



Reduced allergic lung inflammation in rats following formaldehyde exposure: Long-term effects on multiple effector systems

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ABSTRACT

Clinical and experimental evidences show that formaldehyde (FA) exposure has an irritant effect on the upper airways. As being an indoor and outdoor pollutant, FA is known to be a causal factor of occupational asthma. This study aimed to investigate the repercussion of FA exposure on the course of a lung allergic process triggered by an antigen unrelated to FA. For this purpose, male Wistar rats were subjected to FA inhalation for 3 consecutive days (1%, 90-min daily), subsequently sensitized with ovalbumin (OVA)-alum via the intraperitoneal route, and 2 weeks later challenged with aerosolized OVA. The OVA challenge in rats after FA inhalation (FA/OVA group) evoked a low-intensity lung inflammation as indicated by the reduced enumerated number of inflammatory cells in bronchoalveolar lavage as compared to FA-untreated allergic rats (OVA/OVA group). Treatment with FA also reduced the number of bone marrow cells and blood leukocytes in sensitized animals challenged with OVA, which suggests that the effects of FA had not been only localized to the airways. As indicated by passive cutaneous anaphylactic reaction, FA treatment did not impair the anti-OVA IgE synthesis, but reduced the magnitude of OVA challenge-induced mast cell degranulation. Moreover, FA treatment was associated to a diminished lung expression of PECAM-1 (platelet-endothelial cell adhesion molecule 1) in lung endothelial cells after OVA challenge and an exacerbated release of nitrites by BAL-cultured cells. Keeping in mind that rats subjected solely to either FA or OVA challenge were able to significantly increase the cell influx into lung, our study shows that FA inhalation triggers long-lasting effects that affect multiple mediator systems associated to OVA-induced allergic lung such as the reduction of mast cells activation, PECAM-1 expression and exacerbation of NO generation, thereby contributing to the decrease of cell recruitment after the OVA challenge. In conclusion, repeated expositions to air-borne FA may impair the lung cell recruitment after an allergic stimulus, thereby leading to a non-responsive condition against inflammatory stimuli likely those where mast cells are involved.

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1. Introduction

Formaldehyde (FA) is a common indoor and outdoor pollutant, which is found in many products including particleboard, plywood, floor coverings and office furniture (Fló-Neyret et al., 2001; Krakowiak et al., 1998). FA is recognized as toxic at certain doses and the chances of harmful effects are increased at room temperatures due to its volatility (Songur et al., 2003). Employees in industrial and medical areas may be highly exposed to FA. Besides,

domestic exposure to FA is sufficient to provoke sensitization and an aggravation of symptoms in asthmatic patients. Accordingly, the respiratory tract is one of the main targets of FA and after its exposure, an immune reaction with inflammatory mediators release, including histamine, is observed (Salvaggio, 1982). Upon FA exposure, asthmatic patients develop bronchial hyperresponsiveness (Casset et al., 2005) and significantly increased anti-FA antibody titres (Baba et al., 2000). Holding the view that FA is a pro-inflammatory agent, experimental evidences revealed that FA inhalation causes an increase of the number of inflammatory cells in bronchoalveolar lavage of mice upon allergic lung inflammation (Fujimaky et al., 2004). FA inhalation for over 5 consecutive days exacerbates the anti-OVA titres in allergic guinea pigs (Riedel et al., 1996). Repeated exposure to FA led to a worsening of the allergic bronchoconstriction through an antigen sensitization-enhancing

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mechanism (Kita et al., 2003). In spite of these facts, no signs of lower airways inflammation could be demonstrated in FA-exposed guinea pigs after an allergic challenge (Riedel et al., 1996). Interestingly, FA exposure did not cause significant deleterious effects on the airway of patients with intermittent asthma; conversely, its exposure trends toward a protective effect (Ezratty et al., 2007). On the other hand, an exacerbated eosinophilic reaction was observed when allergen and FA exposures occurred simultaneously (Casset et al., 2006). Taken together, these facts indicate that the mechanisms and systems associated to magnitude of allergic lung inflammation, after FA exposure, and are yet to be clarified. In the context of FA effects, we reported that FA inhalation elicits an intense lung neutrophil recruitment and bronchial hyporesponsiveness to methacholine. Both phenomena appear to be mediated by mast cells (Lino dos Santos Franco et al., 2006). Leukocyte mobilization is the first line of the defense against noxious agents and endothelial cell barrier exerts a pivotal role on their migration into inflammatory site (Scalia and Lefer, 1998). A number of inflammatory mediators and adhesion molecules mediate cell traffic into inflammatory site upon allergic reaction, including nitric oxide (NO) and PECAM-1, respectively (Ricciardolo et al., 2006; Payne, 2003; Schleimer and Bochner, 1998). Accordingly, NO is a well-recognized modulator of leukocyte-endothelial cell interaction, controlling the leukocyte adhesion on endothelium during inflammatory process (Ricciardolo et al., 2006), whereas PECAM-1 is involved mainly with cell adhesion and transmigration (Scalia and Lefer, 1998). Moreover, their role on the effects of FA inhalation during an allergic lung inflammation is not yet understood. Taking into account the established knowledge about the major role played by mast cells in allergic events (Galli et al., 2005) and the high rate of indoor and outdoor FA exposure, we were concerned about the NO involvement and endothelial PECAM-1 expression in rodent model of allergic lung inflammation. As FA effects are investigated at the basis of its risk to aggravate a pre-existent airway allergic disorder (Fujimaky et al., 2004), but their influence to mediate the induction of allergic disorder is not yet investigated, we addressed this question by investigating the multiple mediators systems on the effects of FA inhalation and their repercussion to allergic lung inflammation. In this context, data generated could be of interest to understanding the mechanisms orchestrating the airways allergic inflammation.

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 conventional conditions, with free access to food and water. They were housed in plastic cages and manipulated in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the Institute of Biomedical Sciences, University of São Paulo.

2.2. Groups of study

Rats were randomly assigned to 4 groups: (1) FA, identified as rats subjected to short-term daily formaldehyde inhalations, for 3 days. (2) OVA/OVA, rats which were OVA-sensitized and challenged with OVA 2 weeks later. (3) FA/OVA, rats subjected to FA inhalation which were OVA-sensitized immediately after the last FA exposure and challenged with OVA 2 weeks later. (4) Naïve, non-manipulated rats, used to investigate the basal parameters. The rats were killed by sectioning the abdominal aorta under deep chloral hydrate anesthesia (>400 mg/kg, i.p.) 24 h after the last FA inhalation (FA group) or after the OVA challenge (OVA/OVA and FA/OVA groups).

2.3. Exposure of rats to formaldehyde (FA)

A standard glass chamber (20 l) coupled to an ultrasonic nebulizer device (Icel[®], Brazil) was used to generate a constant airstream from an aqueous solution of formalin diluted to 1% FA by wt (Lino dos Santos Franco et al., 2006). Rats (5/chamber) were subjected to daily 90-min sessions of FA inhalation for 3 consecutive days.

2.4. Rat sensitization to ovalbumin (OVA) and antigen challenge

Rats were sensitized by intraperitoneal (i.p.) injection of a suspension containing 10 µg of ovalbumin (OVA) and 10 mg of aluminum hydroxide. One week later the rats received a second sensitization dose of 10 µg OVA in phosphate-buffered solution (PBS) by the subcutaneous route (s.c.). Two weeks after the first sensitization, the rats were subjected to inhaled antigen challenge (aerosolized 1% OVA in PBS, 15 min) using an ultrasonic nebulizer device (Icel[®], SP, Brazil) coupled to a plastic inhalation chamber (18.5 cm × 18.5 cm × 13.5 cm).

2.5. Passive cutaneous anaphylaxis (PCA)

PCA reaction is a typical assay to measure indirectly anaphylactic antibodies levels (Mota and Wong, 1969; Shin et al., 2000; Hong et al., 2003). An IgE-dependent cutaneous reaction was generated by sensitizing the skin of non-manipulated rats with an intradermal injection (100 µl/site) of serially diluted (1:2 up to 1:256) sera from FA/OVA and OVA/OVA rats. Twenty-four hours after the injection, the recipient rats received by intravenous (i.v.) route a solution containing 500 µg OVA plus 2.5 mg Evans blue dye, dissolved in NaCl (0.9%). Thirty minutes later, the rats were killed by deep chloral hydrate anesthesia, the skin was removed and the diameter of the dye stain was measured at the inner surface of the skin. The PCA titres were represented by the highest dilution of the serum which resulted a dye stain of >5 mm in diameter.

2.6. Quantification of cells in bronchoalveolar lavage (BAL)

BAL fluids were taken from killed rats according to De Lima et al. (1992). Briefly, tracheae of rats were cannulated with a polyethylene tubing (1 mm inner diameter) and the lungs were flushed twice with PBS (20 ml total volume). The collected BAL was centrifuged (170 × g for 10 min at 20 °C), and the resulting cell pellet was then 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 microscopically using a Neubauer chamber. The differential cell counts were carried out by cytocentrifuge preparations (Cytospin, Fanem, Brazil) stained with May-Grünwald-Giemsa solution (Mordelet-Dambrine et al., 1984).

2.7. Blood leukocytes and bone marrow cell counts

Leukocytes in tail vein blood samples and bone marrow cells were quantified as described previously (Fialho de Araujo et al., 2003). Blood aliquots were diluted (1:20) in Türk's fluid (3% acetic acid) for total white cell countings in Neubauer chamber, whereas the differential (qualitative) leukocyte countings were performed in blood smears stained according to Mordelet-Dambrine et al. (1984). The recovered lavage of bone marrow (5 ml) was centrifuged (170 × g for 10 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.

2.8. Analysis of mast cells degranulation

Lung fragments were fixed in 4% paraformaldehyde containing 0.1 M Sörensen phosphate buffer (SPB) pH 7.4 at 4 °C for 2 h. The fragments then, were washed with SPB, dehydrated through a graded series of ethanol, and embedded in Paraplast[®] (Sigma, USA). Sections (3-µm thick) were stained with 1% toluidine blue in 1% borax solution. Quantifications of lung mast cells and leukocytes were carried out in 10 serial histological sections for every animal ($n = 3$) (30-µm space between two consecutive sections). Analyses were carried out by two unaware observers using a Zeiss Axioskop II mot plus a high-power objective (40×) and the Axiovision[®] software (Carl Zeiss, Germany).

2.9. Determination of nitrites in BAL cells

Cells recovered from BAL were suspended in RPMI-1640 culture medium enriched with 10% fetal bovine serum. Cell viability was determined by the Trypan blue exclusion test. Aliquots (500 µl) containing 2×10^6 cells/ml were harvested into 96-well plastic microplates (NUNC[®], Naperville, IL) under 5% CO₂ and 95% O₂ atmosphere at 37 °C. Aliquots of the supernatants were collected 24 h later and stored at –80 °C until the analyses. The quantification of nitrites was performed by adding 50 µl of the supernatant to 96-well plastic microplates containing equal volume of Griess reagent at room temperature for 10 min (Misko et al., 1993). The optical density (540 nm) was recorded using a microplate reader (Bio-Tek Instr., USA) and the nitrites levels were obtained using a standard curve of NaNO₂ (5–60 µM). Nitrites concentrations found in samples of BAL-cultured cells from naïve rats were used as controls.

2.10. Immunohistochemical analysis of lung tissue PECAM-1 expression

Lungs of killed rats were removed and immediately frozen in nitrogen-hexan solution and 8-µm thick cryosections were fixed in cold acetone for 10 min for direct immunohistochemistry assay. Sections were incubated with 3% H₂O₂ solution in order to block the endogenous peroxidase and biotin activities. This was followed by an overnight incubation (in a humidified box, 4 °C) with a biotinylated anti-rat

PECAM-1 monoclonal antibody. The sections were then incubated during 60 min with a streptavidin-peroxidase conjugate. Colour development included addition of 3,3'-diaminobenzidine (DAB) followed by lightly staining with haematoxylin and dehydration with ethanol and xylene. The DAB-stained areas in vessels walls were selected and the colour intensity was quantified using image analyser software (Image J). The background reaction was determined in lung sections incubated in the absence of antibody (negative control).

2.11. Pharmacological study

The involvement of nitric oxide (NO) on cellular recruitment to lung after OVA-challenge was investigated in rats treated 30 min before FA inhalation with the non-selective NO synthase (NOS) inhibitor, N^ω-Nitro-L-arginine Methyl Ester (L-NAME), by intraperitoneal route (30 mg/kg).

2.12. Statistical analysis

Data (mean ± S.E.M.) were analysed by ANOVA followed by the Student Newman-Keuls posttest or the Student's tailed paired or unpaired *t*-test, whenever appropriated. A 4.0 version of the GraphPad InStatTM software was used for this purpose. *P* < 0.05 was taken as significant.

2.13. Drugs

Chicken egg ovalbumin (OVA, grade II), Evans blue dye, crystal violet, May-Grünwald-Giemsa dye, acetic acid and 3,3'-diaminobenzidine, N^ω-Nitro-L-Arginine Methyl Ester (L-NAME), were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Aluminum hydroxide (Aldrox[®]) from Wyeth (Whitehall Ltd., São Paulo, Brazil). Chloral hydrate from Merck S.A. (Rio de Janeiro, Brazil).

3. Results

3.1. Effect of previous FA inhalation on allergic lung inflammation induced by OVA challenge

FA inhalation or the OVA-induced allergic reactions were able to cause lung inflammation, as judged by the increased number of cells recruited (see FA and OVA/OVA groups as compared with the naïve group in Fig. 1). When both insults were performed (namely, FA inhalation followed by OVA sensitization/challenge), a significant decrease in cell recruitment was observed regarding the preceding FA and OVA/OVA groups (see the FA/OVA group in Fig. 1). Differential cell analyses indicated that FA inhalation increased the number of recruited mononuclear and neutrophils, but it did not induce eosinophils migration. The OVA-induced allergic reaction (OVA/OVA group) was able to increase the countings of all cell types. It was noteworthy that the allergic reaction induced in rats subjected to FA (group FA/OVA) evoked a BAL cell profile similar to

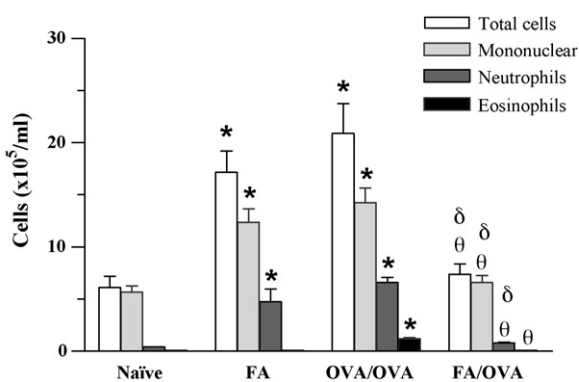


Fig. 1. Effects of previous formaldehyde (FA) inhalation on ovalbumin (OVA)-induced lung inflammation. Group of rats were subjected to FA inhalation for 3 consecutive days (FA). At the last day of FA exposure the rats were OVA-sensitized and then challenged 14 days later (FA/OVA). Lung inflammation was assessed by quantification of the recruited cells present in bronchoalveolar lavage (BAL). The control group consisted of non-manipulated rats (naïve). Data are mean ± S.E.M. of 5 animals per group. **P* < 0.05 related to the naïve group; ^δ*P* < 0.05 related to the OVA/OVA group; ^θ*P* < 0.05 related to the FA group.

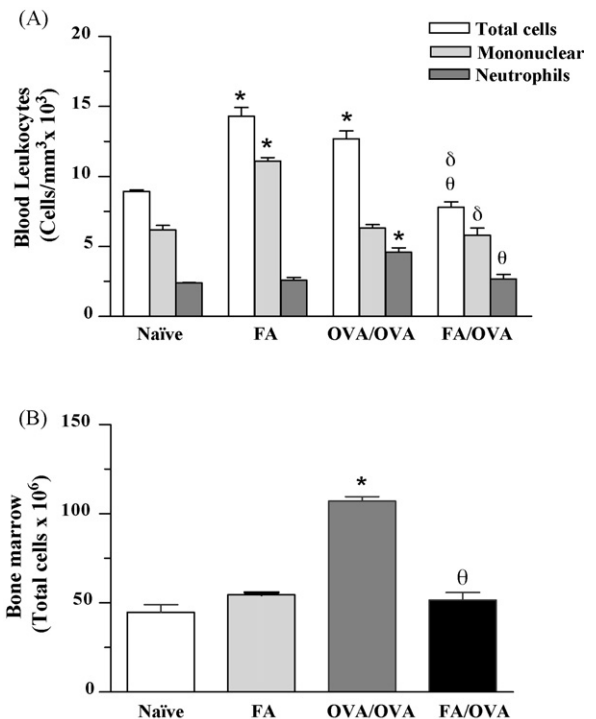


Fig. 2. Effects of previous formaldehyde inhalation on peripheral blood leukocytes (A) and bone marrow cells (B) in OVA-challenged rats. The blood leukocytes counts and the bone marrow cells were determined 1 day after the last FA inhalation or OVA challenge. The control group consisted of non-manipulated rats (naïve). Data are mean ± S.E.M. of 5 animals per group. **P* < 0.05 related to the naïve group; ^δ*P* < 0.05 related to the OVA/OVA group; ^θ*P* < 0.05 related to the FA group.

what was found in the naïve group. As the allergic reaction is mediated by anaphylactic antibodies, and our data also showed reduced allergic response, we decided to investigate whether previous FA inhalation could interfere with antibodies synthesis or not. Data of PCA titres revealed that FA effects did not involve changes in IgE synthesis (PCA titres OVA/OVA = 32.0 versus PCA titres FA/OVA = 28.0; *P* > 0.05).

3.2. Interference of FA exposure on the blood leukocytes and bone marrow cells counts after OVA challenge

Fig. 2 (Panel A) shows that in the FA/OVA group there were no changes in blood total leukocyte number as it was found in the rats of the FA and OVA/OVA groups. The circulating number of neutrophils in the FA/OVA group was significantly lower than that in the OVA/OVA group, but the number of neutrophils in both groups was similar. With regard to the naïve rats, FA inhalation (FA group) increased the number of circulating mononuclear cells, whereas the OVA challenge (OVA/OVA group) increased the number of circulating neutrophils.

In Fig. 2 (Panel B) it is shown that cell numbers quantified in bone marrow of FA/OVA or FA rats were essentially similar to those found in the naïve group. Nevertheless in OVA/OVA rats there was a marked increase of bone marrow cell counts.

3.3. Effect of FA inhalation and OVA challenge on mast cell degranulation

Lung mast cells of naïve rats did not show degranulation (intact: 4.2 ± 0.6 cells/mm²) (Fig. 3A and B). Conversely, lung fragments of the OVA/OVA group showed significant degranulation (intact: 0.5 ± 0.1 ; degranulated: 4.0 ± 0.7 cells/mm²). Degranulation was also evidenced by the presence of granules in the

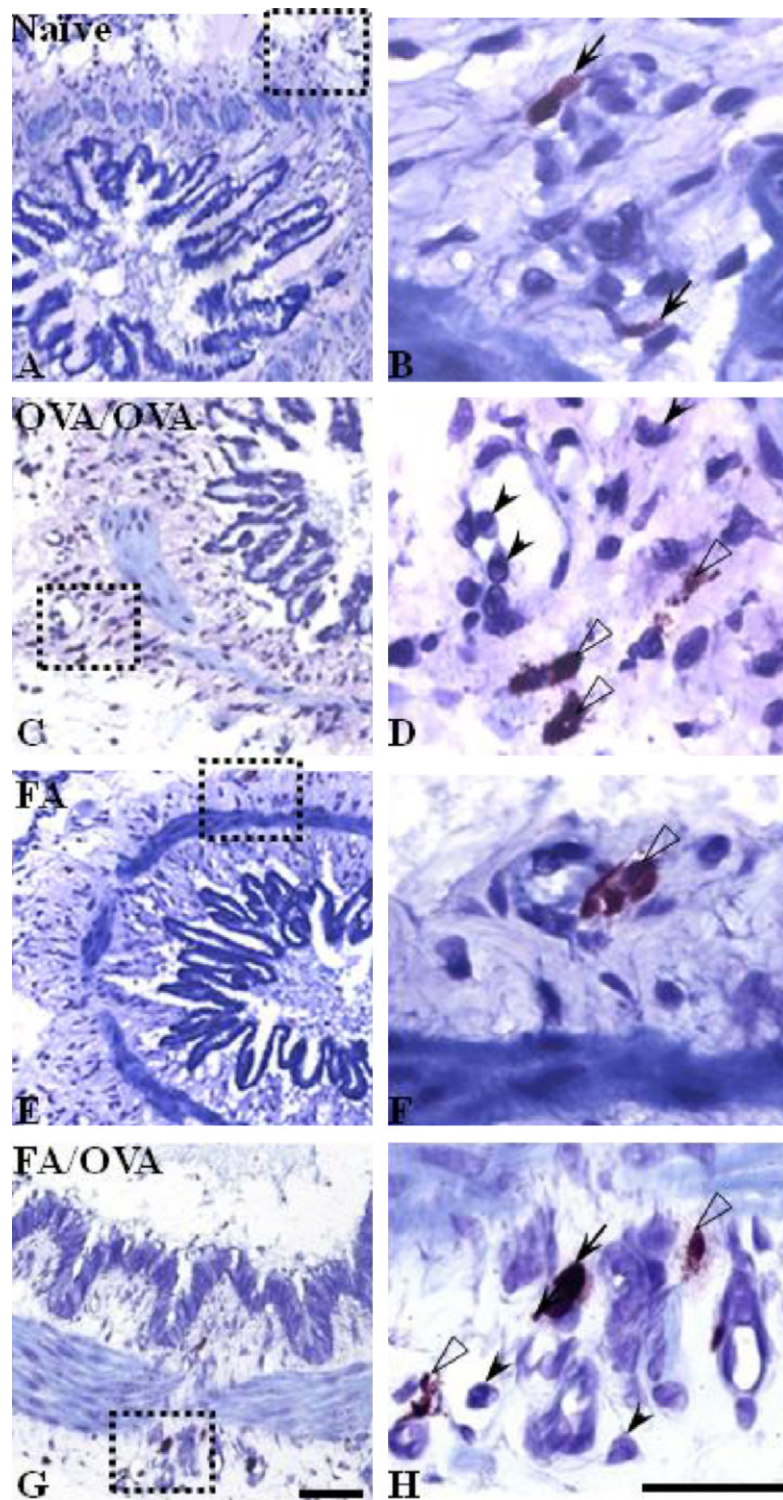


Fig. 3. Morphological analyses of mast cells in rat lungs. Panels A and B: mast cells from naïve rats were intact and metachromatic (arrows), localized near the bronchus. Panels C and D: lung sections from OVA challenged rats (OVA/OVA group) showing a mast cell degranulation (open arrows) and leukocytes (arrowhead) near the bronchus. Panels E and F: FA inhalation induced activation of mast cells (open arrow). Panels G and H: OVA challenge in rats previously subjected to FA (FA/OVA) caused some intact (arrow) and degranulated (open arrow) mast cells and few leukocytes (arrowheads) in the lung ($n = 3$). Toluidine blue stained. Scale bars = 10 μm .

surrounding extracellular space (Fig. 3C and D). Moreover, an increased number of leukocytes close to bronchiole and vessels (6.5 ± 0.5 cells/ mm^2) were also observed (Fig. 3D). It is shown in Fig. 3E and F that FA inhalation caused lung mast cells degranulation (degranulated: 2.0 ± 0.4 cells/ mm^2 ; intact: 0.8 ± 0.5 cells/ mm^2). On the other hand, a less intense mast cell degranulation was

found in FA group as compared with OVA/OVA group. In FA/OVA animals, lung mast cells presented a reduced degranulation (intact: 2.5 ± 0.3 cells/ mm^2 ; degranulated: 1.7 ± 0.5 cells/ mm^2) in addition to few granules extruded and few leukocyte transmigration (2.7 ± 0.4 cells/ mm^2), when compared to the OVA/OVA group (Fig. 3G and H).

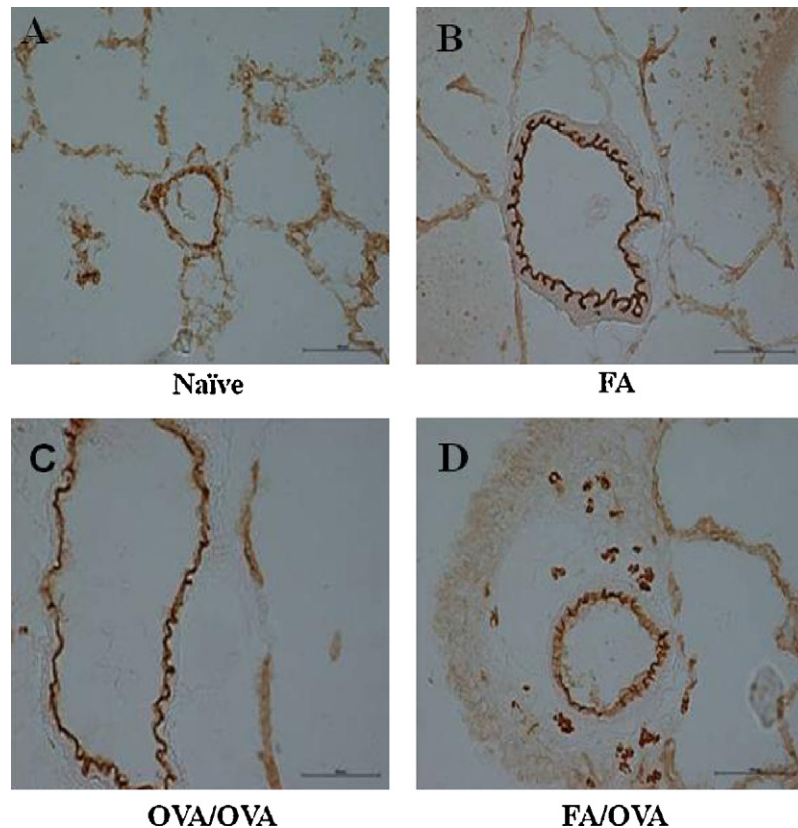


Fig. 4. Expression of PECAM-1 in the lungs of rats. The animal groups were non-manipulated controls (naïve), subjected to FA inhalation (FA), sensitized/challenged with ovalbumin (OVA/OVA) or exposed to FA and sensitized with OVA and, 14 days later, challenged with OVA (FA/OVA). The pictures show positive reaction to PECAM-1 in lung endothelial vessels ($n=3$). Hematoxylin dye ($40\times$).

3.4. Effect of FA exposure on the expression of PECAM-1

Fig. 4 indicates that the separate treatments, either FA inhalation (FA group, Panel B) or OVA challenge (OVA/OVA, Panel C), significantly increased PECAM-1 lung expression as compared to what was observed in the naïve group (Panel A). In the group subjected to both treatments (FA/OVA, Panel D) we observed that PECAM-1 expression actually decreased. These results are shown (as arbitrary units) in Table 1.

3.5. Effect of FA inhalation on nitrites (NO_2) release by BAL-cultured cells

Fig. 5 shows that there was an increased nitrites release by BAL-cultured cells from FA group rats, and that a much higher increase was observed in those of the FA/OVA group, whereas in

BAL-cultured cells of OVA/OVA rats the release of nitrites was not different from basal (naïve group).

3.6. Effect of L-NAME treatment on reduced allergic lung inflammation

Fig. 6 shows that the treatment of animals with L-NAME prevented the reduced allergic lung inflammation due to FA exposure,

Table 1
PECAM-1 expression in lung endothelial cells of rats after formaldehyde (FA) inhalation and sensitized/challenged with ovalbumin (OVA).

Groups	PECAM-1 (arbitrary units)
Naïve	163.0 ± 1.1
FA	$181.0 \pm 0.8^*$
OVA/OVA	$181.0 \pm 1.1^*$
FA/OVA	$162.0 \pm 2.0^\phi$

The animal groups ($n=3$ in every group) were non-manipulated controls (naïve), subjected to FA inhalation (FA), sensitized/challenged with ovalbumin (OVA/OVA) or exposed to FA and sensitized with OVA and, 14 days later, challenged with OVA (FA/OVA). The expression of PECAM-1 was quantified by immunohistochemistry assay.

* $P < 0.05$ related to the naïve group.

ϕ $P < 0.05$ related to the FA and OVA/OVA groups.

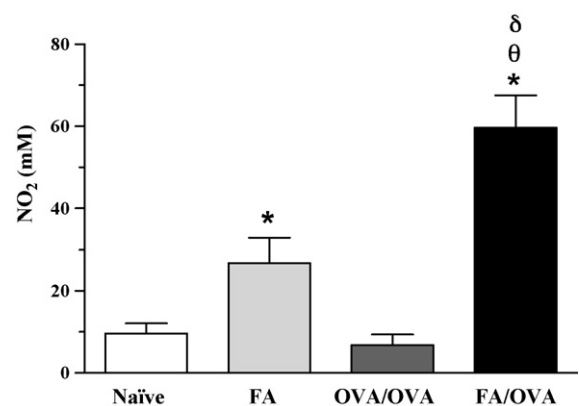


Fig. 5. Nitrites concentration in supernatant of bronchoalveolar lavage (BAL)-cultured cells from ovalbumin (OVA)-induced allergic lung inflammation in rats following formaldehyde (FA) inhalation (FA/OVA). Controls were rats either non-manipulated (naïve) or OVA sensitized/challenged not exposed to FA (OVA/OVA). Aliquots of the supernatants of cultured BAL cells were taken after the scheduled treatments and nitrites levels quantified using Greiss method (see Section 2). Data are mean $\pm$ S.E.M. from 5 animals per group. * $P < 0.05$ related to the naïve group; δ $P < 0.05$ related to the FA group; θ $P < 0.05$ related to the OVA/OVA group.

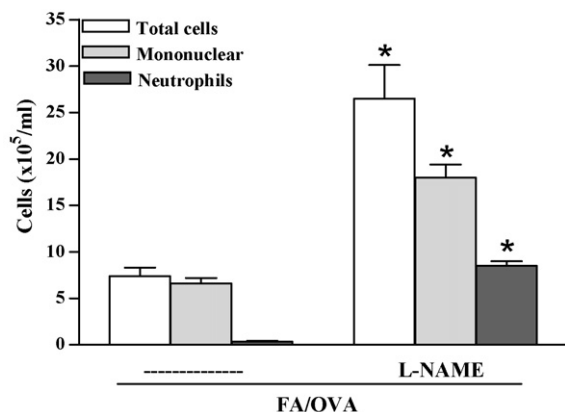


Fig. 6. Role of nitric oxide on reduced allergic lung inflammation induced by FA. Group of rats pretreated or not with L-NAME 30 min before the FA inhalation session and the last day of FA exposure the rats were OVA-sensitized and then challenged 14 days later (FA/OVA). Lung inflammation was assessed by quantification of the recruited cells present in bronchoalveolar lavage (BAL). The control group consisted of non-treated rats. Data are mean $\pm$ S.E.M. of 5 animals per group. * $P < 0.05$ related to the untreated group.

as judged by the increased number of cells collected in BAL of FA/OVA rats relative to non-treated FA/OVA rats. Differential cell analyses indicated that L-NAME treatment increased the number of mononuclear cells and neutrophils.

4. Discussion

In this study, we show that the OVA-induced allergic lung inflammation in rats is blunted by previous exposure to formaldehyde (FA), as evidenced by a reduced number of cells count in BAL (Fig. 1). In these conditions, there was also a reduction of the quantified number of blood leukocytes and bone marrow cells. Being so, since our data indicated that FA inhalation affected not only the lung but also systemic and bone marrow compartments (Fig. 2), we have considered the possibility that a previous FA inhalation might modify the mechanisms involving the induction of allergic immune response. In connection with it, the consequences of FA exposure on OVA-challenged rats were observed as late as 2 weeks after the last FA inhalation. Interestingly, microglial cells of rodents developed strong expression of cyclooxygenase-1 2 weeks after a hind paw formalin injection (Zhang et al., 2007). These data reinforce our suggestion that FA exposure causes a long-lasting effect whose reduced OVA-induced allergic lung inflammation and the cell traffic in distant compartments, such as bone marrow and systemic circulation. In a parallel study, we observed that 2 weeks after the last FA inhalation, rats did not exhibit lung inflammation as revealed by the cell profile in BAL (data not shown). Therefore, our data are consistent with the view that the OVA challenge in rats previously exposed to FA (FA/OVA group) was conducted when lung inflammatory effects due to FA inhalation had already been resolved. Noteworthy rats subjected to OVA sensitization 2 weeks after the last FA inhalation did not develop lung inflammation due OVA challenge (data not shown). Overall, the impaired allergic lung inflammation observed in the FA/OVA group does not appear to be a consequence of the lung inflammation caused by FA inhalation.

In the lungs of FA/OVA group there was a significant reduction of the expression of PECAM-1 (Fig. 4 and Table 1), a molecule involved with neutrophil vascular transmigration (Kelly et al., 2007). These findings were not seen in both FA and OVA/OVA groups, in which there was an increased lung PECAM-1 expression and the inflammatory cell accumulation in the BAL. Thus, we infer that the modest lung inflammation observed in FA/OVA rat group might be associated, at least in part, to a decreased PECAM-1 expression. Our study

did not address what is the exact phase of allergic reaction in which the PECAM-1 expression has been influenced.

The allergic response as that performed herein depends on IgE, Fc ϵ R1 expression and involves mast cell participation (Galli et al., 2005). In FA/OVA and OVA/OVA rats, similar levels of PCA titres were generated, indicating that FA inhalation did not modify the synthesis of anti-OVA IgE. Thus, it is conceivable that the reduced cell migration found in FA/OVA group is not associated to the impaired ability of IgE to bind to mast cells surface. However, we found that FA inhalation caused a differential capacity of lung mast cells to degranulate, regarding what was observed in lung mast cells of the FA/OVA group (Fig. 3G and H). Our data might argue in favor that FA exposure of rats could cause a piecemeal mast cells degranulation (Crivellato et al., 2003) that is defined as a low secretory process in periods of hours or days, interfering with granule-stored materials (Dvorak, 2005). Altogether, these data show that the exposure to FA altered the ability of rat lung mast cells to degranulate.

In contrast with the present results, others authors found that FA inhalation exacerbated allergic lung inflammation in rodents (Sadakane et al., 2002; Fujimaky et al., 2004). However, it is to be noticed that our experimental design consisted of rats subjected firstly to FA inhalation, and subsequently subjected to OVA immunization. This approach differs from the protocols in which the animals are firstly exposed to antigen sensitization and then subjected to a prolonged FA inhalation (Sadakane et al., 2002; Fujimaky et al., 2004). Thus, our data showing that the exposure to FA effectively reduced the allergic lung inflammation might be a consequence of the action of FA on the functional activity of mast cells. These data may be relevant in those cases where individuals are briefly exposed to high levels of FA and later on develop allergic disease to antigen(s) not associated to FA. Upon these conditions, it is likely that the immune system becomes unable to orchestrate a full inflammatory response. We would like to draw attention to the fact that we used a short-term of FA exposure, rats were subjected to higher concentrations of FA than that necessary to induce asthmatic symptoms in indoor condition (Arts et al., 2008). In this context, we have previously shown that FA-induced bronchial hyporesponsiveness to cholinergic stimuli is mediated by NO (Lino dos Santos Franco et al., 2006). Besides, BAL cultured cells of FA/OVA rats also exacerbated the release of NO₂, a marker of NO generation (Fig. 5). Since NO recognizably exerts a negative control on leukocyte-endothelium adhesion (Hickey and Kubes, 1997), it is conceivable that once airways are exposed, and are subjected to the long-lasting effects of FA, there are consistent changes of the functional activity of the cells which are recruited in response to an allergic challenge. Our data showed that the treatment of rats with L-NAME before FA inhalation caused a significant allergic lung inflammatory; this fact might be indicative that NO mediates FA/OVA-induced reduced lung inflammation (Fig. 6). Noteworthy, such effects may be deleterious to the lung once might also impair its responsiveness, as seen by the down-regulated lung inflammation after OVA challenge (Fig. 1). Accordingly, since the inflammatory process is necessary to establish the tissue repair mechanism, the long-lasting interference of FA on the allergic lung inflammation might be a factor of induction of lung dysfunction. Our data did not allow us to infer if the involvement of NO, in this model, is before or after OVA sensitization and challenge. However, as a general hypothesis, we suggest that FA impairs the ability of mast cells to degranulate adequately after an antigen challenge, thence blocking the functions involved with the late allergic response, notably those related to cell recruitment. An exacerbated NO generation and a reduced PECAM-1 expression both appear to exert a major role in order to explain the reduced cell recruitment after the OVA challenge. Overall, keeping in mind that mast cells are immune sentinels (Galli et al., 1999), their blunted activation due to FA exposure might be considered a threat to lung homeostasis.

In conclusion, repeated expositions to air-borne formaldehyde may impair the lung cell recruitment after an allergic stimulus, thereby leading to a non-responsive condition against inflammatory stimuli likely those where mast cells are involved.

Conflict of interest

None.

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References

- Arts, J.H., Muijer, H., Kuper, C.F., Woutersen, R.A., 2008. Setting an indoor air exposure limit for formaldehyde: factors of concern. *Regul. Toxicol. Pharmacol.* (Epub ahead of print).
- Baba, K., Yagi, T., Niwa, S., Sakakibara, A., Hattori, T., Koishikawa, I., Yoshida, K., Yashi, T., 2000. Measurement of formaldehyde-specific IgE antibodies in adult asthmatics. *Arerugi* 49, 404–411.
- Casset, A., Marchand, C., Purohit, A., Le Calvé, S., Donnay, C., Meyer, P., Pauli, G., de Blay, F., 2005. Inhalation de faibles doses de formaldéhyde gazeux: effet sur la réponse bronchique chez des astmatiques sensibilisés aux acariens. *Rev. Fr. Allergol. Immunol. Clin.* 45, 291.
- Casset, A., Purohit, A., Marchand, C., Le Calvé, S., Donnay, C., Uring-Lambert, B., Bahram, G., Pauli, G., de Blay, F., 2006. Le formaldéhyde inhalé et la réponse bronchique. *Rev. Mal. Respir.* 23, 25–34.
- Crivellato, E., Nico, B., Mallardi, F., Beltrami, C.A., Ribatti, D., 2003. Piecemeal degranulation as a general secretory mechanism? *Anat. Rec. A: Discov. Mol. Cell. Evol. Biol.* 274, 778–784.
- De Lima, W.T., Sirois, P., Jancar, S., 1992. Immune-complex alveolitis in the rat: evidence for platelet activating factor and leukotrienes as mediators of the vascular lesions. *Eur. J. Pharmacol.* 213, 63–70.
- Dvorak, A.M., 2005. Piecemeal degranulation of basophils and mast cells is effected by vesicular transport of stored secretory granule contents. *Chem. Immunol. Allergy* 85, 135–184.
- Ezratty, V., Bonay, M., Neukirch, C., Orset-Guillossou, G., Dehoux, M., Koscielny, S., Cabanes, P.A., Lambrozo, J., Aubier, M., 2007. Effect of formaldehyde on asthmatic response to inhaled allergen challenge. *Environ. Health Perspect.* 115, 210–214.
- Fialho de Araujo, A.M., Oliveira-Filho, R.M., Trezena, A.G., Borelli, P., Mathison, R.D., Tavares de Lima, W., 2003. Role of submandibular salivary glands in LPS-induced lung inflammation in rats. *Neuroimmunomodulation* 10, 73–79.
- Fló-Neyret, C., Lorenzi-Filho, G., Macchione, M., Garcia, M.L.B., Saldiva, P.H.N., 2001. Effects of formaldehyde on the frog's mucociliary epithelium as a surrogate to evaluate air pollution effects on the respiratory epithelium. *Braz. J. Med. Biol. Res.* 34, 639–643.
- Fujimaki, H., Kurokawa, Y., Kunugita, N., Kikuchi, M., Sato, F., Arashidani, K., 2004. Differential immunogenic and neurogenic inflammatory responses in allergic mouse model exposed to low levels formaldehyde. *Toxicology* 197, 1–13.
- Galli, S.J., Nakae, S., Tsai, M., 2005. Mast cells in the development of adaptive immune responses. *Nat. Immunol.* 6, 135–142.
- Galli, S.J., Maurer, M., Lantz, C.S., 1999. Mast cells as sentinels of innate immunity. *Curr. Opin. Immunol.* 11 (1), 53–59.
- Hickey, M.J., Kubes, P., 1997. Role of nitric oxide in regulation of leukocyte-endothelial cell interactions. *Exp. Physiol.* 82, 339–348.
- Hong, S.H., Jeong, H.J., Kim, H.M., 2003. Inhibitory effects of Xanthiifrustrum extract on mast cell-mediated allergic reaction in murine model. *J. Ethnopharmacol.* 88 (2/3), 229–234.
- Kelly, M., Hwang, J.M., Kubes, P., 2007. Modulating leukocyte recruitment in inflammation. *J. Allergy Clin. Immunol.*, 3–10.
- Kita, T., Fujimura, M., Myou, Y., Ishiura, Y., Abo, M., Katayama, N., Nishitsuji, M., Yoshimi, Y., Nomura, S., Oribe, Y., Nakao, S., 2003. Potentiation of allergic bronchoconstriction by repeated exposure to formaldehyde in guinea-pigs in vivo. *Clin. Exp. Allergy* 33, 1747–1753.
- Krakowiak, A., Górski, P., Pazdrak, K., Ruta, U., 1998. Airway response to formaldehyde inhalation in asthmatic subjects with suspected respiratory formaldehyde sensitization. *Am. J. Ind. Med.* 33, 274–281.
- Lino dos Santos Franco, A., Damazo, A.S., Beraldo de Souza, H.R., Domingos, H.V., Oliveira-Filho, R.M., Oliani, S.M., Costa, S.K., Tavares de Lima, W., 2006. Pulmonary neutrophil recruitment and bronchial reactivity in formaldehyde-exposed rats are modulated by mast cells and differentially by neuropeptides and nitric oxide. *Toxicol. Appl. Pharmacol.* 214, 35–42.
- Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M., Currie, M.G., 1993. A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.* 214, 11–16.
- Mordelet-Dambrine, M., Arnoux, A., Stanislas-Leguenn, G., Sandron, D., Chretien, J., Huchon, G., 1984. Processing of lung lavage fluid causes variability in bronchoalveolar cell count. *Am. Rev. Respir. Dis.* 130, 305–306.
- Mota, I., Wong, D., 1969. Homologous and heterologous passive cutaneous anaphylactic of mouse antiserum during the course of immunization. *Life Sci.* 8, 813–820.
- Payne, D.N., 2003. Nitric oxide in allergic airway inflammation. *Curr. Opin. Allergy Clin. Immunol.* 3 (2), 133–137.
- Ricciardolo, F.L., Nijkamp, F.P., Folkerts, G., 2006. Nitric oxide synthase (NOS) as therapeutic target for asthma and chronic obstructive pulmonary disease. *Curr. Drug Targets* 7 (6), 721–735.
- Riedel, F., Hasenauer, E., Barth, P.J., Kozirowski, A., Rieger, C.H., 1996. Formaldehyde exposure enhances inhalative allergic sensitization in the guinea pig. *Allergy* 51, 94–99.
- Sadakane, K., Takano, H., Ichinose, T., Yanagisawa, R., Shibamoto, T., 2002. Formaldehyde enhances mite allergen-induced eosinophilic inflammation in the murine airway. *J. Environ. Pathol. Toxicol. Oncol.* 21, 267–276.
- Salvaggio, J.E., 1982. Overview of occupational immunologic lung disease. *J. Allergy Clin. Immunol.* 70, 5–10.
- Scalia, R., Lefer, A.M., 1998. In vivo regulation of PECAM-1 activity during acute endothelial dysfunction in the rat mesenteric microvasculature. *J. Leukoc. Biol.* 64, 163–168.
- Schleimer, R.P., Bochner, B.S., 1998. The role of adhesion molecules in allergic inflammation and their suitability as targets of antiallergic therapy. *Clin. Exp. Allergy* 3, 15–23.
- Shin, H.Y., Lee, C.S., Chae, H.J., Kim, H.R., Baek, S.H., An, N.H., Kim, H.M., 2000. Inhibitory effect of anaphylactic shock by caffeine in rats. *Int. J. Immunopharmacol.* 22 (6), 411–418.
- Songur, A., Akpolat, N., Kus, I., Ozen, O.A., Zararsiz, I., Sarsilmaz, M., 2003. The effects of the inhaled formaldehyde during the early postnatal period in the hippocampus of rats: a morphological and immunohistochemical study. *Neurosci. Res. Commun.* 33, 168–178.
- Zhang, F.Y., Wan, Y., Zhang, Z.K., Light, A.R., Fu, K.Y., 2007. Peripheral formalin injection induces long-lasting increases in cyclooxygenase 1 expression by microglia in the spinal cord. *J. Pain* 8, 110–117.