/kg) given alone or in combination on the corticosterone plasmatic level in OVA-sensitized and challenged mice.

Corticosterone (pg/mL)	Groups				
	Naïve	Allergic	Allergic + AMPH	Allergic + MK-801	Allergic + MK-801 + AMPH
	92.1 ± 46.6	135.5 ± 68.0 ⁽¹⁾	336.3 ± 65.1 ^{(1),(2)}	221.9 ± 93.8 ^{(1),(2)}	315.7 ± 126.8 ^{(1),(2)}

ANOVA followed by Student–Newman–Keuls test.

⁽¹⁾ p < 0.05 compared to the naïve group.⁽²⁾ p < 0.05 compared to the allergic group. Data are expressed as the mean ± SD (n = 6 mice/group).

have been modulated by NMDA glutamate receptors. Moreover, the actions of AMPH and MK-801 seem to occur via an indirect effect produced by the drugs used on endocrine outflow from the CNS (i.e., HPA-axis activity) to the periphery and as a consequence of their direct action on immune cell activity, with emphasis being given to mast cell participation.

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