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# Hyperresponsiveness to adenosine in sensitized Wistar rats over-expressing $A_1$ receptor

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ABSTRACT

Airway hyperreactivity is characterized by increased responsiveness to bronchoconstrictor stimuli and it is hallmark of asthma. Adenosine is an ubiquitous signaling nucleoside resulting from ATP catabolism, whose extracellular levels increase following cellular damage or stress. Adenosine plays a role in asthma; asthmatics, but not normal subjects, present bronchoconstriction following inhalation of adenosine or of its precursor, adenosine-5′-monophosphate, most likely via adenosine $A_{2B}$ receptor on mast cells. However, the mechanism underling the increased airway smooth muscle sensitivity to adenosine in asthmatics remains to be elucidated. Early experimental studies suggested the involvement of $A_1$ receptor; this hypothesis has been confirmed by more recent studies on guinea pigs and is corroborated by the finding of an increased adenosine $A_1$ expression on asthmatic bronchial tissues. Brown Norway rats, the strain usually used to assess asthma models, develop hyperresponsiveness to adenosine 3 h following allergen challenge, but not 24 h thereafter, without involvement of $A_1$ receptor. Here, we investigated the role of adenosine $A_1$ receptor in sensitized Wistar rats showing airway hyperresponsiveness 24 h following allergen challenge. We found that on bronchi of sensitized Wistar rats challenged with allergen there is an increased adenosine $A_1$ receptor expression on smooth muscle that is responsible for hyperresponsiveness to adenosine and ovalbumin.



## 1. Introduction

Airway hyperreactivity, an important feature of bronchial asthma, is characterized by increased responsiveness to a number of bronchoconstrictor stimuli (Hargreave and Nair, 2009). Although a wide variety of mediators and inflammatory cells contribute to the airway inflammatory process and tissue remodeling, mechanisms and signaling molecules that govern the chronic nature of inflammation in asthma and bronchial hyperreactivity are still unknown (Barnes, 2008; Sterk and Bel, 1989).

Adenosine is a ubiquitous signaling nucleoside resulting from ATP catabolism, whose extracellular levels strongly increase following cellular damage or stress (Fredholm, 2007). Adenosine plays a role in bronchial asthma; asthmatics present elevated adenosine levels in bronchoalveolar lavage fluids (Caruso et al., 2006; Driver et al., 1993) and bronchoconstriction following inhalation of adenosine or of its precursor, adenosine-5′-monophosphate (Cushley et al., 1983). Interestingly, in humans, bronchial sensitivity to adenosine reflects allergic asthma and bronchial inflammation better than the sensitivity to other agents, such as methacholine or histamine (De Meer et al., 2002; Manso et al., 2011).

Despite evidence suggesting adenosine as an important mediator in the airways, molecular mechanisms at the basis of its effect as well as receptor subtypes(s) involved are still uncertain. Firstly, it was supposed that bronchial response to adenosine in humans was only due to an indirect mechanism involving $A_{2B}$ receptor activation on mast cells (Forsythe and Ennis, 1999); however, to explain the specific increased sensitivity to adenosine of asthmatic airways, the involvement of a direct mechanism was also investigated. Thereby, early studies demonstrated adenosine $A_1$ receptor involvement in hyperresponsiveness to adenosine in immunized rabbits (Ali et al., 1994; el-Hashim et al., 1996). Successively, Obiefuna et al. (2005) showed that the selective $A_1$ receptor antagonist, L-97-1, inhibited bronchial hyperresponsiveness to histamine and adenosine in the model of allergic rabbits.

The role of $A_1$ receptor in bronchial hyperreactivity remains to be clarified. Interestingly, more recently, increased adenosine $A_1$ receptor expression has been found on asthmatic bronchial tissues (Brown et al., 2008a). Furthermore, in a model of

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sensitized guinea pigs, Smith and Broadley (2010) have demonstrated that adenosine $A_{2B}$ and $A_3$ receptors are involved in cell influx and $A_1$ in the late asthmatic response to allergen.

Brown Norway rats, the strain usually used to assess asthma models, develop hyperresponsiveness to adenosine 3 h following allergen challenge, but not 24 h thereafter. The effect has been suggested to be mediated by adenosine $A_{2B}$ receptor on mast cells, ruling out the involvement of other receptors, such as $A_{2A}$, $A_1$ and $A_3$ (Fozard and Hannon, 2000; Hannon et al., 2001, 2002).

There is evidence that Wistar rats develop bronchial hyperreactivity following sensitization and allergen challenge; hyperresponsiveness to acetylcholine is evident 24 h following allergen challenge, in contrast to what is observed in sensitized Brown Norway rats (Chiba and Misawa, 1993).

On these bases, in the present study we have utilized ovalbumin-sensitized Wistar rats in which bronchial responsiveness to adenosine was evaluated 24 h following allergen or saline challenge. Furthermore, we have analyzed the involvement of adenosine $A_1$ receptor in bronchial hyperreactivity elicited 24 h following allergen exposure.

## 2. Material and methods

### 2.1. Animals

All experiments were performed on male Wistar rats (200–250 g; Harlan Nossan, Italy). Animals were housed in a controlled environment and provided with standard rodent chow and water. All experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associate guidelines in the European Community Council Directive of 24 November 1986 (86/609/).

### 2.2. Sensitization procedure and allergen challenge

Animals were briefly anaesthetized with 4% isofluran (Abbott, Italy) in an anesthetic chamber and injected subcutaneously and intraperitoneally with egg chicken albumin (ovalbumin; Sigma, Italy) 100 mg/kg mixed with aluminum hydroxide gel (13 mg/ml; Sigma, Italy); control rats were injected with only the vehicle. Twenty-one days after sensitization procedure, rats were placed in a restrainer, connected to a nebulizer through a mask and challenged with an aerosol of ovalbumin (5 mg/ml; 2 ml per animal) or saline, at a rate of 0.2 ml/min, under sodium pentobarbital anesthesia (60 mg/kg ip); 24 h thereafter, rats were used for the functional study.

### 2.3. Morphological analysis of lungs

For morphological analysis of lungs, rats were treated as described above and sacrificed 24 h after challenge with aerosolized ovalbumin or saline. The thorax was opened, and the lungs were perfused with phosphate-buffered saline (PBS), pH 7.4, via the pulmonary artery to remove blood. The lungs were distended by instilling 5 ml of 10% buffered formalin, pH 7.4, via the tracheotomy. The trachea was tied closed and the inflated lung was carefully removed to avoid puncturing and placed in 10% formalin for 24 h. Transverse portions, 0.5 cm thick, were cut from the mid- and lower zones of fixed lungs, paraffin-embedded, sectioned at 5 μm and stained with haematoxylin and eosin. Images were taken by a Leica DFC320 video-camera (Leica, Milan, Italy) connected to a Leica DM RB microscope using the Leica Application Suite software V2.4.0.

### 2.4. Functional study

Functional experiments were performed on sensitized rats challenged with aerosolized ovalbumin or with saline, as described above, and on control rats. Animals were anaesthetized with urethane (10 ml/ kg ip.; sol. 10% w/v; Sigma, Italy); the jugular vein and the carotid artery were cannulated respectively for drug administration and for a continuous blood pressure monitoring. Rats were artificially ventilated (60 breaths/min; 1 ml/100 g tidal volume) via a tracheal cannula and connected

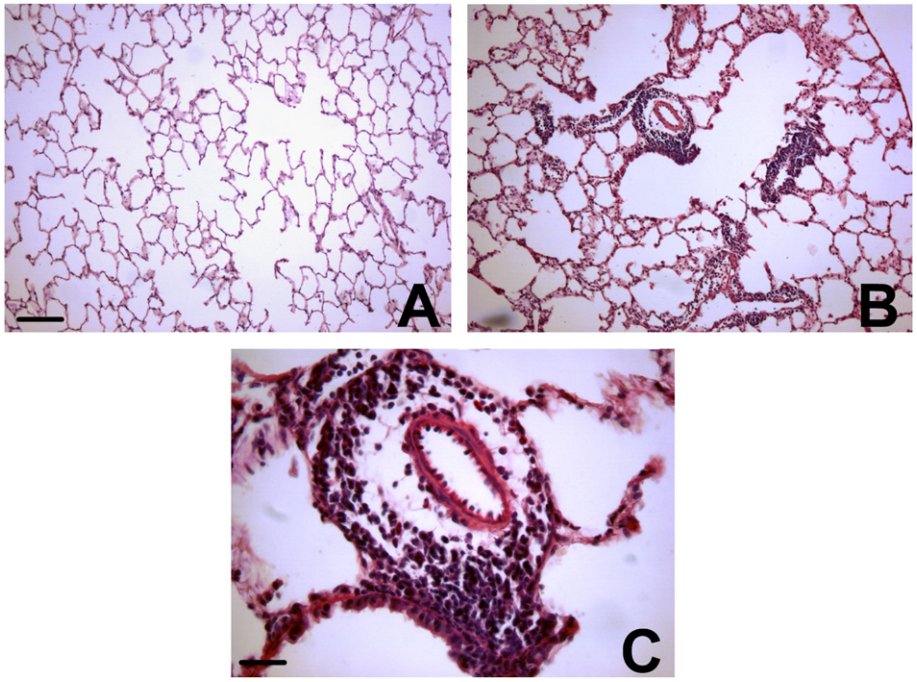


**Fig. 1.** Formalin-fixed, paraffin-embedded and H&E-stained rat lung sections from control rats (A) and sensitized ovalbumin-challenged rats (B and C). Lung sections from control rats are free of inflammation and edema (A). In lungs from sensitized ovalbumin-challenged rats, peribronchial and perivascular inflammatory cell infiltration and edema can be seen (B and C). Pictures shown are representative of three separate experiments performed. Magnification: A and B ×100; scale bar=25 μm and C ×400; scale bar=100 μm.

to a bronchospasm transducer (Ugo Basile, Italy) to monitor change in airway resistance. All data were acquired by a computerized system (BIOPAC Systems, TCI 102). After a stabilization period of 15 min, ovalbumin (1 mg/kg) was intravenously administered and the bronchial response was monitored during the following 1 h; afterward, adenosine (3 mg/kg i.v.; Sigma, Italy) was administered and the bronchial response was monitored for 30 min; in the end, bronchial response to carbachol (10 μg/kg i.v.; Sigma, Italy) was evaluated.

### 2.5. Western blot analysis

In subsets of experiments, adenosine $A_1$ receptor expression on main bronchi obtained from different animal groups was evaluated. For this purpose, rats were sacrificed and lungs were removed. Main bronchi were carefully isolated from the remaining part of the lung, were dissected free of parenchyma and immediately frozen in liquid nitrogen before being stored at −80 °C. On the day of analysis, tissues were crushed into powder and resuspended in 300 μl of lysis buffer (aprotinin, 3.07 μM; EDTA, 100 mM, leupeptin, 2.2 μM; Na-deoxycholate, 10%; NaCl, 150 mM; NaF, 5 mM; NP-40, 10%; ortovanadate, 50 μM; PMSF, 100 μM; Tris–HCl, 65 mM). Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Italy). Protein samples (35 μg) were electrophoresed in an 8% discontinuous polyacrylamide gel and then transferred onto nitrocellulose membranes. The membranes were saturated by incubation with 5% non-fat dry milk in PBS supplemented with 0.1% Tween-20 (PBS-T) for 1 h at room temperature and then incubated with anti-$A_1$ receptor goat antibody (1:200; Santa Cruz Biotechnology, Italy) for 12 h at 4 °C. Successively, membranes were washed and then incubated for 2 h at room temperature with the secondary antibody conjugated with horseradish peroxidase, anti-goat IgG–HRP (1:3000; Dako, Denmark). Immunereactive proteins were visualized by enhanced chemiluminescence using Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Successively, to confirm the equal protein loading, membranes were stripped and incubated with anti β-actin monoclonal antibody (1:3000; Sigma-Aldrich, Italy) and subsequently with anti-mouse IgG–HRP (1:5000; Dako, Denmark), both for 60 min at room temperature. Protein levels were quantified by densitometry of detected bands.

### 2.6. Immunohistochemical analysis

From different groups of animals, the main bronchi were removed as described above, snap-frozen in liquid nitrogen in embedding medium (OCT compound, Sakura Finetek, Europe). Ten cross sections (8 μm) were cut from the approximate middle portion of the bronchi. For staining, sections were fixed in acetone for 5 min, air dried, re-hydrated with PBS, incubated with 0.3% $H_2O_2$ in methanol for 10 min before the incubation in serum-free Protein Block (DakoCytomation, Milan, Italy) for 30 min. After avidin–biotin blocking, sections were incubated for 1 h with adenosine $A_1$ receptor goat polyclonal antibody (1:75 in 1% BSA; Santa Cruz Biotechnology, Italy) or with isotype-matched antibody as negative control. Subsequently, sections were incubated for 15 min in biotinylated anti-goat secondary antibody (1:200; Dako, Denmark), washed and incubated for 5 min with streptavidin–HRP (1:200; Sigma-Aldrich, Milan, Italy). Positivity was detected with 3,3′-diaminobenzidine substrate (DAB) and the nuclei counterstained with haematoxylin and eosin (H&E). The slides were then dehydrated and mounted in Entellan® medium. Images were acquired with Leica DFC320 video camera (Leica, Italy) connected to the microscope (Leica, DMRB) using the Leica Application Suite software V2.4.0.

### 2.7. Rat treatment with $A_1$ adenosine receptor antagonist

To evaluate the role of adenosine $A_1$ receptor in the bronchial response to spasmogens used, different groups of sensitized ovalbumin-challenged animals were pre-treated with $A_1$ adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 100 μg/kg iv.; Sigma, Italy), 5 min before administrating ovalbumin; control animals received the vehicle (DMSO/ distilled water 1:1; 1 ml/kg iv.).

### 2.8. Statistical analysis

All data are expressed as mean ± S.E.M. of at least five experiments and analyzed with a computerized statistical package (GraphPad Prism v. 4.01). Bronchoconstriction is expressed as percentage of bronchoconstriction relative to the maximum percentage (100%) simulated by clamping air piping upstream the tracheal cannula, thereby diverting all pumped air to the transducer. Results are analyzed with one way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons, or with Student's *t*-test when appropriate. A value of $P < 0.05$ was considered significant.

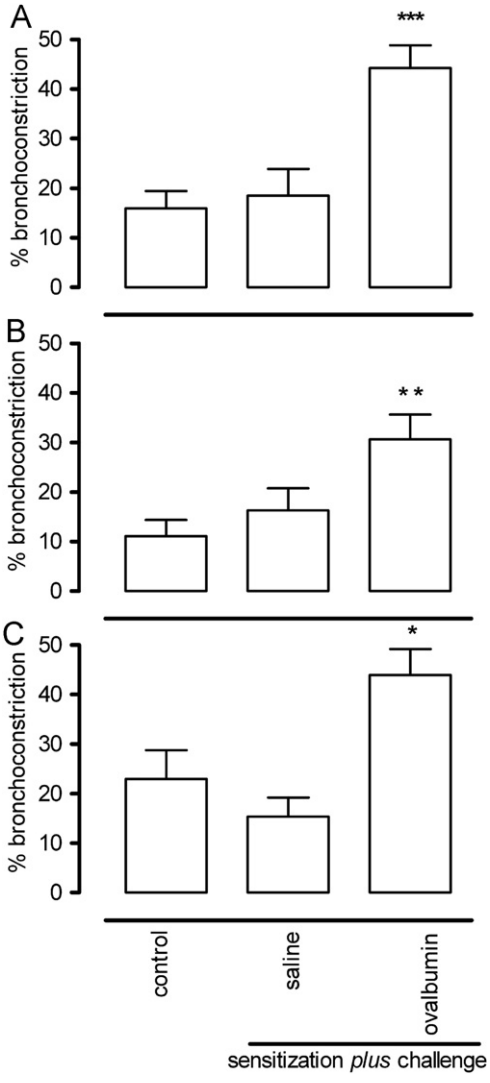


**Fig. 2.** Bronchoconstriction in response to (A) ovalbumin (1 mg/kg iv.); (B) adenosine (3 mg/kg iv.) and (C) carbachol (10 μg/kg iv.) evaluated in sensitized rats challenged with aerosolized saline or with aerosolized ovalbumin and in control rats. Values are mean ± S.E.M. ($n$=9–24); $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* control.

## 3. Results

### 3.1. Morphological characterization of lungs from sensitized rats challenged with ovalbumin shows tissue edema and cell infiltration

Following histological analysis with haematoxylin and eosin staining, peribronchial and perivascular edema and inflammatory cell infiltration was evident in sensitized rats challenged with aerosolized ovalbumin but not in control rats, whose lung sections were free of inflammation and edema (Fig. 1). A similar profile without tissue damage was observed on lung sections from ovalbumin-sensitized rats challenged with aerosolized saline (data not shown).

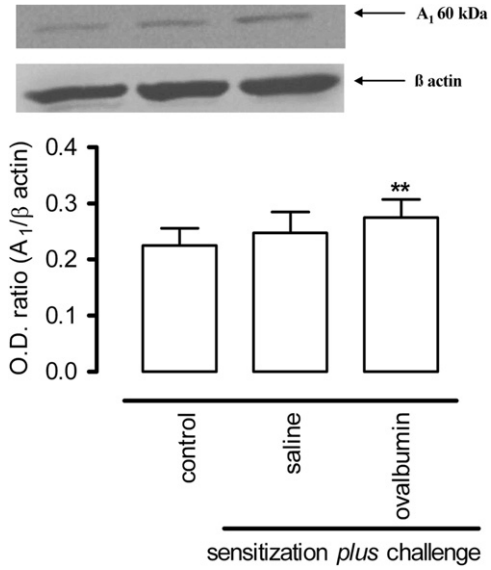


**Fig. 3.** Representative results of Western blot analysis of adenosine $A_1$ receptor expression on bronchial tissue from sensitized rats challenged with ovalbumin or with saline and from control rats. Graph bar represents optical density (O.D.) relative to β actin. $^{**}P < 0.01$ *vs.* control (repeated measures ANOVA, followed by Dunnett's test).

### 3.2. Increased bronchial response to spasmogens in sensitized rats challenged with ovalbumin

In sensitized rats challenged with saline 24 h before the functional study, bronchoconstriction in response to intravenously injected ovalbumin (1 mg/kg), adenosine (3 mg/kg) or carbachol (10 μg/kg) was of 18.53 ± 5.37% ($n=9$); 16.33 ± 4.43% ($n=9$) or 15.33% ± 3.88 ($n=9$) respectively (Fig. 2). These responses were not significantly different from those obtained in control rats (ovalbumin, 15.93 ± 3.49%; adenosine, 11.13 ± 3.27%; carbachol, 23.00 ± 5.00%, $n=15$; Fig. 2). On the contrary, in sensitized rats challenged with aerosolized ovalbumin 24 h before measurements, bronchoconstriction in response to all spasmogens significantly increased (ovalbumin, 44.25 ± 4.61%, $P<0.001$; adenosine, 30.68 ± 5.00%, $P<0.01$; carbachol, 43.95 ± 5.26%, $P<0.05$; $n=19$–24) compared to response obtained in control rats (Fig. 2).

### 3.3. Adenosine $A_1$ receptor is highly expressed on bronchial tissues from sensitized rats challenged with ovalbumin

Western blotting on bronchial tissues from sensitized rats challenged with ovalbumin showed that the adenosine $A_1$ receptor expression was increased compared to the expression evaluated on bronchial tissues from sensitized rats challenged with saline and from control rats (Fig. 3).

Results obtained by Western blot analysis were confirmed by immunohistochemical analysis with bronchial tissues from sensitized rats challenged with ovalbumin showing a positive immunoreactivity for adenosine $A_1$ receptor, predominantly localized on the bronchial smooth muscle, stronger than tissues obtained

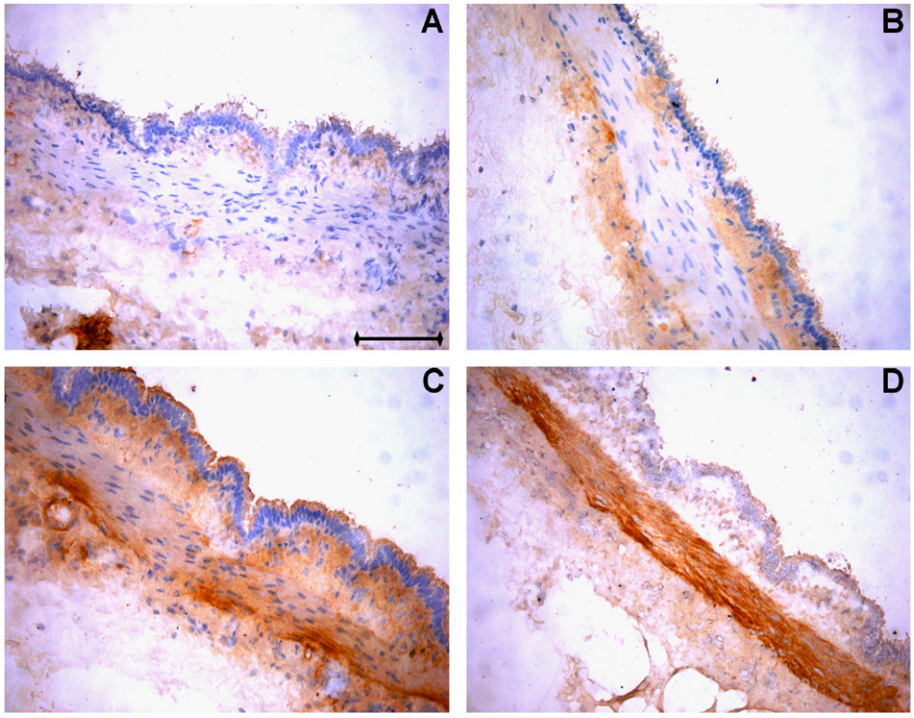


**Fig. 4.** Immunohistochemical localization of adenosine $A_1$ receptor on bronchi control rats (B), and from sensitized rats challenged with saline (C) or with ovalbumin (D); (A) is a negative control. (Original magnification: × 400; scale bar = 50 μm).

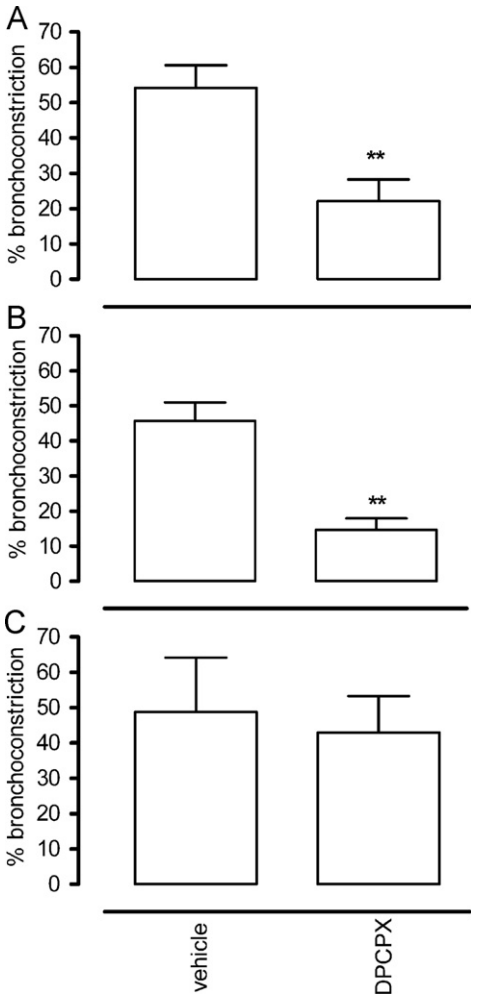


**Fig. 5.** Effect of adenosine $A_1$ receptor antagonist, DPCPX (100 μg/kg iv) on bronchoconstriction induced by (A) ovalbumin (1 mg/kg iv); (B) adenosine (3 mg/kg iv.); and (C) carbachol (10 μg/kg iv.) in sensitized rats challenged with aerosolized ovalbumin. The effect of vehicle (DMSO) is also shown. Values are mean ± S.E.M. ($n$=4–6). $^{**}P < 0.01$.

from sensitized rats challenged with saline and from control animals (Fig. 4).

### 3.4. Adenosine $A_1$ receptor antagonism reduces bronchial response to ovalbumin and adenosine

Rat treatment with $A_1$ adenosine receptor antagonist, DPCPX (100 μg/kg iv.), injected 5 min prior to the administration of spasmogen agents, reduced the bronchial response to ovalbumin and to adenosine ($P < 0.01$), but did not affect the bronchial response to carbachol (Fig. 5).

## 4. Discussion

In this study we show that adenosine $A_1$ receptor mediates increased bronchoconstriction in response to allergen and adenosine observed in sensitized Wistar rats 24 h following challenge with aerosolized ovalbumin. Morphological analysis showed feature of tissue damage and inflammatory cell infiltration in airways from sensitized rats challenged with aerosolized allergen. Results obtained from the functional studies showed that challenge of actively sensitized rats with aerosolized ovalbumin induced bronchial hyperreactivity, evidenced by an increased bronchoconstriction in response to all spasmogens used, that was not observed when sensitized rats were challenged with aerosolized saline. This finding is in accordance with previous experimental works performed on Brown Norway rats (Hannon et al., 2001) and on allergic mice (Fan and Mustafa, 2002) and demonstrates that, in Wistar rats, sensitization *per sè* does not cause bronchial hyperreactivity but allergen challenge is required to establish bronchial hyperreactivity, an important feature of allergic asthma. It is worth noting that this finding is also consistent with clinical observations about asthma; in fact, it must be considered that asthmatics undergo regular exposure to allergen.

Although intriguing findings have suggested the involvement of adenosine $A_1$ receptor in asthma, up to now its role in bronchial hyperreactivity is still unclear (Brown et al., 2008b).

Here, we show that the increased bronchial response to spasmogens, in our experimental model, was paralleled by an increased adenosine $A_1$ receptor expression on bronchi from sensitized rats exposed to aerosolized ovalbumin; on the contrary, sensitized rats exposed to aerosolized saline did not show bronchial hyperreactivity nor increased $A_1$ expression on airways. Adenosine $A_1$ receptor overexpression on airways of sensitized rats challenged with ovalbumin was also confirmed by histological studies; indeed, there was a strong immunopositivity for adenosine $A_1$ receptor on bronchial smooth muscle cells only of sensitized rats challenged with ovalbumin. This finding confirms that adenosine $A_1$ receptor up-regulation is consequent to the exposure to allergen.

$A_1$ receptor was firstly demonstrated to be involved in bronchoconstriction in response to adenosine in a model of allergic rabbits; authors demonstrated specific adenosine $A_1$ receptor binding sites in lung and suggested the possibility of an inducible $A_1$ receptor (Ali et al., 1994). Successively, it was shown that, in the same model, an antisense oligonucleotide targeting $A_1$ receptor mRNA reduced bronchoconstriction induced by either adenosine or allergen (el-Hashim et al., 1996; Nyce and Metzger, 1997). More recently, elevated expression of adenosine $A_1$ receptor has been found localized on epithelium and smooth muscle in bronchial tissue from asthmatic subjects; in contrast, $A_1$ receptors are scarcely expressed on tissues from healthy subjects (Brown et al., 2008a). Interestingly, this is the only adenosine receptor subtype found to be differently expressed on human tissues from asthmatic and normal people.

To evaluate whether the increased adenosine $A_1$ receptor expression also reflected receptor functionality and its involvement in bronchial hyperreactivity, we performed functional experiments also in groups of sensitized rats treated with $A_1$ receptor antagonist, DPCPX, at the dose described in literature (Tigani et al., 2002). We found that DPCPX treatment of sensitized rats challenged with aerosolized ovalbumin reduced not only the increased bronchial response to adenosine, but also the increased response to ovalbumin, while sparing response to carbachol. This finding demonstrates that adenosine $A_1$ receptor is involved in the bronchial response to adenosine and to allergen observed 24 h following challenge in sensitized Wistar rats. Furthermore, evidence that DPCPX does not inhibit the increased response to carbachol indicates no unspecific effect in reducing smooth muscle contractility. Likely, following allergen administration released adenosine contributes to bronchoconstriction through $A_1$ receptor activation, as also suggested by performing experiments in sensitized guinea pigs (Smith and Broadley, 2010). Interestingly enough in this study, authors observed that, in sensitized guinea pigs, $A_1$ antagonist was only able to inhibit the late asthmatic response characterized by bronchial hyperreactivity, 24 h following allergen challenge, consistent with an increased expression of $A_1$ receptor.

In conclusion, our data show that challenge of sensitized Wistar rats with allergen causes bronchial hyperreactivity paralleled by up-regulation of adenosine $A_1$ receptor on airway smooth muscle. In our model, adenosine $A_1$ receptor is involved in bronchial response to adenosine and to allergen, 24 h following allergen challenge, in contrast to what was previously demonstrated in Brown Norway rats, in which hyperresponsiveness to adenosine was observed only 3 h following allergen challenge and did not involve $A_1$ but $A_{2B}$ receptor, consistent with the involvement of mast cells, thus with an indirect adenosine effect (Fozard and Hannon, 2000; Hannon et al., 2001). Thus, it appears that adenosine $A_{2B}$ receptor is involved in an early asthmatic response, while $A_1$ receptor is involved when an increased airway reactivity to adenosine is observed in a late phase.

It is known that to define the mechanism at the basis of airway hyperreactivity to adenosine is made complicated by the fact that the sensitivity to adenosine and receptor(s) involved vary among animal species and rat strains used in asthma models (Fozard and Hannon, 2000). These results represent a further contribution pointing at an important role for the adenosine $A_1$ receptor up-regulation in bronchial hyperreactivity elicited by allergen challenge and also provide a useful rat model to better investigate on the molecular basis of bronchial hyperresponsiveness to adenosine.

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