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Effects of L-arginine and phosphodiesterase-5 inhibitor, sildenafil, on inflammation and airway responsiveness of sensitized BP2 mice

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accepted 29 June 2007*Correspondence and reprints:
amichel@univ-montp1.fr**ABSTRACT**

Nitric oxide (NO) levels are elevated in the exhaled breath of asthmatic patients and NO is considered as a biomarker of airway inflammation. However, the functions of NO in the airways are not completely understood. L-arginine, as the substrate of NO synthases, is the precursor of NO which stimulates guanylate cyclase and leads to the formation of cyclic GMP (cGMP). Sildenafil, a phosphodiesterase-5 (PDE-5) inhibitor, prevents the degradation of cGMP. In this study the effects of L-arginine and sildenafil treatment, alone or in combination, were evaluated in ovalbumin-sensitized BP2 mice. These effects concerning the airway responsiveness to inhaled methacholine (MCh) were evaluated by whole-body plethysmography (WBP), the inflammatory response evaluated by bronchoalveolar lavage fluid (BALF) analyses and lung tissue biopsies (eosinophilic inflammation associated with lung remodelling), and NO metabolite measurements (by Griess reaction) in BALF. Ovalbumin sensitization induced: (a) an inflammatory reaction with eosinophil and neutrophil influx in BALF and lung; and (b) an increased bronchial responsiveness to MCh. L-arginine treatment [50 mg/kg intraperitoneally (i.p.), for 7 days] increased the relative amount of eosinophils and neutrophils in BALF, had a tendency to increase the airway responsiveness to inhaled MCh and increased the NO metabolite level in BAL. Sildenafil treatment (20 mg/kg i.p. for 7 days) did not affect the airway responsiveness to MCh and had a lower effect compared with L-arginine on inflammatory reactions. The combination of the two treatments resulted in a dramatic enhancement of the airway responsiveness to inhaled MCh. The relative amount of eosinophils was increased and lung histology showed obvious worsened tissular lesions such as epithelial shedding and hypertrophy, hyperplasia of smooth muscle cells, and fibrosis. These findings are consistent with the notion that NO production plays a role in the development of airway inflammation and hyperresponsiveness of sensitized mice and highlighted the potential risk of the L-arginine dietary complement or PDE5 treatment in asthmatic patients.

INTRODUCTION

Nitric oxide (NO) was first described as an endothelium-derived relaxing factor because of its activation of guanylate cyclase in vascular smooth muscle cells and

the production of guanosine-3',5'-cyclic monophosphate (cGMP) [1,2]. In addition to its vascular effect, NO was shown to be involved in a variety of biological functions including host defence, platelet aggregation, immune regulation, neurotransmission and inflammation [3,4].

NO is synthesized from the amino acid, L-arginine [5], by the action of either constitutive [6,7] or inducible [8] NO synthases (NOS). There are two constitutive NOS: neuronal (nNOS) and endothelial (eNOS), also known as NOS I and NOS III, respectively, and one inducible NOS: iNOS, also known as NOS II [9]. Constitutive NOS are regulated by intracellular Ca^{2+} calmodulin levels, resulting in the generation of small amounts of NO and are assumed to be involved in physiological regulations. In contrast, iNOS are not only independent of Ca^{2+} concentrations, regulated at transcriptional levels by inflammatory cytokines [10,11] leading to the production of a relatively large amount of NO which may activate guanylate cyclase, but also have cytostatic or cytotoxic effects [12,13].

All three NO synthases are expressed in human lungs and there is increasing evidence that endogenous NO may play an essential role in the physiological regulation of the airway functions. NO is also involved in the pathogenesis of airway diseases such as bronchial asthma [14]. Asthma is a chronic airway disease characterized by airway inflammation and bronchial hyperresponsiveness (BHR) and NO may be involved in both the components of the disease. NO is present in the exhaled air of humans and its level is increased in the exhaled air of asthmatic patients [15,16]. Interestingly, iNOS is expressed in the airway epithelial cells of asthmatic patients but not in nonasthmatics [17]. Concerning BHR, many conflicting results have been reported. In Brown-Norway ovalbumin (OVA)-sensitized rats, Eynott *et al.* [18] reported that SC-51, a selective iNOS inhibitor, inhibited BHR to acetylcholine in sensitized, OVA-challenged animals, while it increased the airway responsiveness in sensitized, saline-challenged rats. OVA-sensitized guinea-pigs challenged with ovalbumin showed an immediate airway response and a late airway response [19]. The treatment of these animals with either N(G)-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, or aminoguanidine, a relatively selective iNOS inhibitor, resulted in a significant reduction of late airway responsiveness with no effect on immediate airway responsiveness, while the treatment of these animals with L-arginine was associated with an increase in late airway responsiveness. Conversely, Tulic *et al.* [20] reported that treatment with NOS inhibitors further increases the hyperresponsiveness of OVA-sensitized rats to methacholine (MCh), and Larson *et al.* [21] showed that the inhibition of NOS enhances antigen-induced contractions and increases the release of cysteinyl leucotrienes in sensitized

guinea-pig lung parenchyma. These researchers presented NO as a protective anti-inflammatory factor.

Concerning airway inflammation, most of the studies reported that NO plays a key role [19,22] mainly in the eosinophilic infiltration of sensitized animals' airways [23–25]. All these studies were performed with selective or non-selective NOS inhibitors or with L-arginine, the NO precursor. Another way to evaluate the involvement of the NO–cyclic GMP (cGMP) pathway in the airway responsiveness of sensitized animals is the study of the effects of selective phosphodiesterase-5 (PDE5) inhibitors that prevent cGMP degradation by PDE5. In fact, most of the studies that evaluated the effects of PDE inhibitors on airways concern the cAMP pathway and only a few studies were performed with PDE5 inhibitors. PDE5 inhibition and the subsequent cGMP increase induced a relaxation of guinea-pigs' trachea *in vitro* and a bronchodilatory activity *in vivo* [26]. But zaprinast, a selective PDE5 inhibitor, was ineffective on antigen-induced airway hyperresponsiveness in sensitized guinea-pigs [27]. Airway epithelial cells play a key role in airway inflammation and have the capacity to produce many inflammatory mediators. Primary cultures of human epithelium expressed PDE5. Zaprinast increased cGMP levels in these cells and reduced Prostaglandin E2 (PGE2) synthesis but had no effect on interleukin (IL)-8 formation [28]. Zaprinast had no effect on antigen-induced pulmonary eosinophil and neutrophil influx in Brown-Norway rats [29]. In contrast, Haddad *et al.* [30] reported that zaprinast reduced, in a dose-independent manner, the proinflammatory cytokine IL-6 and tumour necrosis factor- α (TNF α) production in alveolar epithelial cells. Thus, contrasting results have been reported as regards the effect of zaprinast on airway functions.

The interest in PDE5 inhibitors has been strengthened by the introduction of sildenafil (Viagra®; Pfizer, New York, USA) in therapeutics. Sildenafil is a potent and selective PDE5 inhibitor with efficiency in the treatment of male erectile dysfunction by oral route [31]. Besides its clinical use, sildenafil is a powerful pharmacological tool to study cGMP cell functions. In a recent study, Toward *et al.* [32] evaluated the effects of sildenafil in an animal model of airway disease. Guinea-pigs sensitized and challenged with OVA developed early- and late-phase bronchoconstrictor responses to OVA, bronchial hyperactivity to inhaled histamine and elevated eosinophils in bronchoalveolar lavage fluid (BALF). Treatment with sildenafil [1 mg/kg, intraperitoneally (i.p.), 24 h before OVA challenge] failed to prevent the early and late

bronchoconstrictor responses to OVA but significantly reduced BHR to histamine and eosinophil influx. Thus, all these studies show that the results regarding the mobilization of the NO-cGMP signalling pathway in the airways are conflicting.

The aim of the present study was to further explore the effects of that signalling pathway on airway responsiveness in sensitized animals. BP2 mice were used, because with that strain of hyper-IgE mice and an adequate sensitization protocol, it was possible to obtain animals with both BHR and airway eosinophilia [33]. Mice were sensitized with OVA and the influence of the NO-cGMP pathway on airway reactivity was evaluated through different intraperitoneal (i.p.) treatments: L-arginine, the NO precursor, sildenafil, the PDE5 inhibitor and the combination of the two drugs. Airway smooth muscle responsiveness was assessed by whole-body plethysmography (WBP), and inflammation reaction by BALF analysis and lung histology.

MATERIALS AND METHODS

Animals

Male BP2 mice (weighing 20–25 g, 4 weeks old) were obtained from Elevage Janvier (Le Genest Saint Isle, France) and maintained under standard laboratory conditions. The mice were housed in a temperature-controlled room (21–22°C) with a 12 h light/dark cycle and given food and water ad libitum. The principles of laboratory animal care (NIH Publication No. 85-23, revised 1985) were followed.

Sensitization

The mice were sensitized subcutaneously (s.c.) with 100 µg chicken OVA (0.4 mL of 250 µg/mL OVA in 0.9% NaCl solution) complexed with 1.6 mg of Al(OH)₃. Sham sensitization consisted of 1.6 mg of Al(OH)₃ in 0.4 mL of 0.9% NaCl. That injection was repeated 7 days later. On days 5, 6 and 7 after the second injection, the mice were exposed to an aerosol of 10 mg/mL OVA in 0.9% NaCl (SYST'AM LS nebulizer; Emka Technologie, Paris, France) for 20 min. Control sham-sensitized mice were exposed to 0.9% NaCl for 20 min.

Treatment

Five groups of mice were studied. Each mouse received seven i.p. injections of the respective treatments. Intraperitoneal injections started 2 days after the second s.c. sensitization injection.

- a) Control mice: 10 mL/kg NaCl 0.9%
- b) OVA-sensitized control mice: 10 mL/kg NaCl 0.9%
- c) OVA sensitized mice treated with sildenafil 20 mg/kg
- d) OVA sensitized mice treated with L-arginine 50 mg/kg
- e) OVA sensitized mice treated with sildenafil 20 mg/kg and L-arginine 50 mg/kg

Therefore the protocol may be summarized as follows:

Day 1: first SC sensitization with OVA and Al(OH)₃

Day 8: second SC sensitization with OVA and Al(OH)₃

Day 13 to 15: OVA nebulization

Day 10 to 16: IP treatments

Twenty-four hours after the last OVA exposure, the airway responsiveness to inhaled MCh was evaluated by plethysmography. Then BAL was performed and after BALF collection, the lungs were removed.

Measurement of airway responsiveness

The airway responsiveness in unrestrained, conscious animals was assessed by MCh-induced airflow obstruction evaluated by whole-body barometric plethysmography (Emka Technologie), as described by Hamelmann et al. [34]. Before each experiment, the box containing the mouse was calibrated with a rapid injection of 1 mL of air in the main chamber containing the animal. After the box was calibrated, the pressure difference between the main chamber containing the mouse and a reference chamber was measured. The variation of the box signal results from changes in volume and the resulting pressure in the main chamber during each respiratory cycle of the animal. Inspiration and expiration were processed as a waveform of the box pressure–time signal and recorded by pressure transducers connected to preamplifier modules (Emka Technologie). The changes in early expiration during bronchoconstriction alter the waveform of the box pressure/time curve and can be quantified. These quantified alterations are reflected by the main indicator of airway constriction, enhanced pause: Penh [34,35]. Penh, tidal volume and other respiratory parameters were obtained by a computer data-acquisition system (Emka Technologie), and analysed by IOX software (Emka Technologie). After the mice were acclimated to the main chamber, baseline Penh was measured for 10 min. The measurements of MCh responsiveness were obtained by exposing the mice to ultrasonic nebulization of MCh (6 mg/mL) for 1 min. That dose of MCh was a threshold dose that induced a slight but significant increase in Penh (data not shown) and was the dose used by Lefort et al. [36] and Eum et al. [37] to demonstrate the bronchial hyperactivity to MCh in C57- and BP12-sensitized mice

respectively. The animals' responses to MCh were recorded for 5 min after nebulization. The airway responsiveness was expressed as the percent increase in Penh after MCh administration, compared with Penh for saline challenge. The data are reported as the mean $\pm$ standard error of the mean (SEM); $n = 8$.

BALF analysis

Mice were injected i.p. with a lethal dose of pentobarbital and rapidly exsanguinated by abdominal aorta cutting. That procedure avoids the contamination of the lungs and BALF by circulating blood cells. The trachea was cannulated and the lungs were lavaged with 0.5 mL of saline through the trachea three times and the fluid collected was pooled. After counting the total cells with a Malassez haemocytometer (Bioblock Scientific, Illkirch, France), the preparations were centrifuged (400 rpm for 5 min) with a cytospin centrifuge (Cytospin 2; Shandon, Pittsburgh, PA, USA). The pellets were fixed with methanol at -20°C , and stained with Grünwald–Giemsa for differential cell counting. At least 300 cells were differentiated by light microscopy based on conventional morphologic criteria with an Olympus BH2-RFCA microscope (Olympus France, Rungis, France) connected to a Sony power HAD DXC-950 P colour video camera (Sony France, Clichy, France). Two blinded independent observers counted the number of cells, using the grids of the computer analysis program (Analysis Imaging C software GmbH; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

NO metabolites

The remaining BALF was centrifuged (900 g, 1200 rpm, 6 min) and the supernatant frozen (-70°C) until determination of nitrite and nitrate by the Griess reaction [38]. BALF (100 μL) was incubated (37°C) for 30 min with *N*-2-hydroxyethylpiperazine-*N'*-ethane sulphonate buffer (HEPES) (50 mM, pH 7.4), flavin adenine dinucleotide (5 μM), nicotinamide adenine dinucleotide phosphate (0.1 mM), distilled water (290 μL), and nitrate reductase (0.2 U/mL) for the conversion of nitrate into nitrite. The nitrate reductase was absent for nitrite determination. Unreacted nicotinamide adenine dinucleotide phosphate was oxidized by incubating (25°C , 10 min) with potassium ferricyanide (1 mM). The samples were then incubated (25°C , 10 min) with Griess reagent [*N*-(1-naphthyl)-ethylenediamine: 0.2% (w/v), sulphanilamide: 2% (w/v), solubilized in double-distilled water: 95% and phosphoric acid: 5% (v/v)] and the absorbance measured at 543 nm. The levels of nitrite in

the BALF were calibrated compared to a sodium nitrite (0–150 μM) standard curve.

Lung histology

The trachea and lungs were integrally removed. The left lung lobe was isolated and divided into three parts: the middle part was fixed in formalin, degassed and processed for routine histology in paraffin. Tissue sections, 5 μm in thickness, were cut, mounted on glass slides and stained with haematoxylin/eosin (HE). Observation was made with light microscope (OLYMPUS BH2-RFCA, Japan). The microscope was connected to a camera (Sony power HAD colour video camera, model DXC-950 P). Two blinded independent observers examined the tissues for eosinophil infiltration, epithelial damage, muscular hypertrophy and hyperplasia.

Statistical analyses

The results are expressed as mean $\pm$ SEM, $n = 8$ for each group. The airway responsiveness to MCh was analysed by the non-parametric Friedman test. The BALF cells counts were analysed by the non-parametric Kruskal–Wallis test followed by the non-parametric Mann–Whitney test. A *P*-value < 0.05 was considered to be significant.

Drugs

Chicken OVA (grade V), L-arginine, MCh, HEPES, flavin adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, nitrate reductase, potassium ferricyanide and Griess reagents were obtained from Sigma Chemical (St Louis, MO, USA). Sildenafil was a gift from Prof. Y. Al-Abed, Chemistry Department, Faculty of Science, University of Jordan, Amman, Jordan.

RESULTS

Airway responsiveness

The airway responsiveness to a threshold dose of nebulized MCh (6 mg/mL) for 1 min was assessed as a percent increase of Penh above saline baseline. Data are expressed as mean $\pm$ SEM ($n = 8$ for each group) and presented in *Figure 1*. The baseline Penh for control mice is 0.45 ± 0.02 . MCh induced a slight but significant increase in Penh (0.54 ± 0.04 , $P < 0.01$), 20% above baseline.

In mice sensitized with OVA, the increase in Penh in response to nebulized MCh was significantly enhanced ($57.6 \pm 12.4\%$ above baseline vs. $19.5 \pm 5.2\%$ in control group). In the sensitized mice treated with

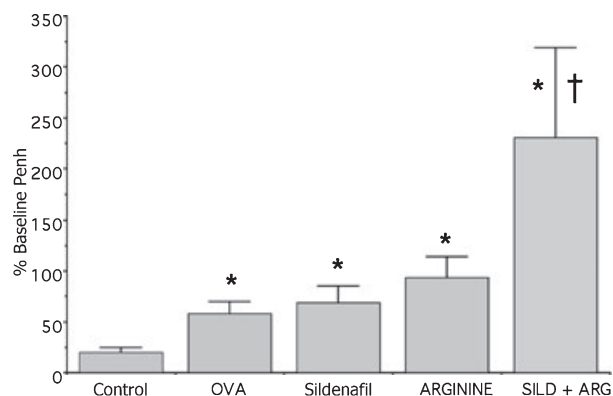


Figure 1 Penh increases after methacholine challenge in control, sensitized and non-treated mice (OVA) and in sensitized mice treated with L-arginine (ARGININE), sildenafil (SILDENAFIL), L-arginine and sildenafil (SILD + ARG). Values are mean $\pm$ SEM ($n = 8$). * $P < 0.05$ vs. control values; † $P < 0.01$ vs. OVA, SILDENAFIL and ARGinine values.

sildenafil or L-arginine, the increase in Penh in response to MCh was also significantly enhanced ($68.2 \pm 17.3\%$ and $93.3 \pm 20.7\%$ above baseline respectively). But the treatment with either sildenafil or L-arginine did not induce any significant hyperresponsiveness to MCh compared with the sensitized but non-treated mice. In contrast, when the sensitized mice were treated with sildenafil and arginine, a significant hyperresponsiveness to MCh was observed ($230.1 \pm 89.0\%$ above baseline).

BALF

The volume of fluid recovered from BAL was not significantly different between the groups (almost 90% recovery, data not shown). The total and differential cell counts from BALF are presented in Table Ia. The total number of cells was significantly increased in the sensitized mice treated with L-arginine. In contrast, with the absence of eosinophils in BALF from control mice, OVA sensitization induced eosinophil infiltration. It must be noted that the number of eosinophils was increased by the different treatments especially when the number of eosinophils was expressed as a percentage of total cells (Table Ib; control OVA: 8.75%, sildenafil: 12.08%, L-arginine: 14.00%; L-arginine + sildenafil: 21.92%). The number of neutrophils was increased by OVA sensitization as well. In contrast, the number of macrophages was significantly reduced in ovalbumin-sensitized mice and the different treatments (sildenafil and or L-arginine) reduced that number of macrophages compared with the control OVA mice.

NO metabolites

The levels of NO metabolites (nitrite and nitrate) in BALF are presented in Table II. The NO metabolite levels were significantly enhanced in the sensitized mice. Treatment with sildenafil did not affect the metabolite levels of the sensitized mice while treatments including L-arginine significantly increased the NO metabolite levels.

Table I (a) Bronchoalveolar lavage fluid cell count^a; (b) bronchoalveolar lavage fluid differential cell count^b

	Total cells	Eosinophils	Macrophages	Neutrophils
(a)				
Control	1.50 $\pm$ 0.19	0.00 $\pm$ 0.00	1.39 $\pm$ 0.18	0.03 $\pm$ 0.01
Control OVA	2.06 $\pm$ 0.43	0.15 $\pm$ 0.02*	0.44 $\pm$ 0.15*	1.42 $\pm$ 0.33*
Sildenafil	1.66 $\pm$ 0.29	0.19 $\pm$ 0.01*	0.11 $\pm$ 0.04*†	1.35 $\pm$ 0.25*
L-arginine	2.69 $\pm$ 0.84*	0.34 $\pm$ 0.09*†	0.12 $\pm$ 0.03*†	2.24 $\pm$ 0.73*
Sildenafil + L-Arg	2.19 $\pm$ 0.40	0.48 $\pm$ 0.10*†	0.13 $\pm$ 0.02*†	1.55 $\pm$ 0.31*
	Macrophages	Eosinophils	Neutrophils	Other cells
(b)				
Control	97.96 $\pm$ 0.51	0.00 $\pm$ 0.00	1.71 $\pm$ 0.51	0.21 $\pm$ 0.11
Control OVA	22.33 $\pm$ 3.89*	8.75 $\pm$ 1.19*	67.79 $\pm$ 4.67*	1.21 $\pm$ 0.09
Sildenafil	5.75 $\pm$ 1.36*†	12.08 $\pm$ 0.82*†	81.63 $\pm$ 1.9*†	0.54 $\pm$ 0.17
L-arginine	5.42 $\pm$ 1.30*†	14.00 $\pm$ 1.10*†	80.13 $\pm$ 2.01*†	0.71 $\pm$ 0.08
Sildenafil + L-Arg	7.08 $\pm$ 1.26*†	21.92 $\pm$ 1.51*†‡	68.88 $\pm$ 3.04*	1.50 $\pm$ 0.43

^aNumber of cells ($\times 10^6$)/mL of BALF. The results are expressed as the mean $\pm$ SEM of cell count from eight mice.

^bPercentage of different cell type. Results are expressed as the mean $\pm$ SEM of cell count from eight mice.

* $P < 0.01$ compared with control values; † $P < 0.05$ compared with control OVA values; ‡ $P < 0.01$ compared with L-arginine and sildenafil alone treated mice values.

Table II: Nitric oxide metabolites (nitrate + nitrite) in BALF (μM)

Control	50.0 $\pm$ 6.7
Control OVA	70.1 $\pm$ 6.4*
Sildenafil	72.4 $\pm$ 7.9*
L-arginine	122.0 $\pm$ 7.2*†
Sildenafil + L-Arg	170.3 $\pm$ 13.9*†

The results are expressed as the mean $\pm$ SEM from eight mice.

* $P < 0.01$ compared with control values; † $P < 0.01$ compared with control OVA values.

Observation of lungs by light microscopy

The lung histology confirmed and the observed inflammatory reaction in sensitized mice and the potentiation of pathological alterations induced by the different treatments (*Figure 2*). Inflammatory cells, mainly eosinophils infiltrated the lungs around the bronchioles. The airway epithelium was thickened, mucus and debris accumulated, and vascular congestion and oedema occurred. Major alterations were observed in the mice treated with both sildenafil and L-arginine: hypertrophy and hyperplasia of bronchial and vascular smooth muscles, vascular congestion, epithelial desquamation and thickness of basal membranes.

DISCUSSION

Reversible airway obstruction in response to allergens, chronic airway eosinophilia and BHR are characteristics of asthma. Different animal models were developed in an attempt to reproduce these human allergic asthma

characteristics but, until now an ideal asthma-like model has not yet been achieved. The mouse model of asthma is used extensively because of the accessibility to genetically established strains. However, the inbred strains of mice display wide differences in susceptibility to develop components of human asthma [39,40]. Particularly, allergen-induced BHR and pulmonary eosinophil influx are not always associated. BP2 mice are a selection of Biozzi mice, which produce high levels of antibodies including IgE [37,41]. A single intranasal challenge with OVA in sensitized BP2 mice induced a severe airway eosinophil influx but did not affect the bronchial reactivity to spasmogens. By contrast, BHR was induced when BP2 mice were challenged with repeated doses of OVA [37]. That is the reason why we chose to sensitize the BP2 mice with multiple doses of OVA (two s.c. injections followed by three nebulizations of OVA solution). In these conditions, a large influx of inflammatory cells (eosinophils and neutrophils) was observed in BALF and lungs. We also observed a decrease in the number of macrophages, which is the main cell type present in BALF of control mice. A similar phenomenon was reported in a mice model of sensitized animal [42]. The mechanisms underlying this decrease in alveolar macrophages are not known, although clinical studies have shown that the number of macrophages in BALF also decrease in asthmatic patients [43]. The inflammatory response was associated with a threefold increase in airway responsiveness to a threshold dose of nebulized MCh (6 mg/mL for 1 min) and with an enhanced level of

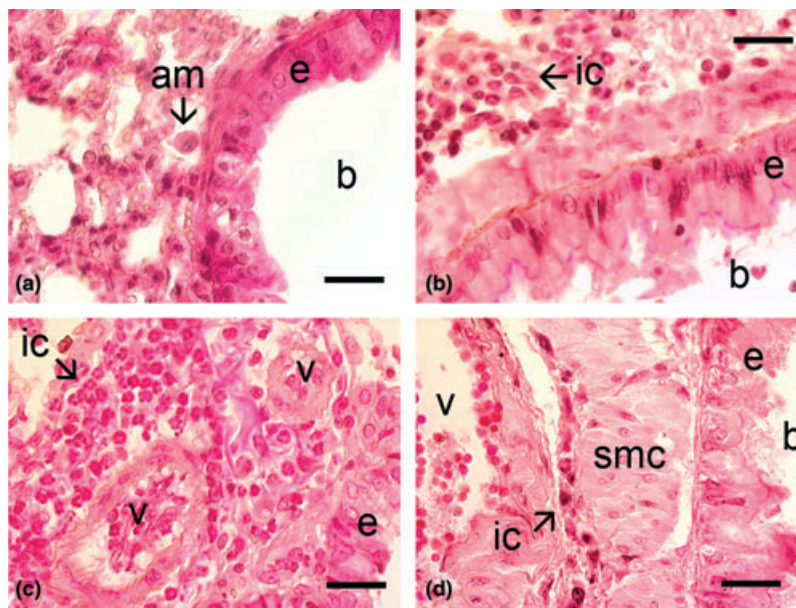


Figure 2 Lung histology of control mice (1), sensitized non treated mice (2), L-arginine (3) and sildenafil plus L-arginine (4) sensitized treated mice. am, alveolar macrophage; b, bronchiole; e, epithelium; ic, inflammatory cells; smc, smooth muscle cells; v, vessels. Inflammatory cell influx can be seen in all sensitized mice. Hyperplasia of smooth muscle can be seen in L-arginine + sildenafil-treated mice. The bar represents 50 μm .

NO metabolites in BALF. Thus, in these experimental conditions, the sensitized BP2 mice developed airway inflammation and increased reactivity to MCh.

The role of NO and related cGMP pathway in human asthma and in sensitized animal models is relatively unclear. In our study, repeated i.p. administration of L-arginine to sensitized BP2 mice induced a large influx of inflammatory cells (eosinophils and neutrophils) in BALF. That enhanced inflammatory reaction is confirmed by lung histology that mainly showed not only an eosinophil infiltration around the bronchioles but also a thickening of epithelium, some vascular congestion and an accumulation of mucus. These data confirmed the critical role of NO in airway inflammation and eosinophilic infiltration that were reported in different sensitized animal models by the use of either NOS inhibitors which decreased the inflammatory reaction [23–25] or L-arginine, the NO precursor which enhanced the eosinophil infiltration [19]. As expected, L-arginine treatments (alone or in combination with sildenafil) significantly increased the NO metabolite levels. It must be noted that the inflammatory reaction and particularly the large influx of neutrophils may contribute to the increase of NO levels as has been recently demonstrated by Fernandes et al. [44]. Considering the airway responsiveness to MCh, the treatment of sensitized mice with L-arginine had no significant effect. That result is in accordance with those of Tulic et al. [20] who reported that MCh responsiveness was not influenced by L-arginine treatment (100 mg/kg, s.c.) in a sensitized rat model.

Most studies on the implication of the NO cGMP pathway in animal models of asthma used L-arginine, the NO precursor or selective and non-selective NOS inhibitors. Another way to study that signalling pathway is to evaluate the effects of selective PDE5 inhibitors which lead to an increase in intracellular cGMP. Conflicting results have been reported for zaprinast, a relatively selective PDE5 inhibitor. Zaprinast is a well-known relaxant of airway smooth muscle both in vitro and in vivo [26], but has been unable to prevent an antigen-induced airway hyperresponsiveness [27]. Moreover, conflicting results were reported for the effects of zaprinast on antigen-induced airway inflammation. The interest in PDE5 inhibitors has been reinforced by the introduction of sildenafil, a more selective and potent PDE5 inhibitor. In our study, a repeated i.p. treatment of sensitized BP2 mice with sildenafil has little effect on the airway responsiveness to MCh, and did not affect the BALF NO metabolite levels but displayed contrasting

effects on the inflammation response. The influx of inflammatory cells in BALF seems to be similar to that observed in control sensitized mice; the only significant difference is observed for a lower macrophage number. But as sildenafil treatment had a tendency to decrease the number of total BALF cells, the percentage of the different cells did not differ from that observed in L-arginine-treated mice. Lung histology did not reveal any perceptible effect of sildenafil treatment, and the lung aspect is similar to that observed in non-treated sensitized mice. Airway responsiveness to nebulized MCh was a little higher but did not significantly differ from that observed in control sensitized mice. These results differed from those of Toward et al. [32], obtained in a guinea-pig sensitized model, which developed early- and late-phase bronchoconstrictor response to OVA. Furthermore, these animals presented hyperreactivity to inhaled histamine and influx of eosinophils in BALF. The administration of sildenafil (1 mg/kg, i.p. 24 h and 30 min before antigen exposure) failed to prevent the early and late bronchoconstrictor responses to OVA, but significantly reduced BHR to histamine and eosinophil influx. Besides the differences linked to the animal models, the mode of administration of sildenafil may explain these discrepancies. In the guinea-pig study, sildenafil was administered at low dose 24 h, 30 min and 6 h after OVA challenge, and in these conditions sildenafil, attenuated bronchial hyperreactivity and inflammation. In our study, repeated administrations of high doses of sildenafil were carried out during the last phase of the sensitization and under these conditions no positive effect was observed. That result is in accordance with those of Clayton et al. [45], who reported that a 10 days of oral treatment with sildenafil at the end of the sensitization period did not produce any significant anti-inflammatory effect in Balb/c OVA-sensitized mice. The relatively less effects of sildenafil treatment in mice with high levels of NO seems paradoxical. Recently, Papapetropoulos et al. [46] showed that lung soluble guanylyl cyclase mRNA levels were severely reduced (60–80%) in sensitized mice with parallel reduction of respective protein expressions. So an increase in NO levels will not necessarily be associated with an increase in cGMP levels. In a preliminary study we observed that the cGMP levels of lung homogenates from OVA-sensitized sildenafil-treated mice were not significantly different from those of non-treated mice (2.53 ± 0.24 and 1.79 ± 0.34 pmol/mg respectively). Toward et al. [32] also reported that sildenafil treatment did not increase lung basal cGMP levels in sensitized guinea-pigs.

The last group of animals studied received a combination of L-arginine and sildenafil. A potentiation of the effects of L-arginine, the NO precursor, by sildenafil that inhibits the degradation of cGMP produced by the stimulation of guanylate cyclase by NO was expected. In fact, the analyses of BALF did not show clear differences in cell counts with the mice that received only arginine: the number of total cells was reduced; the influx of eosinophils increased, while the number of neutrophils was reduced. However, light microscopy observation of the lungs showed that alterations of the tissues were increased in that group of mice with presence of hypertrophy and hyperplasia of bronchial and vascular smooth muscles, vascular congestion, epithelial desquamation and thickness of basal membrane that were not present in the other groups. The airway responsiveness to MCh was dramatically enhanced in that group of mice with a $230.1 \pm 89.0\%$ increase in Penh above baseline compared with the $57.6 \pm 12.4\%$, $68.2 \pm 17.3\%$ and $93.3 \pm 20.7\%$ above baseline observed in saline-, L-arginine- and sildenafil-treated sensitized mice respectively. Thus, if the inflammation reaction is not globally enhanced in terms of inflammatory cell influx, the combination of L-arginine and sildenafil treatment resulted in a large increase in the airway responsiveness to MCh.

Recently cautions about the use of Penh as a measure of lung function have emerged [47,48]. These authors discussed the fact that changes in Penh are not always correlated with airway resistance. Our model had to be improved by the measurement of the airway resistance but it should be mentioned that in our study a significant

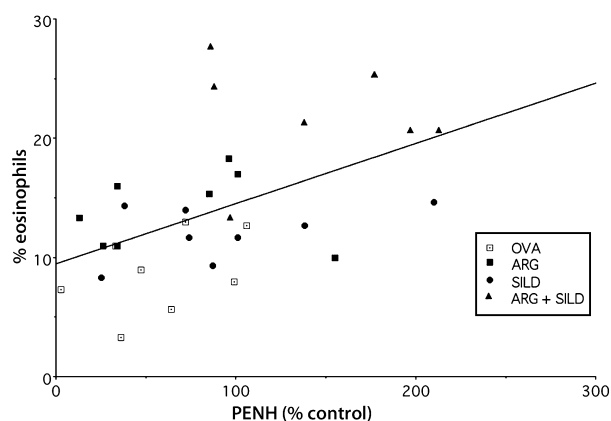


Figure 3 Correlation between Penh increase (% of baseline values) and eosinophil infiltration in BAL of sensitized mice. $Y = 9.46 + 0.05X$; $r = 0.5$; $P < 0.03$.

correlation existed between Penh changes and eosinophil infiltration (Figure 3).

The high dose of L-arginine used was expected to produce high levels of NO for several days and the high levels of NO metabolites in BALF assessed that. But the activation of guanylate cyclase and increased cGMP formation are not the only effects that can be produced by high levels of NO. It is known that under pathological conditions like inflammation, high levels of NO can be produced by iNOS in the body and these high levels of NO can react with concomitantly produced superoxide anions, thereby generating highly toxic compounds such as peroxynitrite and hydroxyl radicals [49,50]. It is well known that NO is involved in bronchial hyperreactivity and airway inflammation present in asthmatic patients, but the relative involvement of the cGMP cascade and the production of toxic radicals is not clearly characterized. The fact that in sensitized BP2 mice, repeated L-arginine treatment associated with a concomitant treatment with a PDE5 inhibitor had differential effects on the inflammation state in contrast with an obvious enhanced responsiveness to MCh need further studies to have better understanding of the relative role of oxidative species and cGMP in NO airway activities.

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