

Formaldehyde inhalation reduces respiratory mechanics in a rat model with allergic lung inflammation by altering the nitric oxide/cyclooxygenase-derived products relationship



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ABSTRACT

Bronchial hyperresponsiveness is a hallmark of asthma and many factors modulate bronchoconstriction episodes. A potential correlation of formaldehyde (FA) inhalation and asthma has been observed; however, the exact role of FA remains controversial. We investigated the effects of FA inhalation on Ovalbumin (OVA) sensitisation using a parameter of respiratory mechanics. The involvement of nitric oxide (NO) and cyclooxygenase-derived products were also evaluated.

The rats were submitted, or not, to FA inhalation (1%, 90 min/day, 3 days) and were OVA-sensitised and challenged 14 days later. Our data showed that previous FA exposure in allergic rats reduced bronchial responsiveness, respiratory resistance (Rrs) and elastance (Ers) to methacholine. FA exposure in allergic rats also increased the iNOS gene expression and reduced COX-1. L-NAME treatment exacerbated the bronchial hyporesponsiveness and did not modify the Ers and Rrs, while Indomethacin partially reversed all of the parameters studied. The L-NAME and Indomethacin treatments reduced leukotriene B₄ levels while they increased thromboxane B₂ and prostaglandin E₂. In conclusion, FA exposure prior to OVA sensitisation reduces the respiratory mechanics and the interaction of NO and PGE₂ may be representing a compensatory mechanism in order to protect the lung from bronchoconstriction effects.

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1. Introduction

Asthma is an allergic lung disease characterised by the influx of leukocytes into the lung, bronchial epithelium damage and airway remodelling (Bochner and Busse, 2005). These events mediate the induction of airway hyperresponsiveness (AHR), which is the major cause of alteration in lung function as observed in asthmatic patients. The social and economic repercussions of AHR as observed in asthmatic patients can be noted by absenteeism at work and at school. AHR is better characterised in patients of whom the forced expiratory volume is reduced (Carr and Peters, 2012) and may be investigated experimentally by the study of respiratory mechanics.

Metropolitan development has determined an increased rate of pollution including that originated by the manipulation of chemical agents. Formaldehyde (FA) is an indoor and outdoor pollutant emitted by car engine exhausts, foam insulation materials, floor coverings, office furniture, sterilising agents, and cigarette side stream smoke (Krakowiak et al., 1998). FA is an irritating agent to the airways and is also to be held in account for carcinogenic effects (Conolly et al., 2004). Therefore, its exposure represents an important risk factor to health.

Some studies show a potential correlation between FA inhalation and the deterioration of asthma symptoms (Green-Mckenzie and Hudes, 2005; Mendell and Heath, 2005), while other demonstrate that FA has not any significant deleterious effects on airway allergen responsiveness in patients with intermittent asthma or that FA impairs the lung cell recruitment after an allergic stimuli (Ezratty et al., 2007).

We have studied the repercussion of FA exposure on a course of lung allergic processes triggered by an antigen unrelated to FA. In

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these studies, we have shown that exposure to FA, prior to Ovalbumin (OVA) sensitisation, evoked a reduction of cell recruitment in the lung, bone marrow and blood, which suggests that the effects of FA are not only localised in the airways (Lino-dos-Santos-Franco et al., 2009). Moreover, FA inhalation has been associated in reducing mast cell degranulation, platelet-endothelial cell adhesion molecule 1 (PECAM-1) expression and the increased release of NO by bronchoalveolar lavage (BAL) cultured cells (Lino-dos-Santos-Franco et al., 2009).

Other studies have recently demonstrated that FA inhalation *per se* increases the oxidative burst, nitration of proteins and it causes a disruption of the physiological balance between oxidant and anti-oxidant enzymes in the lung tissue of rodents. These events support the presumed involvement of oxidative stress on FA-induced lung inflammation (Lino-dos-Santos-Franco et al., 2010, 2011). Using distinct approaches to investigate FA effects, other studies have also identified the FA-induced initiation of oxidative stress in the frontal cortex, hippocampus and heart tissue (Gülec et al., 2006; Gurel et al., 2005). Moreover, FA exposure *per se* decreases the responsiveness of isolated bronchial rings to methacholine (MCh) via NO pathways (Lino dos Santos Franco et al., 2006).

Cyclooxygenase (COX) products (prostaglandins and thromboxanes) and nitric oxide (NO) have received substantial attention regarding their involvement in asthma. Clinical and experimental studies show that products derived from the arachidonic acid metabolism by COX-1 pathways as well as lipoxygenase mediators (leucotrienes) are critical for inducing AHR as observed in allergic diseases (Harrington et al., 2008). In addition, COX-derived products regulate Th17 cells during allergic lung inflammation (Li et al., 2011).

NO is considered a hallmark of asthma. Asthmatic patients show increased levels of exhaled NO (Miraglia Del Giudice et al., 2011). Similarly, we found that FA inhalation *per se* increases NO production in the cultured cells of bronchoalveolar lavage (Lino dos Santos Franco et al., 2006). NO also plays a role in a variety of biological functions including host defense, immune regulation and inflammation (Coleman, 2001). In addition, its effects as a relaxation of smooth muscle is well-established (Li et al., 2005; Nijkamp and Folkerts, 1994).

Because our previous studies have demonstrated that FA exposure, prior to OVA sensitisation, blunted cellular recruitment into the lung, and considering that cellular events mediate the airways responsiveness, in the present work, we investigated the effects of FA exposure, prior to OVA sensitisation, on bronchial responsiveness and respiratory mechanics. Moreover, considering the important role of COX and NOS on immune regulation, and knowing that FA works by activating such pathways, we hypothesised that eicosanoids and NO generated during FA exposure could mediate the functional activity of the airway of smooth muscle after allergic lung inflammation.

2. Materials and methods

2.1. Animals

Male Wistar rats (160–180 g) were obtained from the Institute of Biomedical Sciences, University of São Paulo. The rats were housed in groups (5 rats per cage) in a light and temperature controlled room (12/12-h light–dark cycle, $21 \pm 2^\circ\text{C}$) with free access to food and water. After 10 days of acclimation in departmental facilities, the groups of rats were subjected to experimental procedures. The experiments were approved by the Institutional Animal Care Committee.

2.2. Exposure to formaldehyde (FA) inhalation

The animals were exposed to FA inhalation (1%) as described by Lino-dos-Santos-Franco et al. (2009). In brief, the rats (5/chamber) were exposed to daily 90-min sessions of FA inhalation for 3 consecutive days. For this purpose, we used a standard glass chamber (20 l) coupled to an ultrasonic nebuliser device (Icel[®], Brazil)

that produces an aerosol, with particles of between 0.5 and 1 μm , to generate a constant airstream in an aqueous solution of formalin diluted to 1% FA by weight. The concentration used and the periods of FA exposure were previously found to cause a neutrophilic lung inflammation (Lino dos Santos Franco et al., 2009).

2.3. Experimental model of ovalbumin-induced allergic lung inflammation

In order to investigate the influence of FA exposure on allergic responses, immediately after the last session of FA inhalation, the rats were sensitised with 10 μg of Ovalbumin (OVA) and 10 mg of aluminium hydroxide by subcutaneous routes. After 7 days, the rats received a second sensitisation (booster) with OVA subcutaneously. One week later, the rats were submitted to an inhaled antigen challenge (aerosolised 1% OVA in PBS, 15 min), using an ultrasonic nebuliser device (Icel[®], SP, Brazil), capable of producing an aerosol with particles of between 0.5 and 1 μm , coupled to a plastic inhalation chamber (18.5 cm $\times$ 18.5 cm $\times$ 13.5 cm).

2.4. Experimental groups

Adult male rats were assigned to 4 groups: OVA, identified as rats sensitised and challenged with OVA, but not subject to FA-exposure. FA/OVA, identified as rats subjected to FA-exposure and subsequently sensitised and challenged with OVA. FA, identified as rats subjected to FA-exposure and Naïve, non-manipulated rats, were utilised in order to obtain the basal parameters.

2.5. Determination of ex vivo bronchial responsiveness to methacholine (MCh)

Rings of intrapulmonary bronchus were mounted for the measurement of isometric force contraction using two steel hooks in a 15 ml organ bath (De Lima and Da Silva, 1998). The force contraction was registered using a force displacement transducer and a chart recorder (Powerlab[®], Labchart, AD Instruments).

The bronchus was suspended in an organ bath filled with Krebs–Hanseleit (KH) buffer at 37°C continuously aerated (95% O_2 and 5% CO_2). After the equilibrium period (40 min), the bronchial tension was adjusted to 0.5 g, and the tissue viability was assessed by replacing the KH solution with the KCl buffer. Cumulative dose–response curves to MCh were constructed according to Van Rossum (1963).

2.6. Evaluation of pulmonary mechanics

Each one of the animals was anaesthetised (ketamine, 80 mg/kg ip and xylazine, 10 mg/kg ip), tracheostomised (15 gauge metal cannula) and, mechanically ventilated with a tidal volume of 8 ml/kg, at a frequency of 150 breaths/min, and 3 cmH₂O of positive end expiratory pressure (PEEP) using a small animal ventilator (flexiVent, SCIREQ, Canada). In order to standardise lung volume history, the lungs were first inflated twice to a pressure of 30 cmH₂O (recruitment manoeuvre).

A MCh challenge was performed with increasing doses (intravenously) of MCh dissolved in phosphate-buffered saline (PBS); 8, 16 and 32 $\mu\text{g}/\text{kg}$.

The control group consisted of rats which received an equal volume of PBS only. The single compartment model (respiratory system resistance and elastance) (Bates, 1998) was applied in the experimental data (sinusoidal volume perturbation of 1.5 Hz, peak of 8 ml/kg and corresponding pressure) acquired after 15 s of each injection of MCh or PBS. Between each injection, there was a time interval of 5 min, with regular ventilation and, after this time interval, a recruitment manoeuvre was performed in order to standardise the lung volume history. After the protocol, the animal was submitted to euthanasia (exsanguinations under deep anaesthesia).

2.7. Evaluation of Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase (COX) gene expression

The gene's expressions of iNOS and COX were performed in lung samples, snap-frozen in liquid nitrogen, as utilised by Lino dos Santos Franco et al. (2011). In brief, the total RNA samples were isolated using a Trizol Reagent according to the protocol of Invitrogen[®]. Cycling conditions were: 10 min at 95°C , followed by 45 cycles at 20 s each at 95°C , 20 s at 58°C , and 20 s at 72°C . The analysis was performed using Sequence Detection Software 1.9 (SDS), and the mRNA expression was normalised to the HPRT expression.

2.8. Determination of eicosanoids and nitric oxide in supernatant of lung explants

Enzyme immunoassay kits (Cayman Chemical/Ann Arbor, MI) were used to quantify leukotriene B₄ (LTB₄), thromboxane B₂ (TXB₂) and prostaglandin E₂ (PGE₂), in samples of supernatants of lung explants in culture, according to the methods of Proust et al. (2003). Determinations were made in duplicate for every sample, using standard curves, according to the manufacturer's specifications.

After euthanasia, the chest of the animal was opened and the lungs were flushed through the right ventricle with 5 ml of PBS. Next, the remaining parenchyma was chopped into four small pieces and distributed into 24-well plastic

plates and cultured in 1 ml of DMEN for 4 h at 37 °C with 5% CO₂ and 0.5% Penicillin–Streptomycin (10,000 UI–10 mg/ml). The outcome that was obtained was expressed in pg of eicosanoids per mg of dry-weight of lung tissue.

The nitrite levels were quantified according to the Griess method. The nitrite assay was performed by adding 50 µl of the supernatant of cultured lung tissue, in 96-well plastic microplates, containing an equal volume of Griess reagent for 10 min at room temperature. The optical density (at 540 nm) was recorded using a microplate reader (Bio-Tek Instr., USA) and the nitrite levels were obtained using a standard curve of NaNO₂.

2.9. Pharmacological treatments

The involvement of COX-products and NO on the effects of FA on OVA-induced alterations on bronchial responsiveness and respiratory mechanics were investigated. The basis of this study relies on the role of COX-derived metabolites and the NO pathway on the inflammatory response (Ricciardolo et al., 2006; Ryan et al., 2006). Moreover, we also showed that these mediators are involved with the effects of FA inhalation (Lino-dos-Santos et al., 2006, 2010, 2011). For this purpose, the rats were treated for 30 min before each FA inhalation with Indomethacin (COX inhibitor, 4 mg/kg, ip) and L-NAME (non-specific NOS inhibitor, 30 mg/kg, ip). The control groups were treated with the drug vehicles (PBS for L-NAME and TRIS buffer for Indomethacin). After the last FA inhalation, the treatment of the rats was finalised and the rats manipulated accordingly.

2.10. Statistical analysis

The data is expressed as a mean ± SEM, and comparisons among the experimental groups were analysed by one-way ANOVA, followed by the Student–Newman–Keuls Test for multiple comparisons using GraphPad software V.5. Statistically, significant differences were considered for *P* values lower than 0.05.

3. Results

3.1. FA inhalation prevents ex vivo bronchial hyperresponsiveness and decreases respiratory mechanics after an OVA-challenge

3.1.1. Ex vivo bronchial responsiveness

We showed that an OVA challenge caused bronchial hyperresponsiveness to MCh when compared to the Naïve group. However, we observed that prior FA exposure in allergic rats (FA/OVA group) prevented bronchial hyperresponsiveness to MCh induced by OVA. Moreover, the bronchial responsiveness obtained from the FA/OVA rats was smaller than obtained from the Naïve rats. We also observed that FA *per se* decreased bronchial responsiveness when compared to the naïve group (Fig. 1).

3.1.2. In vivo respiratory mechanics

Our results showed that prior exposure to FA in allergic rats (FA/OVA group) significantly reduced Rrs and Ers when compared to the OVA group. FA inhalation *per se* reduced Rrs and Ers relative to the Naïve group. The Rrs and Ers from the FA/OVA, and Naïve groups were similar. In addition, the OVA challenge (OVA group) increased the Rrs and Ers relative to the Naïve group. (Fig. 2, Panels A and B).

Exposure to FA Inhalation Increases the Gene Expression of Inducible Nitric Oxide Synthase (iNOS) and Reduces the Gene Expression of Cyclooxygenase (COX) 1 without Changing COX-2 in Allergic Rats.

Panel A shows that prior FA inhalation in allergic rats (FA/OVA group) increased the iNOS gene expression in the lung tissue relative to the OVA and Naïve groups and did not differ from FA group. Similarly, FA *per se* increased the iNOS gene expression relative to the naïve group.

Panel B shows that the OVA challenge (OVA group) and FA *per se* increased the COX-1 gene expression in the lung tissue when compared to the Naïve group. However, we observed that FA inhalation, prior to OVA sensitisation (FA/OVA group), reduced the COX-1 gene expression in the lung tissue, when compared to the OVA group and remained elevated relative to the Naïve group.

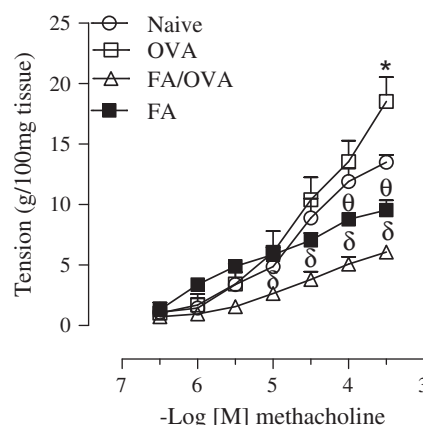


Fig. 1. Previous FA inhalation prevents *ex vivo* bronchial hyperresponsiveness after an OVA-challenge. Groups of rats were submitted, or not, to FA inhalation and sensitised and challenged with OVA. The non-manipulated rats and only exposed to FA were used as control (Naïve and FA groups). The responsiveness to MCh was recorded 24 h after the OVA-challenge. Data is the mean ± SEM from 6 animals. **P* < 0.05 relative to the Naïve group; °*P* < 0.05 relative to OVA and the Naïve groups; °°*P* < 0.05 relative to the Naïve group (ANOVA followed by Student–Newman–Keuls Test).

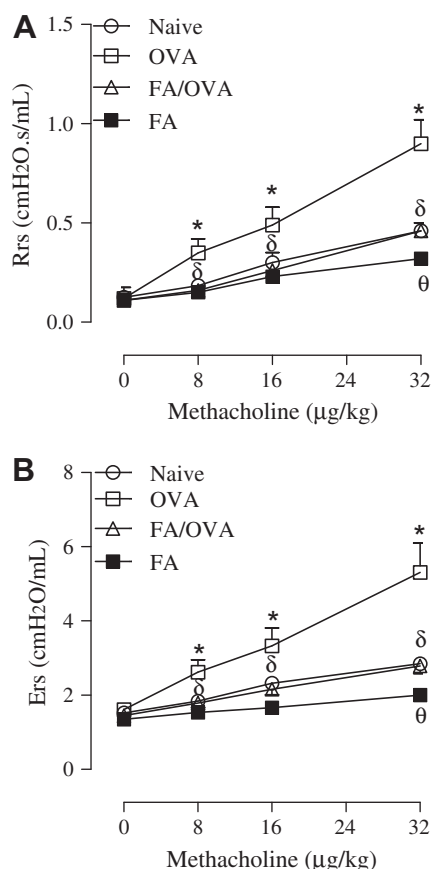


Fig. 2. Prior FA inhalation reduces respiratory resistance (Rrs) (Panel A) and respiratory elastance (Ers) (Panel B) after the OVA-challenge. Groups of rats were submitted, or not, to FA inhalation and sensitised and challenged with OVA. The Naïve group and FA group were used as control. Twenty-four hours after the OVA-challenge, the respiratory mechanics were evaluated. Data is the mean ± SEM from 8 animals. **P* < 0.05 relative to the Naïve group and °*P* < 0.05 relative to the OVA group; °°*P* < 0.05 relative to the Naïve group (ANOVA followed by Student–Newman–Keuls Test).

Panel C shows that the lung tissues of the FA/OVA and OVA groups increased the COX-2 gene expression, when compared to

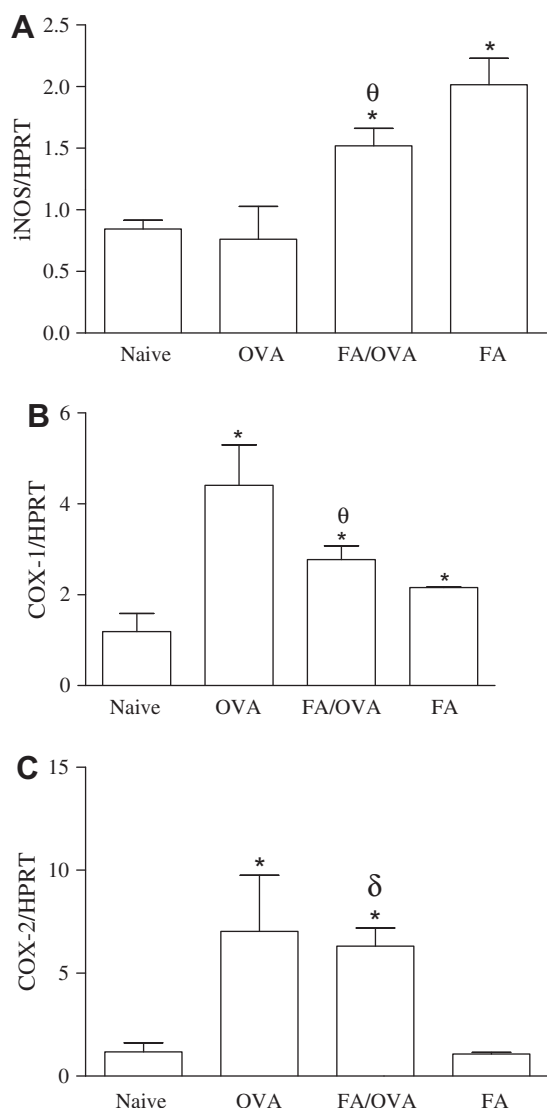


Fig. 3. Exposure to FA increases the gene expression of inducible nitric oxide synthase (iNOS) (Panel A), while it reduces the gene expression of cyclooxygenase (COX) 1 (Panel B), and without change to COX-2 (Panel C) after the OVA-challenge. Groups of rats were submitted, or not, to FA inhalation and sensitised and challenged or not with OVA. The non-manipulated rats were used as control (Naïve group). The evaluation of the gene expression in the lung tissue was performed 24 h after the OVA-challenge. Data is the mean $\pm$ SEM from 5 animals. * $P < 0.05$ relative to Naïve group, ^θ $P < 0.05$ relative to the FA group and ^δ $P < 0.05$ relative to the OVA group (ANOVA followed by Student–Newman–Keuls Test).

the Naïve group. We also observed that FA/OVA group increased the COX-2 gene expression when compared to the FA group (Fig. 3).

3.2. Preventive effects of FA inhalation on bronchial hyperresponsiveness induced by OVA are mediated by COX-derived products and nitric oxide

As shown in Panel A, the treatment with Indomethacin enhanced the bronchial responsiveness to MCh, when compared to the FA/OVA group that was untreated. However, in panel B, the treatment with L-NAME caused an additional reduction in the bronchial responsiveness, relative to the FA/OVA group that was untreated (Fig. 4).

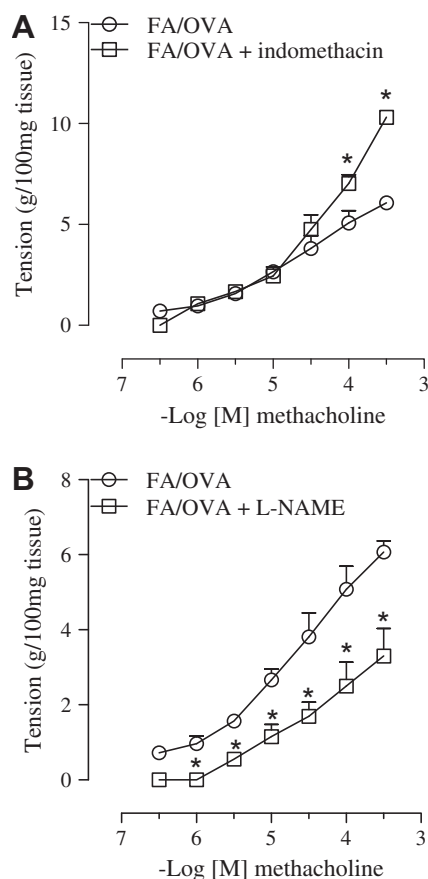


Fig. 4. COX-derived products (Panel A) and nitric oxide (Panel B) mediates the effects of FA inhalation on bronchial hyporesponsiveness to MCh after the OVA-challenge. Groups of rats were treated before each FA inhalation with Indomethacin or L-NAME. The control group consisted of animals treated with a vehicle of drugs. The bronchial responsiveness to MCh was assessed 24 h after the OVA-challenge. Data is the mean $\pm$ SEM from 6 animals. * $P < 0.05$ relative to the FA/OVA group that was untreated (ANOVA followed by Student–Newman–Keuls Test).

3.3. COX-derived products, but not nitric oxide, modulates the effects of FA inhalation on the respiratory mechanics in allergic rats

The treatment with Indomethacin increased the Rrs and Ers to MCh when compared to the FA/OVA group that was untreated (panels A and B). On the other hand, no differences in Rrs and Ers were observed when the rats were treated with L-NAME, prior to FA inhalation (Panels B and D) (Fig. 5).

3.4. COX-derived products modulate nitric oxide release in the lung tissue after FA exposure

The prior exposure to FA in allergic rats (FA/OVA group) increased the NO levels in the lung tissue when compared to the OVA and Naïve groups. Besides, the treatments with Indomethacin or L-NAME reduced the levels of NO in the lung tissue, when compared to the untreated group (Fig. 6).

3.5. The inhibition of nitric oxide and COX-derived products reduces LTB_4 and increases TXB_2 and PGE_2 in the lung tissue of allergic rats exposed to FA

Panel A shows that the rats of the FA/OVA group increased the levels of LTB_4 in the lung explants when compared to the OVA and Naïve groups. The treatment with Indomethacin or L-NAME de-

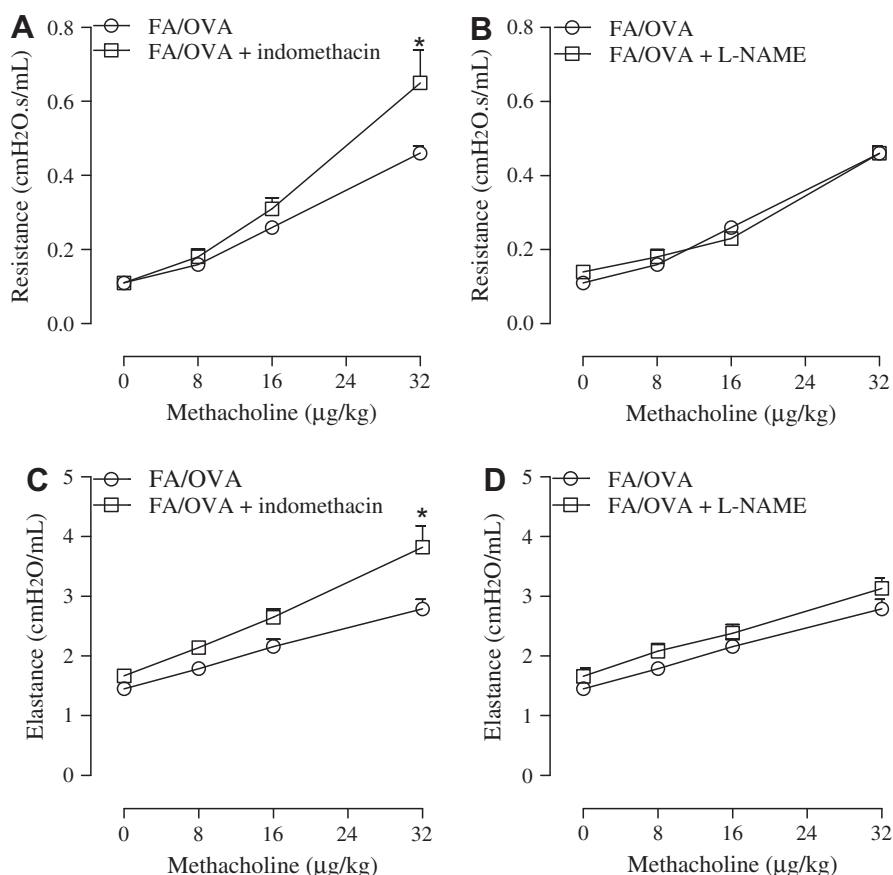


Fig. 5. COX-derived products (Panels A and C) and nitric oxide (Panels B and D) mediate the effects of FA inhalation on Rrs and Ers to MCh after the OVA-challenge. Groups of rats were treated before each FA inhalation with Indomethacin or L-NAME. The control group consisted of animals treated with a vehicle of drugs. 24 h after the OVA-challenge, the respiratory mechanics were evaluated. Data is the mean $\pm$ SEM from 8 animals. * $P < 0.05$ relative to the FA/OVA group that was untreated (ANOVA followed by Student–Newman–Keuls Test).

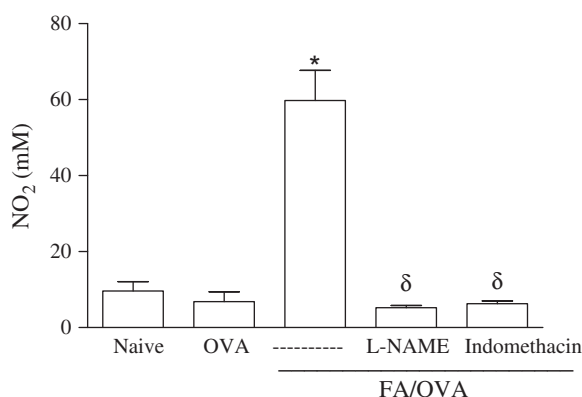


Fig. 6. The effects of treatment with Indomethacin and L-NAME on nitric oxide levels in the lung tissue of allergic rats exposed to FA. Groups of rats were treated before each FA inhalation with Indomethacin or L-NAME. The control group consisted of rats treated with a vehicle of drugs. Levels of NO were determined in the lung tissue of the culture 24 h after the OVA-challenge. Data is the mean $\pm$ SEM from 6 animals. * $P < 0.05$ relative to the Naïve group; ^δ $P < 0.05$ relative to the FA/OVA group that was untreated (ANOVA followed by Student–Newman–Keuls Test).

creased the LTB₄ levels relative to the FA/OVA group that was untreated and increased relative to the Naïve group.

Panel B indicates that the FA/OVA group did not modify the levels of TXB₂ when compared to the OVA and Naïve groups. In contrast, the treatment with Indomethacin or L-NAME increased the

levels of TXB₂ when compared to the FA/OVA group that was untreated and the Naïve groups.

Panel C reveals that both groups, FA/OVA and OVA, increased the levels of PGE₂ generated by the lung tissue when compared to the Naïve group. Moreover, the treatment with Indomethacin or L-NAME increased the levels of PGE₂, relative to the FA/OVA group that was untreated and the Naïve groups (Fig. 7).

4. Discussion

In the present work, we report the effects of FA exposure on the outcome of allergic lung response in rats, notably on the respiratory mechanics. We have used FA, an indoor and outdoor pollutant, widely employed in many industries and laboratories (Fló-Neyret et al., 2001) to investigate the influence of pollutants on the development of allergic lung disease. The relevance of this study relies on the fact that many people are exposed to occupational pollutants and, that these chemical agents can seriously affect the immune system.

There is clinical and experimental evidence that suggests that exposure to FA aggravates those suffering from allergic asthma thus favouring the development of bronchial hyperresponsiveness (Casset et al., 2006; Green-Mckenzie and Hudes, 2005; Mendell and Heath, 2005). Conversely, our data indicate that FA exposure prior to OVA-sensitisation reduced bronchial responsiveness and pulmonary mechanics. Corroborating with our results, Ezratty et al. (2007) utilising a controlled human study, showed that a low dose of FA did not enhance the asthmatic response to allergen

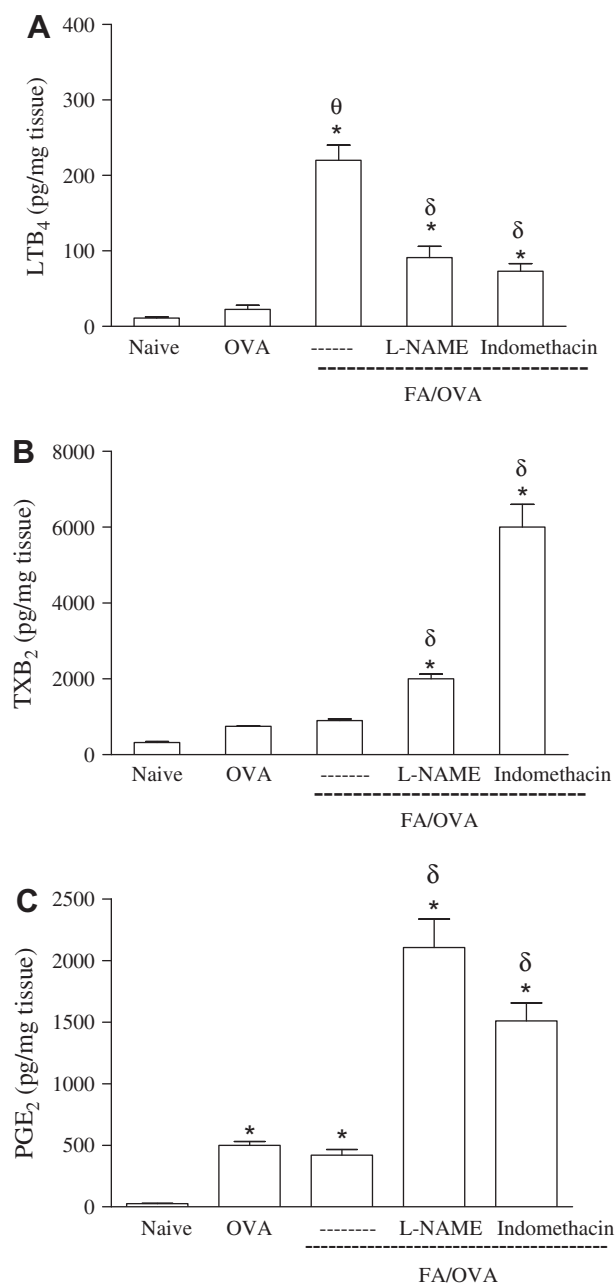


Fig. 7. The effects of treatment with Indomethacin and L-NAME on the levels of arachidonic acid-metabolites of allergic rats submitted to FA with Indomethacin prior to OVA-sensitisation. Groups of rats were treated before each FA inhalation with Indomethacin or L-NAME. The control group consisted of rats treated with a vehicle of drugs. Levels of LTB₄ (Panel A), TXB₂ (Panel B) and PGE₂ (Panel C) were determined in the lung tissue of the culture 24 h after the OVA challenge. Data is the mean $\pm$ SEM from 6 animals. * $P < 0.05$ relative to the Naive group; $^{\theta}P < 0.05$ relative to the OVA group; $^{\delta}P < 0.05$ relative to the FA/OVA group that was untreated (ANOVA followed by Student–Newman–Keuls Test).

in patients with intermittent asthma. They even observed a trend to protect the effect of FA.

Noteworthy, in general, as mentioned above, almost all of the studies have evaluated the effects of FA *per se* or on pre-established allergic response. On the other hand, our study addressed the role of FA inhalation on the development of an allergic response. Thus, this apparent discrepancy can be explained by the differences between the protocols. Another important aspect to consider is the concentration of FA. We have used in our studies, a short-term of FA exposure, and a much higher concentration than necessary, to induce asthmatic symptoms in indoor condition. Our studies did

not address a direct correlation with human exposure, but they did contribute to an understating of the mechanisms involved with acute lung toxicity caused by FA exposure.

It is known that an important aspect of allergic disease is pulmonary dysfunction, represented primarily by alterations in the airway smooth muscle. We used a pharmacological approach, in order to investigate the effects of FA on the alterations in the airway smooth muscle that were induced by allergic stimulus.

Lung function is highly reduced in asthma, and mathematical models have been employed to understand the respiratory mechanics (Bates, 1998), through the use of mechanical ventilators in clinical practice, e.g. in hospitals and in scientific research.

In asthma, structural changes in the parenchyma, due to inflammatory processes, might interfere in the respiratory mechanics and, consequently, in the function of the lung. The alterations in respiratory mechanics can be evaluated by the measurements of air flow/volume and airway pressure. In this context, two important parameters have been investigated: Rrs, which can be defined as the sum of the airways resistance and the lung tissue resistance; and Ers, defined as the force of elastic recoil of the respiratory system tissues.

Our results show that the exposure to FA prior to OVA-sensitisation reduced bronchial hyperresponsiveness as well as Rrs and Ers to MCh. Similar effects were observed when the animals were exposed to FA only. Thus, we might infer that the effects of FA predominated on OVA effects. Besides, we have found in previous studies that FA inhalation, prior to OVA sensitisation, reduced inflammatory cells recruited into the lung and that these effects were modulated by mast cells (Lino-dos-Santos-Franco et al., 2009).

In fact, mast cells are activated by FA (Fujimaki et al., 1992; Norbäck et al., 1995) and we suggest that FA interferes with granule-stored materials of mast cells. Thus, FA inhalation might cause a piecemeal mast cell degranulation which is defined as a low secretory process in periods of hours or days (Crivellato et al., 2003). It is reasonable to admit that this modification in mast cells activity, the lung inflammation and the bronchial responsiveness were reduced. Indeed, at the time of the OVA-challenge, the mast cells were incapable to degranulate and the lung of the rats were not inflamed (Lino-dos-Santos-Franco et al., 2009).

FA is an irritant agent that causes the activation of sensorial fibres (Bianchi et al., 2004) and the release of neuropeptides and nitric oxide. It is known that neuropeptides, such as substance P, induce bronchial relaxation, mediated by prostaglandin E₂ (PGE₂) (Szarek et al., 1998). Similarly, NO promotes the relaxation of airway smooth muscle (Fischer et al., 2002). Thus, we hypothesised that bronchial hyporesponsiveness, induced by FA in allergic rats, could be modulated by NO and PGE₂.

Experimental evidence shows the interaction of COX metabolites and NO. In fact, a relation between NO and prostaglandins (PGs) generation was shown in the uterus (Cella et al., 2006; Franchi et al., 1994). Moreover, the epidermal growth factor and interleukin 1 α enhance PGs production by the stimulation of iNOS activity (Farina et al., 2000).

The interaction between PGs and NO was also found in the present study. FA exposure in allergic rats increased the gene expression of iNOS, decreased the gene expression of COX-1, but did not change the elevated gene expression of COX-2 in the lung tissue. In the present work we confirmed that FA *per se* increased iNOS and COX-1 gene expression, while it did not modify the COX-2 gene expression. These results demonstrated that, in fact, FA interferes with the OVA-induced lung response, and this effect is modulated by COX-derived products and nitric oxide.

In earlier studies, we have demonstrated that Indomethacin treatment exacerbated the bronchial hyporesponsiveness caused by FA inhalation (Lino-dos-Santos-Franco et al., 2006). On the other hand, in the present study, the treatment with Indomethacin

increased the bronchial responsiveness, Rrs and Ers in FA/OVA rats. We believed that these opposite effects of Indomethacin in FA and FA/OVA groups might be as consequence of OVA effects. The effects of FA inhalation in airways smooth muscle could be viewed as the result of balance between the release of bronchoconstriction mediators (e.g. prostaglandin D₂, prostaglandin F₂ α , thromboxane A₂) and those causing bronchodilation (e.g. NO and PGE₂). Thus, the treatment with Indomethacin changed this balance. In fact, our earlier results show that when we prevented the effects of FA with Indomethacin treatment, the OVA challenge elicited the lung inflammation (Lino-dos-Santos-Franco et al., 2010). Nevertheless, the mechanism underlying the FA action seems to involve a kind of complex interaction between FA and the inflammatory components of the allergic response.

The blockade of NO with L-NAME intensified the bronchial hyporesponsiveness, but did not modify the values of Rrs or Ers of the FA/OVA group of rats. However, the treatment with L-NAME, in rats only exposed to FA, caused an increment in the bronchial responsiveness (Lino-dos-Santos-Franco et al., 2006). These differences between FA and FA/OVA groups can be explained by the period of analysis (the FA group parameters were evaluated 24 h after the last inhalation, while in the FA/OVA group, the analysis was performed 14 days after the last inhalation). Other factor to consider is the additional effect triggered by OVA.

Nitric oxide and PGE₂ relax the airway smooth muscle, whereas LTB₄ and TXB₂ play an important role in the contraction of the airway smooth muscle (Li et al., 2005; Henry et al., 2005; Creese and Bach, 1983). We have quantified the levels of these mediators in the lung explants. Our results show that FA exposure, prior to OVA-sensitisation, or FA alone (Lino-dos-Santos-Franco et al., 2006) increased NO levels. On the other hand, the treatments with Indomethacin, or L-NAME, reduced the NO generation. This result shows that, in fact, the effects of FA on allergy are modulated by NO, since when preventing the effects of FA with these treatments, the levels of NO were similar to those found in the OVA group.

But other important mediator is PGE₂, and that can also be involved in bronchial hyporesponsiveness. COX-derived products such as prostaglandins and thromboxanes have received considerable attention regarding their potential role in asthma. Moreover, in previous studies, we have shown that COX-derived products can modulate the effects of FA *per se* and on allergic lung inflammation (Lino dos Santos Franco et al., 2006; Lino dos Santos Franco et al., 2010). Moreover, the effects of FA are also modulated by leukotrienes (lipoxygenase product).

Here, our results have confirmed that FA/OVA interaction increased LTB₄ levels while did not modify the TXB₂ and PGE₂ levels in the lung explants. Thus, we can suggest that reduced contractile response as well as decreased pulmonary mechanics induced by FA exposure in allergic rats might be attributed to increased NO levels, but not to COX-derived products. However, when rats were treated with Indomethacin or L-NAME, before each FA inhalation, the levels of TXB₂ and PGE₂ were elevated. Besides, these treatments reduced the levels of LTB₄. These results seem contradictory, considering that Indomethacin inhibits COX-derived products. However, we must consider that Indomethacin treatment was performed before each FA inhalation and the analysis was performed 24 h after the OVA challenge (14 days later). The basis for such experimental protocol relies on the view that the Indomethacin, or L-NAME treatments, might prevent the effects induced by FA inhalation. Indeed, we also showed reduced iNOS and COX-1 gene expression after pre-treatment with Indomethacin and L-NAME in rats submitted to FA *per se* (Lino-dos-Santos-Franco et al., 2011).

Our data showing an increment in the PGE₂ and TXB₂ after treatment with Indomethacin, can be explained by the following two reasons: (1) the analysis was performed 14 days after the treatment with Indomethacin; (2) during FA exposure, many

mediators are released (oxygen and nitrogen reactive species) which might alter the metabolism of resident and inflammatory cells. Thus, when these cells received the second stimulus (OVA), the responses to mediators released were enhanced (Lino-dos-Santos-Franco et al., 2010, 2011).

Taking to account, our data suggests that the increased bronchial responsiveness, Rrs and Ers in the FA/OVA group, after treatment with Indomethacin, might have been modulated by TXB₂. An additional reduction in bronchial responsiveness was observed in the FA/OVA + L-NAME group which can be attributed to an increment of PGE₂ levels.

The effect of PGE₂ on the airway responses to allergens may be explained in terms of the relaxation of the airway smooth muscle and by the mast cells mediator inhibition (Henry et al., 2005). Accordingly, we showed an interaction between NO and PGE₂, demonstrating the bronchoprotective mechanism in order to maintain the elevated levels of bronchodilator mediators into the lung.

In conclusion, our data has shown that prior exposure to FA in allergic rats reduced the lung responsiveness and the respiratory mechanics to methacholine (MCh). The secretion of NO/COX-derived products might be involved in the sensitisation phase of the immune host defense. In addition, the interaction between NO and PGE₂ may be a compensatory mechanism, in order to protect the lung from bronchoconstriction. This data is helpful in order to understand the mechanisms involved in the effects of pollutants on the development of allergic diseases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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